

Manipulation of the *Nicotiana*
benthamiana immune response to
Agrobacterium tumefaciens to improve
Plant Molecular Farming



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Abstract

Nicotiana benthamiana is used as an expression platform for recombinant protein production using *Agrobacterium tumefaciens*-mediated transient expression. This method has successfully produced multiple recombinant proteins. However, the use of *A. tumefaciens* for transient expression poses a problem because it elicits an immune response from *N. benthamiana* which hampers molecular farming yields. In this research we aimed to improve understanding of the interaction between *N. benthamiana* and *A. tumefaciens* and how this interaction can be manipulated to improve molecular farming. This was achieved firstly by studying how apoplastic alkalization is detrimental to *A. tumefaciens*. Apoplastic alkalization was achieved by manipulating the activity of the H⁺-ATPase named Autoinhibited H⁺-ATPase isoform 2 (AHA2). AHA2 activity was manipulated by over-expression of the RAPID ALKALINIZATION FACTOR-1 peptide (RALF1) and expression of the AHA2-S899A mutant. Both strategies resulted in alkalization of the apoplast. We found that apoplastic alkalization triggered upregulation of pathogenesis-related proteins, mediated by salicylic acid signalling. We demonstrated that these alkalization-induced plant defence responses were detrimental to *A. tumefaciens* and reduced accumulation of recombinant proteins. Next, we developed a high-throughput ‘Traffic Light Screen’ to evaluate new strategies to improve molecular farming and uncover how plant immunity affects *A. tumefaciens*. Using this screen, we investigated the impact of co-expressing 130 RxLR effectors on transient expression and *A. tumefaciens*. We identified six effectors which, when co-expressed, increased fluorescence of secreted RFP. Two of these six effectors also increased accumulation of a recombinantly expressed IgG antibody, VRC01. However, when co-expressed in an industrial scale

agroinfiltration, the most promising effector did not increase final yields of VRC01 produced. We then used the ‘Traffic Light Screen’ to assess the impacts of inhibiting plant defence responses through depletion of genes involved in plant immunity. This identified that silencing of genes *ICS* (encoding isochorismate synthase), *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* and *CHITIN ELICITOR RECEPTOR KINASE 1* improved transient expression. These three genes are promising candidates to pursue further. We also revealed that the receptor *NbCORE* is essential for perception of the MAMP derived from bacterial cold shock protein (CSP), called csp22, in older *N. benthamiana* plants and that depletion of *NbCORE* enables the use of older plants with greater biomass for transient expression. This research demonstrates that manipulating the interaction between *N. benthamiana* and *A. tumefaciens* is an effective strategy to improve molecular farming. We also demonstrate the need to study this interaction further to identify plant defence responses that hamper molecular farming in order to find new strategies to increase recombinant protein yields.

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Chapter 1

Introduction

Molecular farming is the use of plants to produce recombinant proteins. This method of protein production emerged with the first use of transgenic tobacco to produce a functional antibody ([Hiatt et al., 1989](#)). Since then, the industry has progressed into a genuine competitor amongst the typical bacterial and mammalian systems used for recombinant protein production. High profile success stories of molecular farming include the FDA-approved human β -glucocerebrosidase, developed by Protalix Biotherapeutics ([Mor, 2015](#)) and production of ZMapp, a cocktail of three anti-Ebola antibodies, which was used as treatment during the 2014 West African Ebola outbreak ([Na et al., 2015](#)). However, the huge number of *Nicotiana benthamiana* plants required for production of a single patient's dose combined with closing of the trial at the end of the outbreak and before completion led to a Chinese Hamster Ovary-produced antibody cocktail overtaking the plant-produced alternative as the treatment of choice for Ebola infections ([Schillberg and Finnern, 2021](#)). The case of Zmapp production demonstrates the need for more research into how molecular farming can be improved in order to make it a viable competitor in the recombinant proteins market.

One of the most commonly used methods in molecular farming is to use *Agrobacterium*-based transient expression of recombinant proteins in *N. benthamiana*. However, entry of *Agrobacterium* into plants triggers an immune response. This plant immune response may reduce the production of recombinant proteins ([Goulet et al., 2010](#); [Grosse-Holz et al., 2018a](#)). Therefore, in this work, I sought to gain better understanding

of the interaction between *Agrobacterium tumefaciens* and *N. benthamiana* and use this knowledge to reduce the impact of plant immunity on molecular farming.

The underlying question throughout my work is ‘*Does the N. benthamiana immune response hamper Molecular Farming?*’ Specifically, I aim to understand if we can manipulate the *N. benthamiana* immune response to improve molecular farming yields by investigating effects of pH on *Agrobacterium*, how co-expression with pathogen-derived effectors impacts recombinant protein accumulation, and which *N. benthamiana* immunity genes can be silenced to improve molecular farming yields. This introduction will outline the emergence of plant molecular farming as a viable method for recombinant protein accumulation, the issues preventing success of the industry, the molecular biology of *Agrobacterium* infection and transient expression, plant immune responses and the current knowledge of the plant-*Agrobacterium* interaction.

1.1 Production of recombinant proteins by molecular farming

1.1.1 Recombinant protein expression systems

Expression of recombinant proteins is used across a variety of organisms for basic research, biotechnology and production of therapeutics ([Assenberg et al., 2013](#)). The most commonly used expression systems are *E. coli*, insect cells using the Baculovirus Expression Vector System (BEVS) and mammalian cells ([Assenberg et al., 2013](#)). These systems have been used over the last 40 years to produce a wide range of proteins for uses from medicines to food technology and new polymers. Each of these expression systems have their strengths and weaknesses that necessitate their use over another for particular requirements, such as the specific protein being made and the quantity needed ([Burnett and Burnett, 2020](#)). Challenges including expensive media components and risk of human pathogen contamination of mammalian cells, in addition to undesired post-translational modifications (PTM) in insect systems and prokaryotic limitations of size and PTM of recombinant proteins opened space in the market for the development of plant-derived recombinant proteins ([Burnett and Burnett, 2020](#)).

1.1.2 Emergence of Molecular Farming

Plants were first used to produce recombinant proteins for therapeutic use when transgenic tobacco was used as an expression system for antibodies ([Hiatt et al., 1989](#)). Since then, plant-based expression has been tested in many different plant species and using multiple expression strategies and has become known collectively as Molecular Farming ([Kermode and Jiang, 2018](#); [Twyman et al., 2003](#)). Early success was achieved with a wide range of whole plants. For example, avidin was expressed in transgenic maize ([Hood et al., 1997](#)), a herpes antibody in transgenic soybean (Zeitlin 1998), a diagnostic antibody in transgenic alfalfa ([Khouidi et al., 1999](#)), a rabies vaccine in transgenic tomato ([McGarvey et al., 1995](#)) and many other recombinant proteins in transgenic tobacco, *N. benthamiana*, lettuce and other leafy plants ([Twyman et al., 2003](#)). Furthermore, transgenic cereal crops such as wheat and rice were developed for the production of recombinant proteins in seeds. Expression in seeds provides advantageous biochemical conditions for stable protein accumulation and the recombinant proteins produced remain active for multiple years ([Twyman et al., 2003](#); [Stöger et al., 2000](#)). Fruit and vegetable crops such as potato and bananas have been used as potential candidates to produce edible vaccines for use in the developing world ([Mason et al., 2002](#)). To decrease the time needed to generate transgenic lines, many plant cell and tissue culture systems were developed such as tobacco hairy roots, BY-2 cell cultures and cell lines of carrot and maize ([Häkkinen et al., 2014](#)). As well as many plant species being investigated for molecular farming, various expression strategies were also tested. These included transient expression, transgenic plants and transplastomic plants. Over the last twenty years, research and production has focused on a few systems including transgenic tobacco and cereals; expression in mosses and plant cell suspensions and transient expression in *N. benthamiana* ([Kermode and Jiang, 2018](#)).

Transient Expression of Recombinant Proteins

This research has focused on transient expression in *N. benthamiana*. Transient expression for molecular farming is achieved by three methods, which all use a plant host for the expression and accumulation of recombinant proteins by a bacterial or viral vector

(*Kermode and Jiang, 2018*). In the first method, agroinfiltration is used to infiltrate *Agrobacterium* into leaves. This allows *Agrobacterium* to transfer T-DNA into host cells, where it is expressed as the recombinant protein of interest for up to a few weeks (*Kermode and Jiang, 2018*). The second method is to infect plants with recombinant viruses as the vector for transient expression. These viruses carry genes of interest in their genome where insertion will not adversely affect virus replication. (*French et al., 1986; Ibrahim et al., 2019*). Lastly, *Agrobacterium*-delivered replicon-based vectors can be used (*Liu and Timko, 2022*). Instead of directly injecting recombinant viruses, as is done with the second method described, the viral genome is split into multiple modules and are encoded by *A. tumefaciens* T-DNA. The viral modules are then delivered into plant cells as T-DNA. The T-DNA modules assemble into a viral replicon which can replicate and move from cell-to-cell within the leaf. Within this replicon, the coat protein encoding genes are replaced by the gene of interest (GOI) encoding the recombinant protein. This leads to systemic infection of the cells in agroinfiltrated leaves with viral replicons encoding the GOI and consequently systemic expression of the recombinant protein across the leaf (*Peyret and Lomonosoff, 2015; Ibrahim et al., 2019*).

1.1.3 Successful products of molecular farming

Many different proteins have been produced by molecular farming. Strict regulation of proteins for clinical use have resulted in only a few cases of plant-produced therapeutics making it to market, but many plant-derived proteins have become commercial products for non-clinical uses (*Schillberg et al., 2019*). The enzyme known as taliglucerase alfa, produced in carrot cells by Protalix Biotherapeutics is one of very few plant-derived pharmaceuticals that has made it through clinical trials and is approved by the U.S. Food and Drug Administration (FDA) to treat Gaucher's disease (*Schillberg et al., 2019*). Many other pharmaceuticals have been produced in plants but have not yet made it through clinical trials. These therapeutics include vaccines against West Nile virus and Dengue fever, both produced in *N. benthamiana* and reported to induce immunity in mice (*Lai et al., 2018; Ponndorf et al., 2021*).

More success has been achieved in commercialising non-clinical proteins. These include

proteins used for cosmetics such as human epidermal growth factor produced in barley and used by Icelandic company Sif Cosmetics (*Schillberg et al.*, 2019; *Schouest et al.*, 2012). Another common purpose of plant-derived proteins is diagnostics. Companies such as Leaf Expression Systems list diagnostic proteins such as antigens as a commercial product, and maize-produced avidin is distributed by Sigma-Aldrich for the detection of biotin-avidin binding (*Hood et al.*, 1997). Proteins produced by molecular farming also have roles in emerging industries such as alternative proteins and cell cultured meats. Alternative proteins produced in plants are in the process of commercialization by companies such as Moolec, which is producing milk proteins in plants. Cell-cultured meat also provides an opportunity for molecular farming. Components used in production, other than the cell-line itself, need to be derived from non-animal sources. Companies such as Leaf Expression Systems and Core Biogenesis use molecular farming to produce growth factors and cytokines for the cell-cultured meat industry.

Despite a multitude of opportunities for molecular farming there are still issues with plants not meeting the yields produced by conventional methods such as CHO cells, bacterial cultures and insect cells (*Schillberg et al.*, 2019). Research into strategies to increase yields can bring molecular farming into a position to compete with other expression systems to meet requirements for protein expression.

Use of *N. benthamiana* for molecular farming

Prominent molecular farming company Medicago Inc. is amongst many companies and researchers that use *N. benthamiana* as their host plant for transient expression. It has been studied extensively as a model plant (*Bally et al.*, 2018). Molecular farming therefore benefits from extensive investigations published on topics from its immune responses to cell biology and from recent funding through collaborations such as Newcotiana (<https://newcotiana.org>) and Pharma Factory (<https://pharmafactory.org>) (*Jutras et al.*, 2020; *Bally et al.*, 2018). A large amount of genomic information on *N. benthamiana* is available for use, including a chromosome-level assembly of *N. benthamiana* (*Molina-Hidalgo et al.*, 2021).

The leaves of *N. benthamiana* are agroinfiltrated easily, allowing agroinfiltration of

large volumes of leaf tissue ([Jutras et al., 2020](#)). *N. benthamiana* is then able to express transgenes and gene-silencing signals within three days ([Bally et al., 2018](#)). Use of *N. benthamiana* therefore allows for rapid, large-scale expression of recombinant protein. Furthermore, *N. benthamiana* is not used as a food source and therefore its use for research and molecular farming does not endanger production chains and market value of vital food crops.

Extensive research of *N. benthamiana* has revealed another reason *N. benthamiana* produces high yields of recombinant proteins. In the strain used for research and molecular farming (LAB), there is a 72 base-pair insertion into the RNA-dependent RNA polymerase gene, *Rdr1*. This renders the LAB strain hypersusceptible to viral infection and allows high accumulation of transgene transcripts ([Jutras et al., 2020](#); [Bally et al., 2018](#)), making it an ideal plant for molecular farming.

1.1.4 *N. benthamiana* modifications for molecular farming

Various strategies have been reported for engineering *N. benthamiana* to improve the quality and quantity of recombinant protein produced by molecular farming including humanization of protein glycans. One of the most prevalent classes of protein produced is antibodies. A significant area of advance has been made in the glycans of recombinant glycoproteins, which are reported to make up 60% of all approved biopharmaceuticals ([Planinc et al., 2016](#)). Plant glycans include the core $\beta(1,2)$ -xylose and $\alpha(1,3)$ -fucose residues which are not present in endogenous human glycans. To ensure that plant-derived glycoproteins, including antibodies, can be used without being recognised as non-human, transgenic plants have been generated in which the endogenous glycosyltransferases have been knocked out and replaced with their human counterparts. This results in production of antibodies with humanised N-glycans and have been used to produce functional human erythropoietin fusion protein ([Castilho et al., 2013](#)). Additionally, introduction of the mammalian biosynthetic pathway for sialylation of the Fc domain of human antibody 2G12 yielded an antibody that could be efficiently neutralised ([Castilho et al., 2010](#)). These steps have resulted in transgenic *N. benthamiana* lines that are better for production of humanized glycoproteins by molecular farming.

Other strategies involving *N. benthamiana* modifications to improve molecular farming have been explored, including development of a *Nicotiana* hybrid with greater biomass ([Bally et al., 2018](#); [Shamloul et al., 2014](#)), developing plant cell culture and enabling tyrosine o-sulfation of plant produced vaccines ([Singh et al., 2020, 2021](#)).

1.2 Issues facing Molecular Farming

The main problems limiting the success of molecular farming are high costs of downstream processing, issues acquiring regulatory approval for therapeutics, low biological activity of recombinant proteins produced and lower productivity of plants compared to conventional expression systems.

Therapeutics produced in CHO cells and *E. coli* are secreted into the media and easily purified without cell contaminants. In contrast, whole plants must be homogenised before protein extraction, resulting in contamination of the recombinant protein with host proteins and insoluble fibres ([Schillberg and Finnern, 2021](#)). This means that extra steps including homogenisation, filtration and precipitation increase costs and complexities of downstream processing.

Mammalian and bacterial expression systems for biopharmaceuticals have a long history of regulatory approval. Molecular farming on the other hand struggles to find regulation due in part because it has not provided a better method than CHO and *E.coli* cell lines with respect to yields and function ([Buyel, 2019](#)). In addition, good manufacturing processes (GMP) have to be established for whole plant molecular farming because existing requirements for the contained, aseptic environments used for cell culture do not apply when soil is used ([Fischer et al., 2012](#)). Serious problems have been met recently for regulation because molecular farming companies received financial backing from Big Tobacco companies. For example, American molecular farming company KBio are owned by British American Tobacco and Philip Morris International owned equity in Medicago Inc. However, the link between Medicago Inc. and Philip Moris International led to the WHO rejecting the application for approval of their Covid-19 vaccine ([Dyer, 2022](#)). In order to gain WHO approval for plant-derived biotherapeutics, the molecular farming

industry will need to sever ties with the tobacco industry.

Despite protein expression levels in plants increasing dramatically over the last decade to a maximum of approximately 2 g kg^{-1} of plant tissue, Chinese hamster ovary cells are still capable of producing more than double this yield ([Buyel, 2019](#); [Zischewski et al., 2016](#)). A major factor causing low yields is the degradation of recombinant proteins by plant proteases in the secretory pathway and apoplast. Various strategies to avoid this unwanted proteolysis ([Jutras et al., 2020](#)) have been developed including subcellular targeting and co-expression of protease inhibitors, fusion proteins/peptides and native chaperones ([Liu and Timko, 2022](#)). Proteolysis can in some cases be avoided by mutating the cleavage sites of antibodies to prevent degradation ([Puchol Tarazona et al., 2021](#)).

A second factor implicated in low protein yields is the plant defence response induced upon agroinfiltration, indicated by an upregulation in secretion of defence proteins ([Goulet et al., 2010](#)) and a decrease in transcripts of photosynthesis proteins ([Grosse-Holz et al., 2018a](#)). Better understanding of the interaction between plant defence and *Agrobacterium* during transient expression and preventing yield reducing elements of the defence response can improve recombinant protein accumulation.

Understanding the steps of infection and the plant responses to agroinfiltration allows a better assessment of how manipulation of the apoplastic environment may aid *Agrobacterium*-mediated expression. One strategy to avoid the detrimental impact that the defence response to agroinfiltration has on molecular farming is to withdraw *A. tumefaciens* completely by using transgenic plants. Molecular farming using transgenics is very successful for large scale production but the generation of transgenic lines takes a long time and is unsuitable for rapid response and manufacture. A second strategy is to reduce *N. benthamiana* immune responses using a variety of techniques such as effector co-expression, immunity gene silencing and inhibition of pH signalling during plant defence. This is the strategy explored in this thesis.

1.3 Molecular mechanisms of *Agrobacterium* infection

1.3.1 From tumourigenic pathogen to disarmed strain for research and biotechnology.

Molecular farming relies on the natural bacterial phytopathogen *Agrobacterium tumefaciens* to produce recombinant proteins in plants in both academic and biotechnological settings. Its unique ability to transfer DNA material into plant host cells has been extensively studied and has revolutionised plant biology.

A. tumefaciens exists in many genetically distinct strains that vary in their ability to infect plants. Natural isolates of *A. tumefaciens* use hormone regulation to cause tumour formation on the plant, resulting in Crown Gall disease ([Drummond, 1979](#)). The tumours are induced by a distinct *Agrobacterium* plasmid called a ‘tumour inducing’ (Ti) plasmid, of which a fragment can be transferred into host plant cells through the Type IV Secretion System (T4SS) (Figure 1.1) ([Lacroix and Citovsky, 2019](#)). This fragment is called transfer-DNA (T-DNA) and contains two groups of genes necessary for tumourigenesis. The first group encodes enzymes for phytohormone regulation and includes *IaaH* and *IaaM* for auxin biosynthesis and *Ipt* for cytokinin biosynthesis ([Zhu et al., 2000](#); [Zhang et al., 2015](#)). The second group encodes enzymes for opine production, providing a source of carbon and nitrogen ([Zhu et al., 2000](#)). This allows *A. tumefaciens* to produce plant hormones to induce tumour growth and to produce opines as a nitrogen source.

Strains of *A. tumefaciens* used for transient expression have had the T-DNA fragment removed from their Ti-plasmids and therefore no longer cause tumourigenesis ([Hwang et al., 2017](#)). Three strains commonly used in biotechnology are *A. tumefaciens* AGL1, GV3101 and EHA105. These three strains are all derived from the tumourigenic *A. tumefaciens* C58 strain ([Hwang et al., 2017](#)). In these strains, the TiC58 plasmid has been removed. *A. tumefaciens* AGL1, GV3101 and EHA105 no longer harbour the nopaline region of TiC58, resulting in disarmed *A. tumefaciens* strains ([Hwang et al., 2017](#); [Kim et al., 2009](#)).

TiC58 previously encoded the T-DNA and *vir* genes required for transformation. In strains including *A. tumefaciens* GV3101 pMP90, TiC58 has been replaced by a small Ti plasmid encoding the T-DNA and a helper plasmid (pMP90) which harbours the *vir*

genes. This binary vector system minimises the genes needed on the Ti plasmid, allowing for easier manipulation of this plasmid using *E. coli* as host ([Hoekema et al., 1983](#); [Hood et al., 1993](#)). Engineering of *A. tumefaciens* strains has yielded a valuable tool for genetic manipulation: bacteria that no longer cause tumour formation but are still able to transfer T-DNA into plant cells.

1.3.2 *Agrobacterium* infection

In natural infections, *A. tumefaciens* must move through the soil, towards and into the apoplast between plant root cells using flagella and attach to plant cells. In agroinfiltration, we push the bacteria directly into the apoplast in leaves and cell attachment is not required for virulence ([Tomlinson and Fuqua, 2009](#)). A summary of the major steps of the *A. tumefaciens* infection pathway are summarised in Figure 1.1.

Induction of virulence by three plant-derived signals

In order for successful infection, *A. tumefaciens* virulence must first be induced. This occurs by detection of three plant derived signals, using at least three two-component systems (Figure 1.1) ([Guo et al., 2019](#)). The most notable of these two-component systems is the VirA/VirG system, which is involved in the sensing and signalling of all three signals ([Guo et al., 2019](#)). VirA is the sensor kinase and VirG is the response kinase in this master-regulator two-component system (Figure 1.1) ([Thompson et al., 2020](#)). The *virA* and *virG* operons are the only *vir* genes being constitutively expressed by *A. tumefaciens* and mutations in either cause loss of virulence ([Nester, 2014](#)).

The first of these signals are phenolic compounds including acetosyringone ([Stachel et al., 1985](#); [Brencic et al., 2004](#)). Phenolic compounds are detected by one of the linker domains of the dimeric VirA protein, causing activation of the kinase domain of the other subunit which subsequently phosphorylates VirG, which causes activation of *vir* gene transcription (Figure 1.1) ([Chang and Winans, 1992](#); [Turk et al., 1994](#); [Toyoda-Yamamoto et al., 2000](#); [Lee et al., 1995](#); [Roitsch et al., 1990](#); [Subramoni et al., 2014](#)).

The next plant derived signals that induce virulence are sugars and monosaccharides from the plant cell wall. ChvE is a periplasmic protein that binds sugars in acidic conditions

(*Hu et al.*, 2013). After binding sugars, ChvE then binds to the periplasmic domain of VirA, causing phosphorylation of VirG and subsequent *vir* gene expression (Figure 1.1) (*Cangelosi et al.*, 1990; *Shimoda et al.*, 1993; *Nair et al.*, 2011).

The final signal required for virulence is an acidic pH, which is involved in virulence induction through a variety of ways (Figure 1.1). Firstly, acidic pH is needed to upregulate transcription of *virG* so that there is an adequate amount of VirG to induce a necessary level of *vir* gene expression for successful infection (*Mantis and Winans*, 1992). Secondly, as mentioned above, acidic pH is required for the binding of acidic sugars to ChvE and therefore is necessary for sugar-induced virulence. Lastly, acidic pH is the signal responsible for ChvG/ChvI induced virulence (Figure 1.1) (*Wu et al.*, 2012). The ChvG/ChvI two-component system senses and responds to acidic pH. In neutral conditions, the ExoR protein is bound to ChvG and prevents phosphate transfer from ChvG to ChvI. In acidic conditions, ExoR is degraded. This allows phosphate to be transferred from ChvG to ChvI, which promotes expression of upstream acid-inducible genes including *virG* (*Wu et al.*, 2012; *Heckel et al.*, 2014).

T-strand generation and transfer

After induction by VirA/VirG, the other Vir proteins cut, process and traffic T-DNA from the Ti plasmid into the plant nucleus for transcription (Figure 1.1). VirD2 is an endonuclease that nicks the Ti plasmid in the 25 base pair left and right border sequences, excising single-stranded T-DNA out of the Ti plasmid (*Yanofsky et al.*, 1986; *Nester*, 2014). VirD2 then binds to the 5' end of the T-DNA, which protects the T-DNA from degradation as it travels via the T4SS into the plant cell nucleus. This T4SS is comprised of 11 VirB proteins and the VirD4 subunit and is therefore called the VirB/VirD4 T4SS (Figure 1.1).

VirC1 binds to the overdrive, which is a sequence to the right of the right border. This binding enhances VirD2 cutting (*Toro et al.*, 1989) and increases T-strand number by increasing production of T-strand copies, but it is not essential for T-DNA transfer (*Atmakuri et al.*, 2007). VirC1 also directs the T-strand to the VirB/VirD4 T4SS to be taken into the plant cell (*Atmakuri et al.*, 2007). The T-strand, bound to VirD2, forms a T-complex which also includes VirC1, VirC2 and VirD1 (Figure 1.1). This T-complex is

then trafficked into the VirB/VirD4 T4SS and into the plant nucleus because the VirD2 protein includes a nuclear localization signal (Figure 1.1) ([Nester, 2014](#)). VirD4 and VirB11 are channel ATPases and binding of the T-complex to these proteins causes ATP binding and hydrolysis, activating VirB10 and causing transfer of the T-DNA through the VirB/VirD4 T4SS into the plant cell ([Cascales et al., 2013](#)). VirE2 proteins are non-specific single-stranded DNA binding proteins which are trafficked through the VirB/VirD4 T4SS and then attach along the length of the T-DNA, protecting it from degradation in the nucleus (Figure 1.1) ([Christie et al., 1988](#)). Before entering the nucleus, the Vir proteins that have processed and trafficked the T-DNA are most likely removed ([Nester, 2014](#)). An Agrobacterium protein that is involved in this removal is VirF and a plant F-box protein, VBF, can also remove these proteins, to the benefit of Agrobacterium. VirF and VBF cause proteolysis of VirE2 proteins ([Tzfira et al., 2004](#); [Zaltsman et al., 2010](#)). The T-DNA can then be trafficked into the plant cell nucleus. In the case of transient expression, most of the T-DNA is not integrated into the host genome. Instead, it remains as part of the sub-genome, meaning that it is extra-chromosomal and transcribed before being integrated into the host genome. The T-DNA remains in the sub-genome at a high-copy number and is transcribed, resulting in expression of the recombinant protein encoded by the T-DNA (Figure 1.1) ([Guo et al., 2019](#); [Lacroix and Citovsky, 2019](#)).

1.3.3 Using AgroLux to monitor Agrobacterium state

To increase our understanding of the effects of plant immunity on *A. tumefaciens*, in this thesis, a bioluminescent strain of *A. tumefaciens* was used. This strain, *A. tumefaciens* RSPJ07, commonly referred to as AgroLux, produces bioluminescence in proportion to its live density and is a useful tool for *in planta* monitoring of Agrobacterium metabolic state ([Jutras et al., 2021](#)). To generate AgroLux, a *lux* operon was integrated into the linear chromosome of *A. tumefaciens* GV3101. This results in single-copy expression of luciferase in each Agrobacterium cell which can be measured in leaf discs on a plate reader or whole leaves using a long exposure camera (such as used for western blot imaging). AgroLux provides a method to measure how Agrobacterium is affected by plant immune responses and when combined with recombinant protein quantification, gives information

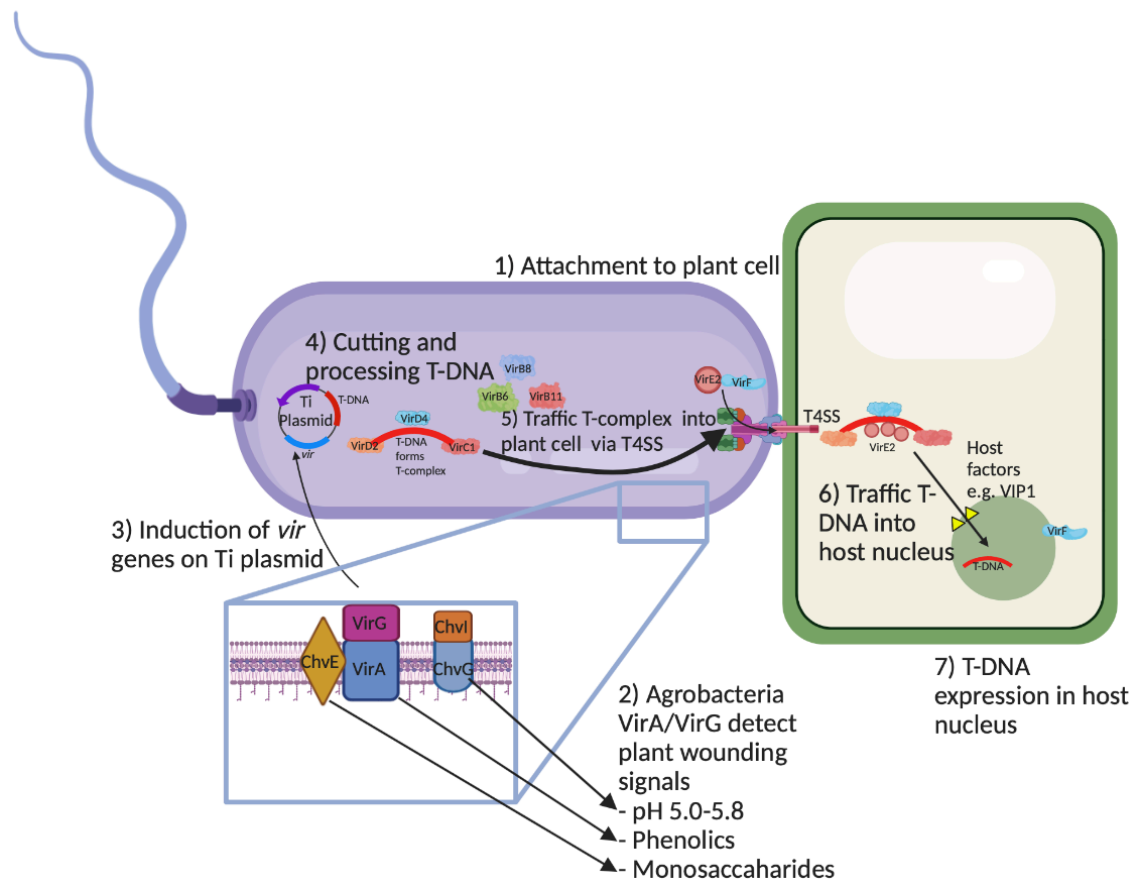


Figure 1.1: The major steps in *Agrobacterium tumefaciens* infection. 1) *A. tumefaciens* moves towards and then attaches to the plant cell in natural infections. In agroinfiltration, the bacteria are delivered straight into the apoplast. 2) *A. tumefaciens* detects phenolics, sugars and acidic pH in the apoplast via membrane proteins. 3) After this, VirG induces expression of *vir* genes. 4) Vir proteins cause cutting of T-DNA out of the Ti plasmid from 25 base pair border sequences and VirD2, C1 and D4 bind to the T-DNA to form the T-complex. 5) VirB proteins and VirD4 traffic the T-complex to the VirB/VirD4 T4SS and the T-complex is injected into the host cell alongside other Vir proteins. 6) VirE2 proteins bind to the T-complex to shield it from host degradation, VirF removes the proteins from the T-complex, leaving the T-DNA, which is trafficked into the nucleus. 7) The T-DNA is integrated into the host sub-genome where it is transcribed.

on the importance of *Agrobacterium* state at various stages of infection for molecular farming. In the twelve hours after agroinfiltration, higher AgroLux correlated to higher GFP accumulation (Jutras *et al.*, 2021). This showed that AgroLux bioluminescence can be used to predict relative recombinant protein accumulation at this early time point when investigating ways to improve molecular farming (Jutras *et al.*, 2021). Observation of how AgroLux and recombinant protein accumulation correlate at various stages of transient expression may provide guidance for strategies to investigate in the future to improve molecular farming.

1.4 Plant Immune Response

Plant defence responses hamper molecular farming (*Zipfel et al., 2006*). In order to prevent this, a good understanding of plant immunity is required. In this section an overview of plant immunity and the specific interactions between host plants and *Agrobacterium* are presented.

1.4.1 PTI

Unlike mammals, plants have only an innate immune response to fight off pathogens (*Zipfel and Felix, 2005*). The first stage of this immune response is PAMP-triggered immunity (PTI). Pathogen-associated molecular patterns (PAMPs) are specific fragments of pathogen components that are recognised by the plant as non-self by surface-localized receptors (*Macho and Zipfel, 2014*). Important PAMPs that are recognised by plants include the flagellin peptide Flagellin 22 (flg22) (*Felix et al., 1999*), elongation factor thermo unstable (EF-Tu) peptide elongation factor 18 (elf18) (*Kunze et al., 2004*), cold shock protein (CSP) peptide cold shock peptide 22 (csp22) (*Felix and Boller, 2003*) and peptidoglycan (*Gust et al., 2007*). PAMPs are recognised by cell surface receptors called pattern recognition receptors (PRRs) (*Zhang and Zhou, 2010*). Plant PRRs are found in two classes: receptor-like kinases (RLKs) and receptor-like proteins (RLPs). Both receptor types contain an ectodomain for ligand binding of the PAMP, a transmembrane domain and in RLKs a cytoplasmic kinase domain or a short cytoplasmic tail in RLPs (*Couto and Zipfel, 2016*). RLPs associate with SOBIR1 or SOBIR-like receptor kinases in order to bypass the need for their own kinase domain (*Liebrand et al., 2013; Gust and Felix, 2014*). PRRs that contain a leucine-rich repeat (LRR) in the ectodomain typically bind protein and peptide PAMPs whereas ectodomains containing lysine motifs (LysM) typically bind carbohydrate PAMPs such as peptidoglycan and chitin (*Couto and Zipfel, 2016; Lu and Tsuda, 2021*). Notable examples of LRR-PRRs are FLAGELLIN SENSITIVE 2 (FLS2) and EF-Tu Receptor (EFR) which bind flg22 and elf18, respectively (*Gómez-Gómez and Boller, 2000; Chinchilla et al., 2007; Felix et al., 1999; Zipfel et al., 2006*). Binding of PAMPs to PRRs activates immune signalling via BAK1 co-receptors, to form activated heteromeric

complexes (*Chinchilla et al.*, 2007). These complexes then phosphorylate receptor-like cytoplasmic kinases (RLCKs) such as BIK1, which then causes phosphorylation of a multitude of different signalling components such as calcium channels, oxidative burst generators (including *NbRbohB*) and mitogen-activated protein kinases. Phosphorylation of these components results in onset of PTI responses such as calcium influx, ROS bursts, callose deposition, stomatal closure and PTI-induced transcriptional reprogramming (*Ngou et al.*, 2022). This transcriptional reprogramming includes upregulation of defence-related genes and biosynthesis of ethylene, salicylic acid (SA) in response to biotrophic pathogens and jasmonic acid (JA) in response to necrotrophic pathogens (Figure 1.2) (*Ngou et al.*, 2022; *Lal et al.*, 2018).

1.4.2 Salicylic acid in plant defence

SA is produced through two pathways in plants (Figure 1.2A). The relative importance of each is still being elucidated in *N. benthamiana* (*Chen et al.*, 2009). Both pathways require chorismate, which derives from the shikimate pathway in plastids. ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) is localized in the chloroplast envelope and is required for transport of SA precursors out of the chloroplasts for use in the isochorismate synthase (ICS) pathway. In this first pathway, chorismate is converted into isochorismate by isochorismate synthase (Figure 1.2A), which is then converted into isochorismoyl-9-glutamate (IC-9-Glu), which then breaks down into SA and 2-hydroxy-acryloyl-N-glutamate (2HNG) or N-pyruvoyl-L-glutamate (NPG). The second pathway is called the PAL pathway, named after its reliance on phenylalanine ammonia-lyase (PAL) to convert phenylalanine into trans-cinnamic acid which can then be converted into SA (Figure 1.2A). Silencing each of these genes in *N. benthamiana* results in significantly reduced levels of SA (*Chen et al.*, 2010; *Catinot et al.*, 2008; *Pallas et al.*, 1996).

SA is a vital plant defence hormone with roles in PTI, effector-triggered immunity (ETI) and systemic acquired resistance (SAR) (*Yan and Dong*, 2014). SA fine-tunes the immune responses it causes through cross-talk with other phytohormones such as ethylene and jasmonic acid (*Spoel and Dong*, 2008). Infection by a biotrophic pathogen induces

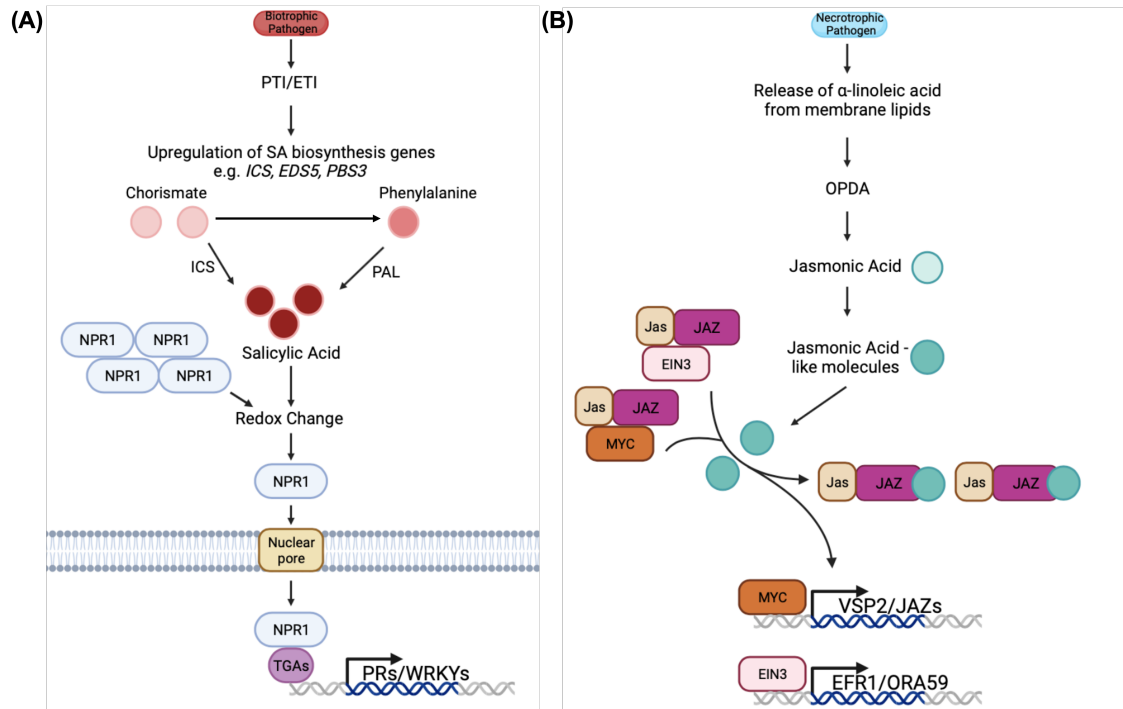


Figure 1.2: Schematic summary of the salicylic acid and jasmonic acid defence pathways. (A) The salicylic acid (SA) pathway. Infection by a typically biotrophic induces PTI and ETI responses, which lead to upregulation of SA biosynthesis genes including *ICS*, *EDS5* and *PBS3*. This leads to an increase in SA biosynthesis through two pathways. The first pathway converts chorismate to salicylic acid through multiple steps including one catalysed by isochorismate synthase (ICS) - this is the ICS pathway. The second pathway is reliant on phenylalanine lyase (PAL), and is hence the PAL pathway. In this pathway, chorismate is converted into phenylalanine, which is converted by PAL to salicylic acid. Salicylic acid production causes a change in the redox state, which causes monomerization of NPR1 oligomers. Once monomerized, the nuclear localisation signal of NPR1 is exposed and NPR1 is translocated to the nucleus, where it binds to TGA transcription factors. TGA transcription factors induce expression of defence genes including pathogenesis related (PR) genes and WRKY transcription factors. (B) Infection by a necrotrophic pathogen causes release of α -linoleic acid from membrane lipids. This is then converted to OPDA, which is converted to jasmonic acid. Jasmonic acid is then converted to jasmonic-like molecules including methyl jasmonate. These molecules then bind to JASMONATE ZIM (JAZ) domain transcriptional repressor proteins, which releases transcription factors including MYCs and EIN3 which were previously bound to JAZ. These transcription factors then induce expression of defence genes including VSP2, JAZ, ERF1 and ORF59. Adapted from a Figure 1, *Pieterse et al. (2012)*.

increases in endogenous SA (*Malamy et al., 1990*) after PTI-induced upregulation of genes involved in SA biosynthesis encoding *ICS*, *PAL* and *EDS5* (*Jones and Dangl, 2006*) (Figure 1.2A). The increased levels of SA then cause transcriptional reprogramming, resulting in upregulation of pathogenesis-related genes (Figure 1.2A) and callose deposition (*Moore et al., 2011; Wang et al., 2013*). SA-dependent transcriptional reprogramming is largely due to effects on the transcription coactivator Nonexpressor of PR genes 1 (NPR1) (*Chen*

et al., 2009) (Figure 1.2A). In the absence of pathogen infection, NPR1 resides as oligomers in the cytosol. Upon infection, SA-accumulation causes cellular reduction. This change in redox state of the cytoplasm reduces the disulfide bonds holding NPR1 as oligomers, and separates NPR1 into monomers (*Moore et al.*, 2011). These monomers are then transported into the nucleus, where they act as transcription coactivators by direct interaction with TGA transcription factors, inducing upregulation of PR protein expression (Figure 1.2A) (*Fan and Dong*, 2002). SA accumulation and signalling can also cause expression of PR proteins in uninfected tissue through NPR1 regulation. This systemic expression of defence genes is called systemic acquired resistance (SAR) and is effective at preventing successful infection of a range of pathogens (*Sticher et al.*, 1997).

1.4.3 The role of extracellular alkalization in plant immunity

PTI includes a variety of responses to pathogens including biosynthesis of plant hormones, ROS bursts, cytosolic calcium influx and extracellular alkalization (*Moroz et al.*, 2017). In cell cultures of various plant species, medium alkalization is one of the earliest responses to elicitor challenge (*Felix et al.*, 1999; *Wu et al.*, 2014b) and pH change in subcellular compartments signals for subsequent defence responses. Elicitor treatment of cultured California poppy (*Eschscholtzia californica*) also resulted in cytosolic acidification and vacuolar alkalization, which led to increased production of antimicrobial compounds called phytoalexins (*Roos et al.*, 2006; *Ahuja et al.*, 2012). Intracellular acidification of tobacco cells resulted in increased PAL accumulation (*Lapous et al.*, 1998).

The mechanism causing pathogen-induced apoplastic alkalization has not yet been fully elucidated. Multiple hypotheses have been proposed including activation of K^+/H^+ antiporters, H^+ /solute cotransporters and regulation of plasma-membrane (PM) H^+ -ATPases (*Elmore and Coaker*, 2011). Phosphoproteomics suggested that the Autoinhibited H^+ -ATPase isoform 2 (AHA2) is regulated by phosphorylation at two residues after flg22 elicitation (*Nühse et al.*, 2007). Phosphorylation of the residue T881 in AHA2 causes upregulation of proton pumping (*Fuglsang et al.*, 2014), whereas phosphorylation of residue S899 in AHA2 causes downregulation of proton pumping into the apoplast (*Haruta et al.*, 2014). After flg22 treatment in *Arabidopsis thaliana*,

phosphorylation of AHA2 at residue T881 was reduced by one third and phosphorylation at residue S899 was increased threefold, suggesting a functional role for AHA2 during pathogen infection (*Nühse et al.*, 2007). Further evidence for AHA2 involvement in PTI is that after flg22 elicitation, AHA2 pumps localise to detergent resistant membrane fractions (DRM). Other important proteins involved in PTI, such as RLKs, are enriched in DRMs (*Shahollari et al.*, 2004; *Elmore and Coaker*, 2011). However, evidence has also been presented that inhibition of AHA2 is not the main cause of apoplastic alkalinization and instead that depolarization of the plasma membrane is due to influx of calcium ions into the cytosol and an efflux of Cl^- and NO_3^- ions causes proton influx, resulting in an alkalized apoplast (*Jeworutzki et al.*, 2010; *Elmore and Coaker*, 2011; *Wu et al.*, 2014b). Therefore it has been proposed that AHA2 downregulation contributes to PAMP-induced apoplastic alkalinization in combination with anion efflux and proton influx (*Elmore and Coaker*, 2011).

P-type H^+ -ATPases including AHA2 are regulated in response to a variety of signals including auxin, blue light, nutrient supply and pathogen perception. Signalling peptides including flg22, Plant peptide containing sulfated tyrosine 1 (PSY1) and Rapid alkalinization Factor 1 (RALF1) regulate AHA2 activity by causing phosphorylation at specific residues, mediated by their respective receptor kinases (Figure 2.1) (*Falhof et al.*, 2016). Binding of RALF1 peptide to its receptor kinase, FERONIA, results in phosphorylation of AHA2 at S899, which inactivates AHA2 and causes apoplast alkalinization (Figure 2.1) (*Haruta et al.*, 2014). PSY1 binds to its receptor, PSY1-R. PSY1-R contains an intracellular kinase domain which, upon PSY1 binding, phosphorylates AHA2 at T881, resulting in increased pumping of protons into the apoplast (Figure 2.1) (*Fuglsang et al.*, 2014). This acidification is necessary for plant growth, cell expansion and hypocotyl elongation. PSY1 and PSY1-R signalling also function in plant defence by balancing production of antagonistic phytohormones JA and SA. PSY1-mediated defence is required to mount JA-mediated defence against necrotrophic pathogens such as *Alternaria brassicicola*, and *psy1r* mutants were more susceptible to *Alternaria brassicicola* infection. In contrast, *psy1r* mutants produced more SA and were less susceptible to *P. syringae* DC3000 infection, suggesting a negative regulatory role for PSY1/PSY1-R in SA-mediated

defence ([Mosher et al., 2013](#)). PSY1/PSY1-R signalling therefore makes an attractive target for pathogen intervention. *Xanthomonas oryzae pv. oryzae* produces a sulphated peptide called Raxx which is a molecular mimic of PSY1. Expression of Raxx results in similar effects as PSY1 expression, including induction of root growth and promotes virulence of *Xanthomonas oryzae pv. oryzae* ([Pruitt et al., 2017](#)).

Effector proteins and ETI

For successful infections, pathogens must overcome plant immunity. Some pathogens secrete effectors into host cells to suppress both PTI and ETI and promote infection, resulting in effector triggered susceptibility (ETS) ([Jones and Dangl, 2006](#)). Effectors can take various forms, with the most prolific classes being proteins and small molecules. Effectors manipulate the host plant by many mechanisms including suppressing immune responses, preventing recognition, manipulating cellular processes and redirecting nutrients. For example, *Pseudomonas syringae* effector, AvrPtoB, is secreted into plant cells and acts as an E3 ligase to cause degradation of vital PTI components including CERK1, BAK1, FLS2 and NPR1 ([Langin et al., 2020](#)). *P. infestans* RxLR effector Pi14054 targets another strand of plant immunity by suppressing RNA silencing in *N. benthamiana* ([Vetukuri et al., 2017](#)). In order to redirect nutrients, several pathogens secrete effectors that induce expression of host *SWEET* genes ([Chen et al., 2010](#)). The encoded SWEET proteins are sugar efflux proteins that are involved in the release of nutrients for the pathogen to take up. A small molecule effector secreted by some *P. syringae* strains, called coronatine, mimicks jasmonic acid. By secreting this jasmonate mimic, the pathogen suppresses salicylic acid signalling and defence responses ([Chen et al., 2010](#)).

Plant pathogens use a variety of effectors to compromise plant immune responses and cause effector triggered susceptibility and enable successful infection. However, in the ongoing molecular arms race between plants and their pathogens, some plants have evolved to recognise these effectors and mount a retaliatory immune response, called ETI. Plants have evolved intracellular nucleotide-binding leucine-rich repeat receptors (NLRs) to detect effectors, which are often encoded by *Resistance (R)* genes. Perception of effectors results in

activation of ETI (*Ngou et al.*, 2022). NLRs detect effectors using a variety of mechanisms, both with and without the aid of helpers and co-receptors (*Wu et al.*, 2018). The responses involved in ETI overlap and interact with those observed during PTI and include ROS production, calicium influx and salicylic acid biosynthesis (*Ngou et al.*, 2022). However, the responses are typically more robust and ETI often modulates the hypersensitive response (HR, a form of programmed cell death) to prevent further spread of biotrophic pathogens (*Cui et al.*, 2015). The evolutionary arms race continues and only when a pathogen can avoid detection or overcome the subsequent immune responses of a host plant can infection be successful.

1.4.4 RNA Silencing

Another layer of plant defence is RNA silencing. Long double-stranded RNA (dsRNA), frequently from viruses, is recognised by plants and triggers transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (*Jay et al.*, 2023). Small RNAs (sRNAs) complementary to the non-self RNA are produced from the dsRNA by Dicer-like (DCL) proteins. The sRNAs then associate with Argonaute proteins, which are nucleases, and either cleave the RNA or trigger translational suppression of the non-self RNA (*Lopez-Gomollon and Baulcombe*, 2022). In addition to detecting virus-derived dsRNA, plants also detect transgene-derived dsRNA, triggering gene silencing of the GOI encoding the recombinant protein produced (*Lopez-Gomollon and Baulcombe*, 2022). Viral suppressors of RNA silencing (VSRs) inhibit this process through a variety of mechanisms including interacting with sRNA to prevent binding of Argonaut proteins, degrading proteins required in RNA silencing and preventing methylation of sRNAs (*Johnson et al.*, 2008; *Ding and Voinnet*, 2007). RNA silencing can also interact with other components of plant immunity (*Lopez-Gomollon and Baulcombe*, 2022). VSRs can inhibit other components of plant immunity. For example, Cauliflower Mosaic Virus VSRs 2b and P6 interfere with salicylic acid-dependent immune responses (*Ji and Ding*, 2001). In contrast, VSRs increase immune responses if they interact with NLRs and induce ETI, as is the case for the tomato spotted wilt virus VSR called Nss (*Lopez-Gomollon and Baulcombe*, 2022; *de Ronde et al.*, 2014).

1.5 The interaction between *A. tumefaciens* and *N. benthamiana*

A. tumefaciens is a natural pathogen of multiple plant species including *N. benthamiana* and plant immunity is induced, even against disarmed strains ([Grosse-Holz et al., 2018a](#); [Goulet et al., 2010](#)). *N. benthamiana* can detect *A. tumefaciens* presence by the PAMP csp22, derived from the cold shock protein (CSP). The receptor responsible for recognition of csp22 is disputed between NbCORE and NbCSPR ([Saur et al., 2016](#); [Wang et al., 2016](#)). Older plants have not been used for molecular farming because they produce low yields of recombinant proteins ([Saur et al., 2016](#)). It has been proposed that older plants do not express recombinant protein well because they are able to perceive the presence of Agrobacterium because the receptors NbCORE and NbCSPR are expressed in older plants, whereas they are not expressed in younger plants ([Saur et al., 2016](#); [Wang et al., 2016](#)).

Upon initial detection of bacteria through recognition of PAMPs, the plant releases reactive oxygen species (ROS) and alkalises the apoplast ([Pitzschke, 2013](#)). As a counter measure to ROS bursts, *A. tumefaciens* strains secrete catalases to detoxify the ROS released by the plants. An example of this is the *katA* catalase, a chromosomal gene implicated in virulence because it detoxifies ROS released by the plant ([Xu and Pan, 2000](#)) (Figure 1.3).

The plant response to agroinfiltration does not stop after PAMP-Triggered Immunity (PTI). The *N. benthamiana* proteome is still affected up to 10 days post infiltration ([Grosse-Holz et al., 2018a](#)). During infection, the plant RNA silencing system is stimulated to cause mRNA degradation of the GOI and methylation of the T-DNA ([Thompson et al., 2020](#)). This problem is mitigated by co-expression of the viral silencing suppressor protein P19. This increases recombinant protein (RP) production significantly and co-expression is commonplace in molecular farming ([Van der Hoorn et al., 2003](#); [Jay et al., 2023](#)).

Agrobacteria have evolved to counter host resistance using a variety of strategies (Figure 1.3) by secreting virulence proteins into plant cells. Hijacking host molecules to aid transfer and transcription of T-DNA is one strategy used. VirE3 proteins continue to aid infection once the T-DNA is inside the nucleus by protecting T-DNA (See Figure 1.1). In *A. thaliana*

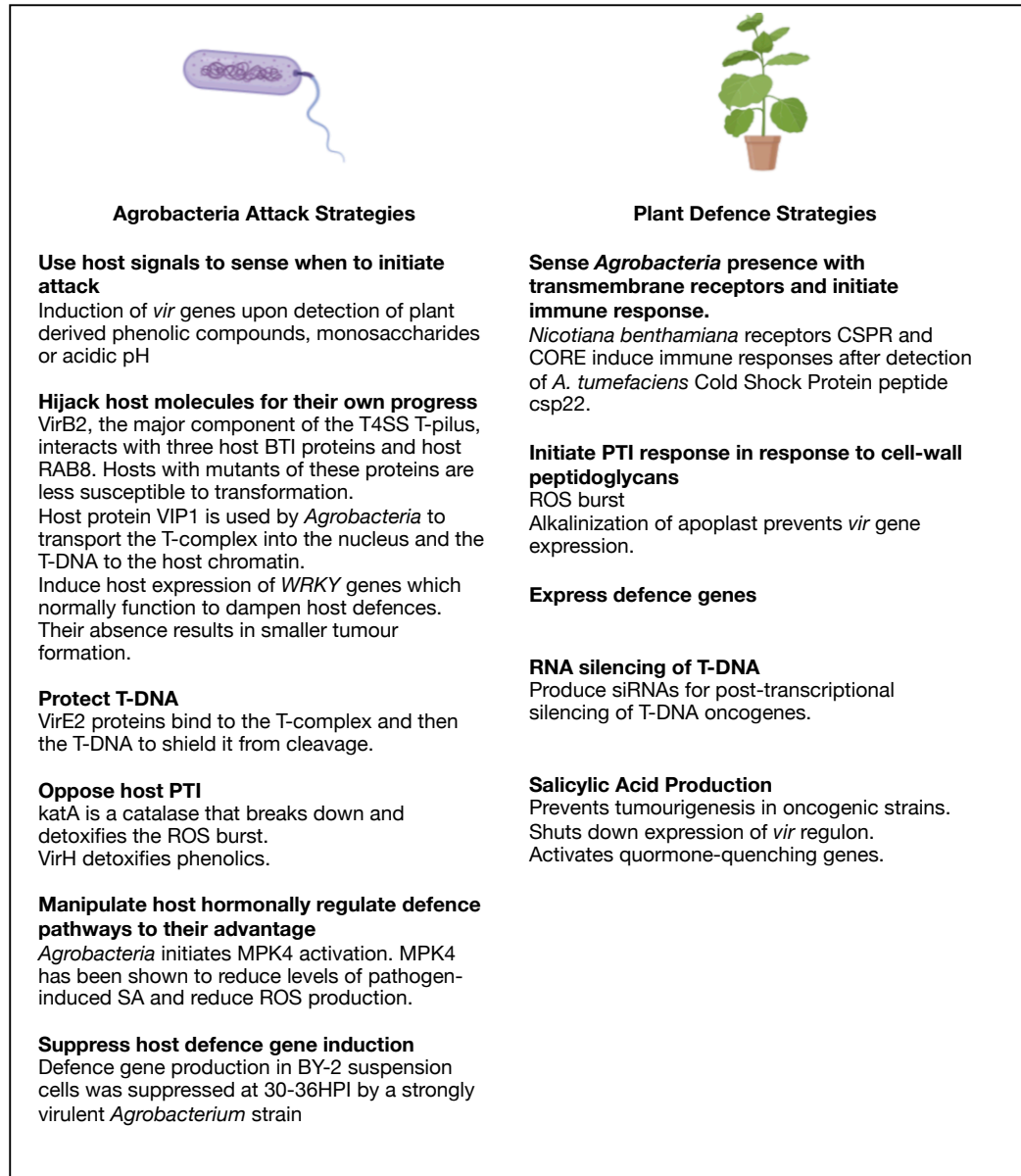


Figure 1.3: Strategies used by *A. tumefaciens* and *N. benthamiana* to aid virulence and immunity, respectively.

VirE3 has been reported to bind to the host transcription factor pBrp and traffic it into the nucleus to stimulate host expression of F-box protein VBF (*Niu et al.*, 2015; *Thompson et al.*, 2020). It has been proposed that VBF then targets the host transcription factor VFP4 to the proteasome for degradation (*Thompson et al.*, 2020; *García-Cano et al.*, 2018). It is suspected that VFP4 is associated with initiating expression of host genes related to defence (*García-Cano et al.*, 2018). Therefore, through expression of VirE3, *A. tumefaciens* may be suppressing the plant immune response (Figure 1.3).

The interactions between *A. tumefaciens* and its host plant during agroinfiltration are summarised in Figure 1.3. Helping *A. tumefaciens* to avoid detection or dampening the plant immune response might be a successful way to improve molecular farming.

1.6 Engineering of *N. benthamiana* and *A. tumefaciens* for molecular farming

In this research, I aim to improve molecular farming by manipulating the interaction between *N. benthamiana* and *A. tumefaciens* by engineering *N. benthamiana* through gene expression and silencing. Some strategies have previously been developed to manipulate both the immune response of *N. benthamiana* and the virulence of *A. tumefaciens* to improve recombinant protein production.

1.6.1 Suppression of plant RNA silencing to improve molecular farming

One element of the plant defence response is transcriptional and post-transcriptional gene silencing of viral genomes (*Baulcombe*, 2004). In counter-defence, viruses encode proteins that suppress host gene-silencing. Plants also respond to T-DNA in *Agrobacterium*-based transient expression and mount the post-transcriptional gene silencing response, degrading the T-DNA transcripts and reducing recombinant protein accumulation (*Feng et al.*, 2022). In order to protect transgenes from post-transcriptional gene silencing, a suppressor protein from *Tomato bushy stunt virus* is transiently co-expressed with recombinant proteins. This viral silencing suppressor, P19, reduces degradation of T-DNA transcripts (*Van der Hoorn et al.*, 2003). Studies have reported that P19 co-expression increases production of a

variety of recombinant proteins including antibodies, allergens, human growth hormone and vaccine candidates ([Chiong et al., 2021](#)). Co-expression of P19 during molecular farming is now common practice and demonstrates that inhibiting the plant immune response is a successful strategy for improving molecular farming ([Jay et al., 2023](#)).

In addition to co-expressing P19, RNA silencing has been reduced by knocking out important components of the RNA silencing mechanisms. Deletion of the RNA-dependent RNA polymerase 6 by genome editing improved the accumulation of GFP upon transient expression and these plants contained lower levels of small interfering RNA against GFP ([Matsuo and Atsumi, 2019](#)).

1.6.2 Inhibition of proteolytic degradation

N. benthamiana encodes at least 1243 putative proteases ([Jutras et al., 2020](#)). These proteases lead to hydrolysis of recombinant proteins, reducing product yields ([Schillberg et al., 2019](#)). Methods used to prevent proteolytic degradation of recombinant proteins includes co-expression of protease inhibitors with IgG antibodies ([Grosse-Holz et al., 2018a](#); [Jutras et al., 2016](#)) and protease depletion by gene silencing ([Mandal et al., 2014](#)). In addition to *in planta* degradation, proteolysis also reduces protein yields during downstream processing. In order to prevent degradation during processing, protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) are added to extraction buffers ([Gengenbach et al., 2018](#)).

1.6.3 Targetting of recombinant proteins to subcellular compartments

Recombinant proteins can be secreted into the apoplast. This is particularly popular for proteins produced in cell culture because the protein can be secreted into the culture media, which facilitates easier downstream processing ([Liu and Timko, 2022](#)). However, the apoplast is a challenging environment for recombinant protein accumulation due to the large numbers of proteases and defence molecules ([Mott et al., 2014](#); [Pillay et al., 2014](#)). In order to mitigate these problems, some recombinant proteins can be targeted to subcellular compartments such as the endoplasmic reticulum (ER), cytosol and chloroplasts. The compartment selected depends on the characteristics of the protein of

interest, including folding, post-translational modifications (PTMs) and its vulnerability to proteases ([Marques et al., 2020](#)). Targeting proteins to the ER has been effective at achieving the highest accumulation levels for proteins including the Dengue virus NS1 antigen ([Marques et al., 2020](#)). The high accumulation of functional protein may be due to the prolonged interaction of the protein with ER-resident chaperones and low proteolytic activity in the ER ([Marques et al., 2020](#)). Recombinant proteins can also be targeted to the chloroplast using an N-terminal fusion of a chloroplast transit peptide. This strategy increased accumulation of a human papillomavirus type 16 therapeutic vaccine candidate ([Yanez et al., 2017](#)). In addition to chloroplastic targeting, recombinant proteins can also be expressed in the chloroplast by engineering the plastid genome, resulting in transplastomic lines. Chloroplast transformation provides advantages including minimal gene silencing, multiple copies of the transgene in a single cell, uniform expression and very low probability of transgene escape into the environment ([Benchabane et al., 2008](#)). However, chloroplast transformation is sometimes an unsuitable strategy. This is because it requires well-characterised chloroplast genomes (which are not available for some plants), translation is constrained by the smaller set of tRNAs in chloroplasts and because chloroplast retained proteins cannot acquire the same PTMs as secreted proteins ([Grabsztunowicz et al., 2017](#); [Liu and Timko, 2022](#); [Zoschke and Bock, 2018](#)).

1.6.4 Engineered *Agrobacterium*

Activation of *vir* genes is essential to successful transient expression. Recent work shows that *vir* gene induction can be increased by addition of components including acetosyringone, acidic buffers and glucose to culture media and agroinfiltration buffers, and results in increased transformation efficiency ([Wu et al., 2014a](#); [Srinivasan and Gothandam, 2016](#); [Xi et al., 2018](#)). *A. tumefaciens* has also been engineered to increase activation of *vir* genes to increase recombinant protein accumulation. An *A. tumefaciens* strain has been developed that over-expresses a constitutively active VirG mutant which increases transformation efficiency and host range of *Agrobacterium*-mediated transformation ([van der Fits et al., 2000](#)). Another engineered *Agrobacterium* strain increases recombinant protein expression by producing enzymes which degrade 1-aminocyclopropane-1-carboxylic

acid (ACC, an ethylene precursor) and Gamma-aminobutyric acid (GABA) (*Nonaka et al.*, 2019, 2017). Both of these phytohormones hinder transient expression. Enabling *A. tumefaciens* degradation of these phytohormones increased transient expression in a variety of plant species, including *N. benthamiana* (*Nonaka et al.*, 2019, 2017).

1.6.5 Barriers to improvement

As discussed in this introduction, various strategies have been found to increase recombinant protein production by molecular farming. However, yields are still lagging behind conventional expression systems (*Buyel*, 2019; *Zischewski et al.*, 2016). In order to find new strategies, many treatments need testing *in planta*. However, investigating strategies is often reliant on expensive and time-consuming techniques such as RNA-seq and mass spectrometry to find candidates and western blotting of many samples to test them. Furthermore, there is large variation in the effectiveness of strategies between host plants, meaning that research is often not translatable between different species. Therefore, in this research, I aim to optimize a new high-throughput screening system to investigate how *A. tumefaciens* state is impacted during molecular farming and to find new strategies to improve yields.

1.7 Research aims and rationale

This thesis contributes a significant advance in the understanding of the effect of plant immunity on molecular farming. As discussed in this introduction, there is significant evidence that there are a variety of interactions between *N. benthamiana* immunity and *A. tumefaciens*. We hypothesised that reducing *N. benthamiana* immune responses to *A. tumefaciens* would increase yields of recombinant proteins.

The aim of this thesis was to improve understanding of how *N. benthamiana* immune responses impact *A. tumefaciens* state and molecular farming. This was achieved firstly by studying how apoplastic alkalinization impacts *A. tumefaciens*. Subsequently, we developed a high-throughput screen to evaluate the effects of new strategies on improving molecular farming. Using this screening technique, we tested the effects of co-expressing

130 *P. infestans* RxLR effectors on recombinant protein accumulation and the impact of silencing a variety of *N. benthamiana* immunity genes on recombinant protein expression.

In **Chapter 2** the effect of apoplastic pH on *A. tumefaciens* was investigated. This was achieved firstly by altering apoplastic pH using chemical buffers. Subsequently, apoplastic pH was controlled by expression of two peptide elicitors which regulate the rate of proton pumping through a plasma membrane H^+ -ATPase called AHA2. Lastly, the apoplast was alkalinized by expressing the mutant, AHA2-S899A. The impacts of apoplastic pH change on AgroLux bioluminescence, recombinant protein accumulation and plant defence responses were assessed and are discussed in this chapter.

In **Chapter 3** a high-throughput screen measuring *A. tumefaciens* state and recombinant protein expression was established. The ‘Traffic Light Screen’ screen measures AgroLux bioluminescence and fluorescent protein expression using a plate reader. This screening system was then utilised in the following two chapters to find new strategies to improve molecular farming and assess the impacts of plant defence responses on *A. tumefaciens*.

Chapter 4 investigates the impact of effector co-expression on transient expression using the ‘Traffic Light Screen’. Using this screen, six positive hits were identified which were then tested with antibody co-expression and subsequently at industrial scale.

Chapter 5 firstly uses the ‘Traffic Light Screen’ to test if immunity gene silencing can be used as a strategy to improve molecular farming. This screen identified three candidates that improved recombinant protein expression which will be investigated further in the future. In this chapter, *NbCORE* was also identified as an essential receptor for induction of the *csp22*-induced ROS burst in older plants. Furthermore, *NbCORE* depletion in older plants increased recombinant protein accumulation and represents a promising strategy for improving molecular farming.

In **Chapter 6**, the implications of these findings are discussed in relation to their impacts on molecular farming and advances in understanding of the interaction between *N. benthamiana* and *A. tumefaciens*.

Chapter 2

Apoplastic alkalization is detrimental to *A. tumefaciens* through induction of salicylic acid-mediated plant defence responses

Isobel L. Dodds, Philippe v. Jutras, Nicholas Holton and Renier A. L. van der Hoorn

Philippe v. Jutras designed and cloned constructs for secreted GFP pHluorin, *AtPSY1*, *NbRALF1*, secreted RFP and cytosolic superfolded GFP. Nicholas Holton provided intellectual advice and support throughout the project. Renier A. L. van der Hoorn supervised and guided the project.

2.1 Abstract

The interaction between *Nicotiana benthamiana* and *Agrobacterium tumefaciens* during transient expression is an integral factor in Plant Molecular Farming. However, the *Agrobacterium* side of this interaction has not been characterised to the same extent as the plant side. *A. tumefaciens* uses pH sensors to induce transformation of plants in both wild and biotechnological settings. Despite common understanding that pH is critical during early infection, long-lasting effects of pH on *Agrobacterium* in agroinfiltrated leaves have not been documented. Here, we manipulated the apoplastic pH of *Nicotiana benthamiana* by targeting the Autoinhibited H⁺-ATPase isoform 2 (AHA2) to monitor the extended effects of pH manipulation *in planta* on *Agrobacterium*. By transiently overexpressing the AHA2-modifying elicitor RALF1, we discovered that extended alkalization of the apoplast is detrimental to *Agrobacterium* and increases the production of pathogenesis-related proteins by *N. benthamiana*. To confirm pH-dependence, we mutated AHA2 to demonstrate the direct impact of alkalization on *Agrobacterium*. We show that expression of AHA2-S899A triggers alkalization of the apoplast, translating into the same disruptive effect on *Agrobacterium* and plant defence as observed with RALF1 overexpression. By contrast, transient expression of *AtPSY1* increased the bioluminescence of AgroLux and decreased accumulation of pathogenesis-related proteins. We show that the changes to AgroLux and PR protein accumulation upon pH change is dependent on salicylic acid signalling. These results demonstrate new insights into the detrimental effects of pH change on *Agrobacterium* and how AgroLux can be used as an effective tool to study the impacts of *N. benthamiana* immune responses on *Agrobacterium in planta*.

2.2 Introduction

Apoplastic pH is a vital physiological factor in plant responses to both biotic and abiotic stresses ([Geilfus, 2017](#)). The leaf apoplast is maintained at approximately pH 5.5 ([Puchol Tarazona et al., 2021](#)) when not under stress and alkalizes transiently in response to pathogen, drought and high salinity ([Geilfus, 2017](#)). The resulting changes to apoplastic pH affect mobility of phytohormones such as ABA, and the transport of nutrients and metabolites across the plasma membrane.

Apoplastic alkalization has been detected during multiple host-pathogen interactions. In one example, potato suspension cells alkalized extracellularly in response to both a PAMP (Chitin) and DAMP (systemin) ([Moroz et al., 2017](#)). Additionally, [Felix et al. \(1999\)](#) showed that purified and boiled flagellin from *P. syringae* caused alkalization in tomato suspension cells. This alkalization is triggered upon the detection of bacterial peptide flg22 ([Felix et al., 1999](#)) by the FLS2 receptor ([Gómez-Gómez and Boller, 2000](#)). However, they found that *A. tumefaciens* flg22^{Atum} is too divergent from *P. syringae* flg22 to cause the alkalization response observed in tomato suspension cells. It was later reported that the flg22^{Atum} elicitor is recognised by wild grape (*Vitis riparia*). This plant species has a previously unknown form of the receptor, FLS2^{XL}, which can recognise flg22^{Atum}. Transgenic *Nicotiana tabacum* expressing FLS2^{XL} were more resistant to *A. tumefaciens* infection. They had significantly less Agrobacterium growth, tumour formation and a lower accumulation of GUS by transient expression ([Fürst et al., 2020](#)). Likewise, agroinfiltration into *A. thaliana* causes extracellular alkalization due to the recognition of the EF-Tu peptide, elf18, by the EFR receptor ([Kunze et al., 2004](#); [Wu et al., 2012](#)). These examples show that apoplast alkalization is part plant PAMP-triggered immunity (PTI) and that this response can be elicited by *A. tumefaciens*.

As part of the PTI response, apoplastic alkalization induces further plant defence responses through regulation of calcium signalling. Calcium ion influx into plant cells causes upregulation of defence response genes. This influx is reliant upon a plasma membrane Ca⁺/H⁺ exchanger, which has an optimal pH of 7.0 ([Zhai et al., 2013](#)). Maintenance of a stable pH of 5.5 using MES buffer during Agroinfiltration in both Col-0

Arabidopsis and *efr1* mutants suppressed the accumulation of transcripts levels of two defence-related genes and increased GUS accumulation ([Wang et al., 2018b](#)). The change in defence-gene transcript is EFR independent and this indicates that the inhibition of alkalization reduces the defence response to agroinfiltration.

Given the lack of both FLS2^{XL} and EFR in *N. benthamiana*, the molecular interaction between *A. tumefaciens* and *N. benthamiana* that causes alkalization has not yet been shown. However, two receptors of Agrobacterium PAMP, csp22, have been reported. These are CSPR and CORE ([Saur et al., 2016](#); [Wang et al., 2016](#)) and their interaction with csp22 may be responsible for alkalization during agroinfiltration of *N. benthamiana*.

In addition to reducing the defence response, preventing alkalization of the apoplast is important for molecular farming because an acidic pH is one of the inducers of *vir* gene expression. Acidic pH is a signal for expression of various acid-inducible genes via the ExoR-ChvG-ChvI regulatory cascade ([Wu et al., 2012](#)). Acidic pH causes degradation of ExoR, which ceases physical interaction between ExoR and ChvG ([Wu et al., 2012](#)), allowing for transfer of phosphate from ChvG to ChvI, thereby activating ChvI and promoting expression of acid-inducible genes that are necessary for virulence. These induced genes include VirG, which is part of the VirA/VirG two-component system which is vital for expression of the Type IV secretion system and of virulence genes secreted for T-DNA transfer ([Mantis and Winans, 1992](#)). A further role of acidic pH in Agrobacterium infection is that it is required for the binding of monosaccharides to ChvE; another required step in the induction of virulence genes by VirA/VirG ([Wu et al., 2012](#)).

Given the importance of apoplastic pH to molecular farming, it is vital to understand how it affects Agrobacterium during its interaction with *N. benthamiana*. A well-characterised regulator of apoplastic pH is Autoinhibited H⁺-ATPase isoform 2 (AHA2). This is a P-type ATPase that controls apoplastic pH by up- and down-regulation of proton pumping across the plasma-membrane in *A. thaliana* ([Falhof et al., 2016](#)). Changes to rate of proton pumping by AHA2 are controlled by three peptide elicitors and interaction with their respective LRR-RK/RLK. These three peptides are flg22, RALF1 and PSY1. Binding of these peptide elicitors to their receptors leads to phosphorylation of AHA2 and a change in proton pumping ([Falhof et al., 2016](#)). For pH manipulation, this

work has focused on the acidifying PSY1 and alkalizing RALF1 peptides (Figure 2.1).

AHA2 pumps protons across the plasma membrane in order to down-regulate apoplastic pH in the absence of peptide elicitors (Figure 2.1A and B). Upon PSY1 binding, the receptor directly interacts with the C-terminal domain of AHA2 and phosphorylates AHA2 on Threonine 881 (T881), in the autoinhibitory domain, increasing proton pumping out of cells (Figure 2.1C) (*Fuglsang et al., 2014*). In contrast, RALF1 binds to its receptor, FERONIA, and through an unknown signal this leads to phosphorylation of AHA2 at Serine 899 (S899) and subsequently a reduction in proton pumping (Figure 2.1D) (*Haruta et al., 2014*). In order to manipulate the pH of the apoplast, we have expressed RALF1, PSY1 and the PSY1 receptor in *N. benthamiana*.

In addition to impacting apoplastic pH, PSY1 signalling also functions in plant defence by regulating production of phytohormones jasmonic acid (JA) and SA. PSY1 signalling is required to mount JA-mediated defence against necrotrophic pathogens such as *Alternaria brassicicola*, and *psy1r* mutants were more susceptible to *Alternaria brassicicola* infection. JA and SA function antagonistically. *psy1r* mutants produced more SA and were less susceptible to *P. syringae DC3000* infection, suggesting a negative regulatory role for PSY1 signalling in SA-mediated defence (*Mosher et al., 2013*).

In order to further understand the effects of apoplastic pH, we also manipulated AHA2 by mutating the two phosphorylation sites. Mutations at these two residues aimed to be phosphomimics (Figure 2.1E and G) and phosphomutants (Figure 2.1F and H). The aim of these mutants was to express AHA2 in forms where it is constitutively in its activated (Figure 2.1E and H) and inactivated (Figure 2.1F and G) forms, aiming at constitutive acidification and alkalinization, independent of PSY1 and RALF1.

The aims of this chapter are to understand how *Agrobacterium* is affected by apoplastic pH at different stages of agroinfiltration and how apoplastic pH affects the plant defence response to *Agrobacterium* infection. Furthermore, this chapter aims to establish the role of apoplastic pH in transient expression and whether apoplastic pH manipulation at different time points is a useful strategy for improving recombinant protein production by plant molecular farming.

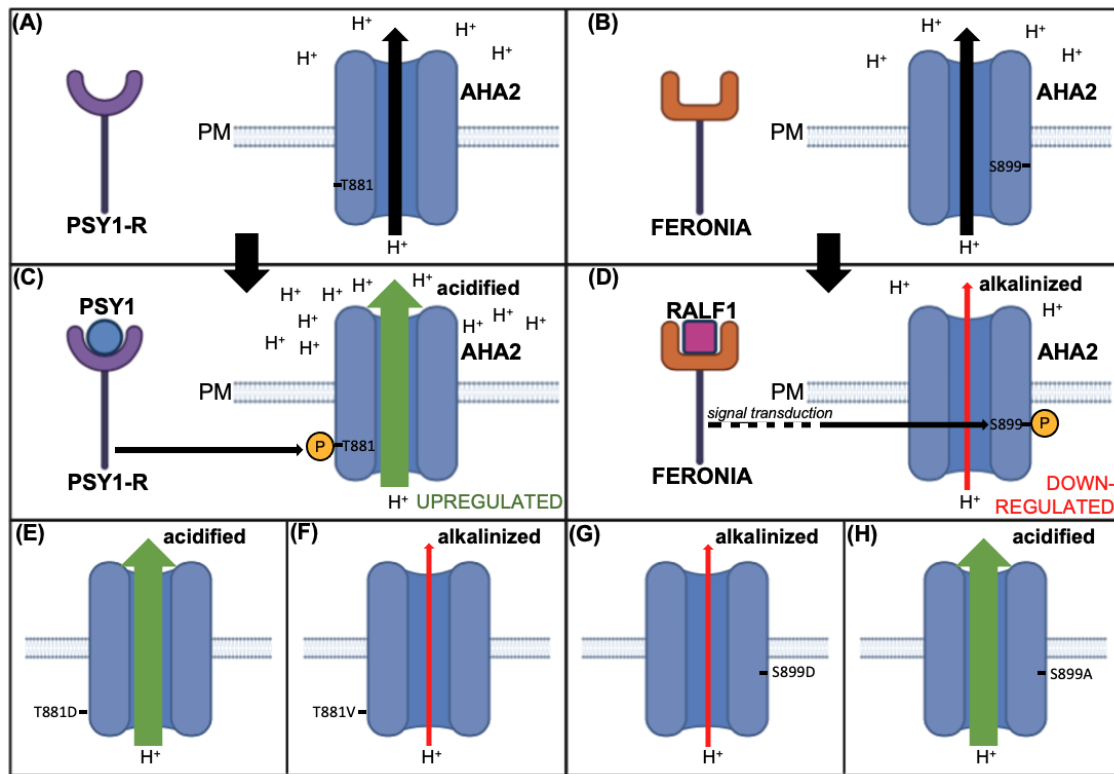


Figure 2.1: AHA2 is a P-type H⁺-ATPase controlling apoplastic pH by up and down-regulation of proton pumping into the apoplast. (A and C) PSY1-R is activated by binding of PSY1. Activated PSY1-R directly phosphorylates AHA2 at position T881, which activates AHA2. This causes AHA2 to pump more protons into the apoplast, causing apoplastic acidification. (B and D) Binding of peptide RALF1 to its receptor FERONIA causes phosphorylation of AHA2 at position S899 by signal transduction, likely through activity of other kinases. It was predicted that substitution of position T881 with aspartic acid would cause acidification (E) and with valine apoplastic alkalization. It was predicted that substitution of position S899 with aspartic acid would cause alkalization (G) and with alanine would cause acidification (H).

2.3 Results

2.3.1 Effect of pH buffering on *Agrobacterium tumefaciens* and recombinant protein production

Buffering of bioluminescent *Agrobacteria* exposes pH sensitivity of *Agrobacterium* state

To establish if *Agrobacteria* are affected by changes in apoplastic pH, we added the bioluminescent strain *Agrobacterium tumefaciens* *RSPJ07* (AgroLux) into a 96-well plate and added phosphate buffers at pH 5.6 or 7.1. AgroLux bioluminescence measured over 100 minutes was significantly higher when buffered at pH 7.1 than pH 5.6 (Figure 2.2A).

By contrast, AgroLux buffered *in planta* was significantly higher when buffered to pH 5.6 than 7.1 (Figure 2.2B). *In planta* buffering of AgroLux was achieved by infiltrating phosphate buffers at pH 5.6 and 7.1 three days after agroinfiltration of AgroLux into leaves. From these results (Figure 2.2) we can see that pH does affect *A. tumefaciens* and this effect differs when administered *in vitro* or *in planta*.

Neutral agroinfiltration buffer affects Agrobacterium state and reduces transient expression of recombinant proteins

To decipher the impact of agroinfiltration pH on *A. tumefaciens* state and fluorescent recombinant protein accumulation *in planta*, we monitored the change in AgroLux bioluminescence and GFP/RFP fluorescence upon agroinfiltration (Figure 2.3). AgroLux was agroinfiltrated alongside *A. tumefaciens* carrying expression cassettes for cytosolic superfolded GFP (cytoGFP) and secreted mRFP (secRFP). At 1, 3, 5 and 7 days post infiltration (dpi) the bioluminescence and fluorescence from GFP and RFP were measured in leaf discs. CytoGFP and secRFP fluorescence are both visible from 3 dpi, representing the beginning of accumulation of these two recombinant proteins. Fluorescence of both proteins is lower in leaves agroinfiltrated with the neutral pH 7.1 buffer, supporting use of an acidic infiltration buffer for transient expression (Figure 2.3). In contrast, the bioluminescence of AgroLux is lower in leaves agroinfiltrated with neutral agroinfiltration buffer from 3dpi, but is the same at 1dpi. The buffering effect of infiltrated buffers lasts only in the range of hours after agroinfiltration (after 300 minutes the difference in pHluorin ration between pH 5.6 and 7.1 has decreased, see Figure 2.4) but has a longer effect on AgroLux (Figure 2.3). The sustained effects of neutral apoplastic pH on recombinant protein accumulation and AgroLux is substantial and therefore led us to test how a sustained alkaline pH *in planta* could affect *A. tumefaciens*. This experiment exposes the long-lasting impact of neutral infiltration buffer pH on Agrobacterium bioluminescence and confirms the importance of acidic pH for recombinant protein expression. Acidic pH triggers infection and T-DNA transfer at the early stage of the plant-bacteria interaction ([Mantis and Winans, 1992](#)). In contrast, a more alkaline apoplastic pH increases AgroLux bioluminescence but results in reduced recombinant protein expression.

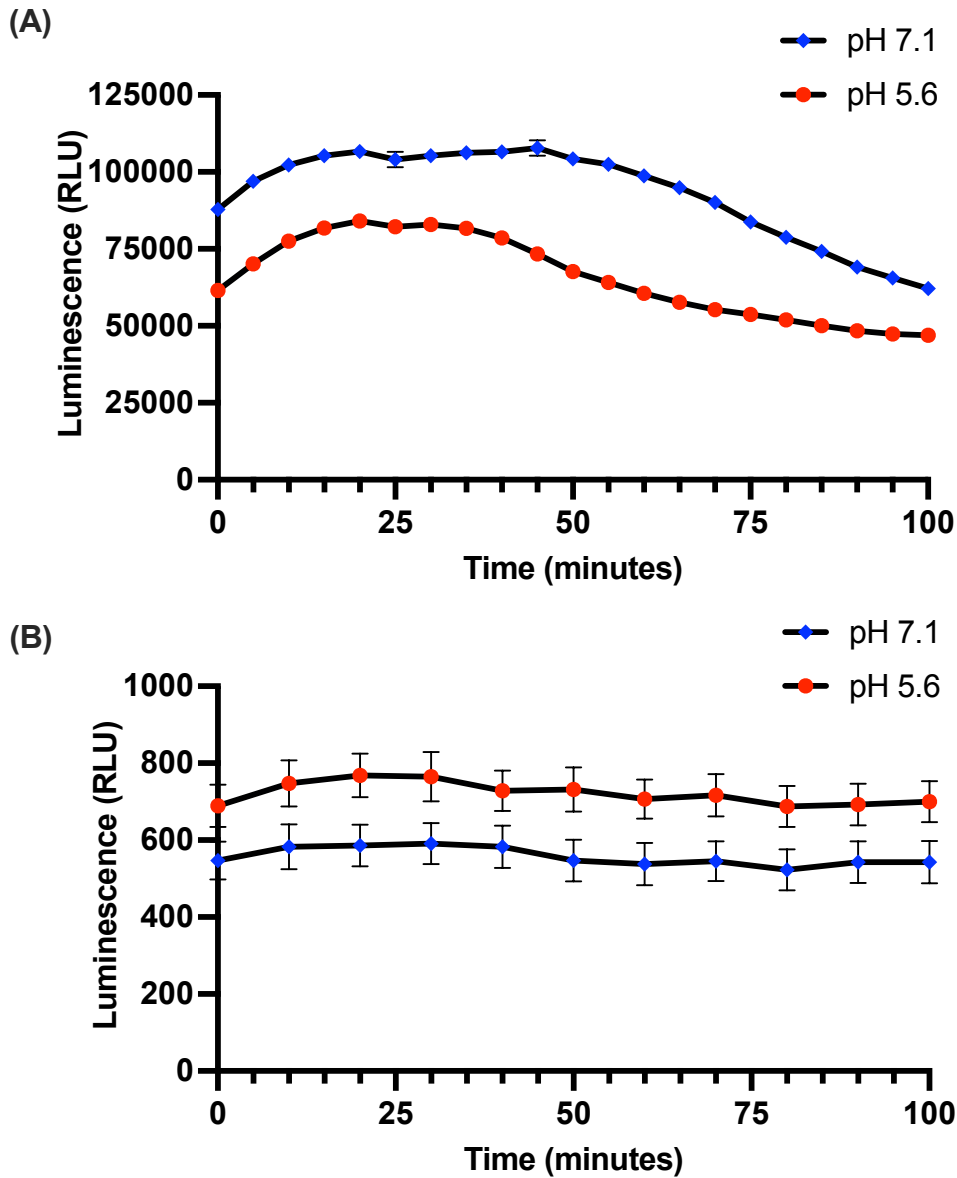


Figure 2.2: Buffering of bioluminescent *A. tumefaciens* at pH 5.6 and 7.1 causes contrasting changes to AgroLux *in vitro* compared to *in planta*. **(A)** *Agrobacterium tumefaciens* RSPJ07 (AgroLux) buffered at pH 7.1 is higher than when buffered at pH 5.6 *in vitro*. *A. tumefaciens* RSPJ07 was mixed to a final OD₆₀₀ of 0.5 with 100mM sodium phosphate buffer at pH 5.6 and 7.1. Bioluminescence of *A. tumefaciens* RSPJ07 was measured over 100 minutes. Twelve replicates for each treatment were measured for which mean and standard error of mean are indicated. Similar results were obtained in one more experimental replicate. **(B)** AgroLux *Agrobacterium tumefaciens* RSPJ07 (AgroLux) buffered at pH 5.6 is higher than when buffered at pH 7.1 *in planta*. *A. tumefaciens* RSPJ07 was agroinfiltrated into four-week-old *N. benthamiana* in MES-MgCl₂ agroinfiltration buffer. Three days post infiltration the same leaf tissue was infiltrated with 100mM phosphate buffer at pH 5.6 and 5.7. Leaf discs were immediately punched and placed into 200μl of water in a 96-well-plate and bioluminescence was measured over 100 minutes. Twelve replicates for each treatment were measured for which mean and standard error of mean are indicated. Similar results were obtained in two more experimental replicates.

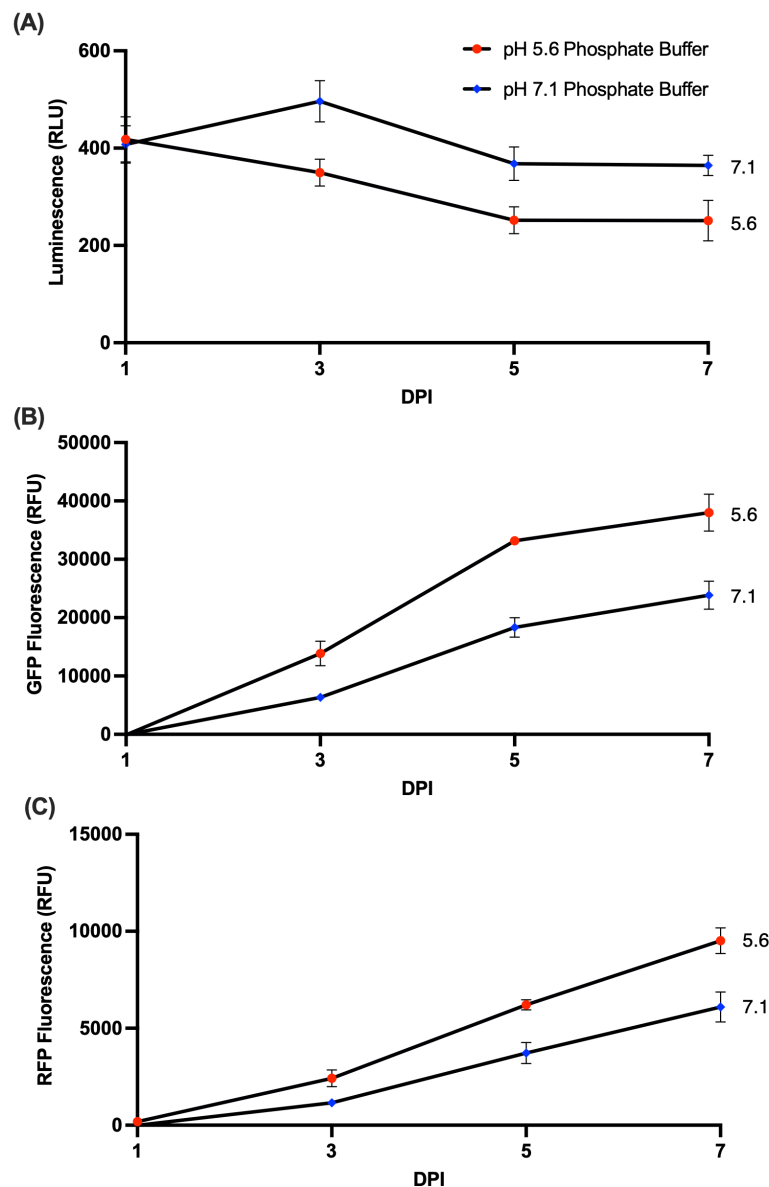


Figure 2.3: Acidic agroinfiltration buffer pH decreases AgroLux bioluminescence and increases transient expression of recombinant proteins. (A) AgroLux bioluminescence is significantly higher in tissue agroinfiltrated at pH 7.1 from 3dpi. (B) cytosolic GFP fluorescence is significantly higher in tissue agroinfiltrated at pH 5.6. (C) secreted RFP fluorescence is significantly higher in tissue agroinfiltrated at pH 5.6. Four-week-old *N. benthamiana* plants were agroinfiltrated with *A. tumefaciens* *RSPJ07* harbouring expression cassettes for cytosolic super-folded GFP (cytoGFP) and secreted RFP (secRFP). *A. tumefaciens* was agroinfiltrated in phosphate buffers at 5.6 and 7.1 instead of standard MES agroinfiltration buffer. At 1, 3, 5 and 7dpi, leaf discs were punched and measured for bioluminescence and fluorescence at Ex. 480nm, Em. 515nm (cytoGFP) and Ex. 550nm, Em. 585nm (secRFP). Twelve replicates for each treatment were measured for which Mean and Standard Error of Mean are indicated. Similar results were obtained in two more experimental replicates.

2.3.2 Techniques used to measure apoplastic pH

Secreted GFP pHluorin can be used to detect apoplastic pH changes

To establish how *Agrobacteria* are affected by apoplastic pH, we used a pH-sensitive GFP mutant that had been used to measure pH in the secretory pathway ([Jutras et al., 2015](#)) but relied on confocal microscopy to establish differences. Four-week-old *N. benthamiana* were agroinfiltrated with *A. tumefaciens* GV3101 harbouring expression cassettes for secreted GFP pHluorin. At 5dpi, apoplastic fluid containing secreted GFP pHluorin was extracted and added to phosphate buffers at pH 5.6 and pH 7.1 (Figure 2.4). The excitation spectrum of secreted GFP pHluorin in apoplastic fluid was measured using a plate reader and followed a similar pattern to that described for neutral and acidic pHs when optimised in previous works (Figure 2.4A) ([Miesenböck et al., 1998](#); [Jutras et al., 2015](#)). For each subsequent experiment in this work using secreted GFP pHluorin, an excitation spectrum measured from 380nm to 480nm was performed on the samples to obtain the two peaks from which to take ratios for that specific experiment because the peaks of the ratios varied slightly between experimental replicates and affected the ratio which the pH measurement was made. The peaks in Figure 2.4B were at 400nm and 480nm and therefore were used to calculate the pHluorin ratio plotted.

Next we tested smaller changes to pH by buffering the apoplastic fluid at intervals and determining the ratio of peaks at wavelength excitations of 400nm and 480nm. This data shows that apoplastic pH can be measured *in vitro* successfully using secreted GFP pHluorin (Figure 2.4B). This method can be performed reasonably high throughput by carrying out apoplastic fluid extractions from agroinfiltrated tissue in small syringes.

A more high throughput pH measurement was to measure secreted GFP pHluorin excitation ratios on a plate reader in leaf discs (Figure 2.5). Four-week-old *N. benthamiana* were agroinfiltrated with *A. tumefaciens* GV3101 harbouring expression cassettes for secreted GFP pHluorin. At 5dpi, leaf discs were punched from agroinfiltrated tissue and infiltrated with 100mM phosphate buffers at pH 5.6 and pH 7.1. They were placed in a 96-well plate and measured for fluorescence at Ex. 400nm and 485nm, Em. 515nm. The ratio of these two fluorescence values was plotted and is significantly different between leaf

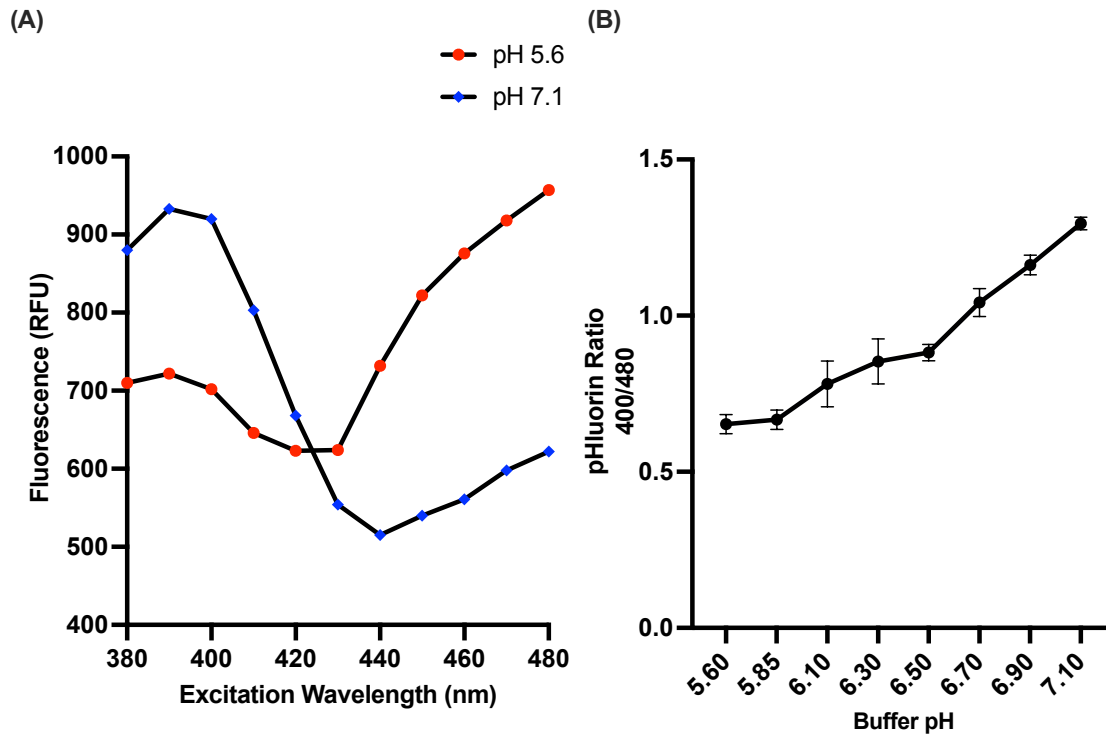


Figure 2.4: GFP pHluorin is pH-sensitive in *in vitro*. (A) Fluorescence excitation scan (Em. 510nm) of secreted GFP pHluorin in AF buffered to pH 5.6 and 7.1. (B) Secreted pHluorin fluorescence ratio (Ex. 400/485nm) in increasing pH Phosphate buffer. Four-week-old *N. benthamiana* plants were agroinfiltrated for expression of secreted GFP pHluorin. At 5dpi, apoplastic fluid (AF) were extracted and diluted in 100mM Phosphate buffers. Nine replicates for each treatment were measured for which Mean and Standard Error of Mean are indicated.

discs infiltrated with phosphate buffer at pH 5.6 and pH 7.1 (Figure 2.5). The ratios of fluorescence of both treatments begins high and then decreases over time (Figure 2.5). The decrease in ratio in both treatments may reflect the gradual return to normal apoplastic pH, which is acidic, after the infiltration of both buffers. However, this hypothesis has not been tested and needs further investigation. These results show that secreted GFP pHluorin does not need to be extracted from leaves to see differences in apoplastic pH and therefore can be used as a high-throughput tool to detect pH changes in the apoplast during our investigations of how apoplastic pH affects *Agrobacterium*.

Confirmation that pHrodo™ Red Dextran can be used to measure pH *in vitro*

The second method to measure the pH of the apoplast was to use pHrodo™ Red Dextran for Endocytosis (pHrodo™ Red Dextran, Invitrogen). This is a fluorescent dextran conjugate

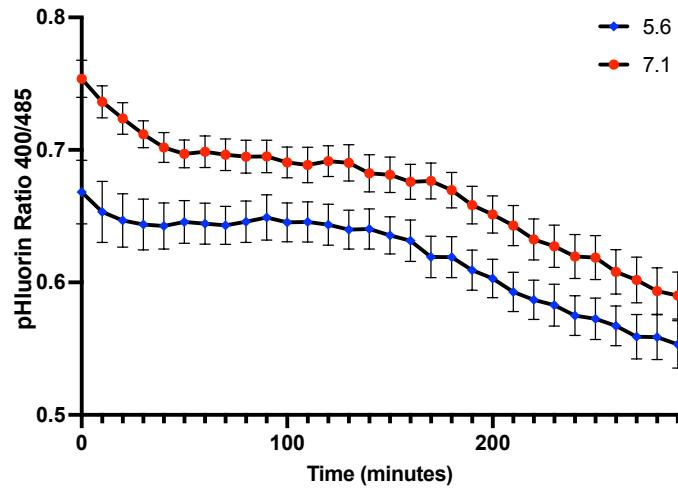


Figure 2.5: GFP pHluorin is pH-sensitive *in planta*. Four-week-old *N. benthamiana* plants were agroinfiltrated for expression of secreted GFP pHluorin. At 5dpi the same tissue was infiltrated with 100mM phosphate buffers at pH 5.6 and 7.1. Leaf discs were punched immediately and fluorescence was measured over 300 minutes at Ex./Em. 400/510 and 485/510nm. Twelve replicates for each treatment were measured for which mean and standard error of mean are indicated. Similar results were obtained in one more experimental replicate.

used to measure the pH of the mammalian endocytic pathway between pH 5 and 8 *in vivo*. This is the pH range that we expect to be measuring in the apoplastic fluid of both naive and agroinfiltrated *N. benthamiana*. Therefore we first tested if pHrodo™ Red Dextran was pH sensitive *in vitro* by adding it to phosphate buffers between pH 5.4 and pH 7.6 (Figure 2.6). The fluorescence of pHrodo™ Red Dextran decreased as expected with increasing alkalinity with an R^2 value of 0.921 (Figure 2.6).

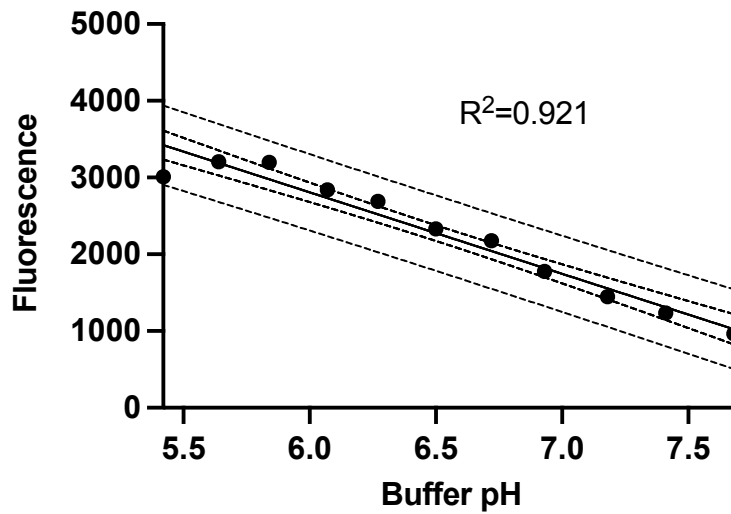


Figure 2.6: pHrodo™ Red Dextran can be used to measure pH *in vitro*. Invitrogen pHrodo™ Red Dextran for Endocytosis was added (20ug/mL) to Phosphate Buffers between pH 5.4 and 7.7 and fluorescence at Ex. 550nm and Em. 585nm. Decreasing fluorescence correlates with increasing pH. Three replicates were used for each measurement, for which Mean and Standard Error of Mean are indicated. Similar results were obtained in two more experimental replicates.

2.3.3 Agrobacterium bioluminescence is reduced by RALF1-induced apoplastic alkalization and increased by PSY1 expression

Agrobacterium bioluminescence is affected by elicitor-generated pH changes in the apoplast

In order to understand how Agrobacterium is affected by pH beyond the changes that are caused by buffers, we transiently expressed two peptide elicitors which have been described as altering the pH of the apoplast antagonistically through regulation of an H⁺-ATPase named Autoinhibited H⁺-ATPase isoform 2 (AHA2) (Falhof *et al.*, 2016). The first peptide is RAPID ALKALINIZATION FACTOR-1 (RALF1) and the second is PLANT PEPTIDE CONTAINING SULFATED TYROSINE 1 (PSY1). The effect of these elicitors was initially measured in *A. thaliana* (Falhof *et al.*, 2016). A homolog of RALF1 was found in the *N. benthamiana* genome and (*NbRALF1*) was cloned into an agroinfiltration vector (Figure 2.17). No homolog of PSY1 was found in *N. benthamiana*, therefore we cloned the *AtPSY1* and its receptor (*AtPSY1-R*) into agroinfiltration vectors.

Four-week-old *N. benthamiana* were agroinfiltrated to express these elicitors and leaf discs were taken and apoplastic fluid extracted at 5dpi. Changes to pH were measured

using secreted GFP pHluorin (Figure 2.7A) and pHrodo™ Red Dextran (Figure 2.7B). Significant alkalization was observed upon overexpression of *NbRALF1* using both pH measurements (Figure 2.7A, B). RALF1 induced alkalization of the apoplast at 5dpi, but cytosolic GFP pHluorin indicated that the cytosolic pH is not affected at 5dpi (Figure 2.8), however this needs further investigation because these results were only collected in one replicate. Although a pattern of acidification was observed in multiple replicates of this experiment upon expression of *AtPSY1* and its receptor, this was not statistically significant using either pH measurement method (Figure 2.7A, B).

AgroLux bioluminescence of leaf discs was also measured and plants expressing *AtPSY1/R* had significantly more AgroLux bioluminescence per leaf disc than EV (Figure 2.7C). Plants expressing *NbRALF1* had significantly less bioluminescence per leaf disc than empty vector (EV) (Figure 2.7C). From this data we show that sustained alkalization by *NbRALF1* causes a large reduction in AgroLux bioluminescence whereas its antagonistic peptide PSY1, although not causing a significant acidification, still causes an increase in AgroLux bioluminescence. This data suggests that Agrobacterium state is sensitive to prolonged pH change in the apoplast.

2.3.4 *NbRALF1*-induced alkalization is detrimental to recombinant protein accumulation

Given the vital role of Agrobacterium in plant molecular farming, we next hypothesised that this prolonged pH effect on Agrobacterium during transient expression would affect recombinant protein production. To investigate this we co-expressed the peptide elicitors with two different proteins. Firstly we co-expressed the HIV neutralising antibody, VRC01, with the pH-changing elicitors (Figure 2.9A). VRC01 accumulation was reduced when co-expressed with *NbRALF1* (Figure 2.9A), suggesting that alkalization may be detrimental to recombinant protein production. Co-expression of *AtPSY1* and its receptor reduced accumulation of VRC01 (Figure 2.9A). To test an additional protein, we expressed RD21, an endogenous *N. benthamiana* Cys proteases. Accumulation of RD21 was detected by western blot using anti-triticain- α and was also reduced with *NbRALF1* expression and slightly reduced with *AtPSY1/AtPSY1-R* expression (Figure 2.9B).

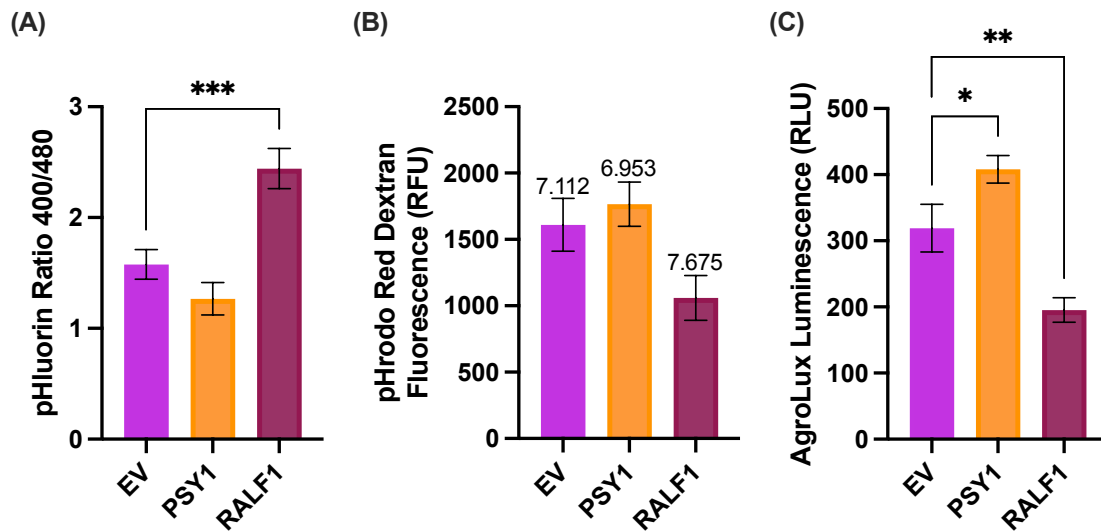


Figure 2.7: *NbRALF1* overexpression increases apoplastic pH and reduces AgroLux bioluminescence. *AtPSY1* expression increases AgroLux bioluminescence. Four-week-old *N. benthamiana* plants were agroinfiltrated with AgroLux and for expression for the following treatments: EV (EV, EV); RALF1 (*NbRALF1*, EV) and PSY1 (*AtPSY1*, *AtPSY1-R*). See Table 7.21. **(A) Ratio of Secreted GFP pHluorin ratio (Ex. 400 and 480, Em. 510) is significantly increased by overexpression of *NbRALF1* and nonsignificantly reduced by *AtPSY1*.** In addition to the constructs above, plants were agroinfiltrated for expression of secreted GFP pHluorin. Leaf discs were taken at 5dpi and measured for fluorescence at Ex. 400 and 480, Em. 510. Twelve replicates for each treatment were measured for which Mean and Standard Error of Mean are indicated. Similar results were obtained in two more experimental replicates. **(B) RALF1-dependent alkalization is measurable using pHrodo™ Red Dextran.** Apoplastic fluid was extracted from leaf tissue expressing the above constructs at 5dpi. pHrodo™ Red Dextran was added at 20ug/mL to the AF and fluorescence at Ex./ Em. 550/585 was measured. Four replicates for each treatment were measured for which Mean and Standard Error of Mean are indicated. A standard curve of phosphate buffers was measured on the same plate and pH quantifications are extrapolated from this. Similar results were obtained in one more experimental replicate. **(C) Bioluminescence of AgroLux is reduced by *NbRALF1* expression and increased by *AtPSY1* expression.** Leaf discs were taken at 5dpi from plants expressing the above constructs and Luminescence was measured on a plate reader. Twelve replicates for each treatment were measured for which mean and standard error of mean are indicated. Mean and Standard Error of Mean are indicated. Data was analysed using ANOVA with a Dunnett's Multiple Comparison test. *P<0.05, **P<0.01, ***P<0.001. Similar results were obtained in two more experimental replicates.

Role of pH in Plant Defence Responses

In order to understand how the elicitor-mediated pH changes affect AgroLux and recombinant protein production, we next investigated the impact on plant defence responses by detecting pathogenesis-related (PR) protein accumulation in total soluble protein. Leaves expressing *NbRALF1* had increased accumulation of β -1,3-glucanase (PR2) and class I chitinase (PR3) in comparison to the EV control (Figure 2.11A and

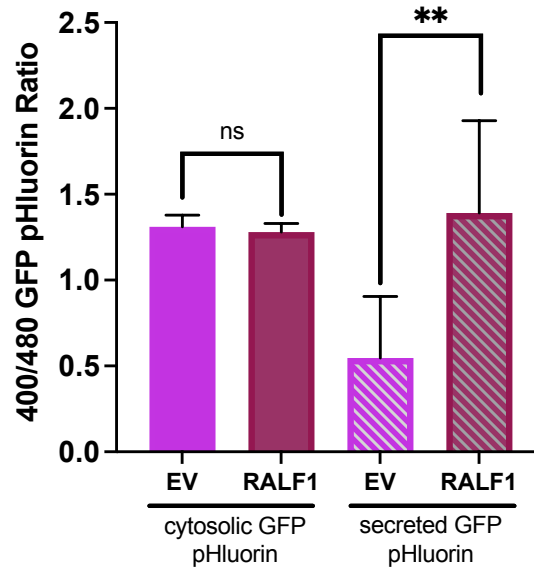


Figure 2.8: *NbRALF1* overexpression increases apoplastic pH but does not impact cytosolic pH. Four-week-old *N. benthamiana* plants were agroinfiltrated with *A. tumefaciens* carrying expression cassettes for either EV or RALF1, each either cytosolic GFP pHluorin or secreted GFP pHluorin. Leaf discs were punched at 5dpi and fluorescence was measured at Ex./Em. 400/510nm and 480/510nm and the ratio between the two plotted. Twelve replicates for each treatment were measured for which mean and standard error of mean are indicated. Data was analysed using ANOVA with a Dunnett’s Multiple Comparison test. **P<0.01. This experiment was performed once and requires further investigation.

B), indicating that the plant defence response is upregulated by RALF1. By contrast, leaves expressing *AtPSY1* and *AtPSY1-R* had decreased accumulation of β -1,3-glucanase (PR2) and class I chitinase (PR3) in comparison to the EV control (Figure 2.11A and B), indicating a downregulation in the plant defence response by PSY1/PSY1-R expression. In addition, leaves expressing *NbRALF1* had lower total protein, especially less of the large RuBisCO subunit (Figure 2.11C). In contrast, leaves expressing *AtPSY1* and *AtPSY1-R* had higher total protein and more of the large RuBisCO subunit (Figure 2.11C). Photosynthesis proteins are transcriptionally downregulated during defence responses, and also upon agroinfiltration (*Grosse-Holz et al.*, 2018a).

To determine if AgroLux bioluminescence changes correlate with *A. tumefaciens* growth, we measured Agrobacterium colonisation by plating out a dilution series of agroinfiltrated leaf extracts and counting CFU. Expression *NbRALF1* and *AtPSY1* with *AtPSY1-R* resulted in no changes to Agrobacterium growth (Figure 2.10), despite the defence responses observed. This is consistent with previous reports that AgroLux

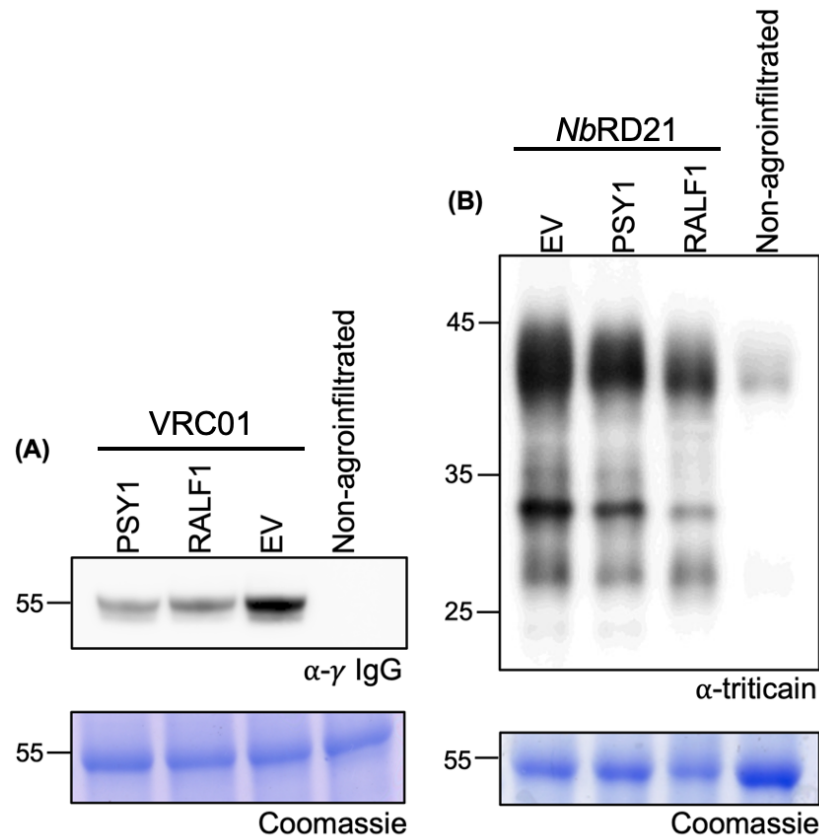


Figure 2.9: *NbRDL1* expression reduces accumulation of antibody VRC01 and protease RD21. Four-week-old *N. benthamiana* plants were agroinfiltrated with AgroLux and for expression for the following treatments: EV (EV, EV); RALF1 (*NbRDL1*, EV) and PSY1 (*AtPSY1*, *AtPSY1-R*) and either VRC01 HC and LC (A) or *NbRD21* (B). See Table 7.21. Leaf discs were taken at 5dpi and total soluble protein extracted. Proteins were separated on a 12% SDS-PAGE gel and detected by western blotting using α - γ IgG (A) and using α -triticain (B) antibodies. Coomassie-stained gels are shown as protein expression and loading controls. All lanes represent samples from four biological replicates in one experiment pooled together. Similar results were obtained in one more experimental replicate.

reduction does not always equate to change in bacterial growth (*Jutras et al.*, 2021).

2.3.5 AHA2-mediated alkalization is detrimental to AgroLux and induces plant defence

AHA2 mutant affects AgroLux

NbRDL1 affects plant defence in a variety of ways (*Zhang et al.*, 2020). We used site-directed mutagenesis of AHA2 to test if the response of AgroLux to *NbRDL1* was pH-dependent. AHA2 was mutated at two phosphorylation sites in the C-terminal

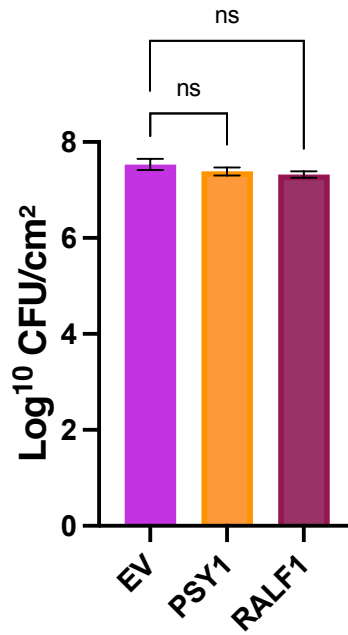


Figure 2.10: *NbRALF1* and *AtPSY1* expression do not affect growth of *A. tumefaciens* in planta. Four-week-old *N. benthamiana* plants were agroinfiltrated with AgroLux and for expression for the following treatments: EV (EV, EV); RALF1 (*NbRALF1*, EV) and PSY1 (*AtPSY1*, *AtPSY1-R*). At 5dpi, bacterial growth was measured by colony count. No significant difference in growth was found. Six biological replicates for each treatment were measured for which mean and standard error of mean are indicated. Data was analysed using ANOVA with a Dunnett's Multiple Comparison test. ns non-significant. Similar results were obtained in one more experimental replicate.

extension because their involvement in regulation of AHA2 have been described ([Falhof et al., 2016](#)). The threonine 881 residue was substituted into aspartate and valine and the serine 899 residue was substituted into aspartate and alanine (Figure 2.1). Four-week-old *N. benthamiana* were agroinfiltrated with AgroLux and expression cassettes for each mutant. Agroinfiltrated leaves expressing these four AHA2 mutants and EV were then analysed by measuring the bioluminescence of AgroLux. Leaves expressing AHA2-T881, AHA2-S899D and AHA2-T881V did not show significantly different AgroLux bioluminescence compared to EV (Figure 2.12A). By contrast, leaves expressing AHA2-S899A had significantly reduced bioluminescence in comparison to the EV control (Figure 2.12).

AHA2 Mutant AHA2-S899A causes alkalization of the apoplast

To determine the effect of AHA2-S899A on apoplastic pH, EV, AHA2-S899A and AHA2 WT were transiently expressed in four-week-old *N. benthamiana* with and without secreted

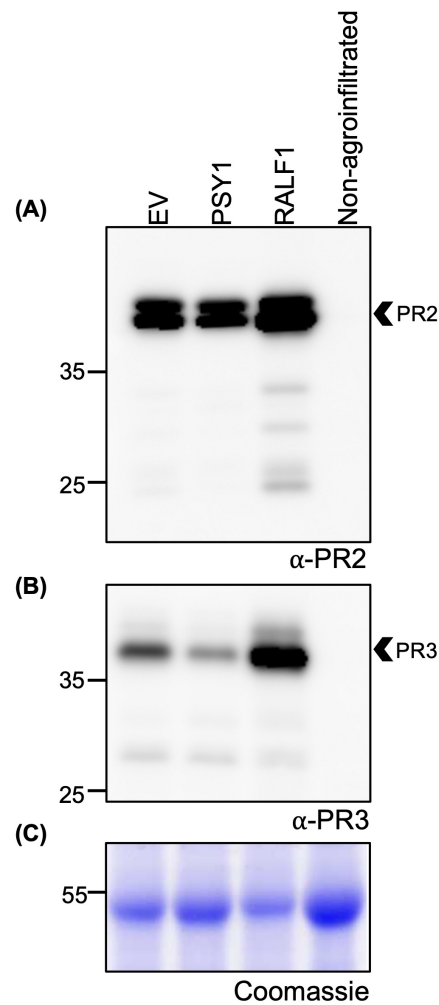


Figure 2.11: *NbRALF1* and *AtPSY1* expression affect accumulation of PR proteins and the total soluble protein profile. (A) PR2 is induced upon *NbRALF1* agroinfiltration. (B) PR3 is induced upon *NbRALF1* and reduced upon *AtPSY1* agroinfiltration. (C) RuBisCO accumulates less in *NbRALF1* and more *AtPSY1* agroinfiltrated leaves. Four-week-old *N. benthamiana* plants were agroinfiltrated with AgroLux and for expression for the following treatments: EV (EV, EV); RALF1 (*NbRALF1*, EV) and PSY1 (*AtPSY1*, *AtPSY1*-R). Leaf discs were taken at 5dpi and total soluble protein extracted. Proteins were separated on a 12% SDS-PAGE gel and detected by western blot using α -PR2 (A) and α -PR3 (B). Coomassie-stained gels (C) show the difference in quantity of RuBisCO large sub-unit upon transient expression. All lanes represent samples from four biological replicates in one experiment pooled together. Similar results were obtained in two more experimental replicates.

GFP pHluorin. We extracted apoplastic fluid for pHrodo™ Red Dextran analysis from tissue agroinfiltrated without secreted GFP pHluorin (see Table 7.21). In the presence of GFP pHluorin, leaf discs expressing AHA2-S899A and AHA2 WT displayed an increased ratio in the peaks of GFP pHluorin fluorescence at excitation wavelengths 400 and 480nm

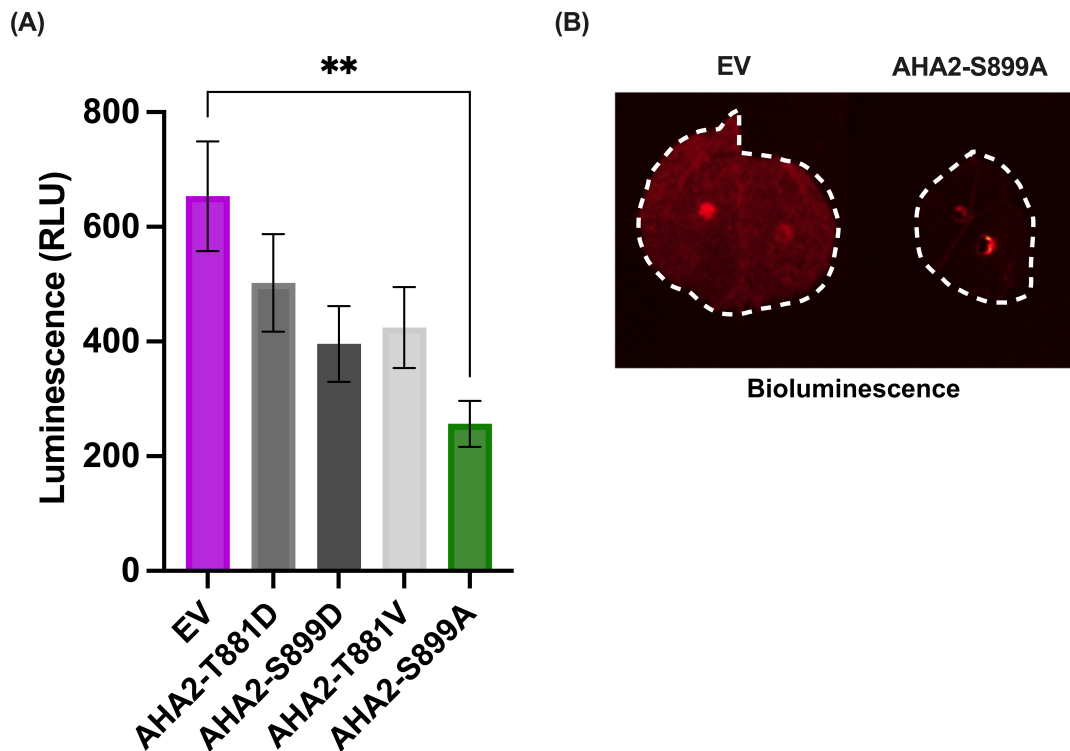


Figure 2.12: S899A mutant of plasma-membrane H^+ -ATPase *NbAHA2* reduces AgroLux bioluminescence. *NbAHA2* was mutated at two residues (S899 and T881) in two ways to form four mutants of *NbAHA2*. Each of these mutants and AgroLux were agroinfiltrated into four-week-old *N. benthamiana*. **(A) Of four mutants, only *NbAHA2*-S899A has a significant impact on AgroLux bioluminescence.** At 5dpi, leaf discs were taken and bioluminescence was measured. Twelve replicates for each treatment were measured for which mean and standard error of mean are indicated. Similar results were obtained in two more experimental replicates. **(B) Reduction in bioluminescence caused by AHA2-S899A expression.** A leaf agroinfiltrated on each side of its midrib with either EV or AHA2-S899A and AgroLux was imaged using a western blot imager. Less bioluminescence is visible on the side of the leaf agroinfiltrated with AHA2-S899A. Leaf areas agroinfiltrated are outlined in a white dashed line. This experiment was performed using three plants and similar results were obtained in one more experimental replicate.

when compared to the EV control, indicating that pH was more alkaline (Figure 2.13B). The increased pHluorin ratio was significant in leaves expressing AHA2 WT but not always significant for AHA2-S899A (Figure 2.13B). Both AHA2-S899A and AHA2 WT had significantly reduced pHrodoTM Red Dextran fluorescence, indicating that pH was more alkaline (Figure 2.13A). Using these two pH measurements in conjunction, we show that expression of both these AHA2- H^+ -ATPases leads to alkalization of the apoplast.

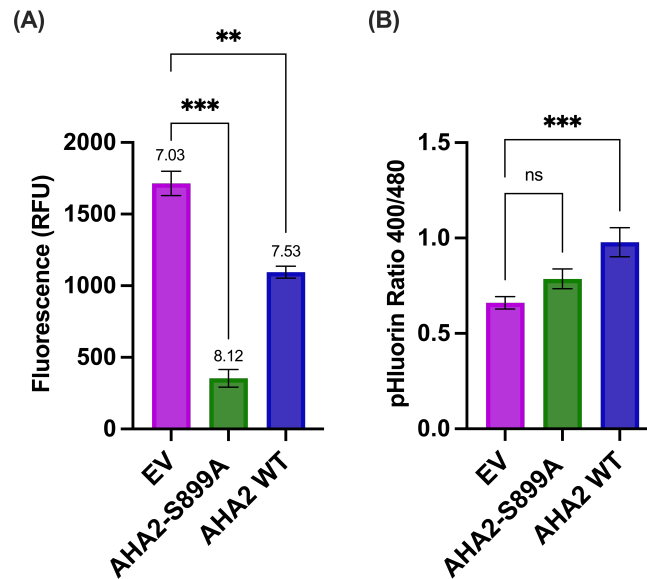


Figure 2.13: Transient *NbAHA2* WT and S899A alkalize the apoplast. (A) *NbAHA2* WT and S899A alkalize the apoplast in pHrodo™ Red Dextran measurements. Four-week-old *N. benthamiana* was agroinfiltrated with either EV or Agrobacteria harbouring expression cassettes for *NbAHA2* WT or *NbAHA2*-S899A. Apoplastic fluid was extracted from leaf tissue expressing the above constructs (See Table 7.21) at 5dpi. pHrodo™ Red Dextran was added at 20µg/mL to the AF and fluorescence at Ex./ Em. 550/585nm was measured. Four replicates for each treatment were measured for which mean and standard error of mean are indicated. A standard curve of phosphate buffers was measured on the same plate and pH quantifications are extrapolated from this. Similar results were obtained in one more experimental replicate. **(B) *NbAHA2* WT and S899A alkalize the apoplast in secreted GFP pHluorin measurements.** The above constructs and secreted GFP pHluorin were agroinfiltrated into four-week-old *N. benthamiana*. 5dpi leaf discs were taken and secreted GFP pHluorin fluorescence (Ex. 400 and 480, Em. 510). Twelve replicates for each treatment were measured for which mean and standard error of mean are indicated. Similar results were obtained in one more experimental replicate.

AHA2-S899A induces plant defence responses

To confirm that alkalization itself, and not other *NbRALF1*-dependent responses, was responsible for the upregulation in plant defence responses and the decrease in AgroLux bioluminescence, we analysed how AHA2-S899A-generated alkalization affects the accumulation of PR proteins. We extracted total soluble protein from the same 5 dpi samples used in Figure 2.12B to detect the accumulation of PR proteins. Leaves expressing AHA2-S899A had increased accumulation of PR2 and PR3 in comparison to the EV control (Figure 2.14), reflecting an upregulation of the plant defence response. These results show that the AHA2-S899A mutant significantly alkalizes the apoplast, which triggers the upregulation of PR proteins (Figure 2.14). This pH-dependent change in the plant defence

response translates into the same disruptive effect on *Agrobacterium* bioluminescence, as observed with RALF1 overexpression.

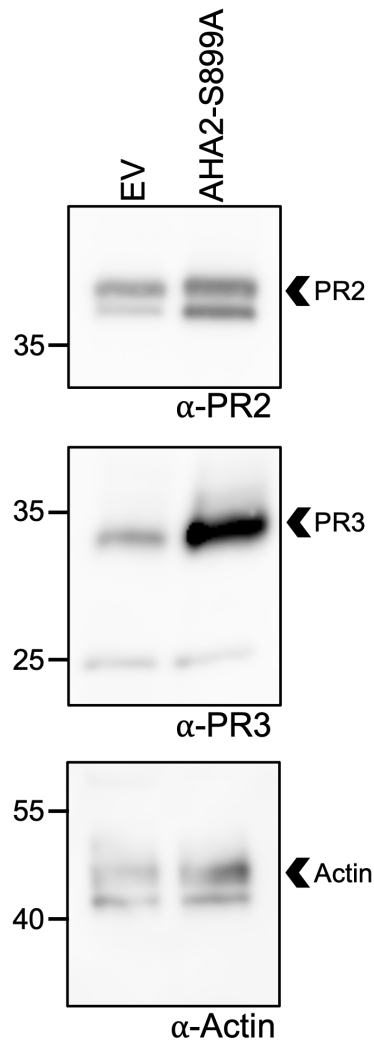


Figure 2.14: Transient *NbAHA2-S899A* expression increases accumulation of PR2 (A) and PR3 (B) proteins. α -Actin western blot is used as a protein expression and loading control (C). Four week-old *N. benthamiana* plants were agroinfiltrated for expression of *NbAHA2-S899A*. Leaf discs were taken at 5dpi and total soluble protein extracted. Proteins were separated on a 12% SDS-PAGE gel and detected by western blotting using α -PR2 (A), α -PR3 (b) and α -Actin (C).

2.3.6 Salicylic acid accumulation is required for the detrimental effect of alkalization on AgroLux

To determine if salicylic acid (SA) is required for the detrimental effect of alkalization on AgroLux, the alkalization causing factors (*NbRALF1*, *NbAHA2* and *NbAHA2-S899A*) were transiently expressed with AgroLux in a *NahG* transgenic line and WT *N. benthamiana*. *NahG* is a bacterial salicylate hydroxylase gene that causes degradation of SA (Figure 2.15). The transgenic line expressing *NahG* has undetectable levels of SA ([Wulff et al., 2004](#)) and accumulates far lower levels of PR2 or PR3 than WT (Figure 2.15B-D). *NahG* plants expressing the alkalization factors and AgroLux were harvested for leaf discs at 5dpi and analysed for bioluminescence. WT plants expressing the alkalization factors demonstrated the expected significant reduction in AgroLux bioluminescence. *NahG* transgenic plants did not have significantly lower bioluminescence of AgroLux upon expression of alkalization factors (Figure 2.15A). This confirms the importance of the SA-regulated immune response during extended alkalization. These data show that the PR protein accumulation in response to alkalization requires SA signalling.

2.3.7 pH-dependent defence response decreases growth rate of the bacterial pathogen *Pseudomonas syringae*

The induction of SA-induced plant defence upon alkalization is severely detrimental to Agrobacteria in the apoplast and led us to question if similar detriment could be imposed upon other bacterial plant pathogens by prolonged alkalization. Furthermore, if the detrimental effects from alkalization in AgroLux are translated into other pathogens, we hypothesised that AgroLux could be used as a proxy for screening new deterrents to plant pathogens.

To examine if the detrimental effects of apoplast alkalization are also reflected in other plant pathogens, we tested the effect of apoplastic alkalization and the related defence responses on *Pseudomonas syringae* growth (Figure 2.16). Agromonas is a technique that combines Agrobacterium-based transient expression with traditional inoculation assays using *P. syringae*. Leaves are agroinfiltrated for expression of a protein, left for three

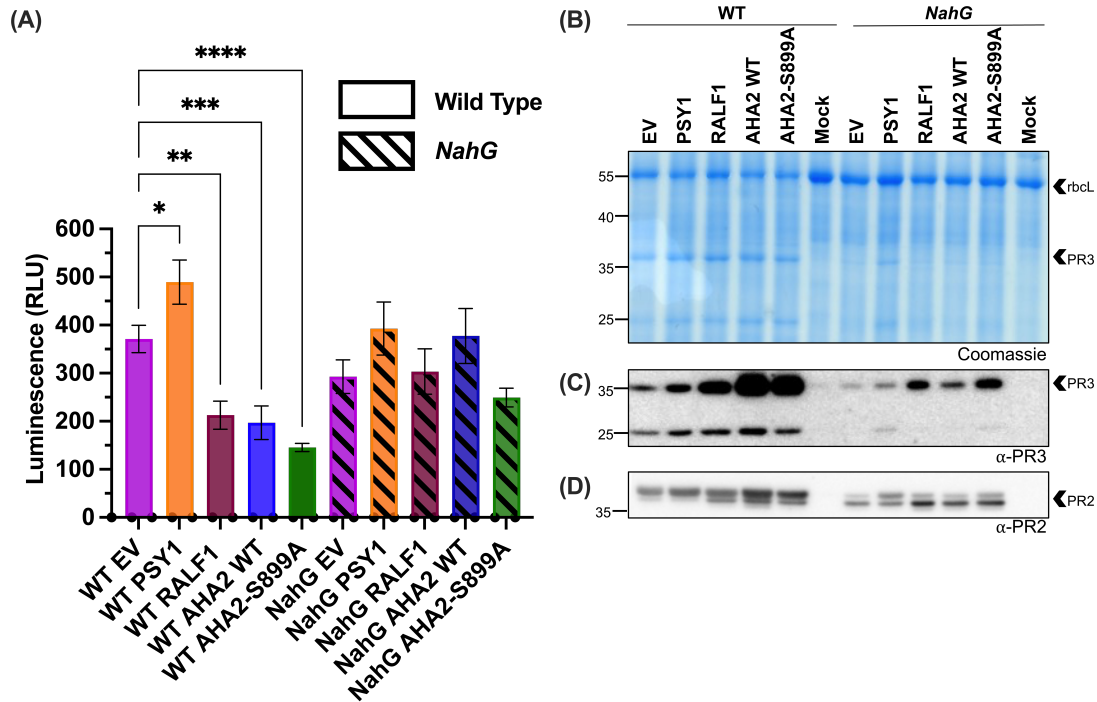


Figure 2.15: SA signaling is required for pH-dependent changes to AgroLux bioluminescence and PR protein accumulation. Four-week old *N. benthamiana* WT and *N. benthamiana* *NahG* were agroinfiltrated for expression of EV, AtPSY1, *Nb*RALF1, *Nb*AHA2 WT and *Nb*AHA2-S899A. At 5dpi, leaf discs were taken and AgroLux bioluminescence was measured (A) and total soluble protein extracted. Proteins were separated on a 12% SDS-PAGE gel and detected by western blotting using α -PR3 (C) and α -PR2 (D). Coomassie stained gel is shown for protein expression and loading control (B). Six replicates for each treatment were measured for which mean and standard error of mean are indicated. Similar results were obtained in one more experimental replicate. (B) Coomassie staining indicates a reduction in PR protein accumulation despite higher accumulation of the *rbcL* large subunit. (C and D) Previously observed patterns of PR3 and PR2 can be seen in the WT plants and *NahG* plants but the quantity of both PR2 and PR3 is much-reduced in *NahG* plants. All lanes represent samples from four biological replicates in one experiment pooled together. Similar results were obtained in two more experimental replicates.

days for expression of the protein and then inoculated with *P. syringae*. This enables investigation of how the protein of interest impacts *P. syringae* growth.

To determine if expression of alkalization factors affects growth of *P. syringae*, 3.5 week old *N. benthamiana* were agroinfiltrated with *A. tumefaciens* carrying expression cassettes for *Nb*RALF1, *Nb*AHA2 and *Nb*AHA2-S899A. 3 days later, the same areas were inoculated (by injection) with *P. syringae* pv. *tomato* DC3000 (ΔhQ). Three days

post inoculation, leaf discs were harvested, surface sterilised and homogenised in water. This homogenate was then plated in serial dilutions on selective plates, incubated for 48 hours and then colonies were counted. *N. benthamiana* plants transiently expressing *NbRALF1*, *NbAHA2* and *NbAHA2-S899A* showed reduced growth of *P. syringae* *pv. tomato* *DC3000* (ΔhQ) (Figure 2.16). This data confirms that the detrimental effect caused by alkalization factors on AgroLux is reflected in the growth of another bacterial plant pathogen.

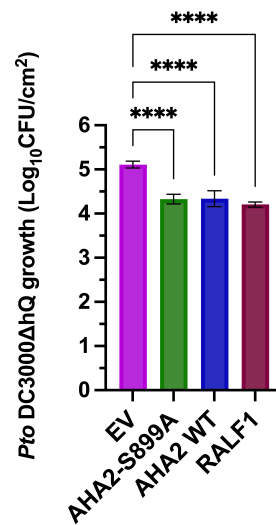


Figure 2.16: Apoplast alkalization also reduces growth of *P. syringae* Three-week-old *N. benthamiana* plants were agroinfiltrated for expression of EV, *NbAHA2-S899A*, *NbAHA2* WT and *NbRALF1*. Four days after agroinfiltration, agroinfiltrated leaves were infected with *P. syringae* *Pto* *DC3000* Δ hQ. Three days after infection, bacterial growth was measured by colony count. Leaves expressing *NbAHA2-S899A*, *NbAHA2* WT and *NbRALF1* had significantly less bacterial growth over three days than EV. Twelve biological replicates for each treatment were measured for which mean and standard error of mean are indicated. Similar results were obtained in two more experimental replicates.

Consensus	ATGGNNRAGTYCYTNNNTCWGKTNNTAAYTCTTWSKRTTCTSRCTCGKNNNNNNNAKCTYT	60
AtRALF1	ATGGACAAGTCTTTTACTCTGTCTTAACCTTACGATTCTCGTCTCTTCATCATCTCT	60
NbRALF1	ATGG--GAGTTCTT--TCAAGT--TTAATCTTGTGTTCTGATCCG-----AGCTTT	47
Consensus	TYNNNTCMKKTCAWGS CGGCNNCGCCRRMGAYMKYGGNGGYSTASSATKGGSTRMKRCY	120
AtRALF1	TCACCTCCGGTCCAAGCCGGCTTCGCCAACGACCTCGGTGGTGTAGCATGGCTTACGACT	120
NbRALF1	TT---TCATTTCATTTGGCGGC--CGCCGGAATAGTGG--GGCCTACGATTGGGTGATGCC	101
Consensus	GGMGASAWNTGGTKSRRGMTGCMAYGGKWSWATMGSAGAGTGYATSGSKGMRGAGGARGA	180
AtRALF1	GGAGACAA--TGGTTCAAGGCTGCCACGGTTCAATACGAGAGTGTATCGGGCCGAGGAAAGA	179
NbRALF1	GGCGAGATCTGGTGGGAGATGCAATGGGAGTATCGGAGAGTGCATGGCTCAAGAGGAGGA	161
Consensus	RNNNGARMTGGACWSWAGWYMAAYAGRMGWATWTRGCKACCAMAAARTACATMAGCTA	240
AtRALF1	A---GAAATGGACTCAGAGATCAATAGAAAGATATTGGCGACCACAAAATACATAAGCTA	236
NbRALF1	GTTTGAGCTGGACAGTGAATCAACAGGCGTATTTAGCTACCAAAAAGTACATCAGCTA	221
Consensus	NCRGTNCKYTGMRMRGAACAGTGTRCCTTGTTCTMMGMMGWGGWGCKTCKTATTACAAYT	300
AtRALF1	TCAGT-CTTTGAAACGGAACAGTGTGCCCTTGTTCAAGAAAGGTTGGCTTATTACAATT	295
NbRALF1	-CGGTGCGCTGCAGAAGAACAGTGTACCTTGTTCTCCGCTGGAGTTCTGATTACAAC	280
Consensus	GNCARAMCNGGAGCTCAGGCTAATCCTTAYWSTCGTGGTTGCAGYRMWATYRCTCGTTGC	360
AtRALF1	GTCAGAAC--GGAGCTCAGGCTAATCCTTATAGTCGTGGTTGCAGCAAAATTGCTCGTTGC	354
NbRALF1	G-CAACCTGGAGCTCAGGCTAATCCTTACTCTCGTGGTTGCAGTGCTATCACTCGTTGC	339
Consensus	AGGAGTTAR	369
AtRALF1	AGGAGTTAG	363
NbRALF1	AGGAGTTAA	348

Figure 2.17: Nucleotide alignment of *AtRALF1* and *NbRALF1*. Nucleotide sequences of the open reading frames of *AtRALF1* (AT1G02900) and *NbRALF1* (Niben101Scf00369:415080..415427) were aligned using Geneious Prime pairwise alignment.

2.4 Discussion

In this chapter, we investigated the role of apoplastic pH in the interaction between *N. benthamiana* and *A. tumefaciens*. Firstly, we confirmed the sensitivity of *A. tumefaciens* to buffers of different pHs (Figure 2.2) and showed how a different pH during agroinfiltration can affect AgroLux bioluminescence and recombinant protein accumulation (Figure 2.3). We then established tools to measure the pH of the apoplast (Figure 2.4, 2.5, 2.6) and to cause prolonged changes to apoplastic pH using RALF1, PSY1 and AHA2 (Figure 2.7, 2.12). We then used these tools to increase the understanding of how apoplastic pH affects Agrobacteria and how plant immunity is involved (Figure 2.11, 2.14, 2.15).

2.4.1 Sensitivity of *A. tumefaciens* to pH

The use of an acidic agroinfiltration buffer for Agrobacterium-based transient expression is commonplace due to the evidence that Agrobacterium *vir* gene expression is induced in acidic conditions and results in higher transformation rates (*Mantis and Winans, 1992*). However, what has not been shown previously is how Agrobacterium state is affected

by pH change *in planta* and *in vitro*. Our data shows that under both conditions, buffering AgroLux changes its bioluminescence (Figure 2.2). However, the effect of a neutral compared to an acidic buffer on bioluminescence is opposite *in vitro* (Figure 2.2A) and *in planta* (Figure 2.2B). Neutral buffering is detrimental to AgroLux bioluminescence *in planta* but is beneficial to AgroLux bioluminescence *in vitro*. This suggests that the detrimental impact of neutral buffering on AgroLux *in planta* is due to compounds produced by the plant in response to the presence of a neutral buffer in the apoplast, including defence-related compounds. Apoplastic alkalization is a plant defence response ([Felix et al., 1999](#)) and therefore the infiltration of a neutral buffer may be detrimental to AgroLux due to an upregulation in plant defence responses. This led to our hypothesis that buffering the apoplast to remain acidic allows Agrobacterium to stay in a more metabolically active state as it is not having to fight off the activated plant immune response.

In order to confirm previous reports of a beneficial effect of pH stabilisation during agroinfiltration on recombinant protein accumulation ([Wu et al., 2014a](#)), we quantified the difference in accumulation of fluorescent proteins when agroinfiltration was performed in an acidic compared to a neutral buffer (Figure 2.3). As expected, we observed significantly more recombinant protein accumulating in tissue agroinfiltrated at pH 5.6 than at pH 7.1 (Figure 2.3B, C). The unexpected result was that AgroLux bioluminescence remained higher in tissue agroinfiltrated at pH 7.1 than pH 5.6 for seven days (Figure 2.3A). The direct buffering effect on AgroLux lasts less than two hours, after which time the plant apoplastic pH control returns. One hypothesis to explain the reduced AgroLux bioluminescence at late time points in the pH 5.6 buffered agroinfiltration is that the larger amount of recombinant protein accumulating in the apoplast leads to changes to apoplastic conditions that are detrimental to Agrobacterium state, but this has not yet been investigated.

This experiment exposes the long-lasting impact of neutral infiltration buffer pH on Agrobacterium bioluminescence and confirms the importance of acidic pH for recombinant protein expression. Furthermore, we show that Agrobacterium is susceptible to conditions in the apoplast at different stages of infection and it is at these later stages of infection that

we did not yet understand. Our data therefore suggested that prolonged pH manipulation should be studied to understand the *Agrobacterium* state at a later stage.

Sustained alkalization caused by both *NbRALF1* overexpression and *NbAHA2* and *AHA2-S899A* overexpression caused significantly reduced AgroLux bioluminescence (Figure 2.7). In contrast, although not causing significant change, the acidification effect observed upon expression of *AtPSY1* and its receptor, led to significant increases in AgroLux bioluminescence (Figure 2.7). This trend in pH effect on AgroLux mirrors that observed *in planta* and led to our hypothesis that prolonged alkalization results in a sustained upregulation in plant defence response that mirrors the induced plant defence response upon initial alkalization by buffering. It is therefore not pH directly, but the pH-induced changes to plant defence that affect AgroLux.

2.4.2 Tools to measure and manipulate apoplastic pH

Investigating how apoplastic pH affects *Agrobacteria* necessitated a robust pH measuring technique. In previous investigations of plant pH manipulation, GFP pHluorin was used, but this required time-consuming and low through-put confocal microscopy to estimate pH. This technique was unsuitable for use in this investigation because multiple different treatments were investigated simultaneously and compared. Therefore we cloned and optimised a secreted form of GFP pHluorin so that its fluorescence could be measured from leaf discs on a plate reader and give an estimate of apoplastic pH (Figure 2.4, 2.5). We did not confirm that this secreted GFP pHluorin was secreted to the apoplast. However, we cloned an identical sequence and signal peptide to that used by [Jutras *et al.* \(2015\)](#), for which confocal microscopy was used to confirm secretion into the apoplast. We compared how the secreted and cytosolic forms of GFP pHluorin were affected by the pH altering peptide elicitors, and could clearly observe that cytosolic GFP pHluorin was not affected where as the secreted form was. Secreted GFP pHluorin measurement using a plate reader provides a fast opportunity to assess if the apoplast is alkalizing. This may be useful in the future to assess if elicitor application to leaf discs is stimulating apoplastic alkalization as a plant defence response in a similar manner to measuring ROS bursts. However, GFP pHluorin is limited because it cannot be used as a measure of absolute pH. Furthermore,

due to the overlapping spectra of GFP pHluorin and leaf autofluorescence, there were large amounts of background fluorescence. This made distinguishing pH possible only when secreted GFP pHluorin was expressed at high levels. Therefore we established a second pH measurement for in apoplastic fluids. pHrodo™ Red Dextran can be used successfully *in vitro* to measure apoplastic fluid pH (Figure 2.6). This second measurement is useful for determining absolute pH as it can be compared to a standard curve. This method can also be used on very small amounts of apoplast fluid extracted, in contrast to a standard lab pH meter which requires several millilitres of apoplastic fluid to extract accurate measurements. However, the use of pHrodo™ Red Dextran requires apoplastic fluid, meaning it cannot be used as a tool in high-throughput experiments, unlike GFP pHluorin. By combining these two techniques, we have optimised a robust method for pH measurement.

In order to understand how prolonged pH change in the apoplast affects *A. tumefaciens*, we used transient expression of RALF1, AHA2, AHA2-S899A to change the pH of the apoplast. Expression of both of these methods resulted in sustained alkalinization of the apoplast (Figure 2.7, 2.13).

The first method resulted in a significant alkalinization from *NbRALF1* overexpression (Figure 2.7). This alkalinization was confirmed by both pHrodo™ Red Dextran and secreted GFP pHluorin fluorescence. Although *NbRALF1* overexpression did result in robust alkalinization which we were able to study further, we sought a second method of alkalization to study in addition to *NbRALF1* because the physiological effects of *NbRALF1* are known to be numerous ([Zhang et al., 2020](#)) and therefore we could not confirm that all changes to AgroLux and plant immunity were mediated through alkalinization. Therefore we next targeted the pump that *NbRALF1* regulates, *NbAHA2*. We mutated *NbAHA2* at two positions. The T881 residue has been described as the position at which PSY1-mediated phosphorylation occurs and causes upregulation of proton-pumping. S899 is the residue phosphorylated for RALF1-mediated alkalinization. Both of these residues were mutated into aspartic acid as phosphomimics (S899D and T881D) (Figure 2.1). They were also both mutated into phosphomutants (S899A and T881V). The only mutant that caused a significant change to AgroLux bioluminescence was AHA2-S899A (Figure 2.12). AHA2-S899A expression reduced AgroLux bioluminescence and alkalized

the apoplast (Figure 2.12, 2.13). This is in fact the opposite of the predicted effect of AHA2-S899A, because we had predicted that a phosphomutant at residue S899 would mean that RALF1-mediated downregulation of proton pumping would be prevented and therefore result in an unregulated stream of protons entering the apoplast and cause acidification. Overexpression of the WT *NbAHA2* also caused alkalization of the apoplast (Figure 2.13). To decipher the mechanisms of how expression of AHA2 and AHA2-S899A cause apoplastic alkalization will uncover more about how AHA2 functions in *N. benthamiana*.

Possible explanations for the alkalization upon expression of AHA2 and AHA2-S899A include that the overexpressed AHA2 and AHA2-S899A are misfolding and are not functional at pumping protons into the apoplast. This would result in regulatory components, including RALF1 and blue light ([Falhof *et al.*, 2016](#)), acting upon both endogenous AHA2 and the transiently expressed, dysfunctional AHA2 pumps. This could mean that fewer functional, endogenous AHA2 pumps are being upregulated. After five days of expression of this misfolded AHA2, a significant reduction in transport of protons into the apoplast would result in apoplastic alkalization. A second possible explanation is that the role of S899 phosphorylation is actually to activate AHA2 because there are contradictory reports of S899 regulation. Although there is evidence that RALF1 causes alkalization through S899 phosphorylation ([Nühse *et al.*, 2007](#)), AHA2 activation through S899 phosphorylation has been demonstrated by expression of S899 phosphomimic, AHA2-S899D ([Rudashevskaya *et al.*, 2012](#)). Expression of AHA2-S899D in yeast increased rate of proton pumping ([Rudashevskaya *et al.*, 2012](#)). This shows that there is possibility that the opposite phosphomutant, AHA2-S899A, could cause downregulation of protons, leading to the alkalization observed in this research. An additional possible explanation is that expression of AHA2 WT and AHA2-S899A do cause acidification initially, which is not captured by the 5dpi measurements taken in this research. This could result in alkalization at 5dpi due to over-corrections occurring during which the plant attempts to restore the cellular membrane potential.

2.4.3 Apoplast alkalization hampers AgroLux and molecular farming by upregulation of SA-mediated defence

Upon finding that AgroLux bioluminescence is hampered by alkalization of the apoplast and that it is increased by *AtPSY1* and *AtPSY1-R* induced acidification, we found that these pH changes correlate with respective up and downregulation of plant defence (Figure 2.7, 2.11, 2.12, 2.14). In all treatments that induced alkalization of the apoplast (*NbRALF1*, *NbAHA2* and *AHA2-S899A* overexpression) there was a clear upregulation in PR proteins and a reduction in the large subunit of RuBisCO in comparison to the EV agroinfiltrated control. Both of these are typical markers of induced plant defence. The opposite trends in the *AtPSY1/-R* tissue and corresponding increase in AgroLux indicates that the sustained apoplastic acidification is reducing the plant defence response, allowing AgroLux bioluminescence in the apoplast to improve. We demonstrate that SA is required in the onset of alkalization-induced defence responses and for the effects on AgroLux bioluminescence (Figure 2.15). Expression of both *RALF1* and *AHA2-S899A* cause alkalization, which then, only in the presence of SA cause upregulation of PR proteins and a significant reduction in AgroLux bioluminescence. *NahG* transgenic plants, which do not accumulate SA ([Wulff et al., 2004](#)), do not affect AgroLux or PR protein accumulation upon expression of *RALF1* and *AHA2-S899A* (Figure 2.15). This demonstrates that SA signalling mediates the detrimental impact of alkalization on AgroLux.

In these investigations, we also show that tissue expressing *NbRALF1* has reduced accumulation of two different recombinant proteins, VRC01 and RD21 (Figure 2.9). This effect may be due to recombinant protein instability at the higher pH, however this is unlikely because in humans, antibodies, such as VRC01, are transported through the blood stream which is a slightly basic environment. Likely explanations for the reduction in recombinant protein are that the alkalization-induced plant defence response is causing degradation of the recombinant proteins, less T-DNA transfer and that the resources required for the plant defence response result in less capacity for recombinant protein production. In Figure 2.9, expression of *PSY1* and *PSY1-R* also reduce accumulation of recombinant proteins. This was unexpected after the significant increase in AgroLux

bioluminescence caused by PSY1/PSY1-R expression (Figure 2.7C). A positive effect on recombinant protein accumulation may be more likely if PSY1/PSY1-R expression is already occurring when agroinfiltration is performed. This could be achieved using transgenic plants or using two agroinfiltrations at least three days apart, agroinfiltrating for expression of PSY1/PSY1-R initially, and then for recombinant protein expression once PSY1/PSY1-R are being expressed. The reason for the negative impact of PSY1/PSY1-R may be because the plant is expressing more recombinant proteins than the EV control. In the PSY1/PSY1-R expressing tissues, the plant is expression four different recombinant proteins (PSY1, PSY1-R, VRC01 and P19). In contrast, the EV, although agroinfiltrated with the same concentration of *Agrobacteria*, is expression only two recombinant proteins (VRC01 and P19). In Chapter 3, we report that co-expression of two recombinant proteins together causes less expression than if either is agroinfiltrated alone (Figure 3.1). The added cost of producing two extra recombinant proteins may be causing a reduction in production of VRC01.

2.4.4 AgroLux seems to be an indicator of SA-mediated plant immunity rather than transient expression

Leaves expressing *AtPSY1* and *AtPSY1-R* have almost double the levels of AgroLux bioluminescence in comparison to the EV controls and more large subunit of RuBisCO accumulating (Figure 2.7C). However, they do not show more recombinant protein accumulating than the EV control (Figure 2.9). Previous investigations reported correlation between AgroLux bioluminescence at 12 hours post infiltration and recombinant protein accumulation at 3dpi. In the experiments we performed in this chapter, AgroLux bioluminescence and recombinant protein measurements were both taken at 5dpi and they do not correlate. This shows that AgroLux cannot be used to predict recombinant protein accumulation when taken at the time of harvest and that at 5dpi, AgroLux state is not important for the success of molecular farming, possibly because this is beyond the time at which T-DNA is transferred. Instead, these results suggest that at this later time-point, reduced AgroLux bioluminescence correlates with high SA levels and increased SA-mediated defence responses (Figure 2.15). alkalization-induced changes to

AgroLux bioluminescence are abolished in *NahG* transgenic plants (Figure 2.15). *NahG* plants do not accumulate SA and have lower accumulation of PR proteins. The abolition of alkalization-induced changes to AgroLux firstly indicates that alkalization of the apoplast induces SA-mediated defence responses. Secondly, this suggests that AgroLux may be useful as a tool to monitor levels of SA-mediated defence. The increase in AgroLux bioluminescence upon PSY1/PSY1-R expression (Figure 2.7) also support our hypothesis that AgroLux can be used to monitor SA-mediated defence. In *A. thaliana*, *psy1r* mutants produced more SA and were less susceptible to *P. syringae* DC3000 infection, suggesting a negative regulatory role for PSY1/PSY1-R in SA-mediated defence ([Mosher et al., 2013](#)). PSY1/PSY1-R signalling functions in plant defence by balancing production of antagonistic phytohormones JA and SA ([Mosher et al., 2013](#)). It is possible that in our experiments, expression of PSY1 and PSY1-R in *N. benthamiana* redresses the balance of JA and SA to reduce SA biosynthesis and increase JA, resulting in reduced SA-mediated defence. This evidence supports our hypothesis that AgroLux can be used as a tool to monitor levels of SA-mediated defence.

2.4.5 Detrimental effect of alkalization on Agrobacterium reveals the use of AgroLux as a proxy for exposing new deterrents to plant pathogens

The data in this chapter support the hypothesis that reduced AgroLux bioluminescence correlates with plant defence responses. This indicated that AgroLux can be as used a screening tool to find new deterrents to plant pathogens as a proxy for immunity assays. The experiments that we currently use to find components to increase plant immunity against *P. syringae* involves using the Agromonas assay ([Buscaill et al., 2021](#)). This assay works well to validate small numbers of transiently expressed proteins to affect plant immunity against *P. syringae*, but it is too labour intensive and time-consuming to screen many different treatments at once. Instead, using AgroLux as a proxy for other bacterial plant pathogens could be an efficient method to screen many different proteins quickly for effects and then validate only positive hits against the relevant pathogen.

I investigated this hypothesis using the over expressed proteins that caused

alkalinization of the apoplast (*NbRALF1*, *NbAHA2* and *AHA2-S899A*). These proteins all caused a significant reduction in AgroLux bioluminescence when measured in the plate reader. We tested these proteins in the Agromonas assay to see if the reduction in AgroLux correlated to a reduction in *P. syringae* growth. All three treatments resulted in a reduction in growth of *P. syringae* (Figure 2.16), providing evidence that AgroLux can be used as a proxy for exposing new deterrents to plant pathogens.

2.5 Conclusions

Over my investigations in this chapter, I sought to better understand how *A. tumefaciens* is affected by the apoplastic pH and how this impacts recombinant protein production. We have successfully shown how apoplastic pH change is associated with changes to the plant defence response and that these changes are dependent on salicylic acid signalling. We have also shown that induction of the plant defence response by alkalization of the apoplast is detrimental to AgroLux bioluminescence and recombinant protein accumulation. Lastly, we have shown that AgroLux is a useful tool to study plant defence responses and as a screening tool for new pathogen deterrents.

Chapter 3

Establishing the Traffic Light Screen

Isobel L. Dodds, Philippe v. Jutras, Nicholas Holton and Renier A. L. van der Hoorn

Philippe v. Jutras designed and cloned constructs for secreted RFP and cytosolic superfolded GFP. Nicholas Holton provided intellectual advice and support throughout the project. Renier A. L. van der Hoorn supervised and guided the project.

3.1 Abstract

In order to improve molecular farming we aim to screen different conditions for high yields of recombinant protein. Simultaneously to finding better protein yields, we also aim to increase our understanding of how elements of *N. benthamiana* immunity affect *A. tumefaciens*. Traditional analysis methods such as western blotting and bacterial growth assays are too time-consuming to screen a large number of conditions. In this chapter, we establish a three-factor ‘Traffic Light’ screening system to measure secreted and cytosolic recombinant protein accumulation and AgroLux bioluminescence using a plate reader. This screen facilitates high-throughput screening of conditions affecting *A. tumefaciens* and recombinant protein accumulation.

3.2 Introduction

Plant Molecular Farming has become a successful method for producing recombinant proteins for various uses such as vaccines, antibodies and growth factors ([Schillberg and Finnern, 2021](#)). For example, in response to the COVID-19 pandemic, plant molecular farming company Medicago has developed a vaccine against COVID-19. This vaccine, COVIFENZ®[®], has been approved by Health Canada for use in 18-64 year-olds ([Medicago, 2022](#)). However, despite some success stories, plant-made recombinant protein yield still lags behind typically favoured systems such as Chinese hamster ovary cells and *E. coli* ([Schillberg et al., 2019](#)).

Many strategies have been found to improve plant molecular farming including co-expression of protease inhibitors and protease gene knock-outs ([Jutras et al., 2020](#)). However, investigating these strategies is frequently reliant on time-consuming techniques to find candidates and test them. In order to find more successful strategies, a high-throughput and inexpensive screening system needed to be developed. Use of fluorescent proteins and analysing their accumulation in unprocessed leaf discs gives a fast and inexpensive strategy for analysing recombinant protein accumulation.

Combining expression of fluorescent proteins with AgroLux bioluminescence facilitates the simultaneous screening of effects on recombinant protein yield and Agrobacterium state *in planta*, repeatedly. Using AgroLux in screens where we use a variety of strategies to alter the *N. benthamiana* immune system gives opportunity to understand in more detail what AgroLux signals represent and how *N. benthamiana* and *A. tumefaciens* interact during Agrobacterium-based transient expression.

In this chapter, I have established a three-factor screen for high-throughput analysis of strategies to improve plant molecular farming and to better understand the interaction between *N. benthamiana* and *A. tumefaciens*.

3.3 Results

3.3.1 Three-Factor Screen Design

In order to establish this three-factor screen, two fluorescent proteins were required. Both a cytosolic and secreted fluorescent protein were sought in order to reflect the production of both secreted and non-secreted recombinant proteins because both are products of molecular farming. Monomeric RFP was selected as the secreted fluorescent protein because it is stable in acidic environments such as the apoplast, in contrast to other fluorescent proteins which are unstable in acidic conditions ([Shinoda H, 2018](#)). Superfolder GFP was chosen as the cytosolic fluorescent protein because its fluorescent spectrum was reported not to overlap with that of RFP and has previously showed robust fluorescence in the cytoplasm ([Dinh and Bernhardt, 2011](#)). Having identified the two fluorescent recombinant proteins of choice, cytosolic superfolded GFP (cytoGFP) and secreted RFP (secRFP), it was important to establish that they were distinguishable by wavelength and therefore could be measured separately on a Typhoon scanner and plate reader. To demonstrate that they are distinguishable, we agroinfiltrated four-week-old *N. benthamiana* with *A. tumefaciens* carrying expression cassettes for either cytoGFP alone, secRFP alone or cytoGFP and secRFP (Figure 3.1A). Into each section of leaf labelled in Figure 3.1A, two *A. tumefaciens* constructs were mixed at equal concentrations: EV+RFP contains *A. tumefaciens* carrying expression cassette for empty vector (EV, no gene between left and right border of the Ti plasmid) and *A. tumefaciens* carrying expression cassette for sec RFP; EV+GFP contains *A. tumefaciens* carrying expression cassette for EV and *A. tumefaciens* carrying expression cassette for cyto GFP; RFP+GFP contains *A. tumefaciens* carrying expression cassette for secRFP and *A. tumefaciens* carrying expression cassette for cyto GFP. Therefore each section contains a mixture of two *A. tumefaciens* constructs at equal concentrations.

At 5dpi, the leaves were scanned on the Typhoon using Cy2 and Cy3 settings and leaf discs were punched and measured for fluorescence on the plate reader. Using the Cy2 setting, fluorescence emission was clearly observed in the agroinfiltrated regions expressing cytoGFP but not secRFP (Figure 3.1B). Scanning with the Cy3 setting showed fluorescence

emission from agroinfiltrated regions expressing secRFP but not cytoGFP (Figure 3.1C). Fluorescence was also quantified from leaf discs using the plate reader (Figure 3.1D, E). Leaf discs measured on the plate reader at Ex./Em. 480/515nm had significantly more fluorescence when expressing cytoGFP than the secRFP/EV agroinfiltrated leaf discs (Figure 3.1D). Leaf discs measured using Ex./Em. 550/585nm had significantly more fluorescence than leaf discs agroinfiltrated for cytoGFP/EV (Figure 3.1E). There was significantly more fluorescence in the regions agroinfiltrated for cytoGFP/EV (at 480/515nm, 3.1D) and secRFP/EV (at 550/585nm, 3.1E) than in regions expressing both fluorescent proteins. This data confirms that secRFP and cytoGFP can be measured separately when co-expressed by using different wavelengths for emission and excitation. Figure 3.4 also shows that the co-expression of the fluorescent proteins negatively affects the accumulation of the the other. This could be because the cellular machinery and energy used to produce one fluorescent protein detracts from the capacity to produce the other, reducing accumulation of each in the agroinfiltration producing both.

3.3.2 Screen Optimisation

Young plants show higher expression levels

To determine the age of plants that allowed for a consistent high level of expression, plants aged 24, 29 and 36 days-old were agroinfiltrated on their third youngest leaf with the traffic light system and at 5dpi, leaf discs were measured for fluorescence and luminescence (Figure 3.2). Over the three experimental replicates, no consistent trend in AgroLux bioluminescence in the different aged plants was observed despite the significant difference in plants aged 29 and 36 days in this replicate (Figure 3.2A). Fluorescence of both cytoGFP (Figure 3.2B) and secRFP (Figure 3.2C) increased with decreasing age of *N. benthamiana*. Increase in fluorescence was significant between every age of plant.

The plants aged 24 days had the highest accumulation of protein but often had very small leaves. To agroinfiltrate multiple constructs into one leaf to avoid inter-plant variation, plants aged 28 days were chosen as the time point for further experiments. This plant age has adequate and consistent expression levels but is not too small for

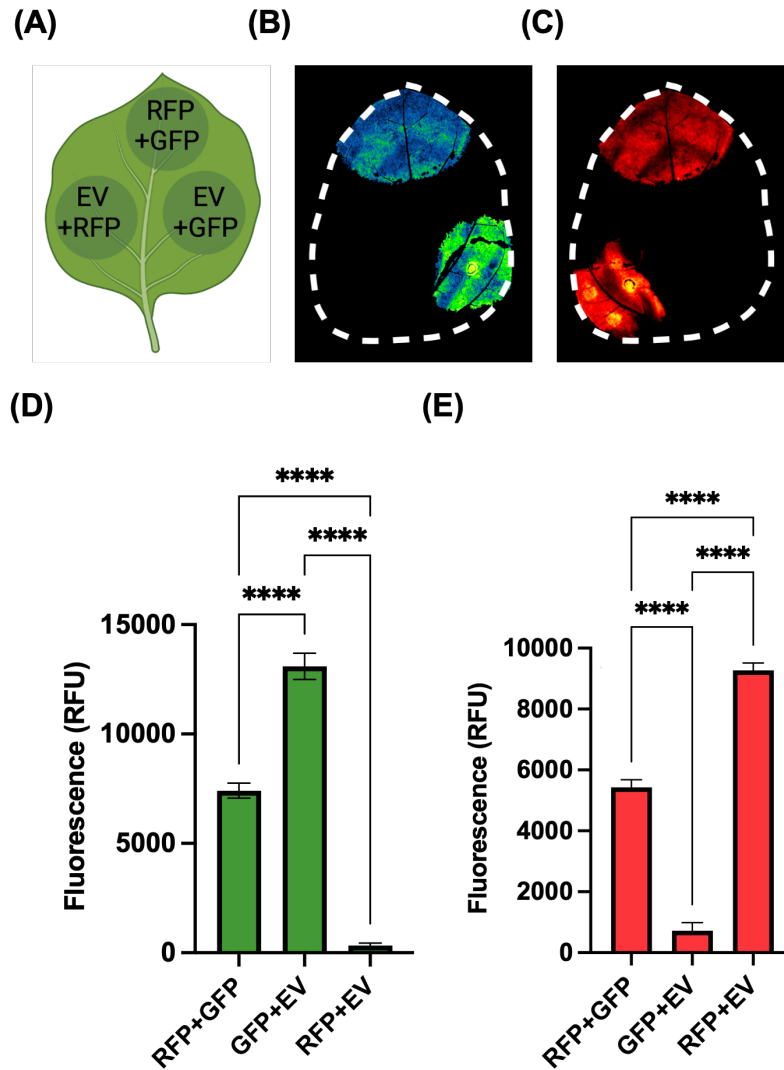


Figure 3.1: Cytosolic GFP and secreted RFP are detected at different wavelengths and can both be measured in the same leaf tissue. Leaves were agroinfiltrated in three regions with either secreted RFP (secRFP) and cytosolic GFP (cytoGFP), EV and secRFP or EV and cytoGFP (A). (B) Agroinfiltrated leaf scanned using Cy2 setting on a Typhoon scanner. Only regions agroinfiltrated with expression cassette for cytoGFP show fluorescence. (C) Agroinfiltrated leaf scanned using Cy3 setting on a Typhoon scanner. Only regions agroinfiltrated with expression cassette for secRFP show fluorescence. Leaf discs were punched from the same leaves and placed in a 96-well plate. Leaf disc fluorescence was measured by a plate reader at Ex./Em. 480/515nm (D) and 550/585nm (E). (D) Only cytoGFP is detected at Ex./Em. 480/515nm. (E) Only secRFP is detected at Ex./Em. 550/585nm. Twelve replicates were used for each measurement, for which mean and standard error of mean are indicated. Similar results were obtained in two more experimental replicates.

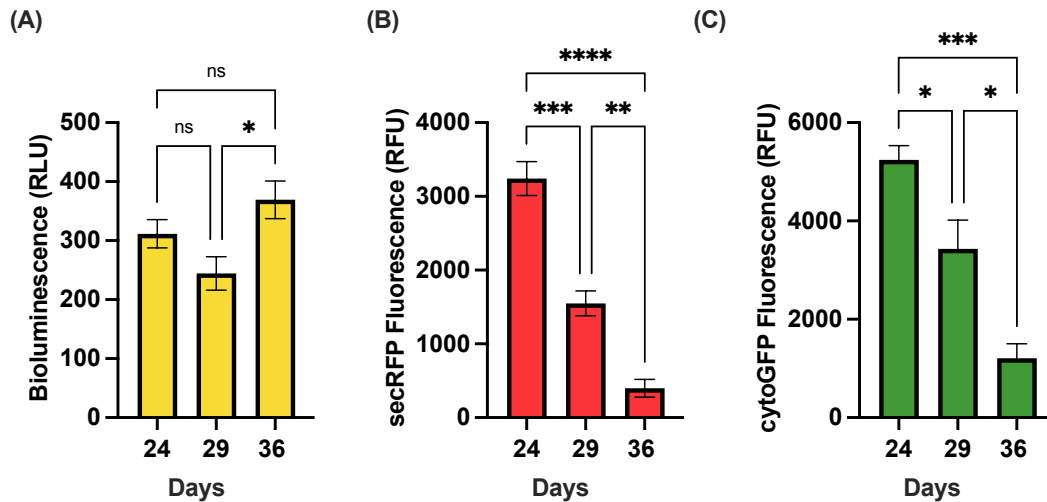


Figure 3.2: Increasing plant age correlates with reduced accumulation of fluorescent recombinant proteins. (A) AgroLux luminescence is lowest in plants agroinfiltrated at 29 days old. (B) cytoGFP accumulation significantly increases with decreasing age of plant at agroinfiltration. (C) secRFP accumulation significantly increases with decreasing age of plant at agroinfiltration. *N. benthamiana* plants aged 36, 29 and 24 days old were agroinfiltrated with AgroLux and for expression of cytoGFP and secRFP. At 5dpi leaf discs were taken and measured for luminescence and fluorescence (Ex./Em. 480/515nm (B) and 550/585nm (C) in a plate reader. Twelve replicates were used for each measurement, for which mean and standard error of mean are indicated. Similar results were obtained in one more experimental replicate.

multiple agroinfiltrations within a leaf. Furthermore, this is the plant age used in industry, increasing likelihood that results obtained in screens will be useful for industrial expression of recombinant proteins in *N. benthamiana*.

Young leaves support high expression levels

To test which leaf was most suitable for screens, five leaves were agroinfiltrated and numbered 1-6 from youngest (most recently emerged) to oldest (Figure 3.3A). AgroLux bioluminescence was similar in leaves 2 and 3 and then bioluminescence increased with each leaf age thereafter, significantly increasing between leaves 4 and 5 (Figure 3.3B). Leaves 2 and 3 showed similar levels of fluorescence of both cytoGFP and secRFP, and then reduced with each leaf number thereafter, with significant differences in accumulation of cytoGFP and secRFP between leaf 4 and 5 and of secRFP also between leaves 3 and 4 (Figure 3.3C and D). Leaf 2 was a lot smaller and more difficult to agroinfiltrate than leaf 3, but the two showed similar, high levels of recombinant protein accumulation. Therefore leaf 3 was

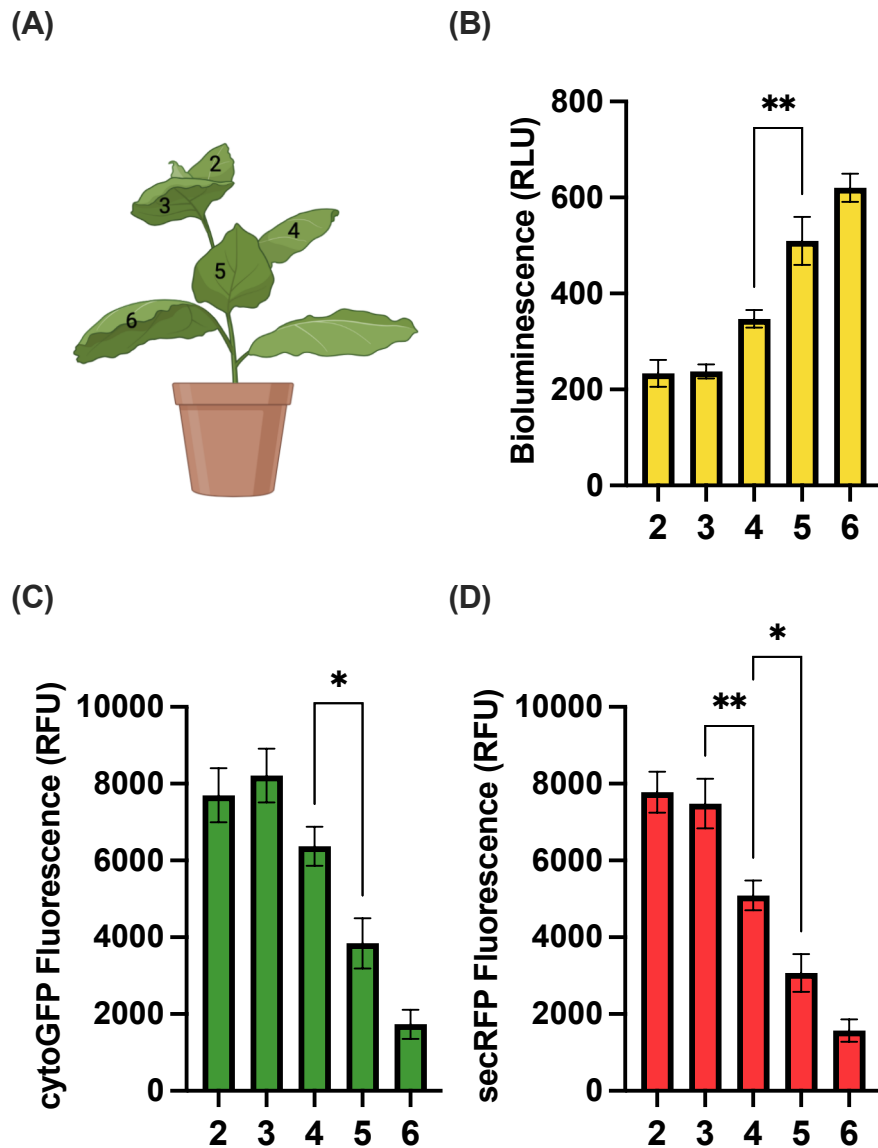


Figure 3.3: Increasing leaf age correlates with reduced accumulation of fluorescent recombinant proteins. (B) AgroLux increases with increasing age of leaf agroinfiltrated. (C) cytoGFP accumulation significantly increases with decreasing age of plant at agroinfiltration. (D) secRFP accumulation significantly increases with decreasing age of plant at agroinfiltration. Four-week-old *N. benthamiana* plants were agroinfiltrated with AgroLux and for expression of cytoGFP and secRFP on five leaves, from the second youngest leaf to the sixth youngest (A). At 5dpi leaf discs were taken and measured for luminescence (B) and fluorescence (Ex./Em. 480/515nm (C) and 550/585nm (D) in a plate reader. Twelve replicates were used for each measurement, for which mean and standard error of mean are indicated. Similar results were obtained in two more experimental replicates.

chosen to be used for screening.

High *A. tumefaciens* culture density increases transient expression

We next tested various optical densities (ODs) of *Agrobacterium* used. For this, we mixed the three constructs used for the screen in equal ratios with each at an OD₆₀₀ of 1.0, 0.5, 0.25, 0.1 and 0.01 and recorded bright field, bioluminescence (using western blot imager) and fluorescence (with Typhoon scanner) of the leaves and took leaf discs (Figure 3.4). All ODs were agroinfiltrated onto one leaf in small areas to avoid inter-leaf variation, as we had seen already how leaf age affects our results (Figure 3.3). AgroLux, cytoGFP and secRFP fluorescence all increased with OD (Figure 3.4). The most significant increase in fluorescence of cytoGFP and secRFP was between ODs 0.25 and 0.5, and this corresponded with a significant increase in AgroLux bioluminescence. The increase from OD 0.5 to 1.0 also resulted in increased AgroLux bioluminescence, cytoGFP and secRFP fluorescence, although not significantly (Figure 3.4). However OD 1.0 also led to slight discolouration of the leaves (Figure 3.4A). Therefore, given the high levels of bioluminescence and fluorescence without detrimental phenotypes, we selected OD 0.5 for further experiments.

Harvest Time-point Optimisation

To determine the time at which to harvest leaf discs, we performed a time course from 1 to 7dpi (Figure 3.5). Agroinfiltrations were done in both the presence and absence of P19 to establish both the difference P19 makes to expression of our two constructs and because part of the silencing screen required the absence of P19 due to the use of Virus-Induced Gene Silencing (VIGS).

In the presence of P19, cytoGFP and secRFP fluorescence increased significantly at 1, 3 and 5dpi and less at 7dpi (Figure 3.5 C, E). In the absence of P19, cytoGFP and secRFP fluorescence increases between 1 and 3 dpi and then plateaus, staying constant from 3 to 7 dpi (Figure 3.5 D, F). Thus, fluorescence was significantly boosted by the presence of P19. However, without P19, fluorescence of both proteins was still significantly above background fluorescence, indicating that sufficient fluorescence is detected without the silencing suppressor. AgroLux bioluminescence decreased at 3 and 5dpi and then stayed similar at 7dpi (Figure 3.5 A, B). These results led us to choose 5dpi for leaf disc harvest

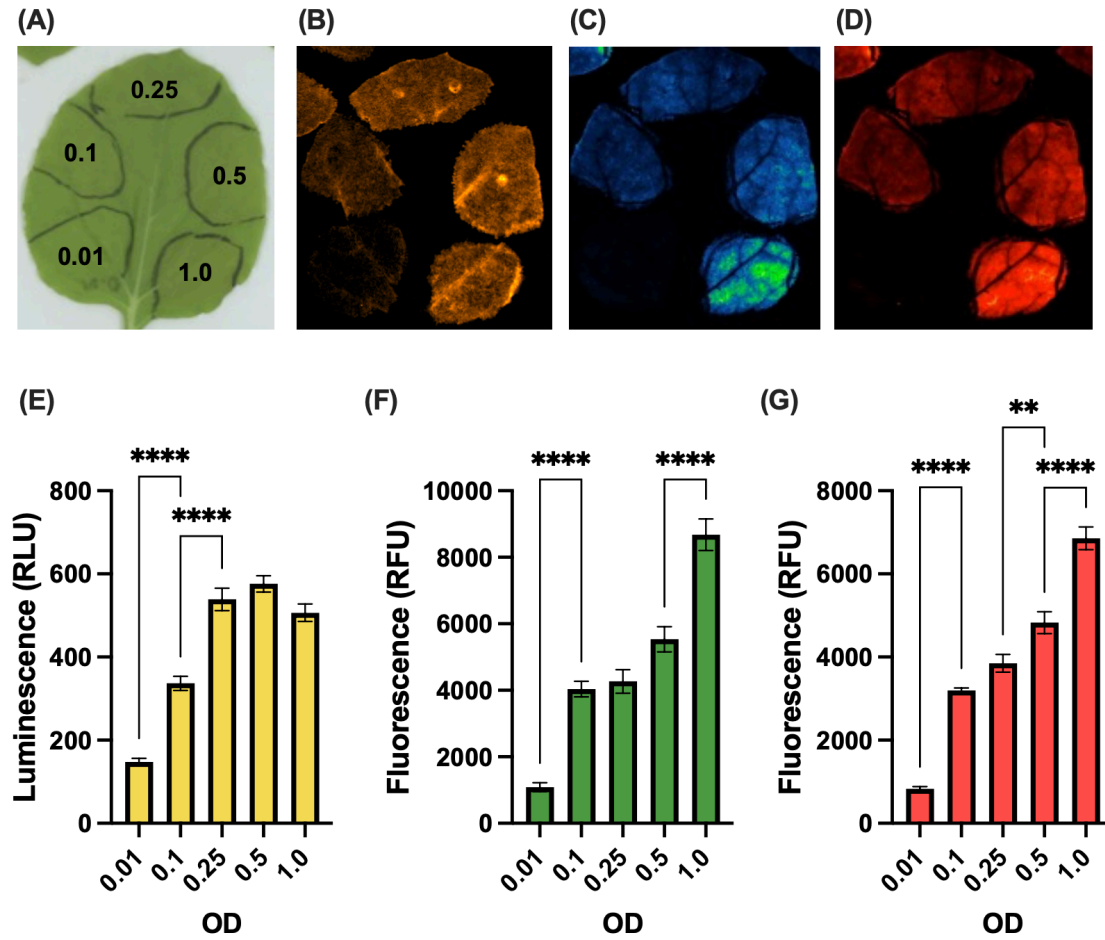


Figure 3.4: Concentration of Agrobacteria agroinfiltrated correlates with AgroLux and fluorescent protein accumulation at 5dpi. Leaves were agroinfiltrated in five areas (A) with AgroLux and for expression of cytoGFP and secRFP at OD₆₀₀ 1.0, 0.5, 0.25, 0.1 and 0.01 in the orientation labelled in panel A. The same orientation of OD₆₀₀ is repeated in panels B-D. At 5dpi, the leaves were scanned on a western Blot imager (B) and Typhoon scanner (C and D) and leaf discs taken were measured on a plate reader for luminescence (E) and fluorescence (Ex./Em. 480/515nm (F) and 550/585nm (G)). (F) cytoGFP accumulation significantly increases with increasing OD of Agrobacteria agroinfiltrated. (G) secRFP accumulation significantly increases with increasing OD of Agrobacteria agroinfiltrated. Twelve replicates were used for each measurement, for which mean and standard error of mean are indicated. Similar results were obtained in two more experimental replicates.

in subsequent experiments.

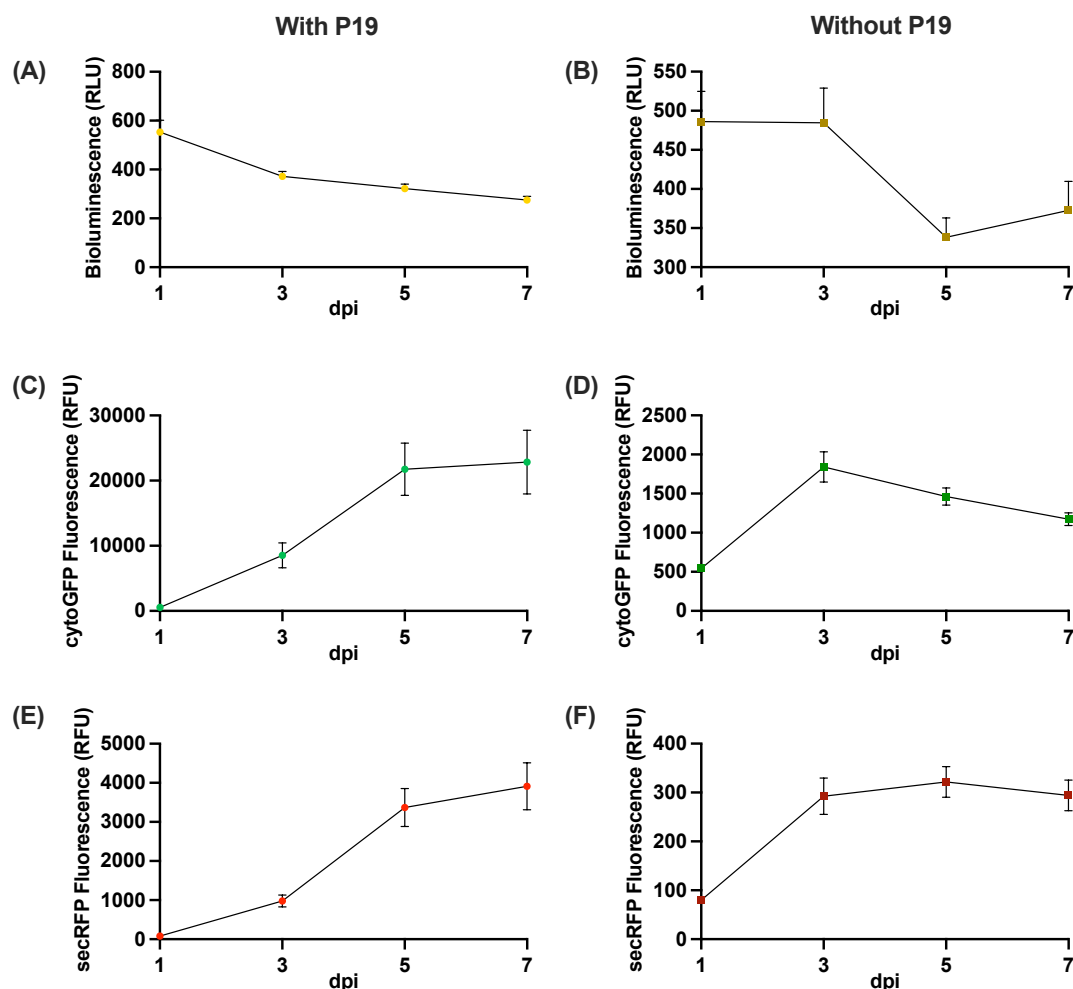


Figure 3.5: P19 boosts cytoGFP and secRFP fluorescence over seven days while bioluminescence decreases. Four-week-old *N. benthamiana* were agroinfiltrated with a 1:1:1 mix of AgroLux and *A. tumefaciens* for expression of cytoGFP and secRFP, with and without P19. At 1, 3, 5 and 7 dpi leaf discs were punched and measured for luminescence (**A, B**) and fluorescence (Ex./Em. 480/515nm (**C, D**) and 550/585nm (**E, F**) on a plate reader. Twelve replicates were used for each measurement, for which mean and standard error of mean are indicated. Similar results were obtained in one more experimental replicate.

3.4 Discussion

In this chapter, we developed a high-throughput screen to identify conditions to improve transient expression. The similar results observed between monitoring accumulation of fluorescent proteins on the Typhoon and leaf discs on a plate reader (Figure 3.1) mean that we can confidently use the faster plate reader method to screen new strategies for increasing recombinant protein accumulation. Using the plate reader is higher through-put than using the Typhoon because only a small area of leaf needs to be agroinfiltrated,

allowing for multiple treatments per leaf and quicker agroinfiltration.

Optimising transient expression has resulted in consistent agroinfiltration conditions that we subsequently use to find new strategies to improve plant molecular farming. In this method, we had to compromise between plant age (Figure 3.2) and leaf age (Figure 3.3) at the time of agroinfiltration for best transient expression and using leaves that are old enough to be easy to agroinfiltrate and fit multiple treatments on. Fitting a minimum of two treatments on one leaf means that a treatment can be compared to a negative control within a leaf to reduce inter-leaf variation. Furthermore, we found an OD of 0.5 results in high transient expression without causing symptoms of plant stress in the leaf such as the discolouration observed at higher OD (Figure 3.4). Lastly, we have shown that 5dpi is the optimal time point at which to take measurements and that these conditions are suitable for use with and without P19 (Figure 3.5).

Manipulation of *N. benthamiana* by various strategies including inhibition of the plant immune response to agroinfiltration, reduction of protease degradation of recombinant proteins and using emerging synthetic biology techniques to introduce new metabolic pathways and expression machinery provides a great opportunity to improve molecular farming yields ([Wang and Demirer, 2023](#); [Jutras et al., 2020](#)). However, as highlighted by [Wang and Demirer \(2023\)](#), in order to take full advantage of these strategies to improve molecular farming, methods for high-throughput screening and mechanistic understanding are required. Previous screens to improve molecular farming have used western blotting to screen 29 conditions ([Grosse-Holz et al., 2018a](#)) and LC-MS/MS to screen 35 conditions ([Wang and Demirer, 2023](#)) to improve molecular farming. Using the ‘Traffic Light’ screen, we were able to screen over 160 conditions for improving molecular farming, as described in the following chapters.

3.5 Conclusions

I have successfully established conditions that can be used to test many different treatments for effects on AgroLux and transient expression. This ‘Traffic Light’ screen is suitable for screening effector proteins, knock-out lines of *N. benthamiana* and in the absence of P19,

also for knock-downs generated by VIGS. Each of these screens will be pursued in the following chapters, using the parameters optimised in this chapter.

Chapter 4

A screen of 130 *Phytophthora infestans* RxLR effectors for improving molecular farming

Isobel L. Dodds, Frederick King, Nicholas Holton and Renier A. L. van der Hoorn

Frederick King performed the agroinfiltrations and analysis for Figures 4.1-4.6 as part of an MBiol project under the supervision of Isobel L. Dodds. Nicholas Holton provided intellectual advice and support throughout the project. Renier A. L. van der Hoorn supervised and guided the project.

4.1 Abstract

Plant molecular farming relies on *Agrobacterium*-mediated transformation for production of recombinant proteins. However, upon agroinfiltration, *N. benthamiana* mounts an immune response against *A. tumefaciens*, which reduces recombinant protein yields. In this chapter, we aim to suppress plant immune responses to agroinfiltration by co-expression with pathogen effectors. We screened 130 *P. infestans* RxLR effectors using the ‘Traffic Light Screen’ for impacts on recombinant protein yields and *Agrobacterium* state. We identified six RxLR effectors from this screen that increased secreted RFP fluorescence. Two of these effectors also increased VRC01 accumulation in bench scale experiments. One of these effectors, E6, was tested at industrial scales but failed to increase the final yield of a purified antibody. This chapter provides a proof of concept for co-expression of pathogen effectors for improving molecular farming and proves that the ‘Traffic Light Screen’ is a powerful tool to monitor the interaction between *A. tumefaciens* and the plant immune system.

4.2 Introduction

Agrobacterium tumefaciens is the causal agent in Crown Gall disease of eudicot plants such as grape vines and walnut trees. It is a plant pathogen with a wide host range that causes large economic damage through crop losses on perennial plants ([Escobar and Dandekar, 2003](#); [Thomashow et al., 1980](#)). The *A. tumefaciens* used for *Agrobacterium*-based transient expression is a disarmed strain lacking the oncogenes, meaning it does not cause tumour formation ([Rogers et al., 1986](#)). Despite this disarmament, the biotechnological strain of *A. tumefaciens* is perceived by the plant as a pathogen and triggers immune responses. Agroinfiltration triggers a transcriptomic and proteomic response ([Grosse-Holz et al., 2018b](#)) in *N. benthamiana*, including upregulation in secretion of defence proteins ([Goulet et al., 2010](#)). Detection of *Agrobacterium* PAMP EF-Tu elicits PTI in *A. thaliana* and restricts transformation rates. This issue is resolved in *A. thaliana efr-1* mutants that are unable to detect EF-Tu and therefore have a reduced PTI response ([Zipfel et al., 2006](#)), demonstrating the beneficial impact of plant defence inhibition on *Agrobacterium*-mediated

transient expression.

As a plant pathogen, *A. tumefaciens* has evolved a variety of mechanisms to suppress plant immune responses by secreting effector proteins that intercept plant defence responses in a variety of ways ([Subramoni et al., 2014](#)). For instance, the *Agrobacterium katA* gene encodes a catalase that converts hydrogen peroxide into water and oxygen and detoxifies the reactive oxygen species (ROS) burst. This allows *Agrobacterium* to hamper plant ROS signalling and subsequent immune responses ([Xu and Pan, 2000](#)). Furthermore, *Agrobacteria* hijack host defences for their own infection. *Agrobacteria* use host protein VIP1 (VirE2-interacting protein 1) to aid transport of the T-DNA into the host nucleus. VIP1 would otherwise induce PR1 expression upon phosphorylation by MAP kinase MPK3 (Figure 1.3) ([Pitzschke and Hirt, 2010](#); [Djamei et al., 2007](#)).

Many other plant pathogens have co-evolved with the plant immune system to reduce immune responses by secreting their own effector proteins ([Asai and Shirasu, 2015](#); [Torto-Alalibo et al., 2009](#); [Göhre and Robatzek, 2008](#); [Alfano and Collmer, 2004](#); [Chang et al., 2004](#)). We hypothesised that expression of effector proteins from other plant pathogens would suppress PTI after agroinfiltration and subsequently increase transformation rates and molecular farming yields. Only a few previous studies have used effectors to improve recombinant protein expression. One such effector is AvrPto. This effector is a kinase inhibitor secreted by *Pseudomonas syringae*, which targets multiple PRRs. One study used transgenic *A. thaliana*, which expressed AvrPto under a dexamethasone (DEX)-inducible promoter. Pre-treatment of these plants with DEX prior to agroinfiltration significantly increased recombinant protein production ([Tsuda et al., 2012](#)). A more recent study engineered *A. tumefaciens* to express a Type III Secretion System (T3SS) and AvrPto, resulting in T3SS-mediated delivery of AvrPto into *N. benthamiana*. Co-expression of the T3SS, AvrPto and GUS resulted in higher GUS accumulation than without the T3SS and AvrPto or in the presence of only one of these proteins ([Raman et al., 2022](#)). Transient co-expression of another five *P. syringae* effectors was tested for increasing recombinant yields in *N. tabacum*. This investigation showed that three of these effectors (HopAO1, AvrPtoB and HopI1) increased recombinant protein accumulation but not significantly ([Buyel et al., 2015](#)). The work in this chapter

expands on these studies by testing the impacts of effector co-expression on recombinant protein accumulation in *N. benthamiana*. In order to do this, we will screen 130 RxLR effectors from plant pathogen *Phytophthora infestans* for effects on recombinant protein accumulation. Simultaneously, we will monitor how plant defence manipulation affects *A. tumefaciens* using AgroLux.

P. infestans is an oomycete pathogen and causal agent of the notorious late blight disease in potato and tomato ([Fry, 2008](#)). *P. infestans* expresses a large suite of effector proteins in order to overcome plant defence responses, up to 563 of which are predicted to be RxLR effectors ([Yin et al., 2017](#)). They are classed as such due to a conserved Arg-X-Leu-Arg (RxLR) amino acid motif. These RxLR effectors are secreted into the plant cell and manipulate host processes at various subcellular locations ([Yin et al., 2017](#); [Whisson et al., 2016](#)). Given that *N. benthamiana* is in the natural host range of *P. infestans*, we concluded that the RxLR effectors are a suitable group of effectors to test if manipulating *N. benthamiana* immunity by co-expression of them would increase recombinant protein yields. A library of 130 cloned but uncharacterised RxLR effectors was provided for our tests by Suomeng Dong (Nanjing Agricultural University, China). Prior to screening this library two positive controls were selected. The first was *P. syringae* AvrPto. This was selected because it had been shown in previous studies to be effective at improving recombinant protein production but through different delivery systems ([Tsuda et al., 2012](#); [Raman et al., 2022](#)). The second was *P. infestans* AvrBlb2, which was selected because it is a characterised RxLR effector that can inhibit secretion of the C14 protease into the apoplast ([Bozkurt et al., 2011](#)).

In this chapter, we aim to improve plant molecular farming yields by suppressing plant immune responses to agroinfiltration by co-expressing RxLR effector proteins. In order to do this, we have screened two selected effectors and a library of 130 *P. infestans* RxLR effectors using the ‘Traffic Light Screen’.

4.3 Results

4.3.1 Effectors AvrBlb2 and AvrPto reduce accumulation of secreted RFP.

To establish controls, two characterised effectors were chosen to be tested with the ‘Traffic Light Screen’. The first, AvrBlb2, is a characterised RxLR effector from *Phytophthora infestans*. AvrBlb2 specifically inhibits secretion of plant protease C14 and reduces plant resistance to *P. infestans*, increasing pathogen growth ([Bozkurt et al., 2011](#)). The second selected effector is the *P. syringae* effector, AvrPto. This effector reduces plant resistance by inhibiting PRRs such as FLS2 and EFR, subsequently interfering with PTI ([Xiang et al., 2008](#)). We selected these two effectors because they both inhibit PTI when transiently expressed in *N. benthamiana*, and therefore we expected them to improve secreted protein expression.

The two effectors were co-expressed using Agrobacterium-mediated transient expression alongside two of the Traffic Light Screen components (AgroLux and secRFP) (Figure 4.1). This was carried out by mixing three *A. tumefaciens* strains: AgroLux carrying an expression cassette for P19, *A. tumefaciens* carrying an expression cassette for secRFP and *A. tumefaciens* carrying an expression cassette for expression of one effector. The RxLR effector plasmids encode GFP, therefore GFP was omitted from the screens in this chapter. We did not check for expression of the effectors by protein or mRNA analysis but instead used the quantified impact on either AgroLux bioluminescence or secRFP fluorescence (Figure 4.1, 4.2, 4.3, 4.4) as evidence that expression of the effectors was taking place. At 5dpi, leaf discs were taken and measured as set out in Chapter 3. AgroLux was increased with the expression of the two effectors but not significantly (Figure 4.1A). Co-expression of both AvrBlb2 and AvrPto resulted in a reduction in secRFP fluorescence (Figure 4.1B). This reduction in transient expression was unexpected because expression of both in *N. benthamiana* suppress PTI and AvrPto expression has been reported to improve transformation of *N. benthamiana* and *A. thaliana* ([Tsuda et al., 2012](#); [Raman et al., 2022](#)). These data indicate that co-expression of AvrBlb2 and AvrPto with the reporters significantly affect secRFP fluorescence and therefore are suitable controls for

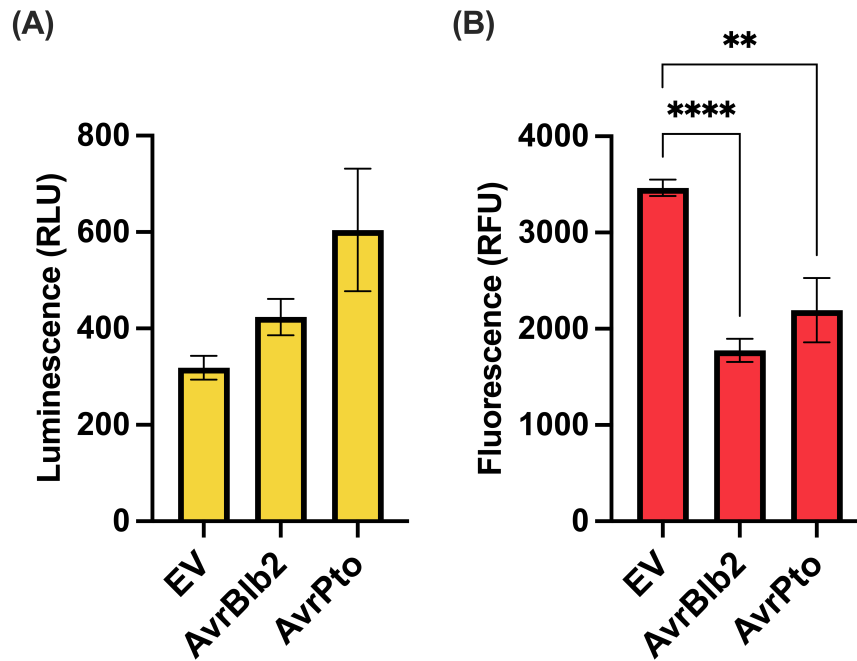


Figure 4.1: Effectors AvrBlb2 and AvrPto reduce secRFP fluorescence. (A) Bioluminescence was not affected significantly by co-expression AvrBlb2 or AvrPto. (B) secRFP accumulation was significantly reduced with coexpression of AvrBlb2 and AvrPto. Four-week-old *N. benthamiana* plants were agroinfiltrated with AgroLux and for expression of secRFP and either *Pi*AvrBlb2 or *Ps*AvrPto. At 5dpi leaf discs were taken and measured for luminescence (A) and fluorescence (550/585nm (B) in a plate reader. Eighteen replicates were used for each measurement, for which mean and standard error of mean are indicated. Similar results were obtained in two more experimental replicates. Data was analysed by ANOVA with Dunnett's post hoc test. This data was collected by Frederick King.

the RxLR effector screen.

4.3.2 'Traffic Light' Screen reveals that five RxLR effectors increase secreted RFP fluorescence

Having established two control effectors, we next screened for effectors capable of improving recombinant protein accumulation using the 'Traffic Light Screen'. We co-expressed a library of 130 *P. infestans* effectors with AgroLux and secRFP (Figure 4.2, 4.3, 4.4). CytoGFP was omitted in this screen because some of the effectors were cloned into a PbinGFP2 binary plasmid. This plasmid encodes GFP as a selection marker, making transiently expressed cytoGFP an unsuitable reporter for recombinant protein accumulation. *P. infestans* can infect *N. benthamiana* (Whisson *et al.*, 2016) and therefore

we hypothesised that co-expression of *P. infestans* RxLR effectors would disrupt *N. benthamiana* PTI responses and reduce the negative effects of immunity on transient expression of recombinant proteins. Furthermore, RxLR effectors have been described to affect *N. benthamiana* PTI in various ways including interacting with proteins in the nucleus, targeting proteins involved in defence signalling ([Wang et al., 2019](#)), affecting vesicle trafficking ([Petre et al., 2021](#)) and impacting secretion ([Wang et al., 2021](#)). *P. infestans* is an oomycete pathogen and hence extremely unrelated to *A. tumefaciens*. We hypothesised that expressing effectors from a very unrelated species of pathogen would give *A. tumefaciens* access to a suite of pathogen effectors so distinct from its endogenous effectors that it could defence itself from different elements of plant defence than if we co-expressed effectors from a more related, bacterial pathogen. For these reasons, we chose to test co-expression of *P. infestans* RxLR effectors.

The effectors were split into five agroinfiltration groups in order to make agroinfiltrations manageable. Fluorescence and bioluminescence were quantified and normalized to the empty vector (EV) control from each group (Figure 4.2, 4.3, 4.4). Analysis of secRFP fluorescence shows that five effectors caused significant increases in accumulation (see Table 4.1 and Figure 4.2A, 4.3A, 4.4A), giving five positive hits from the screen which were renamed E1-5. Eight RxLR effectors caused significant changes to AgroLux bioluminescence (Figure 4.2B, 4.3B, 4.4B), but no effectors increased both secRFP and AgroLux. This confirms that manipulation of *N. benthamiana* immunity by effector expression can affect secRFP accumulation and Agrobacterium metabolic state, but that increased AgroLux bioluminescence at 5dpi does not correlate to increased secRFP fluorescence.

C8P3 co-expression caused a 1.4 fold increase in secRFP fluorescence (Figure 4.2B), however this was not significant. C8P3 shares a highly similar amino acid sequence (99.12% identity) with characterised effector, *PiAvr2*. Given the high similarity with Avr2 and large increase of secRFP with co-expression of it, we decided to pursue C8P3 further and include it as a positive hit (renamed E6). We hoped that the characterisation of E6 would help provide hypotheses for the reasons E6 co-expression increased recombinant protein accumulation that could be tested in the future. However, as described later, E6

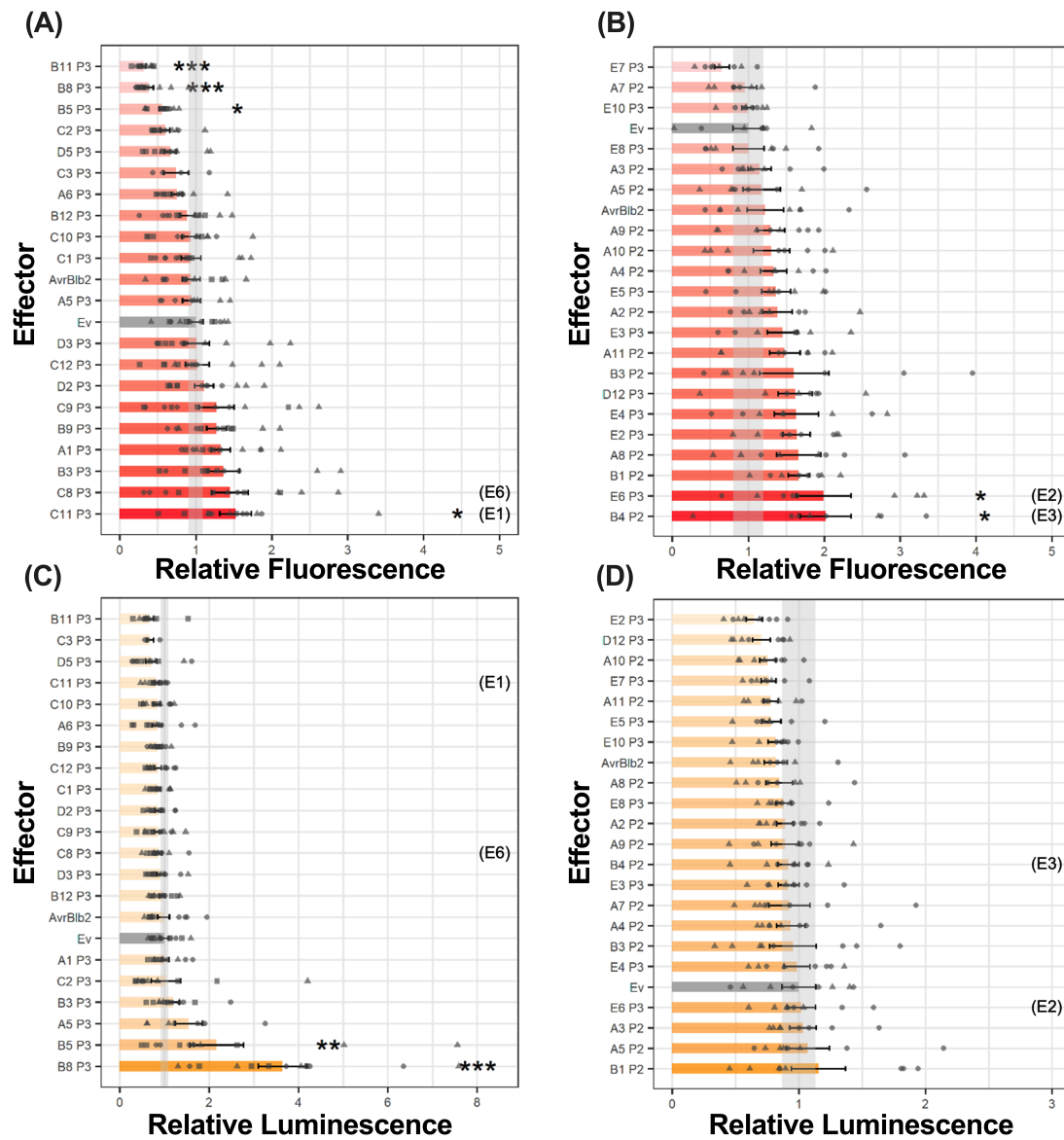


Figure 4.2: Screen of 130 RxLR effectors on AgroLux and secRFP, Groups 1 and 2. Four-week-old *N. benthamiana* plants were agroinfiltrated with AgroLux and for expression of secRFP and one uncharacterised RxLR effector. At 5dpi leaf discs were taken and measured for fluorescence (550/585nm (**A** and **B**) and luminescence (**C** and **D**) in a plate reader. Eight replicates were used for each measurement, for which mean and standard error of mean are indicated. Similar results were obtained in two more experimental replicates. Data was analysed by ANOVA with Dunnett's post hoc test. This data was generated collected by Frederick King.

co-expression did not increase final yield of a recombinant protein.

Candidate Name	Effector Name	Relative secRFP fluorescence
E1	C11P3	1.52

E2	E6P3	1.99
E3	B4P2	2.02
E4	E6P1	1.69
E5	F3P1	1.97
E6	C8P3	1.45

Table 4.1: Six positive hits taken from ‘Traffic Light Screen’ of 130 RxLR effectors.

4.3.3 Confirming positive hit effects on accumulation of an antibody

To test if the positive hits from the screen also had a positive impact on an industrially-relevant recombinant protein, we co-expressed them with the HIV-neutralising antibody VRC01 (Figure 4.5). We co-expressed the six positive hits with the light and heavy chains of VRC01 in four week-old *N. benthamiana*, took leaf disc samples at 5dpi and extracted total soluble protein. Proteins were analysed by western blotting with α - κ IgG antibody to detect the heavy chain of VRC01 (Figure 4.5). The heavy chain (HC) was detected at the expected size of approximately 55kDa in reducing conditions (Figure 4.5). Lanes are grouped based on the plant in which the effectors and VRC01 were agroinfiltrated. However from western blotting it is clear that there is a large amount of variation between plants that is in some cases greater than between the effectors and EV treatments. The western blots show that E6, E2 and E4 are the most promising effectors for increasing accumulation of VRC01 (Figure 4.5). Quantification shows that E6 and E4 both cause a significant increase in VRC01 HC accumulation (Figure 4.6).

4.3.4 E6 did not increase antibody yield in industrial conditions

We next wanted to test if the effectors increased VRC01 accumulation at an industrial scale. As part of an internship with my industry CASE partner, Leaf Expression Systems (LES), I was able to use their facilities to test one of these effectors at industrial scales.

Firstly we tested the impact of all six effectors on VRC01 to check if the same

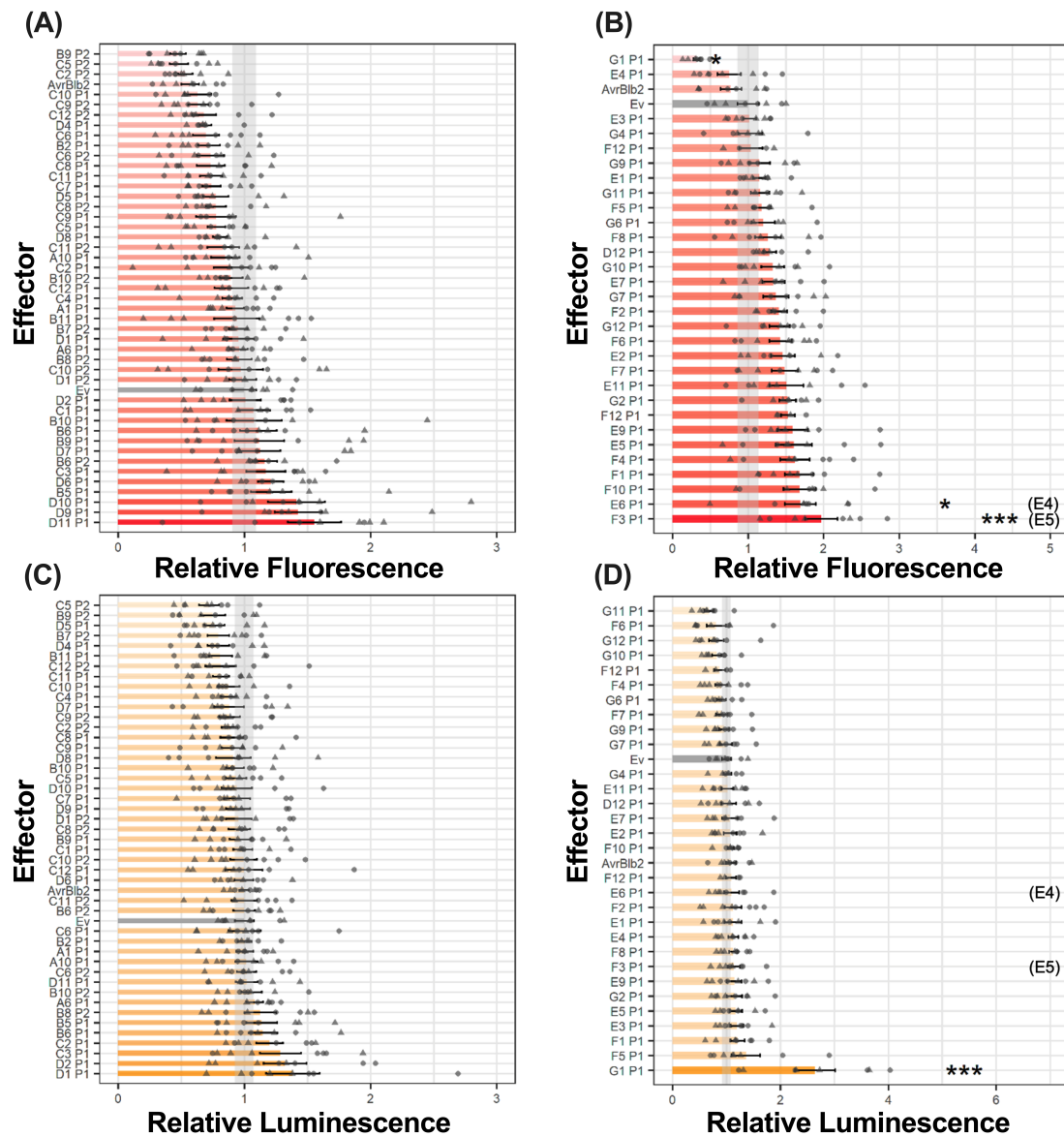


Figure 4.3: Screen for the effects of 130 RxLR effectors on AgroLux and secRFP, Groups 3 and 4. Four-week-old *N. benthamiana* plants were agroinfiltrated with AgroLux and for expression of secRFP and one uncharacterised RxLR effector. At 5dpi leaf discs were taken and measured for fluorescence (550/585nm (**A** and **B**) and luminescence (**C** and **D**) in a plate reader. Eight replicates were used for each measurement, for which mean and standard error of mean are indicated. Similar results were obtained in two more experimental replicates. Data was analysed by ANOVA with Dunnett's post hoc test. This data was generated by Frederick King.

VRC01-increasing effects would be observed at LES (Figure 4.7). The same constructs were used at LES and total protein was analysed by western blotting. At LES both reducing and non-reducing conditions were analysed using an α -IgG antibody that detects both the light and heavy chains of an IgG. Full length VRC01 was detected at the expected

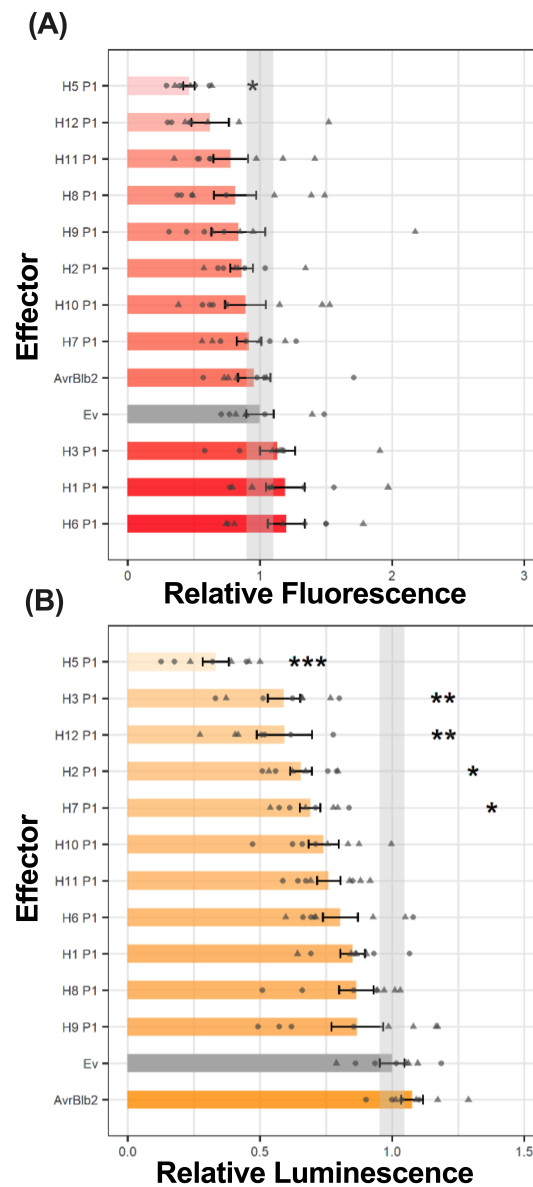


Figure 4.4: Screen for the effects of 130 RxLR effectors on AgroLux and secRFP, Group 5. Four-week-old *N. benthamiana* plants were agroinfiltrated with AgroLux and for expression of secRFP and one uncharacterised RxLR effector. At 5dpi leaf discs were taken and measured for fluorescence (550/585nm **(A)**) and luminescence **(B)** in a plate reader. Eight replicates were used for each measurement, for which mean and standard error of mean are indicated. Similar results were obtained in two more experimental replicates. Data was analysed by ANOVA with Dunnett's post hoc test. This data was generated by Frederick King.

size of 180kDa. We focused on full length VRC01 because our aim was to compare intact, purified VRC01. The effector that caused the highest accumulation of VRC01 was E6 (Figure 4.7). In contrast to the experiments in Oxford, E4 did not increase accumulation of VRC01 (Figure 4.7). This may be caused by the large variation that exists in *N.*

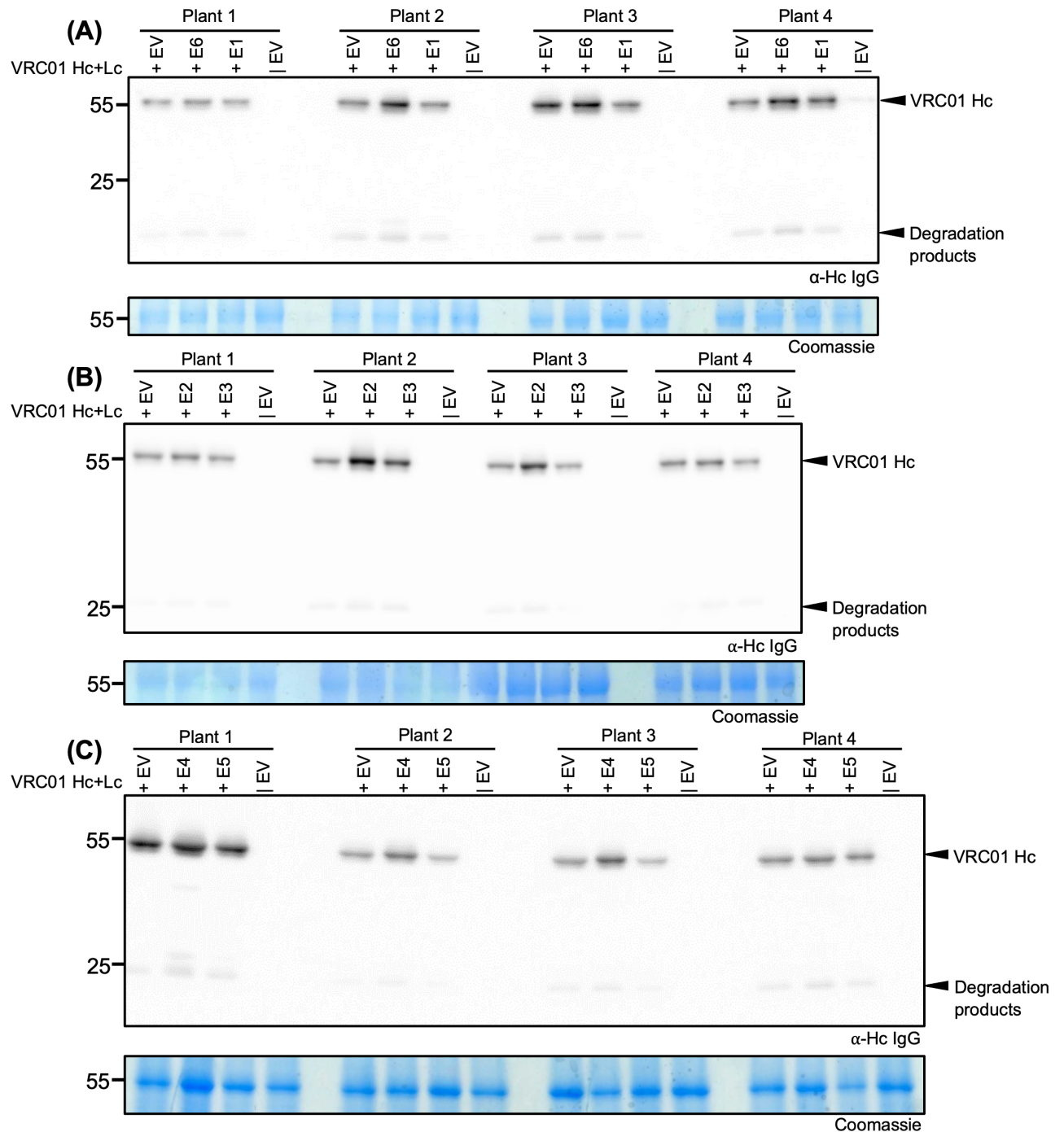


Figure 4.5: Testing six positive hits from RxLR screen on VRC01 accumulation. E6, E2 and E4 appear the most promising effectors for increasing expression of VRC01. Four-week-old *N. benthamiana* plants were agroinfiltrated for co-expression of VRC01 light and heavy chains (LC, HC) and either EV or one of the six positive hits from the effector screen. At 5dpi leaf tissue was taken and its total soluble protein extracted. Proteins were separated on a 12% SDS-PAGE gel and analysed by western blotting using α - γ IgG antibody (to blot for the VRC01 HC) (A, B, C). The lanes are grouped by the plant, all constructs were agroinfiltrated onto a sector of a leaf on four plants. Coomassie stained gel is shown for protein expression and loading control. E6, E2 and E4 appear the most promising effectors for increasing expression of VRC01. Each lane represents one replicate from one experiment. Similar results were obtained in one more experimental replicate. This data was collected by Frederick King.

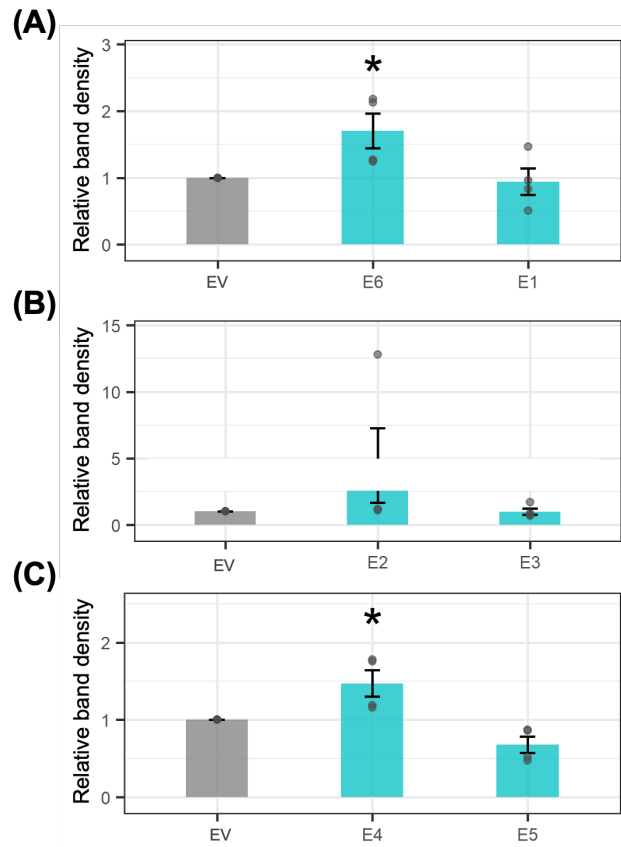


Figure 4.6: Quantified effect of RxLR effector co-expression on VRC01 accumulation. (A) E6 significantly increases VRC01 HC accumulation, E1 has no significant effect. (B) Neither E2 nor E3 has a significant effect on VRC01 HC accumulation. (C) E4 significantly increases accumulation of VRC01 HC, E5 has no significant effect. VRC01 HC bands on blots in Figure 4.5 were quantified using ImageJ Plot Lanes software and normalised relative to the EV treatment band. Four biological replicates were measured, for which mean and standard error of mean are indicated. Data was analysed using a paired t-test, * $P < 0.05$.

benthamiana grown in different conditions, a phenomenon observed in previous efforts to test strategies to improve molecular farming that were successful in Oxford but did not transfer to improving accumulation at LES. This confirms the importance of verifying observed effects in multiple conditions. The differences in VRC01 accumulation were not present when the samples were subjected to reducing conditions (Figure 4.7B). To verify and explain the differences in results in these different condition, in the future these results should be repeated with a total protein loading control and subsequent quantification of bands.

Having confirmed that E6 causes increased VRC01 accumulation in conditions at

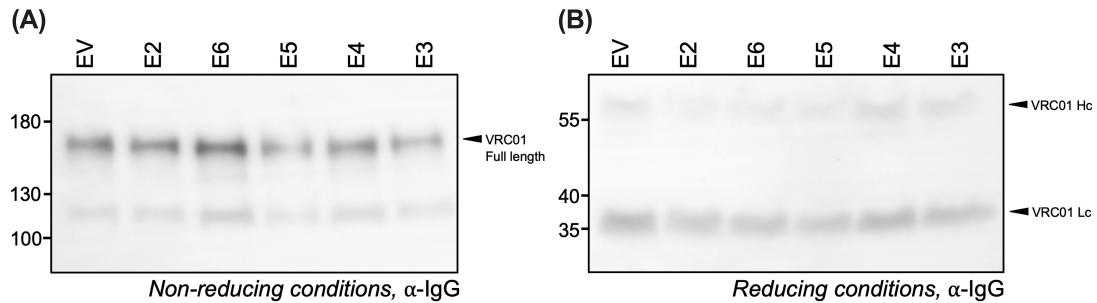


Figure 4.7: E6 causes the highest accumulation of full length VRC01 in comparison to EV. Four-week-old *N. benthamiana* plants were agroinfiltrated for co-expression of VRC01 light and heavy chains (LC, HC) and either EV or one of the six positive hits from the effector screen. At 5dpi leaf tissue was taken and its total soluble protein extracted in either non-reducing (A) or reducing (B) conditions. Proteins were separated on a 12% SDS-PAGE gel and analysed by western blotting using α -IgG antibody. Each lane represents one replicate from one experiment. This experiment was performed only once to check if the same effects on VRC01 accumulation would be observed in conditions at Leaf Expression Systems (Industrial placement host).

LES, we tested co-expression of the effector with VRC01 in a large scale agroinfiltration. *N. benthamiana* were vacuum-agroinfiltrated with *Agrobacterium* constructs carrying expression cassettes for VRC01 LC and HC or for VRC01 LC, HC and E6.

In order to provide the most relevant evidence for E6 use at industrial scale, we compared the E6 agroinfiltration, containing three constructs, to an agroinfiltration lacking a third construct, not an EV construct as had been done previously. This was deemed most suitable because it is how E6 would be used at LES. This method meant that the increase in VRC01 accumulation caused by E6 needs to outweigh the detrimental effects of 50% more *Agrobacteria* entering leaves and the cost of 50% more *Agrobacterium* growth materials and space required.

18 whole plants were vacuum agroinfiltrated for each treatment. The first treatment, labelled 'VRC01 only' (Figure 4.8, 4.9) was agroinfiltrated with a mixture of two *A. tumefaciens* constructs: one with an expression cassette for VRC01 LC and one with an expression cassette for VRC01 HC. The second treatment, labelled 'VRC01+E6' (Figure 4.8, 4.9) was agroinfiltrated with a mixture of three constructs: one with an

expression cassette for VRC01 LC, one with an expression cassette for VRC01 HC and one with expression cassette for effector E6. 150g of leaf tissue was harvested from each agroinfiltration at 5dpi and total protein extracted. VRC01 was purified from the total protein on a Protein A column from the agroinfiltration without an effector (Figure 4.8A) and with E6 (Figure 4.8B). VRC01 was exchanged into PBS buffer (Figure 4.8). The chromatogram showing the detection of VRC01 at 280nm from both the Protein A purification and buffer exchange reveal that more VRC01 was present in the purification from the ‘No effector’ agroinfiltration than from the E6 agroinfiltration (Figure 4.8). This was confirmed by measurement of VRC01 concentration using a NanoDrop and quantification using the Pierce BCA Protein Assay Kit. The ‘No effector’ VRC01 had a concentration of 0.39mg/ml whereas the ‘E6’ VRC01 had a concentration 0.3mg/ml. The smaller peak detected in the VRC01 only Protein A purification chromatogram (Figure 4.8A) is thought to be a contamination and was not collected into a fraction by the ÄKTA.

In order to confirm that the reduced quantity of VRC01 was a true representation of total accumulation, we checked the accumulation of VRC01 in all main fractions of extraction and purification by western blot (Figure 4.9). It is evident that in all fractions, in both reducing and non-reducing conditions, the accumulation of VRC01 is higher in the ‘No effector’ agroinfiltration than with E6 (Figure 4.9).

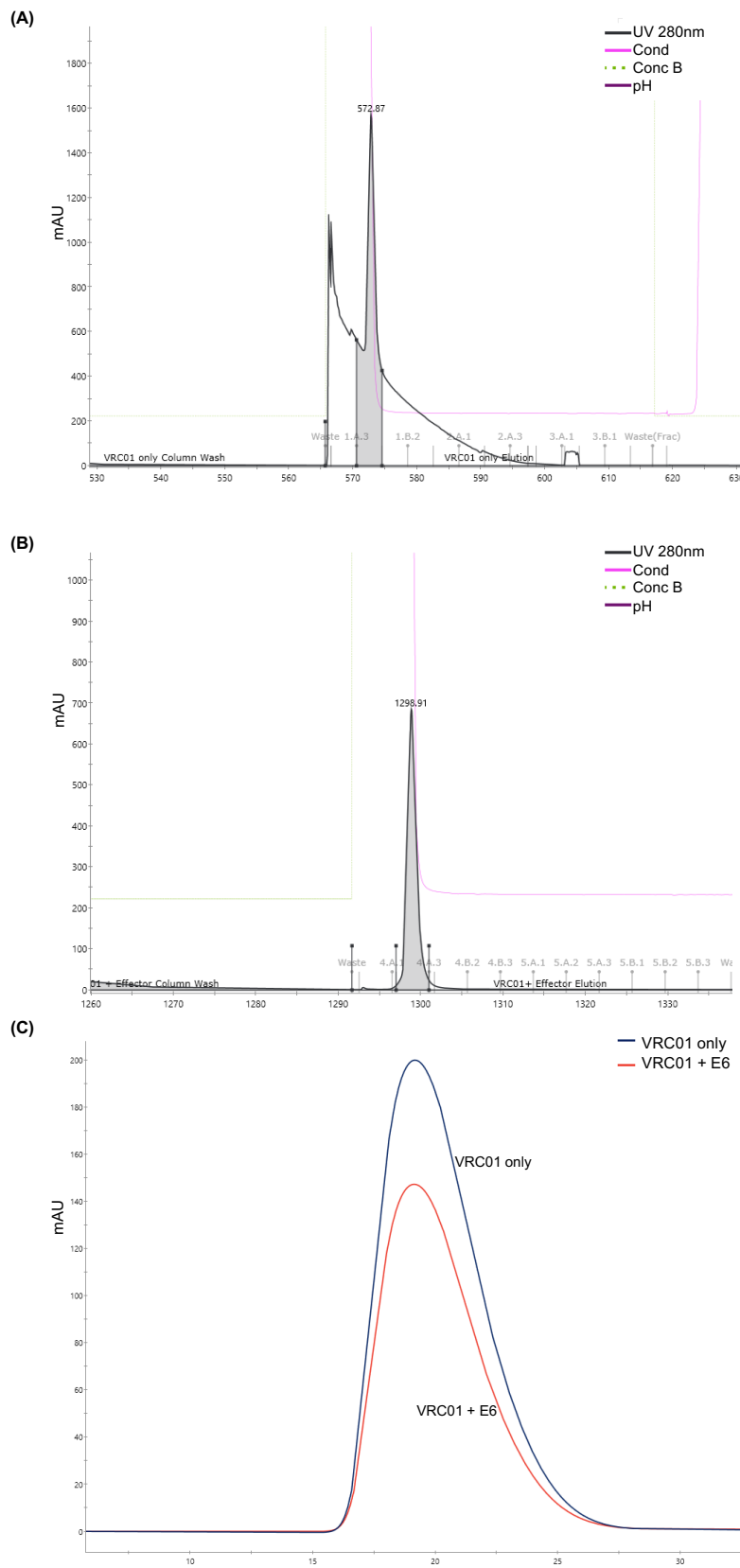


Figure 4.8: (Previous page) Large-scale agroinfiltration and purification of VRC01 from a VRC01 only agroinfiltration in comparison to VRC01 co-expressed with E6. Eighteen whole four-week-old *N. benthamiana* plants were vacuum agroinfiltrated for co-expression of either just VRC01 light and heavy chains or VRC01 (HC and LC) and effector E6. At 5dpi 150g of leaf tissue was taken for each treatment and total soluble protein extracted. Homogenate was filtered and purified on a Protein A column (**A and B**) and exchanged into PBS buffer (**C**) on an ÄKTA. (**A and B**) Chromatogram showing the purification profile of VRC01 from VRC01 only agroinfiltration (**A**) and VRC01 and E6 agroinfiltration (**B**). The dark grey line represents absorbance at 280nm and the light grey shaded zone represents total VRC01 purified by integration. (**C**) Chromatogram showing that VRC01 only (blue line) has more VRC01 purified after buffer exchange than VRC01 and E6 (red line). Blue and red lines represent absorbance at 280nm.

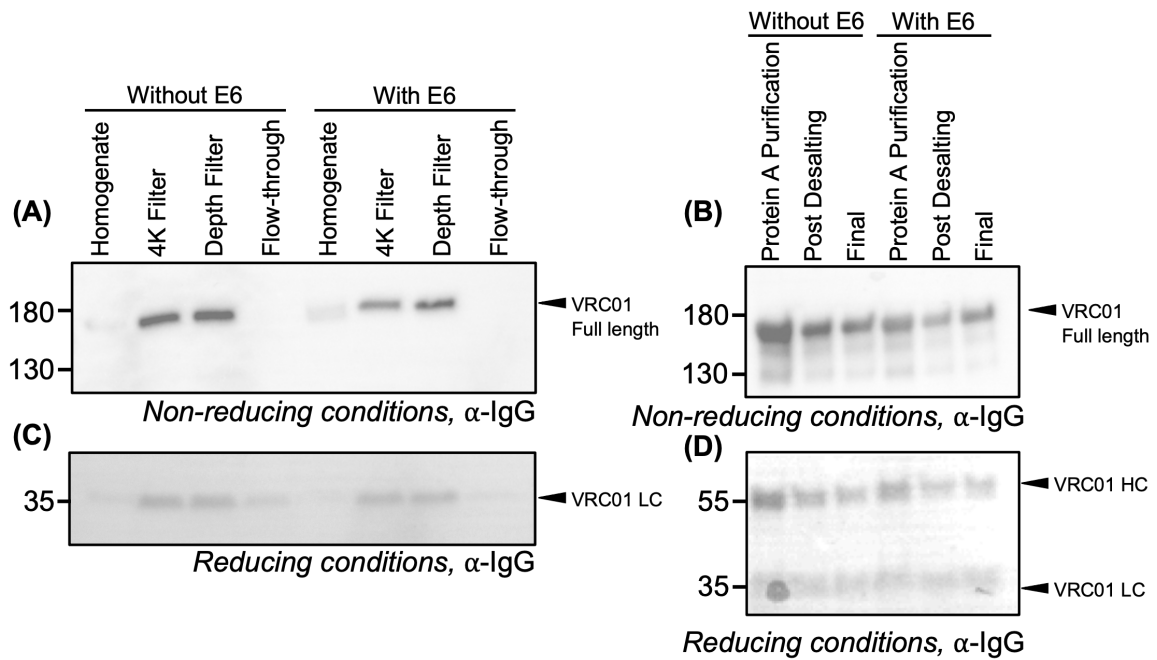


Figure 4.9: α -IgG western blot of all fractions of VRC01 purification with and without E6 show VRC01 only has more VRC01 accumulation throughout extraction and purification. Samples were taken from each of the main stages of extraction and purification. These samples were taken from the fractions before Protein A purification (**A and B**) (after homogenisation, after filtration through a 4K and Depth Filter) and after Protein A Purification (**C and D**) (after purification, after buffer exchange (desalting) and after final miracloth filtration). All fractions were subjected to non-reducing (**A and B**) and reducing (**C and D**) conditions.

4.4 Discussion

In this chapter, we used the ‘Traffic Light Screen’ to investigate the effect of 130 RxLR effectors on transient protein expression and AgroLux bioluminescence. This led us to identify six effectors from this library whose co-expression caused an increase in secRFP fluorescence (Table 4.1). Co-expression of two of these effectors resulted in increased

VRC01 accumulation (Figure 4.5). One of these effectors was then tested at industrial scales for effects on large scale production of pure VRC01, but did not increase production in comparison to a no effector control (Figure 4.8).

The results of this chapter demonstrate the utility of the ‘Traffic Light Screen’ for testing new strategies to improve molecular farming. This work also provides a proof of concept for further testing of co-expressed pathogen effectors to increase yields for plant molecular farming.

4.4.1 Effector co-expression reveals new information about how *A. tumefaciens* responds to plant defence responses.

The ‘Traffic Light Screen’ measured AgroLux bioluminescence after 5 days of effector co-expression. RxLR effectors are delivered by *P. infestans* into *N. benthamiana* cells and affect the plant immune response in a variety of ways to promote virulence ([Whisson et al., 2016](#)). Transient expression of both the characterised positive controls and 130 RxLR effectors affect the plant defence responses occurring in the leaf. Monitoring AgroLux bioluminescence at 5dpi reflects how plant immunity changes caused by effector expression impacts *A. tumefaciens*. ATP, NADP and reduced flavin mononucleotide are required for the bioluminescence reaction produced from the *lux* operon encoded in AgroLux ([Brodl et al., 2018](#)). AgroLux bioluminescence therefore reports the metabolic state of *A. tumefaciens in planta*. Both positive and negative significant changes to AgroLux bioluminescence were detected upon co-expression of the effectors (Figure 4.2B, 4.3B, 4.4B). For example, effector B8P3 caused a 3.8 fold increase in AgroLux bioluminescence. In contrast, effector E7P3 caused a 0.3 fold reduction of AgroLux bioluminescence. Both of these effectors caused cell death in leaves of *N. benthamiana*, representing induction of immune responses. This shows that different mechanisms of cell death cause different changes to AgroLux bioluminescence. Further characterisation of the mechanisms of these effectors and the resulting cell death may reveal the meaning of AgroLux bioluminescence. Increased understanding of plant defence responses that cause changes in AgroLux bioluminescence will make AgroLux a better tool for studying plant immunity. It will also help us to understand what plant defence responses are detrimental to Agrobacterium, and

how to improve plant molecular farming.

4.4.2 Suitability of the Traffic Light Screen for screening strategies to improve recombinant protein production.

Benefits of the ‘Traffic Light Screen’

The main benefit of using this screening system to assess whether the library of effectors would increase recombinant protein yields was the speed at which we could collect data. In only eight weeks it was possible to test all 130 effectors with three experimental replicates for each (Figure 4.2, 4.3, 4.4).

A second benefit of using the ‘Traffic Light Screen’ over traditional recombinant protein analyses was that the ‘Traffic Light Screen’ was much more cost effective. Traditional methods such as ELISA and western blotting require expensive consumables such as specific microtiter plates, antibodies and PVDF membranes. The only consumable used in the ‘Traffic Light Screen’ is a white, opaque 96-well plate which can be sterilised and reused.

The ‘Traffic Light Screen’ was therefore a time and cost effective method to evaluate a library of RxLR effectors for ability to increase recombinant protein yields.

Challenges of the ‘Traffic Light System’

The main challenge faced when using the ‘Traffic Light Screen’ was the high level of variation in secRFP fluorescence within leaves, within plants and between plants (Figure 4.5). To minimise this variation we always used leaves at the same developmental stage, only one leaf per plant and the same plant age for all replicates. In order to account for within leaf variation, at least two leaf discs per agroinfiltrated region were measured.

secRFP as a reporter for recombinant protein accumulation

Another challenge we faced using the ‘Traffic Light Screen’ was that increases in secRFP fluorescence did not always translate to increases in antibody accumulation. There were six positive hits from the screen but only two of these increased VRC01 accumulation (Figure 4.2, 4.3, 4.4, 4.5). One of these two was tested at industrial scale but did not

increase VRC01 production (Figure 4.8, 4.9). At Leaf Expression Systems (LES), in the large scale agroinfiltration, effector co-expression was compared to a control in the absence of an EV construct (Figure 4.8, 4.9). Comparison of the effector to an agroinfiltration including an empty vector control, so that equal ODs were compared, was considered. However, comparison to an infiltration containing no third construct was chosen because this is how it would be incorporated into agroinfiltrations at LES and as a result would give a true indication of the benefit of E6 co-expression to antibody yields in industrial conditions. As a result, we compared the effector treatment agroinfiltrated with 1.5 times the concentration of *A. tumefaciens* than the control. The absence of positive impact caused by effector co-expression in this comparison demonstrates the need for a large improvement to recombinant protein yields in order for the yield increase to outweigh costs of an added construct.

The first possible reason for this inconsistency is that effector expression may change the pH and redox environment in the apoplast, both of which have been observed upon *P. infestans* infection ([Westphal et al., 2019](#); [Chaparro-Garcia et al., 2015](#)). This could lead to altered RFP fluorescence without changing VRC01 accumulation by two mechanisms. The first mechanism is that there may not have been more secRFP but instead that the fluorescence of RFP was increased in altered apoplastic conditions. The levels of fluorescence emitted from fluorescent proteins such as RFP are affected by pH and redox ([Ward et al., 1982](#); [Campbell et al., 2002](#); [Ward et al., 1982](#)). The apoplastic environment may be altered after RxLR effector expression, changing the levels of fluorescence emitted by RFP without affecting actual RFP levels. The second mechanism is due to differences in processing of VRC01 and RFP by apoplastic proteases. A change in the apoplastic environment may cause increased degradation of VRC01 by changing the activity of proteases in the apoplast ([Felle et al., 2005](#)). RFP and VRC01 are structurally distinct and of different sizes ([Campbell et al., 2002](#); [Teh et al., 2014](#)). Their different size and lack of structural similarity may mean that they are processed differently. This could lead to higher stability of RFP in the apoplast in comparison to VRC01, meaning that even if more VRC01 is produced initially, there is less accumulation of intact VRC01 detected.

In future screens, instead of using a stable and well-expressed recombinant protein

like RFP, a better strategy may be to screen for effects of changing conditions on poorly expressed or unstable recombinant proteins. This may yield more positive impacts on protein yield if starting yield is lower. However, using non-fluorescent proteins will reduce the extent to which the screen is high-throughput and limit the number of strategies investigated.

4.4.3 Effector screen is a proof of concept for further screens

This chapter is a proof-of-concept for co-expression of pathogen effectors as a strategy to improve molecular farming. Although the differences to secreted RFP did not translate into changes to purified VRC01 when co-expressed with one of the positive hits (Figure 4.2, 4.3, 4.4, 4.8), this does not mean that the observed increases of secRFP fluorescence were unimportant or that they will not translate into positive effects on other recombinant proteins. Firstly, effector co-expression could only be tested at industrial scales with one effector due to time restraints. Other positive hits may have caused different results and can be investigated in the future when they are characterised further. Secondly, the positive hits may be successful in increasing production of other IgGs and other recombinant proteins. Unpublished data from our lab shows that different IgGs have different sensitivities to proteases (Beritza 2022, personal communication). Thus, the effectors may have had a different effect on an IgG different from VRC01. Plant molecular farming is used to produce a wide range of recombinant proteins that are structurally different to IgGs such as virus-like particles, growth factors and cytokines (*Schillberg and Finner, 2021*). Testing the effectors on different recombinant proteins may show yield increases. Further screens should expand beyond effectors and also include co-expression of proteins such as chaperone proteins, proteins preventing oxidation by polyphenol oxidases and protease inhibitors, to prevent detrimental effects of plant immunity.

The future of effector screens for improving molecular farming yields.

Despite the effectors from this screen providing no clear choice for improving molecular farming, we believe that this screen has provided promise for the theory of using pathogen effectors to improve recombinant yields. Firstly, effects on secRFP and VRC01

accumulation were observed, showing that despite effectors being expressed after T-DNA transfer has occurred, they can still affect recombinant protein production (Figure 4.2, 4.3, 4.4, 4.5). In future it may be a better strategy to test effectors that are known to be involved in PTI three days prior to agroinfiltrating for expression of recombinant proteins. Using this method, the expressed effector could manipulate plant defence while the next agroinfiltration is eliciting the plant immune response.

Secondly, our knowledge and characterisation of plant pathogen effectors is improving. With time and increasing numbers of functional screens of pathogen effectors, a more targeted approach to choosing effectors that will be part of screens will be possible and may yield better results.

4.5 Conclusions

This chapter presented a screen of 130 *P. infestans* RxLR effectors for effects on transient RFP expression and AgroLux bioluminescence, resulting in six positive hits. From these positive hits, two had significant effects on accumulation of IgG antibody, VRC01. One of these effectors was then tested in industrial settings and scale but it did not increase yield of VRC01 produced compared to a no effector control. From this chapter we present evidence that the ‘Traffic Light Screen’ is a useful way to screen pathogen effectors for improving molecular farming and for better understanding of *A. tumefaciens* during transient expression.

Chapter 5

A screen of *N. benthamiana* depleted of immunity genes reveals four promising strategies to improve molecular farming yields

Isobel L. Dodds, Changlong Chen, Pierre Buscaill, Nicholas Holton
and Renier A. L. van der Hoorn

Changlong Chen helped to perform the first experimental replicate for data presented in Figure 5.7. Pierre Buscaill helped to design experiments for data presented in Figure 5.7. Nicholas Holton provided intellectual advice and support throughout the project. Renier A. L. van der Hoorn supervised and guided the project.

5.1 Abstract

Production of recombinant proteins is challenged by the plant immune response to agroinfiltration. In this chapter, we screened *N. benthamiana* with impaired expression of genes known to be involved in the plant immune response in order to produce *N. benthamiana* for increased transient expression. Four week-old plants silenced for *NbICS*, *NbCERK1* and *NbNPR1* accumulated more recombinant protein than the control plants and offer promising strategies for improving molecular farming. The cold shock protein peptide, csp22, is thought to be the elicitor of PTI in *N. benthamiana* by *Agrobacterium*. In this chapter we show that the perception of csp22 in *N. benthamiana* requires CORE and that depletion of *NbCORE* abolishes the csp22-induced ROS burst. Furthermore, we show that depletion of CORE in 6 and 8 week-old *N. benthamiana* enables successful transient expression of eGFP, whereas 6-8 week-old plants typically produce low yields of recombinant proteins. Enabling use of older plants with more biomass for agroinfiltration by CORE depletion can increase yields of recombinant proteins.

5.2 Introduction

In addition to an innate response, mammals have evolved complex, adaptive immune responses and mobile defender cells that allow them to fight off infection by pathogens (*Zipfel and Felix, 2005*). However, plants rely solely on an innate immune system. This involves perception of pathogen-associated molecular patterns (PAMPs) by stationary cells and subsequently released immune signals from the infection site (*Chisholm et al., 2006*). Some plants recognise pathogen virulence molecules, called effectors, to cause further immune signalling and defence responses. Despite lacking an adaptive response, there are still many elements of immunity in plants which facilitate successful resistance to a pathogen. Furthermore, an evolutionary arms race is in constant battle as pathogens evolve to evade recognition and plants evolve to recognise pathogens once more (*Martel et al., 2021*).

For the immune response to occur, perception of pathogen presence is vital. This occurs through recognition of PAMPs by PRRs, located in the plasma-membrane (*Zipfel, 2014*). Notable PAMPs include flagellin peptide, flg22, and EF-Tu peptide, elf18, which are detected by PRRs FLS2 and EFR, respectively (*Gómez-Gómez and Boller, 2000; Chinchilla et al., 2007; Felix et al., 1999; Zipfel et al., 2006*). Upon recognition of PAMPs by these receptors, PRRs recruit co-receptor BAK1, and these receptor complexes then induce immune signalling via phosphorylation, resulting in PAMP-triggered immunity (PTI) (*Chinchilla et al., 2007; Heese et al., 2007; Yuan et al., 2021*). PTI then comprises responses including ROS bursts, callose deposition, Ca^{2+} influx and salicylic acid signalling (*Li et al., 2014; Tian et al., 2019; Sreekanta et al., 2015; Lal et al., 2018*). Successful pathogens suppress PTI by secreting virulence molecules called effectors, resulting in susceptibility of the plant to infection (*Jones and Dangl, 2006*). However, in some cases plants have evolved to recognise these effectors as non-self by specific disease resistance (*R*) genes (*Dangl and Jones, 2001*). *R* genes typically encode nucleotide-binding and leucine-rich repeat (NB-LRR) proteins, and their perception of effectors causes Effector-Triggered Immunity (ETI) (*Jones and Dangl, 2006*). A huge suite of immunity genes encode proteins required for each of these steps and their depletion by silencing increases susceptibility to pathogen

infections (*Balaji et al.*, 2011).

An important part of plant defence is the upregulation of salicylic acid biosynthesis (Figure 1.2). Salicylic acid is a vital phytohormone with roles in PTI, ETI and systemic-acquired resistance (SAR) (*Yan and Dong*, 2014). Salicylic acid is produced through two pathways in plants. In the first pathway, SA is produced through conversion of chorismate to isochorismate by isochorismate synthase (ICS) (Figure 1.2). Isochorismate is then converted into isochorismoyl-9-glutamate (IC-9-Glu), which then breaks down into SA and 2-hydroxy-acryloyl-N-glutamate (2HNG) or N-pyruvoyl-L-glutamate (NPG). This is the ICS pathway. The second pathway is the PAL pathway. Phenylalanine ammonia-lyase (PAL) converts phenylalanine into trans-cinnamic acid which can then be converted into SA (Figure 1.2). Silencing of *ICS* and *PAL* results in significantly reduced levels of SA (*Chen et al.*, 2010; *Catinot et al.*, 2008; *Pallas et al.*, 1996). Pathogen infection induces increases in endogenous SA (*Malamy et al.*, 1990) after PTI-induced upregulation of genes including *ICS*, *PAL* and *EDS5*, all three of which are involved in SA biosynthesis (*Jones and Dangl*, 2006). Increase in SA causes transport of Nonexpresser of PR genes 1 (NPR1) monomers into the nucleus, where NPR1 acts as a transcription coactivator by direct interaction with TGA transcription factors, inducing upregulation in transcription of pathogenesis-related genes (Figure 1.2) (*Moore et al.*, 2011; *Fan and Dong*, 2002). SA accumulation and signalling can also cause expression of PR proteins in uninfected tissue through NPR1 regulation. This systemic expression of defence genes is called systemic acquired resistance (SAR) and is effective at preventing successful infection of a range of pathogens (*Sticher et al.*, 1997).

N. benthamiana elicits an immune response upon agroinfiltration, indicated by upregulation of secreted defence proteins (*Goulet et al.*, 2010) and a decrease in transcripts of photosynthesis proteins (*Grosse-Holz et al.*, 2018a). We hypothesised that identification of important immunity genes that trigger defence against *Agrobacterium* and subsequent silencing of these genes would increase molecular farming yields because the defence response to agroinfiltration would be hindered.

Despite having larger biomass for agroinfiltration, older, fully developed *N. benthamiana* plants are not used for producing recombinant proteins because they support

low levels of transient gene expression ([Saur et al., 2016](#)). It is thought that this is because they are able to perceive Agrobacterium cold shock protein (CSP), where as younger plants cannot ([Saur et al., 2016](#)). A 22 amino acid long fragment of CSP, called csp22, elicits a ROS burst in older plants but not younger plants, suggesting it is recognition of this peptide that enables mature *N. benthamiana* to perceive Agrobacterium and hamper molecular farming.

The receptor that recognises CSP is disputed between CSPR and CORE ([Saur et al., 2016](#); [Wang et al., 2016](#)). Both *NbCSPR* and *NbCORE* have been suggested as the receptor of csp22. Transcriptomics showed that expression of both are induced in older plants and there has been experimental evidence supporting the case for each ([Saur et al., 2016](#); [Wang et al., 2016](#)). *NbCSPR* depletion by virus-induced gene silencing suppressed the csp22-induced ROS burst ([Saur et al., 2016](#)). An RLK from tomato, CORE, was overexpressed in young *N. benthamiana* and enabled csp22 responsiveness. Furthermore, the *NbCORE* ortholog conferred csp22 responsiveness to the csp22-insensitive *A. thaliana* ([Wang et al., 2016](#)). Evidence exists for both receptors, but we hypothesised that confirmation of the essential receptor for Agrobacterium recognition and subsequent silencing of it, would enable prevention of the mature plant immune response to agroinfiltration. These csp22-insensitive *N. benthamiana* could then be used when mature for molecular farming, and agroinfiltration of their larger biomass would enable increased recombinant protein yields.

The plant immune response is multifaceted and controlled by expression of many immunity genes. In this chapter, we aim to reduce the *N. benthamiana* defence against agroinfiltration by silencing immunity genes involved in a variety of stages of the immune response. By silencing the defence response to agroinfiltration, we aim to improve yields of recombinant protein produced.

5.3 Results

5.3.1 Screen of plants with impaired expression of characterised plant immunity genes

Screen of VIGS plants reveals three positive hits

In order to uncover the *N. benthamiana* immunity genes which hamper recombinant protein production and Agrobacterium state, twenty characterised genes were silenced using Virus-Induced Gene Silencing (VIGS). These genes are characterised as having functions in a variety of plant defence responses. To test the role of PTI receptors, we silenced the PRRs *CERK1*, *CSPR* and *CORE* ([Willmann et al., 2011](#); [Saur et al., 2016](#); [Wang et al., 2016](#)); the RLK that facilitates kinase domain-lacking RLPs called *SOBIR1* ([Liebrand et al., 2013](#); [Gust and Felix, 2014](#)); the co-receptor *BAK1* ([Chinchilla et al., 2007](#)) and the wall-associated kinase *WAK1* ([Rosli et al., 2013](#)). We tested the role of transcriptional reprogramming by silencing WRKY transcription factors ([Wani et al., 2021](#)). We have also investigated the role of nucleotide-binding–leucine-rich-repeat (NLR) receptor-mediated defence responses by silencing *EDS1*, *SAG101* ([Tran et al., 2016](#)) and three *NRC* genes (*TRV::NRC234*) ([Wu et al., 2017](#)). The role of calcium signalling was tested by silencing *CDPK* ([Romeis et al., 2001](#)), MAP kinase signalling by silencing *MPK3/6* ([Liu et al., 2021](#)), ROS burst production by silencing *RbohB* ([Asai et al., 2008](#)) and the role of apoplastic pH by attempting to silence *AHA2*. *R* gene-mediated defence was investigated by silencing *ACIF1* and *CRT3a* ([van den Burg et al., 2008](#); [Liebrand et al., 2012](#)). Finally, phytohormone signalling was tested by silencing *ICS*, *PAL*, *NPR1* and *EIN2* ([Chen et al., 2009](#); [Tran et al., 2016](#)).

In order to knock-down characterised immunity genes in *N. benthamiana*, VIGS was carried out. The protocol used in this thesis used Tobacco Rattle Virus (TRV) based vectors. TRV is a bipartite RNA virus composed of TRV RNA1 (hereon referred to as TRV1) which encodes the viral replicase, RNA-dependent RNA polymerase and movement protein ([Hayward et al., 2011](#)). TRV RNA2 (hereon referred to as TRV2) encodes the viral coat protein and has been engineered to incorporate fragments of host genes (TRV2::host gene) name ([Hayward et al., 2011](#)). TRV1 and TRV2 (encoding fragments of different

host genes) can be delivered to plants by *Agrobacterium* mediated transient expression and results in silencing of the host gene encoded by the TRV2::fragment ([Hayward et al., 2011](#)).

Two week-old *N. benthamiana* were agroinfiltrated for co-expression of TRV1 with TRV2 constructs for each gene of interest (see Table 5.1). At three weeks post agroinfiltration the *TRV2::PDS* silencing control line showed significant bleaching, reflecting successful silencing. Plants were then agroinfiltrated with AgroLux and strains carrying expression cassettes for cytoGFP and secRFP, the ‘Traffic Light’ reporters. After 5 days, leaf discs were punched and measured in the plate reader for bioluminescence and fluorescence of cytoGFP and secRFP. The screen was performed in two groups in order to make both agroinfiltrations a manageable size. From this screen, we found that *TRV2::RbohB* plants increased bioluminescence of AgroLux (Figure 5.1), but this was not consistent across the three experimental replicates. *TRV::PAL* plants have increased AgroLux bioluminescence in this repetition (Figure 5.2) which is repeated across the other experimental replicates and is a significant increase in one replicate. This leads us to conclude that *PAL* silencing possibly leads to increased AgroLux bioluminescence. We found that *TRV::WRKY7/8/9/11/12/14* plants and *TRV::ICS* plants had significantly reduced bioluminescence of AgroLux (Figure 5.1). *TRV::ICS*, *TRV::NPR1* and *TRV::CERK1* plants all had significantly increased fluorescence of both cytoGFP and secRFP (Figure 5.1, 5.2). *TRV::EIN2* plants had significantly more cytoGFP fluorescence but not more secRFP fluorescence (Figure 5.1). Given that most recombinant protein produced from molecular farming is secreted into the apoplast, we therefore concluded that the positive hits from this screen to test again for effects on recombinant protein production were *TRV::ICS*, *TRV::NPR1* and *TRV::CERK1* plants.

Some phenotypic differences were observed in VIGS plants. *TRV::ICS* and *TRV::EIN2* plants had pale, yellowing leaves and were smaller than *TRV::GUS* plants (Figure 5.3). *ICS* is required to produce phyloquinone, a vital component for photosynthesis. The reduction in phyloquinone may cause the pale leaf phenotype observed in *TRV::ICS N. benthamiana* plants. Ethylene over-production results in reduction of chlorophyll ([Foo et al., 2006](#)). If this is *EIN2*-independent it is possible that in *TRV::EIN2* plants, ethylene overproduction

is occurring as a result of ethylene insensitivity, then leading to a reduction in chlorophyll production and the pale leaf phenotype. *TRV::CRT3a*, *TRV::EDS1*, *TRV::PAL* and *TRV::SOBIR1* plants were smaller than *TRV::GUS* plants (5.3). *TRV::CDPK* plants were very small and had pale green leaves (5.3). *TRV::BAK1* plants were smaller with dark green, thick and elongated leaves (5.3). See Figure 5.3 for pictures of all VIGS lines.

Screen of knock-out plants reveals no positive hits

In addition to the VIGS *N. benthamiana* plants, we also screened a group of immunity gene knock-out lines (Figure 5.4). These were all pre-existing lines obtained either through recent experiments in our lab or from collaborators (See Table 5.2). We used plants that lack the EDS1 family proteins EDS1, SAG101, and PAD4 in a variety of combinations (see Table 5.2) and *nrg-1*, *nrg1/adr1* and *nrc234* knock-out plants to test the role NLR-mediated immunity. We used *bak1-1* and *sobir1/sobir1-like* knock-out lines to investigate the effects of PTI. *ics* and *rbohB* knock-out lines were used to test the effects of reducing SA signalling and ROS burst generation. However, these lines were recently generated and were found to be incomplete knock-outs. The *ics* mutant contains a single-nucleotide deletion mutant of one copy of the *ICS* gene (NbD005352) but contains an intact form of the other copy of *ICS* (NbD032217) (Felix Homma, personal communication). The *rbohB* mutant also silenced only one of two *rbohB* homeologues and *rbohB* leaf discs still produced a ROS burst in response to flg22 (NbD019394.1 was edited but not NbD039395.1). The *rbohB* ROS burst was weaker than elicited from WT plants, but not abolished as it is in *TRV::RbohB* plants (personal communication, Mariana Schuster). Furthermore, *bak1-1* lines contain two edited *BAK1* genes (Niben101Scf02128g00022.1; Niben101Scf02513g11004.1). However, there are four *BAK1* encoding genes in the *N. benthamiana* genome, two of which are not edited. This is therefore also not a complete knock-out (personal communication, Renier van der Hoorn).

Surprisingly, from this screen there were no positive hits for increased AgroLux bioluminescence or cytoGFP/secRFP fluorescence across all three repetitions of this screen (Figure 5.4).

Table 5.1: List of VIGS plants generated.

Gene(s) Silenced	Reference
<i>GUS</i>	<i>Duggan et al. (2021a)</i>
<i>WAK1</i>	<i>Rosli et al. (2013)</i>
<i>CDPK</i>	This chapter
<i>CORE</i>	This chapter
<i>RbohB</i>	Zachary Savage, unpublished
<i>MPK3/6</i>	Tolga Bozkurt, unpublished
<i>ACIF1</i>	This chapter
<i>CERK1</i>	Tolga Bozkurt, unpublished
<i>CSPR</i>	<i>Saur et al. (2016)</i>
<i>EIN2</i>	<i>Tran et al. (2016)</i>
<i>WRKY7/8/9/11/12/14</i>	Tolga Bozkurt, unpublished
<i>AHA2</i>	This chapter
<i>ICS</i>	This chapter
<i>NPR1</i>	<i>Tran et al. (2016)</i>
<i>CRT3a</i>	<i>Liebrand et al. (2012)</i>
<i>EDS1</i>	<i>Tran et al. (2016)</i>
<i>NRC234</i>	<i>Wu et al. (2017)</i>
<i>SAG101</i>	<i>Pombo et al. (2014)</i>
<i>PAL</i>	Tolga Bozkurt, unpublished
<i>BAK1</i>	<i>Wang et al. (2018a)</i>
<i>SOBIR1</i>	<i>Liebrand et al. (2013)</i>

Table 5.2: List of knock-out lines.

Mutant names	Reference
<i>bak1-1</i>	Unpublished, provided by Johannes Stuttmann
<i>nrg-1</i>	Unpublished, provided by Jonathan D G Jones
<i>nrg1/adr1</i>	provided by Jonathan D G Jones
<i>nrc234</i>	<i>Wu et al. (2020)</i>
<i>ics</i>	This chapter
<i>pad4/eds1</i>	<i>Gantner et al. (2019)</i>
<i>sobir1/sobir1-like</i>	<i>Huang et al. (2021)</i>
<i>pad4</i>	<i>Gantner et al. (2019)</i>
<i>sag101b/pad4</i>	(<i>Gantner et al., 2019</i>)
<i>eds1</i>	<i>Gantner et al. (2019)</i>
<i>sag101a-1/b-1</i>	<i>Gantner et al. (2019)</i>
<i>eds1a, pad4, sag101a, and sag101b (epss)</i>	<i>Lapin et al. (2019)</i>
<i>rbohB</i>	This chapter

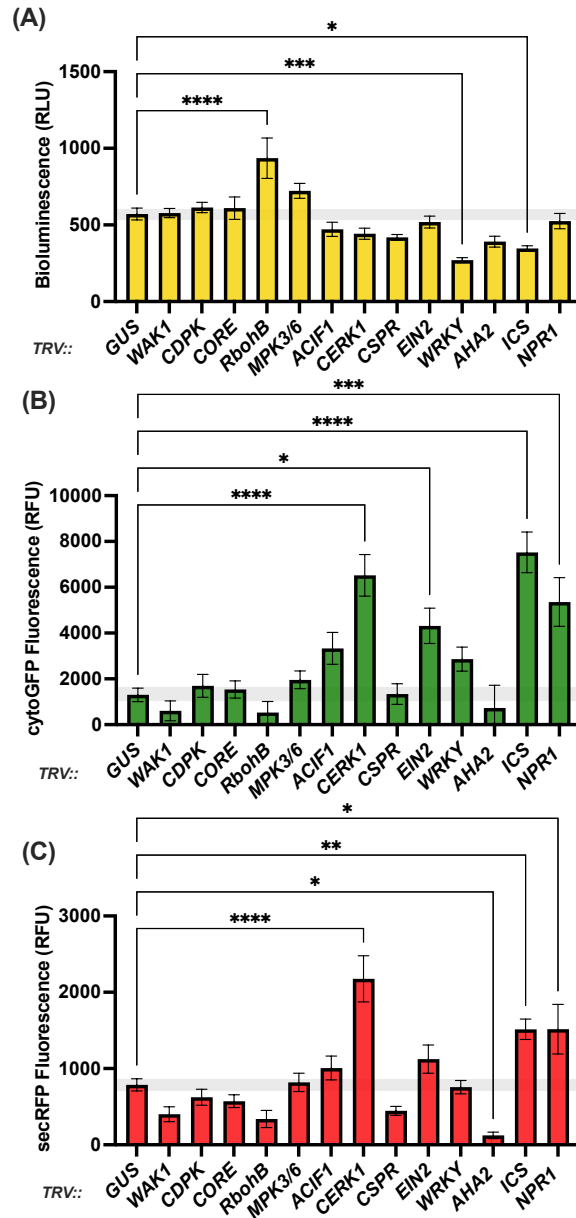


Figure 5.1: Screen of *N. benthamiana* gene knock-downs for effects on AgroLux bioluminescence and cytoGFP and secRFP fluorescence (Group 1). Two week-old *N. benthamiana* plants were agroinfiltrated for expression of TRV1 and a TRV2:: construct for Virus-Induced Gene Silencing. After three weeks and upon bleaching of *TRV::PDS* (silencing control) plants, the plants were agroinfiltrated with AgroLux and for expression of secRFP and cytoGFP. At 5dpi, leaf discs were punched and measured for luminescence and fluorescence (Ex./Em. 480/515nm (B) and 550/585nm (C) in a plate reader. (A) Some significant changes were observed in this experimental replicate but these were not reproduced in further experiments. (B and C) *TRV::ICS*, *TRV::NPR1* and *TRV::CERK1* plants had increased accumulation of cytoGFP and secRFP. Twelve replicates were used for each measurement, for which mean and standard error of mean are indicated. Data was analysed by ANOVA with Dunnett's post hoc test. Similar results for cytoGFP and secRFP were obtained in two more experimental replicates.

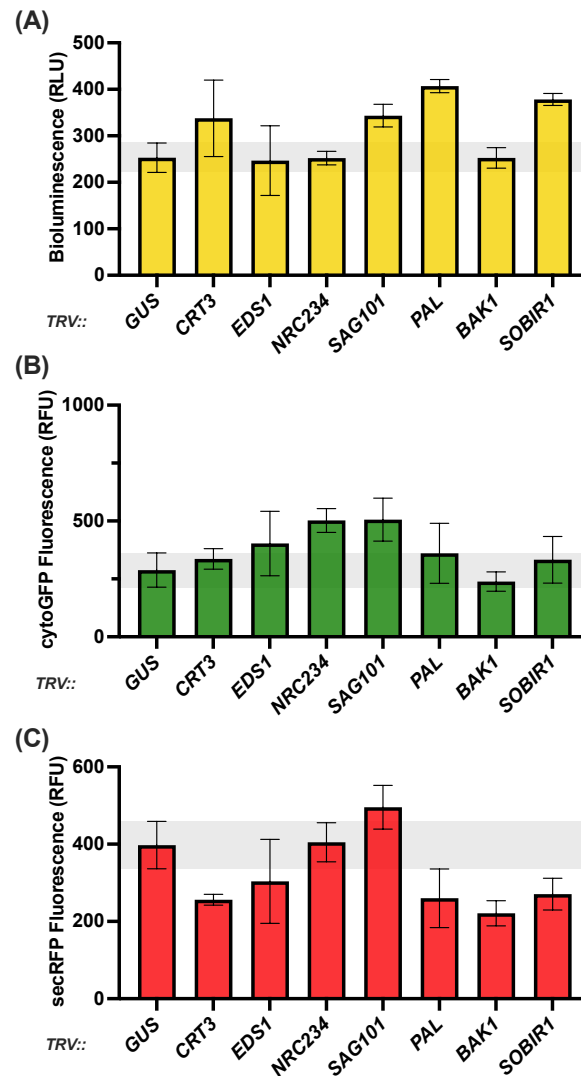


Figure 5.2: Screen of *N. benthamiana* gene knock-downs for effects on AgroLux bioluminescence and cytoGFP and secRFP fluorescence (Group 2). Two week-old *N. benthamiana* plants were agroinfiltrated for expression of TRV1 and a TRV2:: construct for Virus-Induced Gene Silencing. After three weeks and upon bleaching of *TRV2::PDS* (silencing control) plants, the plants were agroinfiltrated with AgroLux and for expression of secRFP and cytoGFP. At 5dpi, leaf discs were punched and measured for luminescence (A) and fluorescence (Ex./Em. 480/515nm (B) and 550/585nm (C) in a plate reader. Twelve replicates were used for each measurement, for which mean and standard error of mean are indicated. Data was analysed by ANOVA with Dunnett's post hoc test. Similar results were obtained in two more experimental replicates.



Figure 5.3: Virus-Induced Gene Silenced plants pictured before agroinfiltration.

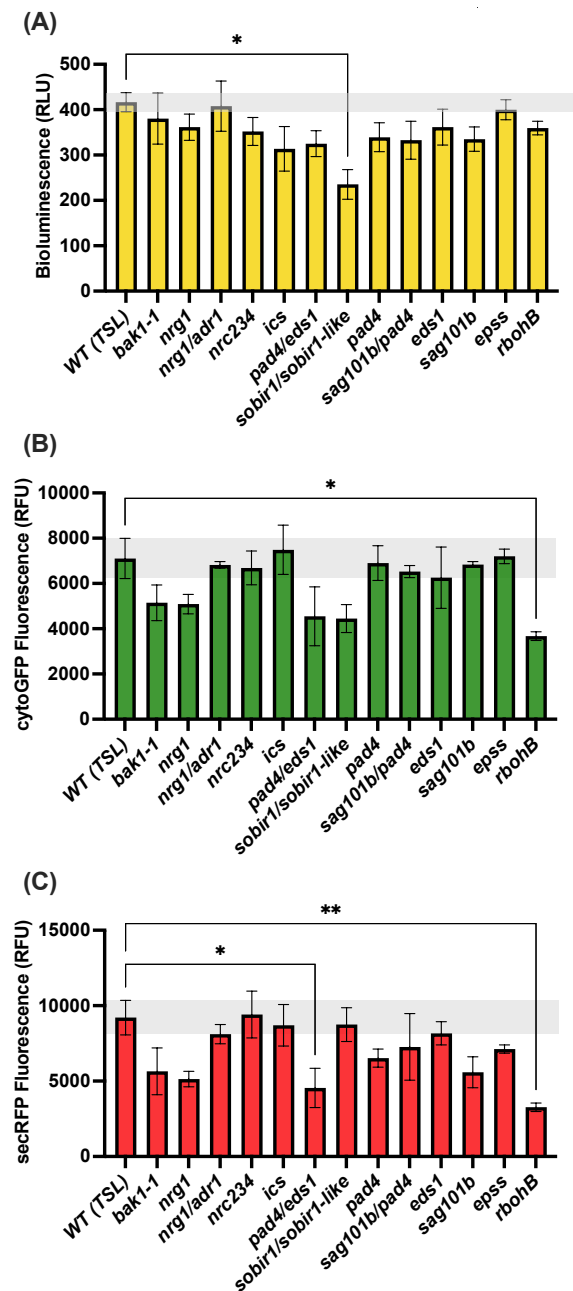


Figure 5.4: Screen of *N. benthamiana* knock-out plants for effects on AgroLux bioluminescence and cytoGFP and secRFP fluorescence. Wild-type *N. benthamiana* and each mutant were sown out at the same time and agroinfiltrated with AgroLux and for expression of cytoGFP and secRFP at four weeks-old. At 5dpi, leaf discs were punched and measured for luminescence (A) and fluorescence (Ex./Em. 480/515nm (B) and 550/585nm (C) in a plate reader. No significant changes were observed consistently in this group of knock-outs. Twelve replicates were used for each measurement, for which mean and standard error of mean are indicated. Data was analysed by ANOVA with Dunnett's post hoc test. Similar results were obtained in two more experimental replicates.

5.3.2 *NPR1*, *CERK1* and *ICS* depletion increases eGFP accumulation

Having screened the VIGS library, we then tested if silencing the three positive hits also improves recombinant protein accumulation over the whole leaf and not only in the leaf discs that were measured in the screen so that random variation in recombinant protein expression within leaves was accounted for. Therefore VIGS of *NPR1*, *ICS* and *CERK1* was repeated and then whole leaves were agroinfiltrated for eGFP expression. Four leaves from four plants were scanned using the Cy2 setting on a Typhoon scanner, which detects GFP fluorescence (Figure 5.5A). GFP from four leaves was then quantified using ImageJ (Figure 5.5B). *TRV2::ICS*, *TRV2::CERK1* and *TRV2::NPR1* plants all had significantly more eGFP fluorescence than the control *TRV2::GUS* plants (Figure 5.5). This data confirms that silencing of *ICS*, *CERK1* and *NPR1* increases transient eGFP expression.

5.3.3 Confirmation of VIGS Silencing

Five genes required confirmation of silencing because these constructs were not used previously (*NbACIF1*, *NbCDPK*, *NbCORE*, *NbICS* and *NbAHA2*). We used semi-quantitative RT-PCR with gene specific primers and normalized to actin as a control. Significant reduction in respective gene of interest transcript levels in comparison to *TRV::GUS* was shown in *TRV::ACIF1* (Figure 5.6A), *TRV::CDPK* (Figure 5.6B), *TRV::CORE* (Figure 5.6C) and *TRV::ICS* (Figure 5.6D). Significant reduction in *NbAHA2* transcripts was not observed in *TRV::AHA2* plants (Figure 5.6E). From this data, we can confirm that silencing was successful for *NbACIF1*, *NbCDPK*, *NbCORE* and *NbICS*, but not for *NbAHA2*. The failure of *AHA2* silencing in *TRV::AHA2* plants is surprising and contradicts the strong negative impact observed on cytoGFP and secRFP fluorescence in *TRV::AHA2* (Figure 5.1. The possibility of co-silencing other P-type ATPases has been investigated but no possibilities were identified. To identify the cause of the detrimental impact on recombinant protein expression in *TRV::AHA2* plants will require further investigations.

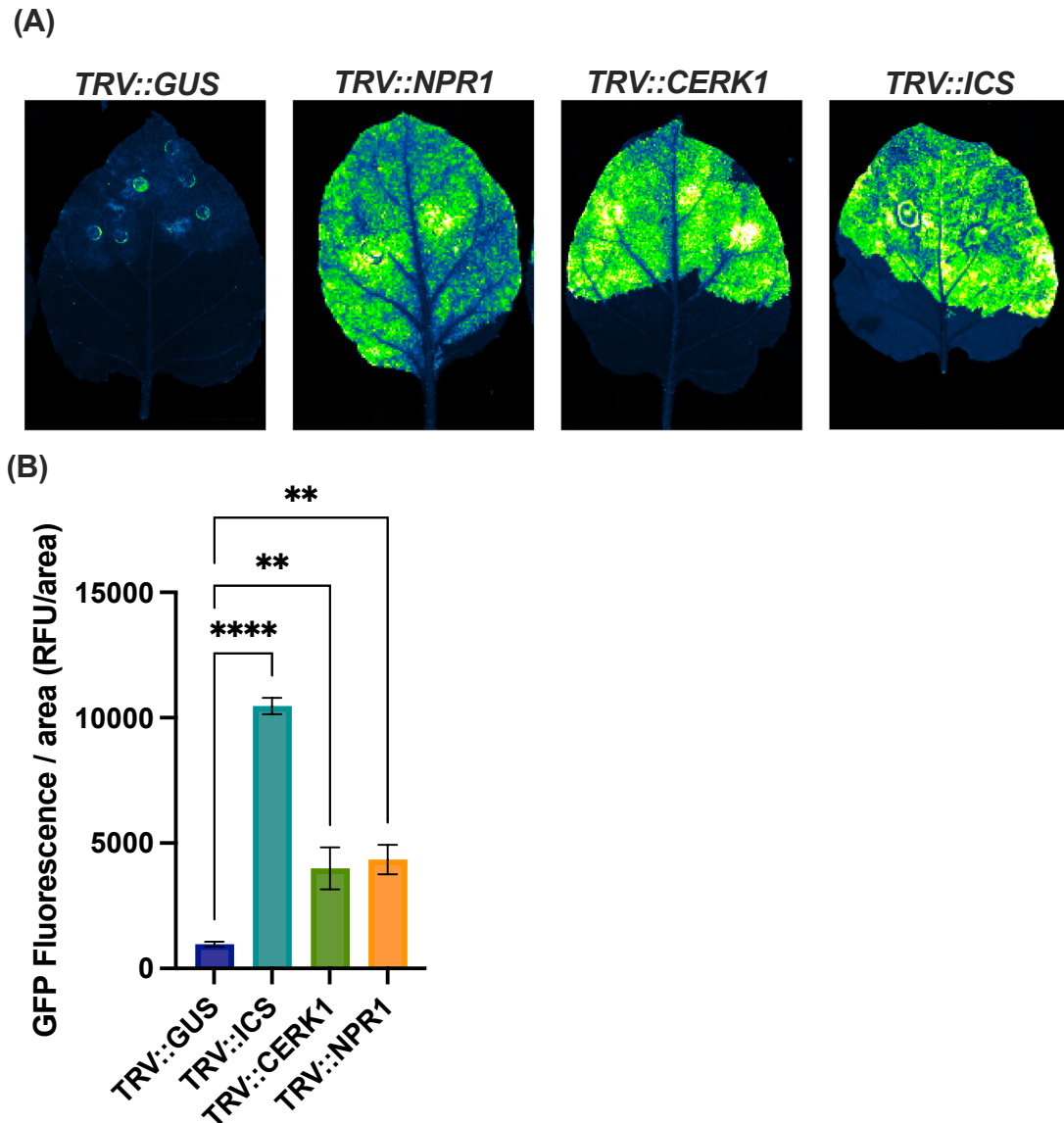


Figure 5.5: Virus-Induced Gene Silencing of NPR1, CERK1 and ICS increases accumulation of eGFP. Two week-old *N. benthamiana* plants were agroinfiltrated to express *TRV1* with *TRV2::GUS*, *TRV2::NPR1*, *TRV2::CERK1*, *TRV2::ICS* or *TRV2::PDS* for Virus-Induced Gene Silencing. After three weeks and upon bleaching of *TRV2::PDS* (silencing control) plants, the plants were agroinfiltrated with for expression of eGFP. At 5dpi, leaves were scanned on a Typhoon scanner for fluorescence using the Cy2 laser setting (Ex./Em. 480/515nm) (B). Quantification of eGFP using ImageJ confirms more eGFP fluorescence is observed in VIGS knock-downs of NPR1, CERK1 and ICS in comparison to the *TRV2::GUS* control, mean and standard error of mean are indicated. This was observed on four leaves from four different plants. Similar results were observed in another experimental replicate for *TRV2::ICS* and *TRV2::CERK1* and in two more experimental replicates for *TRV2::NPR1*.

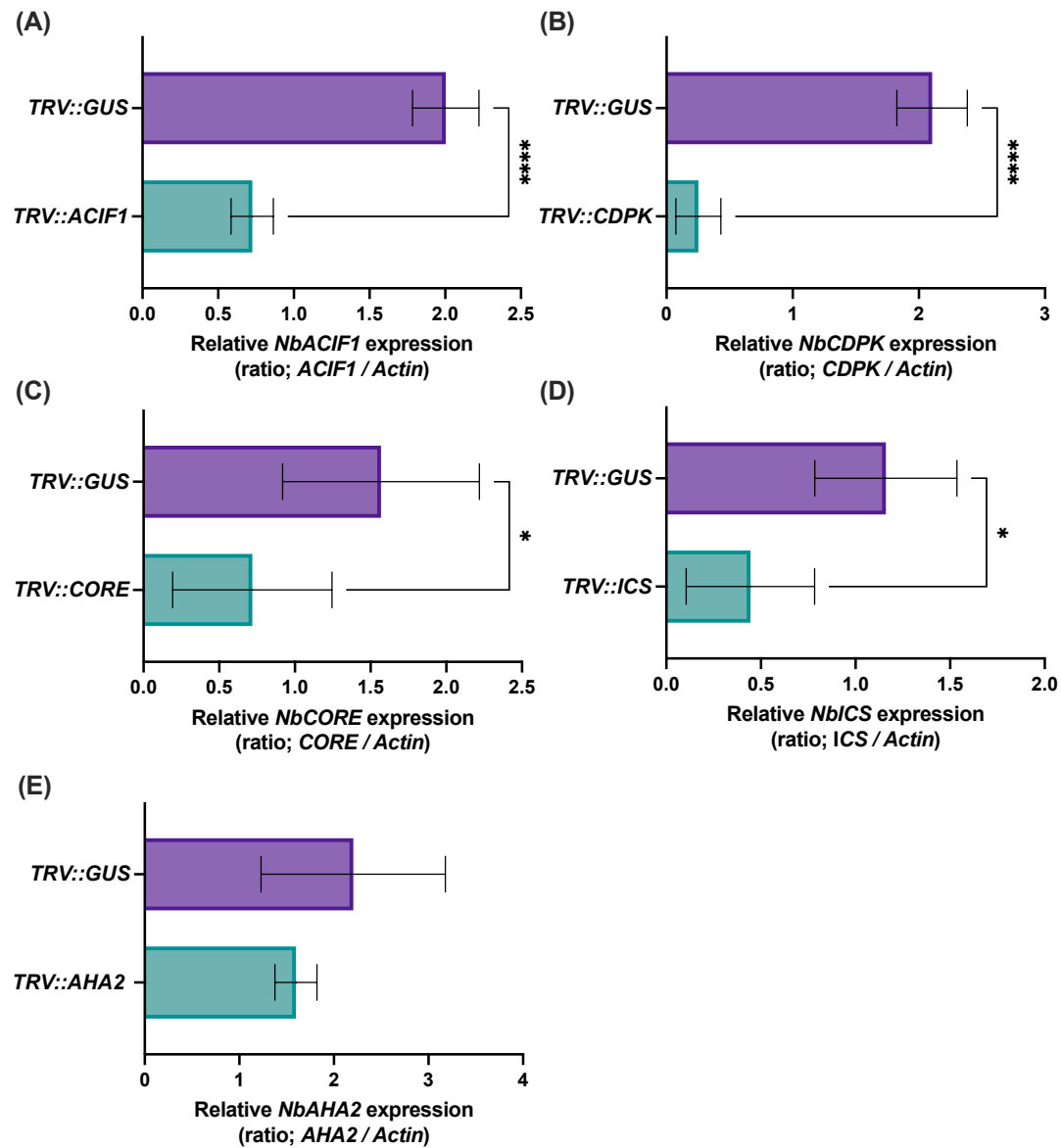


Figure 5.6: Virus-Induced Silencing reduces transcript levels of ACIF1, CDPK, CORE and ICS in *N. benthamiana*. Three weeks after agroinfiltration for VIGS, leaf tissue was collected, total RNA was extracted and cDNA generated by reverse transcription. PCR was performed on the cDNA using specific primers for *Actin*, *ACIF1*, *CDPK*, *CORE*, *ICS* and *AHA2*. The PCR product was separated by size on a 3% agarose gel and imaged. Bands were quantified using ImageJ Plot Lanes software and normalised in comparison to a housekeeping gene (*Actin*). Transcripts of *ACIF1*, *CDPK*, *CORE* and *ICS* were significantly reduced in their respective VIGS plants in comparison to *TRV2::GUS* controls. No silencing of transcripts was observed in *TRV2::AHA2* plants. Four biological replicates were used for RT-PCR, mean and standard error of mean are indicated. Data was analysed using unpaired t-tests.

5.3.4 *NbCORE* is required for *csp22* perception

Older plants support only low transient expression upon agroinfiltration (Chapter 3), which is thought to be caused by the perception of the *csp22* peptide (of CSP) from *A. tumefaciens*. Two receptors of *csp22* have been proposed to perceive *csp22* in older plants and both are transcriptomically upregulated in older *N. benthamiana* ([Saur et al., 2016](#); [Wang et al., 2016](#)). *NbCSPR* has been reported to be the receptor of *csp22* after depletion of it reportedly suppressed the *csp22*-induced ROS burst. The receptor *CORE* has been detected in tomato plants and overexpression of this *SlCORE* in *A. thaliana* enabled the ROS burst response to *csp22*-elicitation and resistance to pathogen infection ([Wang et al., 2016](#)). In order to confirm which of these receptors is responsible for Agrobacterium detection in older *N. benthamiana*, we silenced expression of each receptor by VIGS. This method had already been used for silencing of *NbCSPR* ([Saur et al., 2016](#)) and therefore we recloned the exact fragment used previously for silencing *NbCSPR*. No previous attempts to silence *NbCORE* were published and therefore we cloned a new fragment for *NbCORE* silencing (see 5.6C). Two week-old *N. benthamiana* plants were agroinfiltrated for expression of TRV carrying the silencing fragments. Four weeks later, when the plants were six weeks-old, leaf discs were taken and tested for the *csp22*-induced ROS burst. Plants silenced for *NbCSPR* had a similar ROS burst to the *TRV::GUS* control plants (Figure 5.7A). However, the *csp22*-induced ROS burst was completely abolished in *TRV::CORE* (Figure 5.7A). The area under the curve for each leaf was calculated and was significantly different between *TRV::GUS* and *TRV::CORE* plants (Figure 5.7B). From these data we can conclude that *NbCORE* is essential for the *csp22*-induced ROS burst.

5.3.5 *NbCORE* depletion increases recombinant protein accumulation in older plants

VIGS of *NbCORE* increases eGFP accumulation in six week-old *N. benthamiana*

csp22 is thought to be the PAMP responsible for the induction of plant immunity by Agrobacterium in *N. benthamiana* and that this perception is the reason for poor

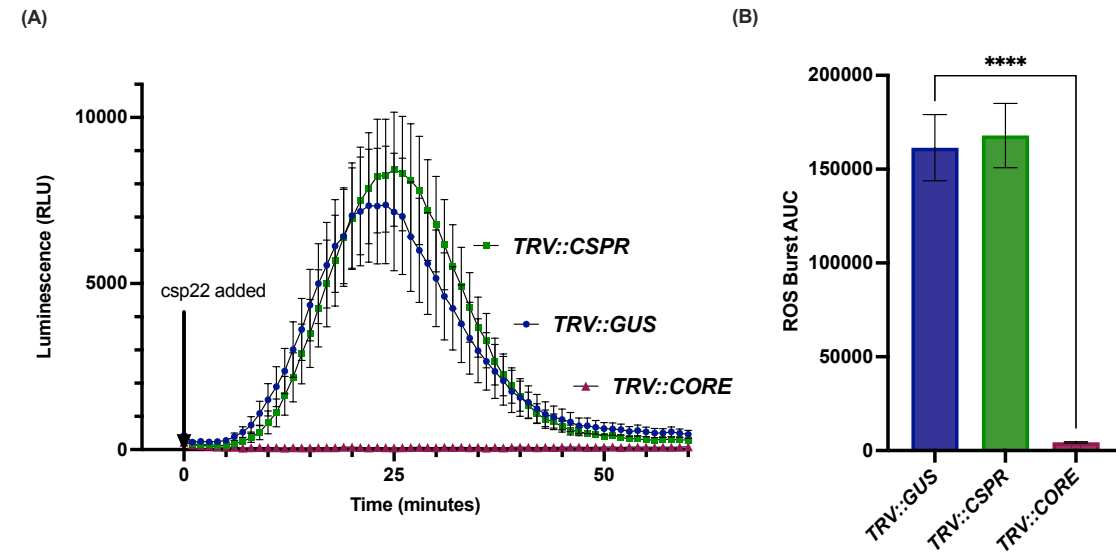


Figure 5.7: The *csp22*-induced ROS burst is absent in *NbCORE*-silenced plants. Leaf discs taken from 6 week-old plants (4 weeks after VIGS agroinfiltration) were placed in a solution containing luminol and horseradish peroxidase. *csp22* was added to the leaf discs and the ROS burst was detected by chemiluminescence over 100 minutes (A). The area under curve (AUC) was measured by integration of the detected ROS bursts. (A and B) indicate a ROS burst in CSPR silenced plants and an absence of ROS burst in CORE silenced plants. 12 replicates were measured, for which mean and standard error of mean are indicated. Data was analysed by ANOVA with Dunnett's post hoc test. Similar results were obtained in two subsequent experimental replicates.

agroinfiltration productivity in older *N. benthamiana*. Upon finding that *NbCORE* silencing in six week-old plants abolishes the *csp22*-induced ROS burst (Figure 5.7), we next hypothesised that these older *TRV::CORE* plants would improve recombinant protein production in older plants. We tested this hypothesis by agroinfiltrating six week-old *TRV::GUS* and *TRV::CORE* plants for expression of eGFP. After 5 days we scanned the leaves for eGFP for fluorescence. There was clearly more fluorescence emitted from *TRV::CORE* plants than *TRV::GUS* plants (Figure 5.8A). To confirm that eGFP accumulation was higher, and not just that fluorescence was increased, total soluble protein was extracted from leaf discs from the agroinfiltrated leaves and analysed by western blot. Western blotting analysis confirmed higher eGFP accumulation in *TRV::CORE* plants than *TRV::GUS* (Figure 5.8B).

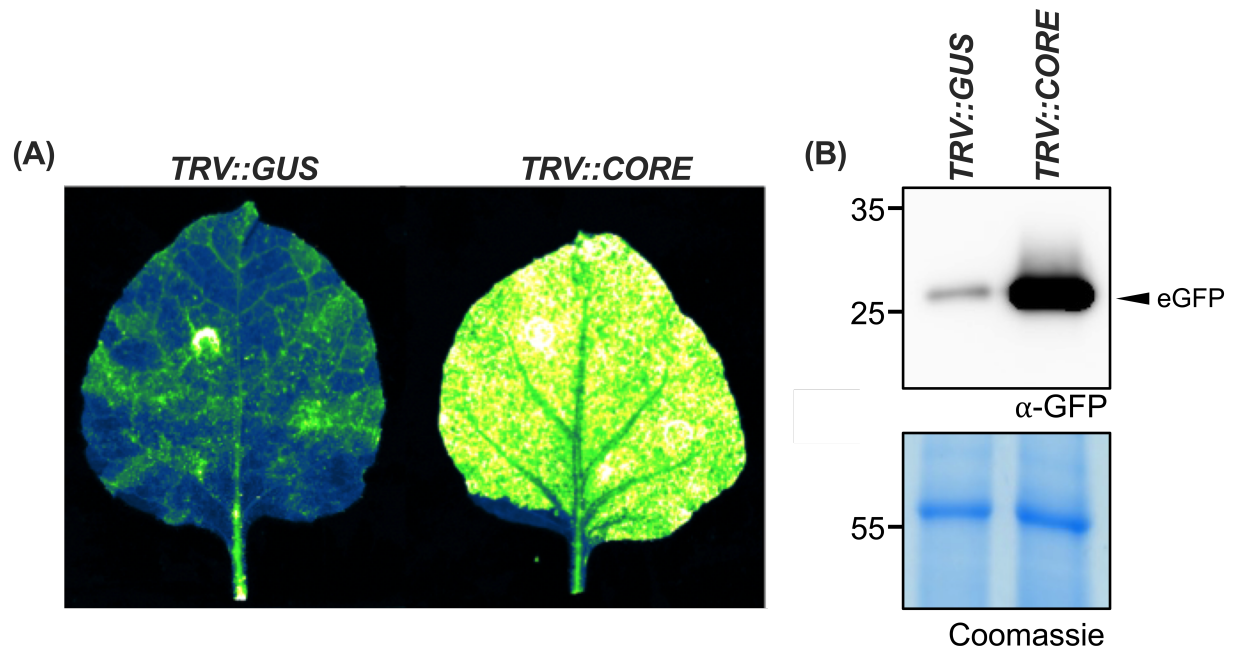


Figure 5.8: *NbCORE* silencing increases recombinant protein production in six week old *N. benthamiana*. Six week-old *TRV2::GUS* and *TRV2::CORE* plants were agroinfiltrated for expression of eGFP. At 5dpi the agroinfiltrated leaves were scanned on a Typhoon scanner using the Cy2 setting (A). Total soluble protein was extracted and separated by size on a 12% SDS-PAGE gel and analysed by western blotting using α -GFP antibody (B). The gel was also Coomassie stained for loading controls. Four replicates were pooled in each lane. Similar results were obtained in two subsequent experimental replicates.

NbCORE* silencing allows recombinant protein accumulation in eight week-old *N. benthamiana

I next tested if *NbCORE* silencing would also improve recombinant protein production in even older plants. Six and eight week-old *TRV::CORE* and *TRV::GUS* were agroinfiltrated for expression of eGFP. After 5 days, eGFP was detected by scanning agroinfiltrated leaves using the Cy2 setting on a Typhoon scanner. eGFP fluorescence was quantified using ImageJ. In both six and eight week-old *N. benthamiana* significantly more eGFP was produced in *TRV::CORE* plants than *TRV::GUS* plants (Figure 5.9). Depletion of *NbCORE* therefore renders even eight-week old *N. benthamiana* amenable to recombinant protein accumulation by agroinfiltration. Furthermore, no developmental phenotype was observed in *TRV::CORE* at four, six and eight weeks-old, meaning that *NbCORE* silenced plants are suitable for use in molecular farming (Figure 5.10). Using older plants that are still amenable to agroinfiltration allows more recombinant protein production because it

enables use of plants with more biomass.

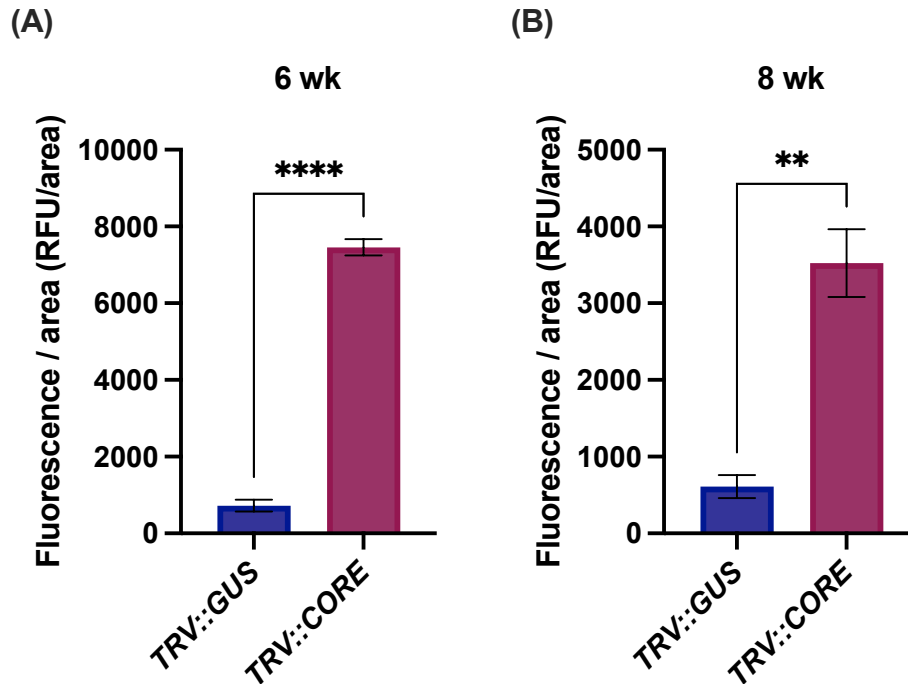


Figure 5.9: A significant increase in eGFP accumulation is measured in both six and eight week old *NbCORE* silenced plants. Six (A) and eight (B) week-old TRV::GUS and TRV::CORE *N. benthamiana* were agroinfiltrated for expression of eGFP. At 5dpi the agroinfiltrated leaves were scanned on a Typhoon scanner using the Cy2 setting. eGFP fluorescence was quantified from images using ImageJ and normalised by leaf area. In both ages there is a significant increase in eGFP accumulation upon CORE silencing. Four biological replicates were measured and data was analysed using Welch's t-test.

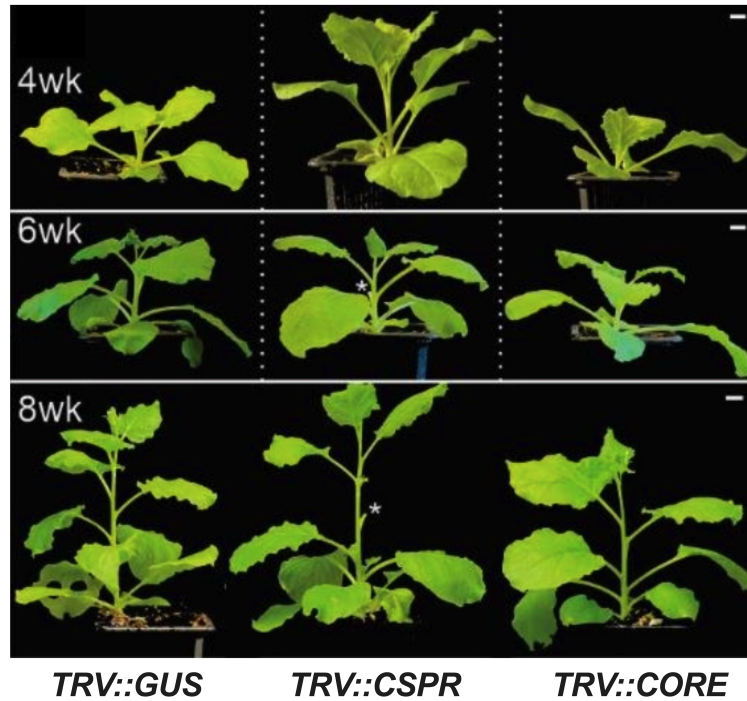


Figure 5.10: *TRV::CSPR* and *TRV::CORE* plants do not have any additional developmental phenotypes in comparison to *TRV::GUS* plants at 4,6 and 8 weeks old.

5.4 Discussion

In this chapter we screened *N. benthamiana* silenced for a variety of immunity genes for effects on AgroLux bioluminescence and expression of fluorescent recombinant proteins (Figure 5.1, 5.2, 5.4). This identified three candidate genes for silencing to improve recombinant protein yields (Figure 5.1, 5.2). These three genes were confirmed to improve eGFP accumulation (Figure 5.5) and offer promising strategies to improve molecular farming. Although ineffective at increasing recombinant protein production in the screen of four-week old *N. benthamiana*, depletion of *NbCORE* in older plants significantly increased eGFP production (Figure 5.8, 5.9). This difference in impact on recombinant proteins corroborates previous reports that *CORE* is only expressed in older plants ([Wang et al., 2016](#); [Wei et al., 2018](#)) and shows that the reason *CORE* silencing did not impact recombinant protein in four week-old plants (Figure 5.1) is that *CORE* is not strongly expressed in the control, *TRV::GUS* plants either. These six week-old *TRV::CORE* plants could also not mount a ROS burst against *csp22* (Figure 5.7). From this chapter,

we therefore propose that four week-old *N. benthamiana* silenced for *NbICS*, *NbNPR1* and *NbCERK1* be tested for increased production of other recombinant proteins such as antibodies. We also suggest that six week-old *N. benthamiana* *NbCORE* knock-out plants be generated and tested for increased accumulation of recombinant proteins and that this may be an opportunity to use older plants with greater biomass in order to improve yields. Furthermore, that an *A. tumefaciens* be engineered with an unrecognisable csp22 peptide that can be used for agroinfiltration.

5.4.1 AgroLux bioluminescence is affected by salicylic acid induced immunity

The screen of both VIGS and knock-out plants resulted in only two reproduced changes to AgroLux bioluminescence (Figure 5.1, 5.2). The two genes silenced for these two changes were *NbPAL* and *NbICS*. These two genes are essential for salicylic acid biosynthesis by encoding the two enzymes responsible for the two pathways by which SA is produced (Figure 1.2) ([Pallas et al., 1996](#); [Catinot et al., 2008](#); [Chen et al., 2009](#)). However, despite both encoding genes that are responsible for SA biosynthesis, silencing of these two genes resulted in opposite effects on AgroLux bioluminescence. *NbPAL* encodes phenylalanine ammonia lyase, which converts phenylalanine into cinnamate as part of one biosynthetic pathway for salicylic acid production ([Chen et al., 2009](#)). *NbICS* encodes isochorismate synthase, which is the crucial enzyme for conversion of chorismate into isochorismate, which can then be converted into SA ([Wildermuth et al., 2001](#)). Silencing these genes has been shown previously to reduce SA accumulation and silencing of *ICS* and inhibition of *PAL* have been shown to increase plant susceptibility to pathogen infection ([Catinot et al., 2008](#); [Mauch-Mani and Slusarenko, 1996](#); [Anand et al., 2008](#); [Chen et al., 2009](#)). Given the reduced SA accumulation and increase in plant susceptibility, it is not surprising that *TRV::PAL* plants have increased AgroLux bioluminescence (Figure 5.2). The reduced SA biosynthesis in *TRV::PAL* plants may result in reduced exposure of AgroLux to plant immune responses, leading to higher AgroLux bioluminescence. However, the contrasting reduction of AgroLux bioluminescence in *TRV::ICS* plants was unexpected (Figure 5.1). Furthermore, recombinant protein accumulation in *TRV::ICS* plants was

increased, suggesting that plant defence was not upregulated. It is possible that the reduction in AgroLux bioluminescence was due to the accumulation of products in the SA biosynthetic pathway, in particular of the isochorismate precursor, chorismate. Preventing the conversion of isochorismate to chorismate by ICS depletion may cause accumulation of chorismate. No studies have investigated the effect of chorismate on Agrobacteria but there is a possibility that it is detrimental to AgroLux bioluminescence. Furthermore, *ICS*-silenced *N. benthamiana* have very low production of phyloquinone, a photosystem I (PSI) cofactor, because ICS is required for its synthesis ([Catinot et al., 2008](#)). *TRV::ICS* plants were small and had pale green leaves ([Catinot et al., 2008](#)) (see 5.3). It is possible that the low levels of PSI and the subsequent lowered rates of photosynthesis will limit nutrient availability to Agrobacterium and result in low AgroLux bioluminescence after 5 days *in planta*.

5.4.2 Effect of PTI on AgroLux bioluminescence remains to be elucidated

When performing the ‘Traffic Light Screen’ on *N. benthamiana* silenced for immunity genes in this chapter, measurements were only taken for bioluminescence and fluorescent proteins at 5dpi. However, many of the genes silenced in this work are known to be involved in PTI, which we would observe in the initial hours after agroinfiltration. By the late time point at which we took samples, Agrobacterium state in *TRV::GUS* plants may have recovered from any PTI induced effects on AgroLux bioluminescence. This might be why we have not detected the effects of PTI gene depletion on AgroLux bioluminescence using this screen. In order to show the effects of PTI on AgroLux bioluminescence, leaf discs should be taken at a much earlier time point. Investigation of this earlier time point will inform on the importance of Agrobacterium state for rates of transient expression ([Jutras et al., 2021](#)).

5.4.3 Silencing of three immunity genes improves recombinant protein accumulation

In this chapter we screened the depletion of 30 immunity genes for effects on recombinant protein accumulation. Silencing of *NbICS*, *NbNPR1* and *NbCERK1* increased

accumulation of both secreted RFP and cytosolic sfGFP in the screen (Figure 5.1, 5.2). This benefit to recombinant protein accumulation across leaves was confirmed by whole leaf agroinfiltration of silenced plants with eGFP (Figure 5.5). The effects of silencing these three genes provide evidence that manipulation of a range of plant immune responses give new strategies for improving molecular farming yields.

Salicylic Acid-induced immunity is detrimental to molecular farming

Both *NbICS* and *NbNPR1* are both involved in SA-induced immune responses (Figure 1.2) ([Mou et al., 2003](#)). *NbICS* converts chorismate into isochorismate which is then converted into salicylic acid as part of the phenylalanine-independent SA biosynthetic pathway. *NbNPR1* is involved in SA signalling when it interacts with TGA transcription factors to regulate expression of systemic acquired resistance (SAR) genes such as PR1 ([Fan and Dong, 2002](#); [Wildermuth et al., 2001](#)). SA has been reported to reduce *Agrobacterium* virulence by preventing cell attachment, reducing *vir* gene expression and reducing growth of *Agrobacterium in vitro* at high concentrations. The SA-associated roles of these two genes have previously been reported to affect *Agrobacterium* infectivity ([Anand et al., 2008](#)). Silencing of *ICS*, *NPR1* and another SA-associated protein, SABP2 (SA-binding protein 2) all caused increased tumour growth after infection of tumourigenic *Agrobacterium* and increased GUS activity after transient transformation. The results from this screen corroborate with this report that SA-dependent plant immunity affects *Agrobacterium*. We show that silencing of these genes increases accumulation of three different recombinant proteins (Figure 5.1, 5.2, 5.5), indicating that silencing of *NbICS* and *NbNPR1* might be a promising strategy to improve recombinant protein yields in molecular farming. Furthermore, the incidence of two genes required for SA-dependent immunity showing detrimental impacts on recombinant protein production indicates that SA, and its many associated pathways of plant defence, are an important area to consider for future attempts to improve molecular farming yields.

***NbCERK1* silencing improves recombinant protein yields**

NbCERK1 silencing significantly improved production of eGFP (Figure 5.5) and provides evidence that hampering the PTI response can improve molecular farming. CERK1 is most commonly known as a critical RLK in the perception of fungal chitin and eliciting PTI in response to the MAMP (*Miya et al.*, 2007). However, CERK1 is also involved in sensing bacterial peptidoglycan in *A. thaliana* (*Willmann et al.*, 2011). CERK1 does not directly bind peptidoglycans but is instead a vital part of the LYM1/LYM3/CERK1 perception system. All three of these proteins are required for *A. thaliana* defence responses to peptidoglycan and for resistance to bacterial infection (*Willmann et al.*, 2011). CERK1 is also the target of *P. syringae* TIII effector protein AvrPtoB (*Gimenez-Ibanez et al.*, 2009), showing that CERK1 is a natural target for pathogenic bacteria. Silencing of CERK1 may therefore provide an environment in which there is a reduced PTI response to agroinfiltration, explaining the increased success of transformation and improved yields of recombinant proteins.

Future directions to improve molecular farming

This chapter has shown that silencing genes involved in plant immunity is a promising strategy to improve molecular farming. To continue this work, the next investigations should include expression of alternative recombinant proteins such as antibodies and growth factors in *TRV::ICS*, *TRV::NPR1* and *TRV::CERK1* plants. Following these investigations, knock-out lines that do not express these three genes should be generated and tested for increased recombinant protein expression. Knock-out lines are more useful than VIGS plants for industrial-scale production of recombinant proteins because it avoids the need for two agroinfiltrations and a license to work with TRV.

5.4.4 *NbCORE* as the receptor for csp22

In this chapter we show that the receptor CORE is required for the csp22-induced oxidative burst in *N. benthamiana* (Figure 5.7). This corroborates previous studies which reported that transient expression of *NbCORE* in young *N. benthamiana*, in which CORE is not

yet expressed to high levels, confers responsiveness to csp22 and enables production of a ROS burst in response to elicitation ([Wei et al., 2018](#)) and that *NbCORE* binds csp22 with high affinity ([Wang et al., 2016](#)). This data, however, contradicts a previous study that presented *NbCSPR* as the receptor of csp22 ([Saur et al., 2016](#)). In this work we show that *NbCSPR* is not essential for the csp22-induced ROS burst because *TRV::CSPR* plants are still able to mount a response to elicitation (Figure 5.7). More recent studies have instead presented evidence that *NbCSPR* is identical to RE02, which is the receptor for the MAMP VmE02 ([Nie et al., 2021](#)). In this study, they showed that RE02 (CSPR) did show low affinity to csp22 but its affinity to another PAMP, VmE02, was 160 times higher than RE02 affinity for csp22 ([Nie et al., 2021](#)). This therefore suggests that the work of [Saur et al. \(2016\)](#) may have been observing a small role of CSPR in the csp22 response, but latter evidence from this thesis, [Wang et al. \(2016\)](#), [Wei et al. \(2018\)](#) and [Nie et al. \(2021\)](#) show that CSPR/RE02 is instead the receptor of VmE02 and CORE is the receptor responsible for csp22 perception.

5.4.5 *NbCORE* silencing for improved molecular farming

NbCORE is only expressed to high levels in older plants, resulting in csp22 response only in older *N. benthamiana*. We hypothesised that this csp22-responsiveness was the cause for poor expression of recombinant protein in older *N. benthamiana*. In this chapter we tested this hypothesis by transiently expressing a recombinant protein, eGFP, in six and eight week-old *TRV::GUS* and *TRV::CORE* plants (Figure 5.9). The *TRV::CORE* plants were unable to perceive csp22 and had significantly more accumulation of eGFP when both six and eight weeks old. This shows that prevention of Agrobacterium detection by *NbCORE* silencing improves recombinant protein accumulation. This discovery offers an opportunity to use mature plants with larger biomass for molecular farming.

5.4.6 Next steps for CORE work

In order to use this technology in industry, the next step is to produce *NbCORE* knock-out plants by genome editing. This will allow use of silenced plants without the need for two agroinfiltrations and a TRV license. Following production of knock-out plants, these should

be tested in industrial conditions and scales to assess if use of older, *NbCORE* knock-out plants can improve molecular farming yields.

The interaction of *Agrobacterium* csp22 and *NbCORE* can also be avoided by another method. Engineering *A. tumefaciens* strains lacking csp22 or harbouring an unrecognisable csp22 will provide another strategy to avoid *NbCORE* elicitation in older plants. Use of such a strain would also allow efficient transient expression in older *N. benthamiana*.

5.5 Conclusions

Manipulation of plant immunity through gene silencing is a successful strategy to improve molecular farming. We have identified three genes that offer promising strategies to improve recombinant yields when silenced in four week-old *N. benthamiana*, as is currently used in industry. Furthermore, we show that the receptor *NbCORE* is essential for the csp22-induced ROS bursts in older *N. benthamiana* and that avoiding the interaction between *NbCORE* and csp22 in older *N. benthamiana* drastically improves transient expression. Future work should continue to investigate manipulation of the plant immune response to improve recombinant protein expression by screening more immunity genes and producing knock-out lines by gene editing for promising candidates.

Chapter 6

General Discussion

The aims of this work were to investigate how *N. benthamiana* immunity impacts *A. tumefaciens* and to manipulate the interaction between these two species to improve molecular farming yields. In the experimental chapters of this thesis I have advanced understanding of how *A. tumefaciens* is affected by *N. benthamiana* and have identified multiple strategies that reduce the immune response and increase recombinant protein accumulation. Within each experimental chapter there are detailed discussions which are not repeated here. Instead, this chapter reflects on the main findings of this thesis and provides a broader discussion of the implications on molecular farming, as well as directions for future research.

In **Chapter 2** I investigated how *A. tumefaciens* state is impacted by apoplastic pH at different time points after agroinfiltration. *A. tumefaciens* bioluminescence was significantly affected by apoplastic pH both immediately after agroinfiltration (Figure 2.2) and also after sustained apoplastic pH change (Figure 2.7, 2.13). The sustained pH change was imposed by expressing either peptide elicitors which regulate proton pumping into the apoplast (Figure 2.7), or mutation of the H⁺-ATPase pump (Figure 2.13) which extrudes cellular protons into the apoplast. These sustained pH changes correlated with pathogenesis-related (PR) protein accumulation (Figure 2.11, 2.14). The change in AgroLux bioluminescence after expression of pH-modifying proteins was abolished in *NahG* lines (Figure 2.15). *NahG* mutants are transgenic *N. benthamiana* expressing a bacterial salicylate hydroxylase, resulting in *N. benthamiana* which do not accumulate salicylic acid

(SA). This indicates that SA is implicated in regulation of pH-induced plant defence responses. In parallel with reducing AgroLux bioluminescence, apoplastic alkalization also reduced the accumulation of recombinant proteins (Figure 2.9). This suggests that the alkalization-derived plant defence response which was detrimental to *A. tumefaciens* is also detrimental to molecular farming. This demonstrates the value for molecular farming in understanding the interaction between *N. benthamiana* and *A. tumefaciens*.

In **Chapter 3** I developed a high-throughput screen measuring *A. tumefaciens* state and recombinant protein production. I optimized the expression of cytosolic GFP and secreted RFP as read outs for recombinant protein expression and accumulation alongside *A. tumefaciens* *RSPJ07* bioluminescence to monitor *A. tumefaciens* state. These three read-outs were measured in leaf discs on a plate reader, providing a fast and quantitative method to compare treatments. The resulting ‘Traffic Light Screen’ was used in the subsequent two chapters and yielded multiple strategies to improve molecular farming.

In **Chapter 4** I aimed to improve recombinant protein yields by inhibiting the plant immune response through co-expression of pathogen effectors. I also aimed to gain insights into how *A. tumefaciens* is affected by plant immunity by observing how different pathogen effectors impacted AgroLux bioluminescence. We screened 130 pathogen effectors using the ‘Traffic Light Screen’ (Figure 4.2, 4.3, 4.4). Changes to AgroLux were observed upon effector expression (Figure 4.2, 4.3, 4.4) and we discovered new leads to elucidate the meaning of AgroLux changes. Two effectors that caused cell death led to opposing effects on AgroLux bioluminescence (Figure 4.2, 4.3, 4.4), suggesting that AgroLux can detect different mechanisms of cell death. Future work should investigate the mechanisms of these effectors to uncover how they change AgroLux bioluminescence. The screen also yielded six positive hits that increased secreted RFP fluorescence (Figure 4.2, 4.3, 4.4). Co-expression of two of these effectors then caused an increase in antibody accumulation (Figure 5.5, 4.6). However, when tested in industrial conditions and scale, the most promising effector candidate did not increase production of a purified antibody (Figure 4.8). Effector co-expression was compared to a control in the absence of an EV construct because this is how it would be incorporated into industrial agroinfiltrations at Leaf Expression Systems (my industry CASE partner company). As a result, we compared the effector

treatment agroinfiltrated with 1.5 times the concentration of *A. tumefaciens* than the control. The absence of positive impact caused by effector co-expression in this comparison demonstrates the need for a large improvement to recombinant protein yields in order for the yield increase to outweigh costs of an added construct.

In **Chapter 5** I improved recombinant protein yields by inhibiting plant defence by depleting immunity genes and transcripts. In order to identify which genes are promising candidates to improve molecular farming, I generated silenced lines by Virus-Induced Gene Silencing (VIGS) and used knock-out plants. I then performed the ‘Traffic Light Screen’ in these silenced plants (Figure 5.1, 5.2, 5.4). This screen provided three candidates for which VIGS of these genes increased protein fluorescence (Figure 5.5). These are promising strategies to pursue further. Genome edited knock-out plants are being generated and will be assessed for improved recombinant protein accumulation. In addition to these three candidates, we also identified that the receptor *NbCORE* is required for the *csp22*-induced ROS burst in 6 week-old *N. benthamiana* (Figure 5.7). VIGS of *NbCORE* subsequently significantly increased the accumulation of recombinant protein in 6 and 8 week-old plants (Figure 5.8, 5.9). This provides a method by which older plants with larger biomass can be used for agroinfiltration in molecular farming. The findings of this chapter further reinforce the importance of understanding the interaction between *N. benthamiana* and *A. tumefaciens*, and also demonstrate that manipulation of *N. benthamiana* immunity is an effective approach to improve molecular farming.

6.1 Advances made in the uses and interpretation of AgroLux

AgroLux was developed as a tool to monitor the bioluminescence of *A. tumefaciens* during agroinfiltration. This was achieved by the integration of a single copy of the *lux* operon into the genome of *A. tumefaciens* *GV3101* (pMP90) and resulted in a strain useful for monitoring the state of *Agrobacterium in planta*. The relevance to molecular farming was demonstrated by the correlation between Agrolux bioluminescence and recombinant protein expression after incubation at different light intensities for twelve hours after

agroinfiltration. AgroLux could also be used to detect Avr/Cf-induced immune responses before visible tissue collapse, demonstrating its utility as a rapid quantitative assay for the hypersensitive response (*Jutras et al., 2021*). This is a new tool developed in our lab. Research is ongoing to establish the utility of AgroLux in research and to understand how AgroLux changes can be interpreted.

In Chapter 2, I demonstrated the correlation between AgroLux bioluminescence and the accumulation of pathogenesis-related (PR) proteins after changing apoplastic pH by expression of RALF1, PSY1, AHA2 and AHA2-S899A (Figure 2.7, 2.12, 2.11, 2.13, 2.14). Subsequently, the pH-induced change to AgroLux was abolished in plants that were unable to accumulate salicylic acid (SA) (Figure 2.15). AgroLux residing in plant tissue that was expressing PSY1 and PSY1-R had significantly higher bioluminescence than EV controls (Figure 2.7). Abolition of PSY1 signalling in *A. thaliana* increases the accumulation of SA and decreases growth of *P. syringae* DC3000 (*Mosher et al., 2013*). We hypothesise that the increased bioluminescence of AgroLux in PSY1/PSY1-R expressing leaves is due to the reduction of SA and possibly an accompanying increase in jasmonic acid (JA) levels (*Mosher et al., 2013*). This research provides evidence that AgroLux may be an indicator for the strength of SA-mediated defence responses underway. However, interpretation of AgroLux from the screen of VIGS plants does not currently support this hypothesis (Figure 5.1, 5.2). Plants with depleted transcripts of *ICS*, *NPR1* and *PAL* did not show consistent increases in AgroLux bioluminescence (Figure 5.1, 5.2). We speculate that the lack of increased AgroLux bioluminescence in this situation may be due to plant defence compounds in response to the presence of TRV virus in the leaves, however this is yet to be investigated. Future research should be carried out to confirm that AgroLux bioluminescence is a reflection of SA-mediated defence response. The opposing AgroLux phenotypes observed in leaves expressing PSY1/PSY1-R and RALF1 or AHA2-S899A can be used to understand the mechanism underlying contrasting AgroLux signals by performing proteomics and transcriptomics of pH-manipulated leaf tissue. This could reveal interesting changes to the proteome and transcriptome that caused the contrasting effects to AgroLux observed in Chapter 2.

The increase in AgroLux in PSY1/PSY1-R expressing tissue (Figure 2.7) and

corresponding decrease in recombinant protein accumulation (Figure 2.9) demonstrate that AgroLux measured at the time of recombinant protein harvest does not correlate to accumulated levels of recombinant protein. Therefore, to use AgroLux as a tool in molecular farming, measurements should be taken at an early time point ([Jutras et al., 2021](#)). In order to assess if PSY1/PSY1-R expression is an effective strategy to increase recombinant protein accumulation, transgenic *N. benthamiana* expressing these two *A. thaliana* genes should be generated. The change in balance of SA and JA in these transgenics could be assessed with AgroLux at an early time point. Subsequently, accumulation of recombinant protein can be measured and compared to AgroLux bioluminescence.

Although AgroLux is unsuitable for predicting recombinant protein production at late time points, AgroLux bioluminescence is a useful proxy for predicting utility of new deterrents to plant pathogens. In Chapter 2 I show that the detrimental effects of apoplastic alkalinization on AgroLux bioluminescence are reflected in the growth of *P. syringae* DC3000 (Figure 2.16). Current methods for finding strategies to reduce growth of bacterial plant pathogens include the Agromonas assay ([Buscaill et al., 2021](#)). This assay is useful to validate small numbers of transiently expressed proteins to increase plant immunity, but is too time-consuming and labour intensive to screen many different treatments. Therefore, using AgroLux to screen many different transiently expressed proteins for impacts on bioluminescence and then validating only promising candidates against the relevant pathogen provides a faster and cheaper alternative.

6.2 The importance of csp22 in the interaction between *N. benthamiana* and *A. tumefaciens* - advances made and future investigations

In Chapter 5 the centrality of *NbCORE* to csp22 responsiveness was demonstrated. We showed that depletion of *NbCORE* in six week-old *N. benthamiana* abolished the csp22-induced ROS burst (Figure 5.7) and increased accumulation of recombinant protein (Figure 5.8, 5.9). This data is consistent with reports that CORE binds csp22 with high

affinity ([Wang et al., 2016](#)) and that expression of the *Solanum lycopersicum* CORE enables csp22-responsiveness in the leaves of young *N. benthamiana* ([Wei et al., 2018](#)). However, our data refutes claims that *NbCSPR* is the underlying receptor for csp22 responsiveness ([Saur et al., 2016](#)) because we still detect a csp22-induced ROS burst in *TRV::CSPR* plants (Figure 5.7). Older *TRV::CORE* plants are efficient for recombinant protein production (Figure 5.9). Previously, older plants did not produce sufficient levels, likely due to their ability to perceive csp22 ([Saur et al., 2016](#)).

Having established the importance of the detection of csp22 by CORE in the interaction between *N. benthamiana* and *A. tumefaciens*, we have identified important leads for future research. The next stage of research into this interaction has multiple promising strategies. Firstly, testing accumulation of recombinant proteins in *NbCORE* knock-out plants may provide a serious opportunity to change the age, and therefore size, of plants used for agroinfiltration. Secondly, engineering *A. tumefaciens* strains that harbour an unrecognisable csp22 is another strategy that can be used to increase transient expression in older plants. This strategy has been used to produce an engineered *A. tumefaciens* strain expressing EF-Tu variants that are not recognised by *A. thaliana* EFR. This resulted in an *A. tumefaciens* strain which elicited a reduced plant defence response and increased transient expression efficiency in *A. thaliana* ([Yang et al., 2023](#)).

In addition to helping molecular farming, using plants that do not detect the presence of *A. tumefaciens* is beneficial to studies of plant immunity. As has been shown in this research, *A. tumefaciens* itself elicits plant defence responses. This may cause confounding effects when studying specific elements of plant defence through transient expression, as is done frequently. Avoiding detection of *A. tumefaciens* during agroinfiltration may help provide conditions for testing immunity without these confounding effects. Lastly, in Chapter 2 I demonstrated the role of extracellular alkalinization in SA-mediated plant defence. Extracellular alkalinization has been demonstrated due to the interaction of plant PRs with PAMPs, including flg22 with FLS2 and elf18 with EFR ([Felix et al., 1999](#); [Kunze et al., 2004](#)). However, these PRR:PAMP relationships do not occur in the interaction between *N. benthamiana* and *A. tumefaciens* because *N. benthamiana* does not encode EFR and *A. tumefaciens* flg22^{Atum} is too divergent from *P. syringae* flg22 to

cause an alkalinization response ([Fürst et al., 2020](#)). Therefore, having identified that *A. tumefaciens* and *N. benthamiana* do interact by CORE perception of csp22 (Figure 5.7), it is plausible that csp22 may cause extracellular alkalinization in older *N. benthamiana*. This can easily be tested using csp22 peptide and *N. benthamiana* cell culture. The csp22 peptide can then be mutated to identify important residues for which mutation prevents extracellular alkalinization, and to inform on further attempts to inhibit csp22-induced plant defence.

Another PAMP that may prove important in this interaction is peptidoglycan. Chapter 5 presents evidence that peptidoglycan detection might be an important element of this interaction for molecular farming because *CERK1* depletion led to increased recombinant protein production (Figure 5.5). *CERK1* detects bacterial peptidoglycan in *A. thaliana* as part of the LYM1/LYM3/*CERK1* perception system ([Willmann et al., 2011](#)). Further investigation into how *A. tumefaciens* peptidoglycan is detected in *N. benthamiana* and how this detection can be avoided may provide further strategies to improve molecular farming.

6.3 Stable pH is essential for successful transient expression of recombinant proteins

The data in Figure 2.3 demonstrate that maintaining an acidic pH during agroinfiltration significantly increases accumulation of recombinant proteins. This evidence supports previous reports that stable pH enhances transformation efficiency in *A. thaliana* seedlings ([Wu et al., 2014a](#)). In Chapter 2 I attempted to control the pH of the apoplast by expressing peptide elicitors (Figure 2.7A, B) that regulate a proton pump and the proton pump itself (AHA2) (Figure 2.13). This strategy was successful at causing apoplastic alkalinization in comparison to the EV control but not acidification. *N. benthamiana* which retain an acidic apoplast after agroinfiltration may improve molecular farming yields. This might be achieved by producing plants that are constitutively activated at proton pumping into the apoplast. I attempted this by overexpressing phosphomutants of AHA2, but these did not cause acidification, as is discussed in Chapter 2. Further research to manipulate AHA2 is

required to create a stable acidic pH in the apoplast.

6.4 Strategies and methods to investigate in future screens

In this thesis the ‘Traffic Light Screen’ was used to search for new strategies to improve molecular farming. In these two screens, over 160 treatments were tested for effects on recombinant protein production. In the screen of RXLR effectors, six positive hits were identified which increased RFP fluorescence between 1.4 and 2.02 fold (Figure 4.2, 4.3, 4.4). However, the increase in RFP fluorescence did not translate into higher yields when the most promising candidate was tested at Leaf Expression Systems (LES) (Figure 4.8). As described above, one reason that this benefit did not translate into higher yield at LES is that 1.5 times the concentration of *A. tumefaciens* was used in the effector treatment than the control. This was considered a more realistic test because mixing constructs is the protocol followed while testing new strategies as opposed to cloning the co-expressed gene into the same backbone as the GOI. This is because it allows recombinant proteins to be tested in multiple conditions without recloning many times. The increase in recombinant protein therefore needs to be substantial enough to outweigh the cost both physiologically to the plant and economically for growing the extra construct at scale.

From 160 treatments tested in the ‘Traffic Light Screen’ used in Chapter 4 and 5, only nine positive hits were found (Table 4.1, Figure 5.5). In Figure 3.5 both fluorescent proteins maintain high fluorescence at 7 dpi. This demonstrates the high expression level and stability of secreted RFP and cytosolic GFP in *N. benthamiana*. Screening for effects on a poorly expressed or unstable recombinant protein in future may yield more positive results. However, this may reduce the extent to which the screen is high-throughput and limit the number of strategies investigated.

A consistent issue faced when using co-expression of proteins to inhibit the effects of plant immunity on *A. tumefaciens* as a strategy to increase molecular farming is that plant immunity is suppressed too late to impact *A. tumefaciens* infection. As seen in Figure 3.5, transiently expressed proteins have not accumulated at 1 dpi. The T-DNA for the recombinant protein and the treatment protein are transferred at approximately the

same time because the constructs are co-agroinfiltrated. Therefore, the infectivity of *A. tumefaciens* is less relevant to molecular farming yields by the time the treatment protein is well expressed at 3 dpi. Future expression of effectors and other proteins to inhibit plant defence should be expressed by *A. tumefaciens* itself instead of by the plant via *Agrobacterium*-mediated transient expression. This strategy has been successful previously when *A. tumefaciens* was engineered to express the Type III Secretion System and AvrPto ([Raman et al., 2022](#)) and results in the effector being expressed by *Agrobacterium* at the same timepoint as its own virulence proteins, as soon as agroinfiltration occurs ([Raman et al., 2022](#)). This could be achieved in a high-throughput screen if the treatment proteins are placed on an accessory plasmid as part of a ternary vector system. Ternary vectors have been used successfully for expression of a constitutive virG mutant and for improving maize transformation ([Kikuchi et al., 2005](#); [Anand et al., 2018](#)). Using this system, the treatments would be expressed earlier and have a greater chance to inhibit PTI at a time point that impacts molecular farming yields.

In this thesis we have uncovered the detrimental role of SA-mediated defence in molecular farming and on *A. tumefaciens* state. This was first shown in Chapter 2, when AgroLux was significantly affected by pH-induced changes to PR proteins only in plants which accumulated SA (Figure 2.15). *NahG* transgenics, which do not accumulate SA, did not produce conditions for altered AgroLux bioluminescence upon expression of PSY1, RALF1, AHA2 WT or AHA2-S899A (Figure 2.15). The defining role of SA was also shown by the increase in recombinant protein expression after silencing of *ICS* and *NPR1* (Figure 5.5). *ICS* is the enzyme responsible for biosynthesis of SA by the *ICS* pathway ([Chen et al., 2010](#); [Catinot et al., 2008](#)) and *NPR1* is the transcription coactivator that regulates SA-induced upregulation of defence genes ([Moore et al., 2011](#); [Fan and Dong, 2002](#)). Depletion of genes involved in SA-mediated defence caused increased recombinant protein production (Figure 5.5), demonstrating that SA biosynthesis and signalling is detrimental to molecular farming. Silencing *EIN2* also increased accumulation of recombinant protein (Figure 5.1), although below the threshold set to be selected for further investigation. *EIN2* is a membrane protein involved in ethylene signalling and is required for ethylene-induced defence responses ([Liu et al., 2011](#)). Application

of a chemical ethylene biosynthesis inhibitor or chemical ethylene perception inhibitor increase T-DNA transfer frequency in plants including tomato ([Davis et al., 1992](#); [Ezura et al., 2000](#); [Han et al., 2005](#); [Nonaka and Ezura, 2014](#)) and transient expression using *A. tumefaciens* expressing an ethylene precursor degrading enzyme improved recombinant protein production, but not in *N. benthamiana* ([Nonaka et al., 2017](#)). Preventing ethylene signalling by *EIN2* depletion provides an alternative method to reduce ethylene-mediated defence which does not require chemical application, which may compromise Current Good Manufacturing Practice (cGMP) regulations, and is effective in *N. benthamiana*.

These results demonstrate significant effects of phytohormone signalling on molecular farming. A future screen should therefore focus on strategies that impact the balance of phytohormones. For example, effectors that decrease SA or increase JA can be tested. The *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) effector, called RaxX, is a mimic of peptide elicitor PSY1 and the two show high similarity ([Pruitt et al., 2015](#)). PSY1 signalling decreases levels of SA in *A. thaliana* and reduces plant defence to *P. syringae* DC3000. *A. tumefaciens* expression of RaxX and other treatments that interfere with phytohormone-mediated defence is a promising strategy to use in future screens.

The increase in recombinant protein after VIGS of *CERK1* (Figure 5.5) shows that reducing PTI can also be a useful strategy. Using *A. tumefaciens* strains which express PTI-inhibiting effectors themselves is a promising strategy. Expression of AvrPtoB targets CERK1 signalling and inhibits PTI ([Gimenez-Ibanez et al., 2009](#)). Expression of effectors, such as AvrPtoB, which inhibit PTI should be screened using *A. tumefaciens* expression because they need to be expressed very early into infection. Other promising treatments to screen include expression of chaperone proteins, protease inhibitors and silencing suppressors.

6.5 The wider implications of this research on molecular farming

I have demonstrated that the interaction between *N. benthamiana* and *A. tumefaciens* is an important event for molecular farming. Research into the plant side of this interaction

is abundant, but there has been less focus on *A. tumefaciens* state during agroinfiltration (*Jutras et al.*, 2021). The results from this research reveal that the state of *A. tumefaciens* in *N. benthamiana* and the immune response that acts upon *A. tumefaciens* play a major role in defining the success of transient expression of recombinant proteins. This research provides evidence to scientists using *A. tumefaciens*, both in the molecular farming industry and those studying immunity genes conducted through agroinfiltration, that the presence of *A. tumefaciens* in the apoplast after agroinfiltration impacts plant defence responses that should not be ignored. This work also demonstrates that manipulation of the plant defence response elicited upon agroinfiltration is a promising strategy that could have far-reaching impact across the whole industry of molecular farming. This is because the impacts of inhibiting plant defence to *A. tumefaciens* can improve production of many different recombinant proteins produced by molecular farming. This is in contrast to the majority of molecular farming research presented at the most recent International Society of Plant Molecular Farming meeting, where more than half of the presentation talks described research to improve the production of a specific recombinant protein. Manipulating the interaction between *N. benthamiana* and *A. tumefaciens* therefore provides a promising strategy to improve *Agrobacterium*-mediated transient expression across a whole industry and in academic research.

The discovery that depletion of *NbCORE* transcripts enables recombinant protein accumulation in six and eight week-old plants (Figure 5.9) offers new opportunities for molecular farming to use older plants with greater biomass. A more robust disruption of this immune response can be achieved by knock-out of *NbCORE* by genome editing or development of a strain of *A. tumefaciens* harbouring csp22 peptides that cannot be recognised by CORE. Use of these approaches to avoid the *N. benthamiana* immune response to agroinfiltration will improve transient expression of recombinant proteins in older plants. Use of older plants with greater biomass may enable increased production of a variety of recombinant proteins.

6.6 Conclusion

Agrobacterium-mediated transient expression in *N. benthamiana* is an efficient expression system for recombinant protein production. However, *A. tumefaciens* is a natural pathogen of *N. benthamiana* and agroinfiltration elicits an immune response from *N. benthamiana*. *N. benthamiana* immunity hampers molecular farming by causing a reduction in recombinant protein yields. This research confirms that manipulating the interaction between *N. benthamiana* and *A. tumefaciens* by inhibiting plant defence is an effective strategy to improve molecular farming. We demonstrate the need to study the interaction between *N. benthamiana* and *A. tumefaciens* to identify plant defence responses that hamper recombinant protein yields. There are several directions for future research to build on the work of this thesis and improve molecular farming. These include using AgroLux to understand effects of plant defence on *A. tumefaciens* state *in planta*, engineering *N. benthamiana* and *A. tumefaciens* to prevent detection of *A. tumefaciens*, manipulating phytohormone balance and screening new strategies to inhibit plant defence using high-throughput screens.

Chapter 7

Materials and Methods

7.1 Plant lines and growth Conditions

N. benthamiana plants were grown at a constant 21°C and approximately 60% relative humidity under a 16 hour light ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$) and 8 hour dark regime. Names and references of the knock-out lines used in Chapter 5 are listed in Table 5.2. Transgenic *NahG* *N. benthamiana* were kindly provided by Jonathan Jones and were described previously ([Wulff et al., 2004](#)).

7.2 Bacterial strains, culture conditions and media

Bacterial strains and respective antibiotic selection used in this research are listed in Table 7.1. Cultures of *A. tumefaciens* and *P. syringae* were cultured at 28°C and cultures of *E. coli* were cultured at 37°C, with shaking at 220rpm for liquid cultures. LB media was used for all cultures and was composed of 10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl. Antibiotics were used at the following concentrations: 10mg/L gentamicin, 25mg/L rifampicin, 50mg/L kanamycin and 100mg/L streptomycin.

7.3 Construction of plasmids

A. tumefaciens strains carrying binary plasmids delivering *cytosolic superfolded GFP*, *AvrBbb2*, *AvrPto* and *eGFP* have been described previously ([Jutras et al., 2021](#); [Bozkurt](#)

et al., 2011; *Rathjen et al.*, 1999; *Kourelis et al.*, 2023) and are listed in Table 7.3. VRC01 LC and VRC01 HC were recloned into pJK268c from plasmids described by *Grosse-Holz et al.* (2018a) using Golden Gate Cloning (*Engler et al.*, 2014) in a BsaI reaction. cDNA fragments of *NbAHA2*, *AtPSY1*, *AtPSY1-R* and *NbRALF1* were amplified using the primers described in Table 7.5. *NbAHA2* and *AtPSY1* were amplified into two fragments to remove a BsaI recognition site by introduction of a synonymous mutation in the PCR. The secreted RFP gene (pPJ056 and pID042) was obtained from AddGene (<http://www.addgene.org>) and amplified using the primers in Table 7.5. For production of binary vectors for overexpression, gene fragments were inserted into either pJK268c pL1V2-P19-F2 (*Kourelis et al.*, 2020) or pJK001c using Golden Gate Cloning (*Engler et al.*, 2014) in a BsaI reaction, resulting in the plasmids listed in Table 7.3. Both pJK268c and pJK001c contain an open reading frame driven by a double cauliflower mosaic virus (CaMV) 35S promoter and followed by the 35S terminator on the T-DNA. The T-DNA of pJK268c also carries a gene encoding the viral silencing suppressor P19. AHA2 mutants were made by introduction of a single amino acid substitution by site directed mutagenesis of the AHA2 plasmid using the plasmids listed in Table 7.3. For the production of binary vectors for VIGS, gene fragments (see Table 5.1) were obtained from Twist Biosciences and cloned into the golden-gate compatible vector TRV2gg (*Duggan et al.*, 2021b) also using Golden Gate Cloning with a BsaI reaction. Fragments for silencing *NbCORE*, *ACIF1*, *ICS*, *CDPK* and *AHA2* were designed using the SolGenomics VIGS tool (Table 7.4, SGN-VIGS (*Fernandez-Pozo et al.*, 2015)). All gene constructs were verified by Sanger sequencing performed by Source Bioscience (<https://sourcebioscience.com>) and analysed using Geneious Prime (<https://www.geneious.com>). Plasmids were amplified in *E. coli* DH10 β and purified using Machery-Nagel NucleoSpin Plasmid kit. Plasmids were then transformed into *A. tumefaciens* GV3101 or *A. tumefaciens* RSPJ07 by heat shock (see Table 7.1). Transformants were selected on plates of lysogeny broth (LB) agar medium containing 25mg/L rifampicin, 10mg/L gentamycin and 50mg/L kanamycin or 100mg/L streptomycin. A single colony of transformant was cultured in liquid LB containing the same antibiotics. The plasmids encoding *P. infestans* RXLR effectors were provided by our collaborators the Dong Group from Nanjing Agricultural University,

China. Putative effector sequences were cloned from cDNA of *P. infestans* T30-4 and inserted into pBinGFP2 plasmids, see Table 7.2.

7.4 Virus-Induced Gene Silencing (VIGS)

A. tumefaciens containing *TRV1* and *TRV2* were grown overnight at 28°C in LB medium containing 25mg/L rifampicin, 10mg/L gentamycin and 50mg/L kanamycin. The cultures were centrifuged at 3500xg for 10 minutes at room temperature and then resuspended at OD₆₀₀=1.0 in agroinfiltration buffer (10 mM MES, 10 mM MgCl₂, 100μM acetosyringone at pH 5.7). *TRV2* cultures were mixed at a 1:1 ratio with *TRV1* cultures. Two week-old *N. benthamiana* were agroinfiltrated with the bacterial suspension mixture using a 1ml needleless syringe. Three or five weeks later plants were assessed for silencing by observing if the *TRV::PDS* plants had bleached leaves. Silenced plants were then agroinfiltrated with *A. tumefaciens* carrying expression cassettes for recombinant proteins.

7.5 Agroinfiltration for protein expression

For each agroinfiltration experiment a mixture of constructs was injected into *N. benthamiana* leaves (Figure 7.1). In this thesis, *A. tumefaciens* constructs refers to *A. tumefaciens* which has been transformed to contain a binary, Ti plasmid encoding a gene of interest for expression of a recombinant protein, over-expression of an endogenous protein or expression of the tobacco rattle virus (TRV) RNA1 and RNA2 used for VIGS (Figure 7.1A). *A. tumefaciens* strains used in this thesis are *A. tumefaciens* GV3101 (*pMP90*) and *A. tumefaciens* RSPJ07 (*pMP90*)(AgroLux). *A. tumefaciens* RSPJ07 (*pMP90*) is classed as a different strain because the *lux* operon has been integrated into the *A. tumefaciens* chromosome and is therefore expressed by *A. tumefaciens* itself, in contrast to cytoGFP and secRFP which are on a binary plasmid and are expressed by *N. benthamiana*, not *A. tumefaciens* (Figure 7.1A). In this thesis, *A. tumefaciens* carrying an expression cassette are referred to as different ‘constructs’. These ‘constructs’ are not deemed as different strains because no editing of the linear or circular chromosome has taken place (Figure 7.1A).

Table 7.21 gives a list of the mixtures of constructs used for experiments in Chapter 2. *A. tumefaciens* cultures were grown overnight at 28°C in LB medium containing 25mg/L rifampicin, 10mg/L gentamycin and 50mg/L kanamycin or 100mg/L streptomycin. The cultures were centrifuged at 3500xg for 10 minutes at room temperature and then resuspended in agroinfiltration buffer (10 mM MES, 10 mM MgCl₂, 100μM acetosyringone at pH 5.7). Agroinfiltration for experiments using different pH buffers were resuspended in 100mM phosphate buffer instead of standard agroinfiltration buffer. The constructs used to express different recombinant proteins are grown as separate cultures overnight and then mixed to each be at a final OD₆₀₀ of 0.5 in the final mixture, meaning that for an experiment using a mixture of three constructs (e.g for the Traffic Light Screen), the final OD₆₀₀ of the agroinfiltrated mixture is 1.5 (Figure 7.1B). Standard agroinfiltrations used each construct at an OD₆₀₀ of 0.5 unless otherwise stated. Bacterial suspension mixtures were agroinfiltrated into *N. benthamiana* using a 1ml needleless syringe into the third youngest leaf unless specifically stated.

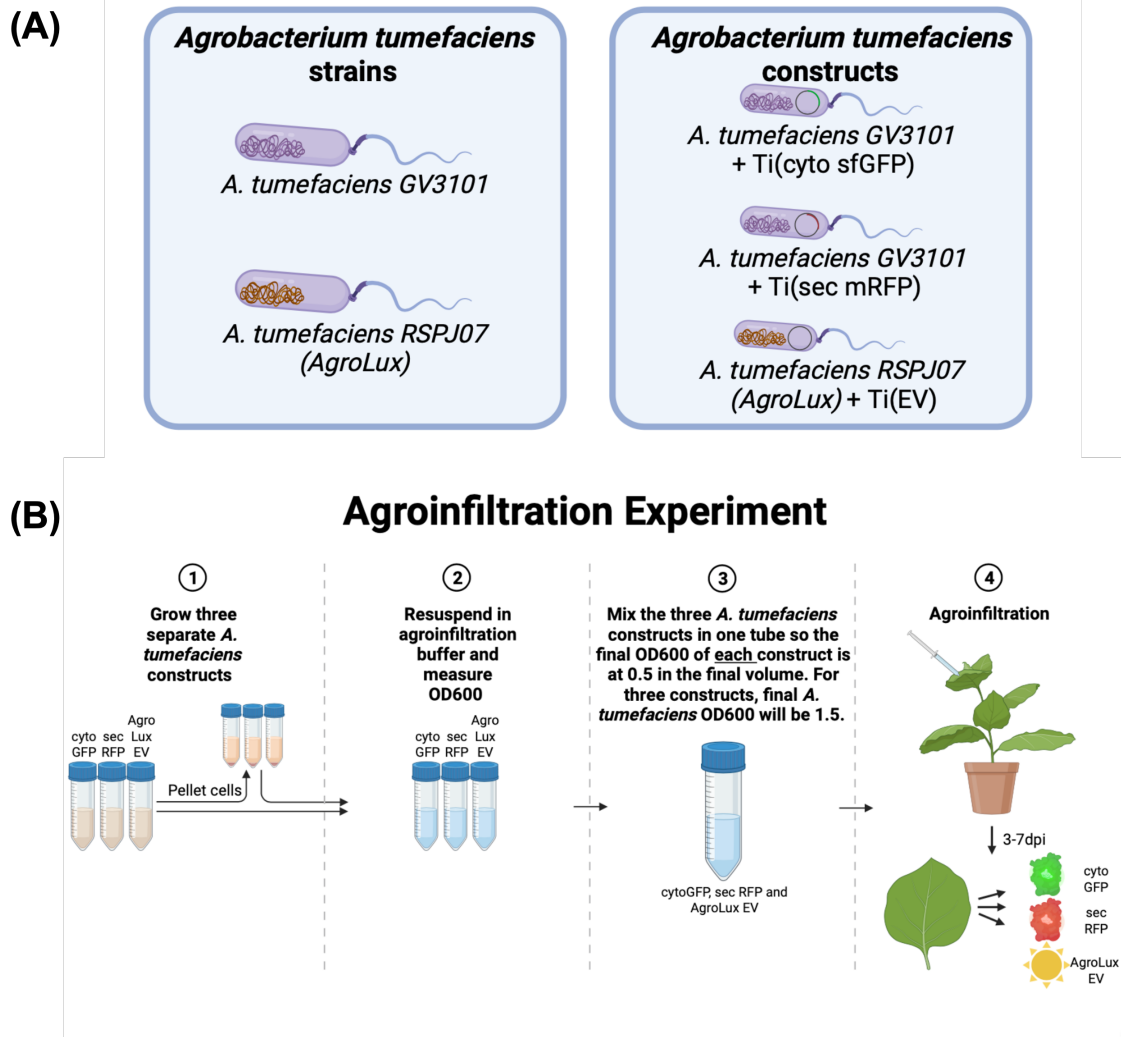


Figure 7.1: A) Explanation of the nomenclature used in this thesis. *A. tumefaciens* strains used in this thesis are *A. tumefaciens* GV3101 (pMP90) and *A. tumefaciens* RSPJ07 (pMP90) (AgroLux). In this thesis, *A. tumefaciens* carrying an expression cassette are referred to as different ‘constructs’. These ‘constructs’ are not deemed as different strains because no editing of the linear or circular chromosome has taken place. (B) **The agroinfiltration process.** The constructs used to express the recombinant proteins required in a given experiment are grown overnight as separate liquid cultures. These cultures are centrifuged to cell pellets and then are resuspended in agroinfiltration buffer. The OD₆₀₀ of the resuspended cultures are measured and then the volume of each construct required for a final OD₆₀₀ of 0.5 of each culture in the final volume is calculated. The constructs are then mixed into a single tube so that each is at an OD₆₀₀ of 0.5. Therefore, if three constructs are mixed, the final OD₆₀₀ of the mixture is 1.5. This mixture is agroinfiltrated into *N. benthamiana*, leading to transient expression of recombinant proteins after 3-7 days post infiltration (dpi).

7.6 Agromonas inoculation assay

Agromonas assays were carried out using *P. syringae* DC3000 ΔhQ as previously described by Buscaill *et al.* (2021) and Sueldo *et al.* (2023). Briefly, leaves of 3.5 week-old *N.*

benthamiana were agroinfiltrated for protein expression as described above. At three days post agroinfiltration, the agroinfiltrated leaf tissue was infected with *P. syringae* DC3000 ΔhQ at an OD₆₀₀ of 0.0001. Three days later, 10mm diameter leaf discs were punched from the agroinfiltrated/infected leaf tissue, which were surface sterilised with 15% hydrogen peroxide solution for two minutes, washed twice in sterile MilliQ water and then dried for 30 minutes. Leaf discs homogenized in 1ml sterile MilliQ using sterile metal beads in a tissuelyser for 5 minutes at 30 Hertz⁻¹. 200 μ l of the lysed tissue was transferred to the first row of a sterile 96-well plate. Serial 10-fold dilutions were made until the last row of the plate. 20 μ l of each serial dilution was plated on LB-agar plates containing *Pseudomonas* CFC Agar Supplement (10 μ g ml⁻¹ cetrimide, 10 μ g ml⁻¹ fucidin and 50 μ g ml⁻¹ cephaloridine, SR0103; Thermo Fisher Scientific). Plates were dried and incubated at 28°C for two days, before colonies were counted.

7.7 *A. tumefaciens* colony count

Growth of *A. tumefaciens* was analysed by colony count. Four week-old *N. benthamiana* were agroinfiltrated with *A. tumefaciens* carrying expression cassettes for EV/EV, PSY1/PSY1-R and RALF1/EV. At 5dpi, 10mm diameter leaf discs were punched from the agroinfiltrated leaf tissue, which were surface sterilised with 15% hydrogen peroxide solution, washed twice in sterile MilliQ water and then dried. Leaf discs were then pulverised in 1ml sterile MilliQ using sterile metal beads in a tissuelyser for 5 minutes at 30 Hertz⁻¹. 200 μ l of the lysed tissue was transferred to the first row of a sterile 96-well plate. Serial 10-fold dilutions were made until the last row of the plate. 20 μ l of each serial dilution was plated on LB-agar plates containing 10mg/L gentamicin and 25mg/L rifampicin. Plates were dried and then incubated at 28°C for two days, before colonies were counted.

7.8 Apoplastic fluid extraction

N. benthamiana leaves were rolled into a 50ml syringe filled with ice-cold water. The leaves were infiltrated with water by pulling the plunger, thereby applying negative pressure by

drawing the air out of the leaves, and then pushing the plunger back to apply positive pressure and force water in the spaces where the air had been drawn out. The water infiltrated leaves were then carefully blotted dry, rolled up and placed into the barrel of a 20ml syringe, which was inserted into a 50ml conical centrifuge tube. These were then centrifuged at 500 $\times g$ for 25 minutes at 4°C and apoplastic fluid accumulating in the 50ml conical centrifuge tube was collected.

7.9 AgroLux bioluminescence measurements

7.9.1 AgroLux measurements using a plate reader

Leaf discs (6mm diameter) were punched from leaves agroinfiltrated with *A. tumefaciens* *RSPJ07*. Leaf discs were placed onto 200 μ l sterile water in a white, opaque 96-well plate (Corning). The plate was placed into an Infinite M200 plate reader (Tecan) and kept in the dark for five minutes prior to bioluminescence measurements with an integration time of 1 second. Background bioluminescence was accounted for by measurement of leaf discs from leaves agroinfiltrated with *A. tumefaciens* *GV3101*.

7.9.2 AgroLux leaf imaging using Image Quant LAS4000 imaging system

Agroinfiltrated leaves were kept in the dark for five minutes and then imaged using an Image Quant LAS4000 imaging system (GE Healthcare, <https://www.gehealthcare.com>) to detect bioluminescence. Image data were analysed using ImageJ and the Orange Hot LUT was applied to colour images.

7.10 pH Measurement using secreted GFP pHluorin

N. benthamiana were agroinfiltrated with *A. tumefaciens* carrying expression cassettes for secGFP pHluorin as described above. Either apoplastic fluid or leaf discs were taken from agroinfiltrated leaves and placed in a white, opaque 96-well plate (Corning). The plate was placed in an Infinite M200 plate reader (Tecan) and a fluorescence excitation scan was performed using excitation wavelengths of 380nm to 480nm, emission wavelength 510nm.

Peaks of fluorescence emission were identified from the fluorescence excitation scan and fluorescence emissions at these wavelengths were measured with Gain 100. Background fluorescence was accounted for by measuring fluorescence from leaf discs agroinfiltrated with *A. tumefaciens* carrying an empty vector expression cassette and subtracting this fluorescence from pHluorin measurements. Ratios of the two peaks were calculated and then statistical analysis was performed using a One-way ANOVA followed by Dunnett's multiple comparisons using GraphPad Prism 9 for macOS.

7.11 Buffering AgroLux and GFP pHluorin *in planta* and *in vitro*

7.11.1 Buffered AgroLux measurement

A. tumefaciens was grown overnight in liquid culture in LB medium containing 25mg/L rifampicin and 10mg/L gentamycin. Cultures were then centrifuged at 2500xg for 10 minutes at room temperature. *A. tumefaciens* cell pellet was then resuspended in 100mM phosphate buffers (pH 5.6-7.1) to a final OD₆₀₀ of 0.5. For *in vitro* experiments, bacterial suspensions were immediately transferred to a white, opaque 96-well plate (Corning) and bioluminescence measured on a Infinite M200 plate reader (Tecan). For *in planta* experiments, bacterial suspensions were agroinfiltrated into leaves. Leaf discs (6mm diameter) were punched and placed onto 200μl sterile water in a white, opaque 96-well plate (Corning) and bioluminescence measured on an Infinite M200 plate reader (Tecan).

7.11.2 Buffered GFP pHluorin measurement

Secreted GFP pHluorin was expressed in *N. benthamiana* as described above. *In vitro* measurements were taken from apoplastic fluid isolated from leaves expressing secGFP pHluorin and buffering them with 100mM phosphate buffers from pH 5.6 to 7.1 in a white, opaque 96-well plate (Corning). *In planta* measurements were taken by transiently expressing secGFP pHluorin and injecting the agroinfiltrated tissue with 100mM phosphate buffers, punching leaf discs (6mm diameter) and placing these onto 200μl sterile water in a white, opaque 96-well plate (Corning). Fluorescence emitted from secGFP pHluorin was

measured both by a fluorescence excitation scan to identify the two peaks of fluorescence emission and fluorescence emitted at the excitation peaks, from which a ratio was taken (pHluorin Ratio 400/480).

7.12 pH measurement using pHrodo™ Red Dextran

Apoplastic fluids were collected from leaves transiently expressing pH-modifying proteins or the EV control. Extracted apoplastic fluids and 200mM phosphate buffers from pH 5.6 to 7.1 were added into a transparent, 384-well plate. To each well, 20µg/mL pHrodo™ Red Dextran (Thermofisher, P10361). The plate was immediately placed into an Infinite M200 plate reader (Tecan), where fluorescence at at Ex./Em. 550/585nm was measured. A standard curve was produced from the fluorescence emissions from pHrodo™ Red Dextran in phosphate buffers and the pH of the apoplastic fluid was interpolated from this standard curve using the ‘Interpolate a Standard Curve’ statistical function of GraphPad Prism version 9 for macOS.

7.13 Western Blot

Recombinant proteins were extracted in total extracts at five days post infiltration and visualised using western blotting, as described by [Jutras et al. \(2021\)](#). Briefly, leaf discs (10mm diameter) were punched from agroinfiltrated tissue, flash-frozen in liquid nitrogen and pulverised using a cold pestle. The resulting leaf powder was mixed 3:1 with 1x phosphate-buffered saline (PBS:fresh weight leaf tissue) and briefly vortexed. This mixture was centrifuged at at 16,000xg for 10 minutes at 4°C. The supernatant was then mixed 3:1 with gel loading buffer (200 mM Tris-HCl (pH 6.8), 8% SDS, 0.4% bromophenol blue, 40% glycerol) and samples were heated for 5 minutes at 95°C. Gel loading buffer added to proteins analysed in reducing conditions also contained 400mM DTT. Proteins were separated on Bis-Tris gels at 200V. Proteins were then transferred to a PVDF membrane using the TransBlot Turbo system (Bio-Rad, Hercules, US). Membranes were then blocked in 5% skim milk in 1x PBS containing 0.025% Tween-20 overnight at 4°C. VRC01 was detected using Anti-IgG (Fc)-HRP antibody (Binding Site, AP004) at a dilution of 1/5000

in 5% skim milk in 1x PBS containing 0.025% Tween-20 overnight at 4°C. At LES, VRC01 was detected using Anti-Human IgG H&L (HRP) antibody (abcam, ab102429) at a dilution of 1/5000 in 5% skim milk in 1x TBS-T (50 mM Tris-Cl, pH 7.6; 150 mM NaCl, 0.025% Tween-20), incubated at RT for 1 hour. GFP was detected using an anti-GFP HRP-conjugated antibody (Abcam, Ab66663) at a dilution of 1/5000 in 5% skim milk in 1x PBS containing 0.025% Tween-20 overnight at 4°C. RD21 was detected using a wheat anti-triticain- α primary antibody (produced and provided by the Andrey Zamyatin lab) at a dilution of 3/10000 in 5% skim milk in 1x PBS containing 0.025% Tween-20 for two hours at room temperature (RT). α -PR2 and α -PR3 (Agrisera, AS07 208 and AS07 207) were used to detect PR2 and PR3 at a dilution of 1/2500 in 5% skim milk in 1x PBS containing 0.025% Tween-20, incubated overnight at 4°C. Detection of RD21, PR2 and PR3 required use of an HRP-conjugated secondary antibody (α -Rabbit IgG (HRP), GE Healthcare, 399275 NA934V) used at a dilution of 1/5000. Chemiluminescent signals were detected using the Clarity ECL western blotting detection kit (Bio-Rad). Images of membranes were taken using an Image Quant LAS4000 imaging system (GE Healthcare). For each PVDF membrane, an identical SDS-PAGE gel was stained in InstantBlue Coomassie Protein Stain (Abcam) as a protein loading control.

7.14 Traffic Light Screen

For the effector screen (Chapter 4), four week-old *N. benthamiana* were agroinfiltrated with *A. tumefaciens* GV3101 carrying expression cassettes for secreted RFP and *A. tumefaciens* RSPJ07 (two constructs mixed together, see Figure 7.1). Cytosolic GFP could not be added because GFP was already encoded in the pre-cloned binary vectors. For the silencing screens (Chapter 5), VIGS or knock-out plants were agroinfiltrated with a mixture of three constructs: *A. tumefaciens* GV3101 construct carrying expression cassettes for cytosolic GFP, *A. tumefaciens* GV3101 carrying expression cassette for secreted RFP and *A. tumefaciens* RSPJ07 EV (Figure 7.1B). At 5dpi, leaf discs (6mm diameter) were punched and placed onto 200 μ l sterile water in a white, opaque 96-well plate (Corning). AgroLux, cytosolic GFP and secreted RFP were monitored by measuring the bioluminescence and

fluorescence of agroinfiltrated leaves on a plate reader. AgroLux was measured as described earlier. Secreted RFP fluorescence was measured using 550 nm excitation and 585 nm emission wavelengths at gain 100. GFP fluorescence was measured using 500 nm excitation and 530 nm emission wavelengths at gain 100. For each agroinfiltrated sector, at least two leaf discs (technical replicates) were taken to reduce the effect of variability in expression within leaves.

Statistics of the effector screen (Chapter 4) were performed by Frederick King using *R* version 4.1.2 and the *tidyverse* collection of packages ([Wickham et al., 2019](#)). Treatment and control means of the traffic light reporter data were compared using a two-way analysis of variance (ANOVA) and Dunnett's post-hoc test to account for multiple comparisons. Statistics of the silencing screen (Chapter 5) and comparisons of AgroLux and fluorescent proteins in Chapter 2 were performed using a One-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad Prism9 for macOS.

7.15 Large-scale agroinfiltration at Leaf Expression Systems

A. tumefaciens carrying expression cassettes for VRC01 LC, VRC01 HC and Effector E6 (C8P3, see Table 7.2) were grown in liquid culture with the corresponding antibiotics overnight at 28°C. The OD₆₀₀ of each liquid culture was measured. Liquid cultures were diluted in sterile water (containing 100µM acetosyringone) so that each construct was at a final OD₆₀₀ of 0.5 in a final volume of 18L. In the effector treatment bacterial suspension, constructs for VRC01 HC, VRC01 LC and effector E6 (three constructs) were each mixed to a final OD₆₀₀ of 0.5, giving a total OD₆₀₀ of 1.5 in the 18L bacterial suspension. In the control treatment, constructs for VRC01 HC and VRC01 LC (two constructs) were mixed to a final OD₆₀₀ of 0.5, giving a total OD₆₀₀ of 1 in the 18L bacterial suspension. 18 whole plants were vacuum infiltrated for each treatment and then incubated for five days.

7.16 Purification of VRC01 at Leaf Expression Systems

150g of leaf tissue was harvested for each treatment at 5 dpi and blended at 3:1 (buffer:tissue) weight ratio with extraction buffer (50mM Tris, 150mM NaCl, 10mM

Ascorbic acid, pH 8.0). This homogenate was then centrifuged at 4000rpm for 30 minutes at 4°C. The supernatant was then filtered through miracloth and then a 1µm bag filter. The resulting filtrate was then filtered using a 0.22µm PolyCap filter followed by a 0.22µm PES bottle top filter. The filtrate was then loaded onto a 5ml HiTrap™ Protein A HP column equilibrated in 20mM Tris, 150mM NaCl, pH 7.75 using an AKTA Avant (GE Healthcare). The column was washed in 20 column volumes (CVs) of equilibration buffer (20mM Tris, 150mM NaCl, pH 7.75). VRC01 was eluted with 10CVs of elution buffer (100mM citrate, 0.2M Tris). The eluted antibody fractions were pooled and exchanged into 1x PBS buffer (137mM NaCl, 2.7mM KCl, 20mM phosphate) using HiPrep™ 26/10 Desalting was performed using AKTA Avant (GE Healthcare). VRC01 fractions were pooled (with E6, without) and syringe filtered. Concentrations were quantified using the Pierce™ BCA Protein Assay Kit. Samples of all fractions were taken and added to gel loading buffer (in reducing and non-reducing conditions) and analysed using western blotting as described above.

7.17 Fluorescent protein leaf imaging and quantification

Leaves transiently expressing eGFP and cytosolic GFP were scanned on an Amersham Typhoon 5 Biomolecular Imager (GE Healthcare Life Sciences, Little Chalfont, UK) using the 488nm laser and Cy2 filter at PMT300 to detect *in planta* fluorescence at 5dpi. The ImageJ green fire blue LUT was applied to images. Leaves transiently expressing secreted RFP were scanned on the same Typhoon imager using the 532nm laser and Cy3 filter at PMT300 to detect *in planta* fluorescence at 5dpi. The ImageJ red hot LUT was applied to images. Quantification of fluorescence (Chapter 5, Figure 5.9) was performed using ImageJ and normality was tested by a Shapiro-Wilk test and statistically analysed with a Welch's t-test using GraphPad Prism 9 for macOS.

7.18 Semi-quantitative RT-PCR

100µg of leaf tissue was flash-frozen in liquid nitrogen and ground to a fine powder using a frozen pestle. The Qiagen RNeasy Mini Kit (including the Qiagen Shredder columns) was

used to extract total RNA. The extracted RNA was treated using the Qiagen RNase-free DNase and purified using the Qiagen RNeasy Mini Kit. The RNA was measured using a NanoDrop. Reverse transcription (RT) was performed on 1 μ g RNA by following the First-Strand cDNA Synthesis using the SuperScript[™] II RT protocol, using Oligo-dT primers to produce cDNA. PCR was performed using Q5 polymerase with gene-specific primers (See Table 7.5). PCR products were run on a 3% agarose gel and imaged using a G:Box imaging system. Quantification was performed using ImageJ. Data was statistically analysed using an Unpaired t-test using GraphPad Prism 9 for macOS.

7.19 ROS assays

Leaf discs (4mm diameter) were punched from VIGS plants and incubated overnight in a white, 96-well plate (Corning) containing 100 μ l of sterile water. Water was removed from the wells and replaced with 100 μ l of solution containing 25 ng/ μ l L-012, 25 ng/ μ l Horse Radish Peroxidase (HRP) and 500 nM csp22. Chemiluminescence was measured immediately using an Infinite M200 plate reader (Tecan). Luminescence readings were taken every minute for one hour. The *PtoDC3000* csp22 peptide sequence was used (LNGKVKWFNNAKGYGFILEDGK) and was synthesized by Genscript. The Area Under the Curve (AUC) integration and unpaired t-test was performed using GraphPad Prism 9 for macOS.

7.20 Photos of VIGS plants

Photos taken of VIGS plant phenotypes were taken using an iPhone 11 Pro.

7.21 Bacterial strains, plasmids and primer tables

Table 7.2: *P. infestans* RXLR effectors used in the screen. The effectors were named according to the position of their plasmids within three 96-well plates (P1-3). The gene names represent the predicted RxLR effector genes that were previously cloned into the plasmids from *P. infestans* T30-4 genomic DNA.

Effector	Gene name	Antibiotic resistance
A6 P1	PITG_06092	gentamicin, rifampicin, kanamycin
A8 P1	PITG_18685	gentamicin, rifampicin, kanamycin
A10 P1	PITG_01934	gentamicin, rifampicin, kanamycin
B2 P1	PITG_22757	gentamicin, rifampicin, kanamycin
B5 P1	PITG_07451	gentamicin, rifampicin, kanamycin
B6 P1	PITG_23014	gentamicin, rifampicin, kanamycin
B9 P1	PITG_15287	gentamicin, rifampicin, kanamycin
B10 P1	PITG_02830	gentamicin, rifampicin, kanamycin
B11 P1	PITG_04074	gentamicin, rifampicin, kanamycin
C1 P1	PITG_05983	gentamicin, rifampicin, kanamycin
C2 P1	PITG_06375	gentamicin, rifampicin, kanamycin
C3 P1	PITG_09218	gentamicin, rifampicin, kanamycin
C4 P1	PITG_06478	gentamicin, rifampicin, kanamycin
C5 P1	PITG_00366	gentamicin, rifampicin, kanamycin
C6 P1	PITG_02843	gentamicin, rifampicin, kanamycin
C7 P1	PITG_04099	gentamicin, rifampicin, kanamycin
C8 P1	PITG_04373	gentamicin, rifampicin, kanamycin
C9 P1	PITG_06059	gentamicin, rifampicin, kanamycin
C10 P1	PITG_07533	gentamicin, rifampicin, kanamycin
C11 P1	PITG_11484	gentamicin, rifampicin, kanamycin
C12 P1	PITG_04196	gentamicin, rifampicin, kanamycin
D1 P1	PITG_05750	gentamicin, rifampicin, kanamycin
D2 P1	PITG_00821	gentamicin, rifampicin, kanamycin
D4 P1	PITG_04314	gentamicin, rifampicin, kanamycin
D5 P1	PITG_05076	gentamicin, rifampicin, kanamycin
D6 P1	PITG_07558	gentamicin, rifampicin, kanamycin
D7 P1	PITG_09216	gentamicin, rifampicin, kanamycin
D8 P1	PITG_04266	gentamicin, rifampicin, kanamycin

D9 P1	PITG_19617	gentamicin, rifampicin, kanamycin
D10 P1	PITG_06432	gentamicin, rifampicin, kanamycin
D11 P1	PITG_07597	gentamicin, rifampicin, kanamycin
D12 P1	PITG_09160	gentamicin, rifampicin, kanamycin
E1 P1	PITG_07594	gentamicin, rifampicin, kanamycin
E2 P1	PITG_01904	gentamicin, rifampicin, kanamycin
E3 P1	PITG_02918	gentamicin, rifampicin, kanamycin
E4 P1	PITG_04169	gentamicin, rifampicin, kanamycin
E5 P1	PITG_06087	gentamicin, rifampicin, kanamycin
E6 P1 (E4)	PITG_06485	gentamicin, rifampicin, kanamycin
E7 P1	PITG_02387	gentamicin, rifampicin, kanamycin
E9 P1	PITG_12761	gentamicin, rifampicin, kanamycin
E11 P1	PITG_19994	gentamicin, rifampicin, kanamycin
F1 P1	PITG_09503	gentamicin, rifampicin, kanamycin
F2 P1	PITG_09223	gentamicin, rifampicin, kanamycin
F3 P1 (E5)	PITG_21933	gentamicin, rifampicin, kanamycin
F4 P1	PITG_23046	gentamicin, rifampicin, kanamycin
F5 P1	PITG_20940	gentamicin, rifampicin, kanamycin
F6 P1	PITG_08317	gentamicin, rifampicin, kanamycin
F7 P1	PITG_09224	gentamicin, rifampicin, kanamycin
F8 P1	PITG_13509	gentamicin, rifampicin, kanamycin
F10 P1	PITG_15337	gentamicin, rifampicin, kanamycin
F12 P1	PITG_09836	gentamicin, rifampicin, kanamycin
G1 P1	PITG_10347	gentamicin, rifampicin, kanamycin
G2 P1	PITG_14294	gentamicin, rifampicin, kanamycin
G4 P1	PITG_21288	gentamicin, rifampicin, kanamycin
G6 P1	PITG_09861	gentamicin, rifampicin, kanamycin
G7 P1	PITG_12731	gentamicin, rifampicin, kanamycin
G9 P1	PITG_13306	gentamicin, rifampicin, kanamycin

G10 P1	PITG.22675	gentamicin, rifampicin, kanamycin
G11 P1	PITG.15297	gentamicin, rifampicin, kanamycin
G12 P1	PITG.09935	gentamicin, rifampicin, kanamycin
H1 P1	PITG.13847	gentamicin, rifampicin, kanamycin
H2 P1	PITG.18405	gentamicin, rifampicin, kanamycin
H3 P1	PITG.04388	gentamicin, rifampicin, kanamycin
H5 P1	PITG.15038	gentamicin, rifampicin, kanamycin
H6 P1	PITG.14360	gentamicin, rifampicin, kanamycin
H7 P1	PITG.14046	gentamicin, rifampicin, kanamycin
H8 P1	PITG.18981	gentamicin, rifampicin, kanamycin
H9 P1	PITG.22722	gentamicin, rifampicin, kanamycin
H10 P1	PITG.12952	gentamicin, rifampicin, kanamycin
H11 P1	PITG.16242	gentamicin, rifampicin, kanamycin
H12 P1	PITG.15940	gentamicin, rifampicin, kanamycin
A2 P2	PITG.06094	gentamicin, rifampicin, kanamycin
A3 P2	PITG.06076	gentamicin, rifampicin, kanamycin
A4 P2	PITG.13125	gentamicin, rifampicin, kanamycin
A5 P2	PITG.22896	gentamicin, rifampicin, kanamycin
A7 P2	PITG.19232	gentamicin, rifampicin, kanamycin
A8 P2	PITG.16275	gentamicin, rifampicin, kanamycin
A9 P2	PITG.05846	gentamicin, rifampicin, kanamycin
A10 P2	PITG.04049	gentamicin, rifampicin, kanamycin
A11 P2	PITG.10654	gentamicin, rifampicin, kanamycin
B1 P2	PITG.07569	gentamicin, rifampicin, kanamycin
B3 P2	PITG.00582	gentamicin, rifampicin, kanamycin
B4 P2 (E3)	PITG.14965	gentamicin, rifampicin, kanamycin
B6 P2	PITG.16427	gentamicin, rifampicin, kanamycin
B7 P2	PITG.15930	gentamicin, rifampicin, kanamycin
B8 P2	PITG.17316	gentamicin, rifampicin, kanamycin

B9 P2	PITG_16195	gentamicin, rifampicin, kanamycin
B10 P2	PITG_18670	gentamicin, rifampicin, kanamycin
C2 P2	PITG_21740	gentamicin, rifampicin, kanamycin
C5 P2	PITG_19302	gentamicin, rifampicin, kanamycin
C6 P2	PITG_17218	gentamicin, rifampicin, kanamycin
C8 P2	PITG_17309	gentamicin, rifampicin, kanamycin
C9 P2	PITG_22922	gentamicin, rifampicin, kanamycin
C10 P2	PITG_23054	gentamicin, rifampicin, kanamycin
C11 P2	PITG_23215	gentamicin, rifampicin, kanamycin
C12 P2	PITG_22825	gentamicin, rifampicin, kanamycin
D1 P2	PITG_23074	gentamicin, rifampicin, kanamycin
A1 P3	PITG_00707	gentamicin, rifampicin, streptomycin
A5 P3	PITG_07414	gentamicin, rifampicin, streptomycin
A6 P3	PITG_07947	gentamicin, rifampicin, streptomycin
B1 P3	PITG_11384	gentamicin, rifampicin, streptomycin
B3 P3	PITG_13044	gentamicin, rifampicin, streptomycin
B5 P3	PITG_13047	gentamicin, rifampicin, streptomycin
B8 P3	PITG_14685	gentamicin, rifampicin, streptomycin
B9 P3	PITG_14787	gentamicin, rifampicin, streptomycin
B11 P3	PITG_15110	gentamicin, rifampicin, streptomycin
B12 P3	PITG_15123	gentamicin, rifampicin, streptomycin
C1 P3	PITG_15125	gentamicin, rifampicin, streptomycin
C2 P3	PITG_15235	gentamicin, rifampicin, streptomycin
C3 P3	PITG_15278	gentamicin, rifampicin, streptomycin
C8 P3 (E6)	PITG_19655	gentamicin, rifampicin, streptomycin
C9 P3	PITG_20301	gentamicin, rifampicin, streptomycin
C10 P3	PITG_20336	gentamicin, rifampicin, streptomycin
C11 P3 (E1)	PITG_20934	gentamicin, rifampicin, streptomycin
C12 P3	PITG_21362	gentamicin, rifampicin, streptomycin

D2 P3	PITG_22604	gentamicin, rifampicin, streptomycin
D3 P3	PITG_22870	gentamicin, rifampicin, streptomycin
D5 P3	PITG_22935	gentamicin, rifampicin, streptomycin
D12 P3	PITG_23206	gentamicin, rifampicin, streptomycin
E2 P3	PITG_02860	gentamicin, rifampicin, streptomycin
E3 P3	PITG_06094	gentamicin, rifampicin, streptomycin
E4 P3	PITG_04089	gentamicin, rifampicin, streptomycin
E5 P3	PITG_22757	gentamicin, rifampicin, streptomycin
E6 P3 (E2)	PITG_15142	gentamicin, rifampicin, streptomycin
E7 P3	PITG_10835	gentamicin, rifampicin, streptomycin
E8 P3	PITG_21190	gentamicin, rifampicin, streptomycin
E10 P3	PITG_21388	gentamicin, rifampicin, streptomycin

Table 7.1: List of bacterial strains used.

Strain	Antibiotics	Reference
<i>Pseudomonas syringae</i> pv. tomato DC3000 ΔhQ	rifampicin	(Wei et al., 2007)
<i>Escherichia coli</i> DH10 β	rifampicin	(Grant et al., 1990)
<i>Agrobacterium tumefaciens</i> GV3101 (pMP90)	rifampicin, gentamicin	(Koncz and Schell, 1986)
<i>Agrobacterium tumefaciens</i> RSPJ07 (pMP90)	rifampicin, gentamicin	(Jutras et al., 2021)

Table 7.3: List of binary vectors used.

Name	Description	Reference
pJK268c	EV, containing P19	Kourelis et al. (2020)
pJK001c	pJK001c	Kourelis et al. (2020)
TRV1	RNAi of TRV	Liu et al. (2002)
pPJ039	NbRALF1	Unpublished, Philippe v. Jutras
pPJ054	AtPSY1	Unpublished, Philippe v. Jutras
pPJ055	AtPSY1-R	Unpublished, Philippe v. Jutras
pPJ059	secreted GFP	Unpublished, Philippe v. Jutras
	pHluorin	
pPJ041	VRC01 LC	Unpublished, Philippe v. Jutras
pPJ040	VRC01 HC	Unpublished, Philippe v. Jutras
pB034	RD21	Unpublished, Pierre Buscaill
pID005	AHA2 WT	This work
pID006	AHA2-S899A	This work
pID007	AHA2-S899D	This work
pID008	AHA2-T881V	This work
pID009	AHA2-T881D	This work
pPJ048	cytosolic	Jutras et al. (2021)
	superfolded GFP	
pPJ056	secreted RFP	Unpublished, Philippe v. Jutras
pT50	AvrPto	Rathjen et al. (1999)
pTRBO::FLAG:	AvrBlb2	Bozkurt et al. (2011)
Avrblb2		
TRV::GUS	TRV2gg::GUS	Duggan et al. (2021b)
pID021	TRV2gg::WAK1	Rosli et al. (2013)
pID032	TRV2gg::CDPK	This work
pID024	TRV2gg::CORE	This work
pID011	TRV2gg::BAK1	Wang et al. (2018a)
pID010	TRV2gg::CSPR	Saur et al. (2016)
pID013	TRV2gg::EDS1	Tran et al. (2016)
pID014	TRV2gg::SOBIR1	Liebrand et al. (2012)
pID015	TRV2gg::NPR1	Tran et al. (2016)
pID017	TRV2gg::CRT3a	Liebrand et al. (2012)
pID018	TRV2gg::EIN2	Tran et al. (2016)
pID025	TRV2gg::SAG101	Pombo et al. (2014)
pID028	TRV2gg::ACIF1	This work
pID031	TRV2gg::AHA2	This work
pID034	TRV2gg::ICS	This work
pJK-B2-022	eGFP	Kourelis et al. (2023)
TRV2::PDS	TRV2::PDS	Liu et al. (2002)
TRV2gg::MAPK3/6	TRV2gg::MAPK3/6	Unpublished, Tolga Bozkurt
TRV2gg::PAL	TRV2gg::PAL	Unpublished, Tolga Bozkurt
TRV2gg::WRKY7/ 8/9/11/12/14	TRV2gg::WRKY7/ 8/9/11/12/14	Unpublished, Tolga Bozkurt
TRV2::NBCERK1	TRV2::NBCERK1	Unpublished, Tolga Bozkurt
pID041	cytoGFP no P19	This work
pID042	secRFP no P19	This work

Table 7.4: List of new VIGS fragments cloned into TRV2::gg vectors.

Gene(s) Silenced	Fragment sequence
<i>CDPK</i>	GAAGAAATTGCTGGTCTGAAAGAAATGTTCCGGATG ATAGATACAGACAATAGTGGTCAAATAACCTTTGAG GAGCTCAAAGTTGGATTAAAAAGAGTCGGTGCTAAT CTCAGGGAGTCTGAGATCTATGATCTTATGCAGGCA GCGGATGTTGATAACAGCGGCACAATTGATTATGGG GAATTTATAGCAGCAACATTACATTTTAAACAAAATT GAAAGAGAAGATCATCTATTTGCTGCTTTCTCTTAC TTTGATAAAGATGGCAGTGGCTACATTACTGCAGAC GAGCTTCAACAAGCTTGTGAGGAATTTGGCATTGGG GACGTCCATCTAGAAGATATGATAAGAGACGCCGAT CAGGACAATGATGGACGAATTGATTACAATGAGTTC GTTGCTATGATGCAAAAAGGA
<i>CORE</i>	TACAGACGAGTCACTGTTTTTGGATCTTAGTTCTCTG AAACTGAGAGGTACTTTTTTACCAAGTATTGGAAATC TGAGCTTTCTTCGTGTCCTTGAGCTCCAAAATAATA GCTTTTCAGGTGAAATCCCATCAGAGCTTGGCTATT TGCATAAGTTACAGGTTTTTACGCCTTGACAATAACT CATTCACCTGGTCATATTCCTTCAAACATTTCTGGTT GCTTCAACCTTTTTTTCTGTTGTTCTTTCATATAATA TGCTGGTAGGCATGATTCCAGCAGAATTAGGCACCT TGTCGAAACTT
<i>ACIF1</i>	TGTAAGAGTTTGAAGAAATTCTCATGTGGATCTTG CATGTTTGGAGCCAAAGGTATGAATGCTTTGCTTGAT CACTGTTCTACCCTTGAGGAATTATCTGTGAAGCGCC TACGTGGCATCAATGATGGGTTTGCAGCTGACCCAAT TGGCCCTGGAGCTGCGGCTTCATCGCTTAAATCTATA TGTTTAAAAGAGCTTTATAATGGCCAGTGCTTTGGGC CTTTGATTATTGGGTCCAAAAATTTGAGGACTTTGAA GTTGTTGCGATGTTTAGGTGATTGGGATAGGCTTTTT GAGACA
<i>AHA2</i>	GGCTGAAATTGCTAGGCAACGTGAGCTGCATACACTG AAAGGTCACGTCAATCAGTGGTGAAGTTGAAAGGTC TTGACATAGAGACAATTCAGCAATCATACTGTTTG AAAGGAGAGTATGTTTCGACAAGTGTGATTTAACCGGT GAAGACATAATGAGGATGTGATTGTGAGTTACCTGTG GAAGAGACGAGAAATTAATAGTAGCAACATCTTAAAA AGTGCCTCATATATTTGATATATATATATTCTTTAAG TTGGTCTTGCGTTTCCTATTTTCAAGTCTTGGCTCCA TGAG
<i>ICS</i>	AGTTTAAGATCTTGTCGTCCATTCACCCTACTCCAGC AGTTTGTGGGTATCCTACAGAAGATGCACGGGCTTTT ATTTCAGAAACCGAAATGTTTGACCGAGGAATGTATG CTGGTCCTGTTGGTTGGTTTGGGGGGGAAGAGAGTGA ATTTGCTGTTGGAATAAGGTCAGCTTTGGTTGAAAAG GGTCTTGGCGCATTAATTTATGCGGGAAGTGGGATAG TGGAAGGAAGTGATTCATCTCTAGAATGGGAAGAGCT CGAGCTGAAGACTTCACAGTTCACCAAATTGATGAAA CTTG

Table 7.5: Primers used in this research.

Primer Name	Sequence 5'-3'
35S_Promoter	CTATCCTTCGCAAGACCCCTTC
35S_Terminator	CTCAACACATGAGCGAAACC
35S-P_BasI_For	CTCTGTGGTCTCAGGAGGTCAACATGGTGGA
35S-T_BasI_Rev	TTCGTGGTCTCAAGCGATCTGGATTTTAGTACTGG
pPJ039_For	TGGTGGTCTCAAATGGGAGTTCCCTTCAGGTTT
pPJ039_Rev	TTCGTGGTCTCAAAAGCTTAACTCCTGCAACGAGTGA
pPJ040_For	TGGTGGTCTCAAATGGCTAAACAAGCACCTGTC
pPJ040_Rev	TTCGTGGTCTCAAAAGCTTACTTACCAGGAGACAGAGAC
pPJ041_For	TGGTGGTCTCAAATGGCTAAACAAGCACCTGTC
pPJ041_Rev	TTCGTGGTCTCAAAAGCTTAGCACTCTCCCCCTATTATAAAGAC
Cyto_GFP_For	TGGTGGTCTCAAATGCGTAAAGGCGAAGAG
Cyto_GFP_Rev	TTCGTGGTCTCAAAAGCTCATTTGTACAGTTCATCCATACC
PSY1_For1	TGGTGGTCTCAAATGACTTTTGTAGTTCGTCTTCTTGTG
PSY1_Rev1	TTCGTGGTCTCACTGAAACGGAACACAGGGTTGC
PSY1_For2	TGGTGGTCTCATCAGGTGGGTTTGAGAAATTCGTGGA
PSY1_Rev2	TTCGTGGTCTCAAAAGCTCAGCCTCTGCCGACGA
AtPSY1R_For	TGGTGGTCTCAAATGATTGACGAGAAGATGAGATC

AtPSY1R_Rev	TTCGTGGTCTCAAAGCTTACGTCTCTTCTTCTTCTCAG
RFP-sec_For	TGGTGGTCTCAAATGAAGACTAATCTTTTCTCTTTTCTCATCTTTTCAC
RFP-sec_Rev	TTCGTGGTCTCAAAGCCTAGGCGCGGTGGAG
TRV2 ^{ggg} -sequencing-Fw	TGTTCAGGCGGTTCTTGTGTGTCA
TRV2 ^{ggg} -sequencing-Rv	CCGTAGTTTAATGTCTTCGGGACATGC
MEK2-RT-new-Fw	GCGGCTGCGACATACAAAT
MEK2-RT-new-Rv	TCCAGAGAGAAATCAACACAGATC
ICS-RT-new-Fw	ATTTTCATGGTCCCTCAGGTTG
ICS-RT-new-Rv	TCTGCACCCCTCATAAGAACGG
CSPR-RT-new-Fw	ACTGGGCTTTCTCTCTTCTG
CSPR-RT-new-Rv	ACCCAAAGGCTTCAGGGATG
CORE-RT-new-Fw	TGGCATTCGACAGTTTGGTG
CORE-RT-new-Rv	CAGACCCAAAACCAACCCATG
AHA2_Fw	GTCTGAGTGCCGAGGAAAGGA
AHA2_Rv	CCCATGTAGTGTCTCTGAGCAAG
ACIF1_Fw	CCTTCACCCGAGCTCACCTC
ACIF1-RT-new-Rv	TTCATCGGGAAAGCTCATATGTGT
CDPK_Fw	CTATGGTCCCGAAGCTGATG
CDPK-RT-new-Rv	GGCCATGGATCTGATGAGAAAGTC

PBinGFP2-F	AAGACCCCAACGAGAAAGC
PBinGFP2-R	GAACCCCTAATTCCCTTATCTG
PK7-NGFP-F	GTCCTGCTGGAGTTTCGTGAC
PK7-NGFP-R	GGTGATTTTTCGGGACTCTAGCA
PK7-NGFP-2018F	GTCCTGCTGGAGTTTCGTGAC
PK7-NGFP-2018R	TTGCGGACTCTAGCATGGCC
AHA2_GG_Frag1_Fwd	GGTGGTCTCAAATGGCAAAAAGCTATCAGCCTC
AHA2_GG_Frag1_Rev	TTCGTGGTCTCAATGGTTTTCAGCACTATCATGCC
AHA2_GG_Frag2_Fwd	GGTGGTCTCACCATCCGTAGAGCTCTCAACCTT
AHA2_GG_Frag2_Rev	TTCGTGGTCTCAAAGCTCAAACTGTATAATGCTGC
AHA2_T881D_For	CTTGTCTAGAGAGACCTACATGGGCTTCAACCACGAGAGCC
AHA2_T881D_Rev	GCCCATGTAGGTCTCTCTGAGCAAGTGCCCATTTGAGC
AHA2_S899D_For	TGAAAAGAACGACTACAGGGAACCTGTCTGAAATCGCTGAACAAGCAAGAGG
AHA2_S899D_Rev	CAGTTCCCTGTAGTCGTTCTTTTCATTGAAGAGATTGTGGCTTCTGGTGG
AHA2_T881V_For	GCTCAGAGAGTACTACATGGGCTTCAACCACGAGAGCC
AHA2_T881V_Rev	GCCCATGTAGTACTCTCTGAGCAAGTGCCCATTTGAGC
AHA2_S899A_For	GAAAAGAACGCCCTACAGGGAACCTGTCTGAAATCGCTGAACAAGCAAGAGGAG
AHA2_S899A_Rev	GTTCCCTGTAGGCGTTCTTTTCATTGAAGAGATTGTGGCTTCTGGTGG
NbPR1_For	AATATCCCACTCTTGCCG

NbPR1_Rev	CCTGGAGGATCATAGTTG
NbActin_For	TGGACTCTGGTGATGGTGTC
NbActin_Rev	CCTCCAATCCAAACACTGTA

Table 7.6: Constructs used in each agroinfiltration of Chapter 2.

Figure	Treatment Name	Construct Names	Gene of Interest	Strain
2.7	EV	Empty Vector	Empty Vector	GV3101
		Empty Vector	Empty Vector	GV3101
		secreted GFP pHluorin	GFP pHluorin	RSPJ07
2.7	RALF1	RALF1	<i>NbRALF1</i>	GV3101
		Empty Vector	Empty Vector	GV3101
		secreted GFP pHluorin	GFP pHluorin	RSPJ07
2.7	PSY1	PSY1	<i>AtPSY1</i>	GV3101
		PSY1-R	<i>AtPSY1-R</i>	GV3101
		secreted GFP pHluorin	GFP pHluorin	RSPJ07
2.9(A)	EV	Empty Vector	Empty Vector	GV3101
		Empty Vector	Empty Vector	GV3101
		VRC01 Lc	VRC01 Lc	GV3101
		VRC01 Hc	VRC01 Hc	GV3101
2.9(A)	RALF1	RALF1	<i>NbRALF1</i>	GV3101
		Empty Vector	Empty Vector	GV3101
		VRC01 Lc	VRC01 Lc	GV3101
		VRC01 Hc	VRC01 Hc	GV3101
2.9(A)	PSY1	PSY1	<i>AtPSY1</i>	GV3101
		PSY1-R	<i>AtPSY1-R</i>	GV3101
		VRC01 Lc	VRC01 Lc	GV3101
		VRC01 Hc	VRC01 Hc	GV3101
2.9(B)	EV	Empty Vector	Empty Vector	GV3101
		Empty Vector	Empty Vector	GV3101
		RD21	<i>NbRD21</i>	GV3101
2.9(B)	RALF1	RALF1	<i>NbRALF1</i>	GV3101
		Empty Vector	Empty Vector	GV3101
		RD21	<i>NbRD21</i>	GV3101

2.9(B)	PSY1	PSY1	<i>AtPSY1</i>	GV3101
		PSY1-R	<i>AtPSY1-R</i>	GV3101
		RD21	<i>NbRD21</i>	GV3101
2.11	EV	Empty Vector	Empty Vector	GV3101
		Empty Vector	Empty Vector	GV3101
2.11	RALF1	RALF1	<i>NbRALF1</i>	GV3101
		Empty Vector	Empty Vector	GV3101
2.11	PSY1	PSY1	<i>AtPSY1</i>	GV3101
		PSY1-R	<i>AtPSY1-R</i>	GV3101
2.12	EV	Empty Vector	Empty Vector	RSPJ07
2.12	AHA2-T881D	AHA2-T881D	<i>NbAHA2-T881D</i>	RSPJ07
2.12	AHA2-S899D	AHA2-S899D	<i>NbAHA2-S899D</i>	RSPJ07
2.12	AHA2-T881V	AHA2-T881V	<i>NbAHA2-T881V</i>	RSPJ07
2.12	AHA2-S899A	AHA2-S899A	<i>NbAHA2-S899A</i>	RSPJ07
2.13(A)	EV	Empty Vector	Empty Vector	RSPJ07
2.13(A)	AHA2-S899A	AHA2-S899A	<i>NbAHA2-S899A</i>	RSPJ07
2.13(A)	AHA2 WT	AHA2 WT	<i>NbAHA2</i>	RSPJ07
2.13(B)	EV	Empty Vector	Empty Vector	RSPJ07
		secreted GFP pHluorin	GFP pHluorin	GV3101
2.13(B)	AHA2-S899A	AHA2-S899A	<i>NbAHA2-S899A</i>	RSPJ07
		secreted GFP pHluorin	GFP pHluorin	GV3101
2.13(B)	AHA2 WT	AHA2 WT	<i>NbAHA2</i>	RSPJ07
		secreted GFP pHluorin	GFP pHluorin	GV3101
2.14	EV	Empty Vector	Empty Vector	RSPJ07
2.14	AHA2-S899A	AHA2-S899A	<i>NbAHA2-S899A</i>	RSPJ07
2.15	WT EV	Empty Vector	Empty Vector	RSPJ07
		Empty Vector	Empty Vector	GV3101
2.15	WT RALF1	RALF1	<i>NbRALF1</i>	RSPJ07
		Empty Vector	Empty Vector	GV3101

2.15	WT PSY1	PSY1	<i>AtPSY1</i>	RSPJ07
		PSY1-R	<i>AtPSY1-R</i>	GV3101
2.15	WT AHA2 WT	AHA2 WT	<i>NbAHA2</i>	RSPJ07
		Empty Vector	Empty Vector	GV3101
2.15	WT AHA2-S899A	AHA2-S899A	<i>NbAHA2-S899A</i>	RSPJ07
		Empty Vector	Empty Vector	GV3101
2.15	NahG EV	Empty Vector	Empty Vector	RSPJ07
		Empty Vector	Empty Vector	GV3101
2.15	NahG RALF1	RALF1	<i>NbRALF1</i>	RSPJ07
		Empty Vector	Empty Vector	GV3101
2.15	NahG PSY1	PSY1	<i>AtPSY1</i>	RSPJ07
		PSY1-R	<i>AtPSY1-R</i>	GV3101
2.15	NahG AHA2 WT	AHA2 WT	<i>NbAHA2</i>	RSPJ07
		Empty Vector	Empty Vector	GV3101
2.15	NahG AHA2-S899A	AHA2-S899A	<i>NbAHA2-S899A</i>	RSPJ07
		Empty Vector	Empty Vector	GV3101
2.16	EV	Empty Vector	Empty Vector	RSPJ07
2.16	AHA2-S899A	AHA2-S899A	<i>NbAHA2-S899A</i>	RSPJ07
2.16	AHA2 WT	AHA2 WT	<i>NbAHA2</i>	RSPJ07
2.16	RALF1	RALF1	<i>NbRALF1</i>	RSPJ07

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