The chloroplast: A key component of plant defence and target of plant pathogens

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signed  (Hannah Brown)

Abstract

Plant pathogens secrete a diverse range of effector proteins as part of their virulence strategy that work to supress plant immune responses. These effectors target and manipulate a multitude of key host defence cellular signalling pathways and organelles in the plant cell. The chloroplast represents a prime candidate for such manipulation by phytopathogen effector proteins being not only the site of photosynthesis and many defence hormone biosynthetic pathways in the plant cell, but also for its capabilities of producing the important signalling molecules reactive oxygen species (ROS). This study looked to examine how *Pseudomonas syringae* effector proteins manipulate the chloroplasts of host *Arabidopsis thaliana*. Here we show with use of fluorimagery that *P. syringae* is capable of supressing host Arabidopsis photosynthesis upon infection in an effector protein dependent manner. Furthermore, prior activation of host basal immunity through PAMP pre-treatment of Arabidopsis leaves induces a mechanism of protecting chloroplasts against bacterial suppression of host photosynthesis upon subsequent *P. syringae* challenge. These fascinating results indicate the chloroplast is a key player in the *A. thaliana* PTI (PAMP-triggered immunity) response. This study examined this facet of PTI through analysing the bacterial induced suppression of photosynthesis in a variety of PTI signalling mutants and confocal imaging of ROS production post *P. syringae* inoculation in order to better understand the signalling events linking PTI, phytobacterial virulence strategies and the chloroplast. Knowledge of such host-pathogen interactions will prove crucial for the future engineering of effective sustainable intervention strategies to protect host chloroplasts' from bacterial effector protein manipulation and enhance plants' resistance to pathogens.

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IV. Aknowlegdments

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III. Table of Abbreviations

Abbreviation	Definition
Avr	Avirulence factor
BAK1	Brassinosteroid insensitive 1- associated kinase 1
BKK1	BAK1-Like1
BR	Brassinosteroid
BRI1	Brassinosteroid insensitive 1
CAS	Calcium sensing receptor
CDPK	Calcium-dependant protein kinase
CERK1	Chitin elicitor receptor kinase1
CFU	Colony forming unit
CIp	Chloroplast isolation buffer
DAMP	Damage associated molecular pattern
DCF	Dichlorofluorescein
DTT	Dithiothreitol
EDTA	Ethylendiaminetetraacetic acid
EF-tu	Elongation factor- Tu
EFR	Elongation factor-Tu receptor
EGTA	Ethylene glycol tetraacetic acid
ETI	Effector-triggered immunity
FIN4	Flagellin-insensitive 4 (Aspartate oxidase)
FLS2	Flagellin sensing 2
H_2DCF-DA	2'7'-dichlorodihydrofluorescein diacetate
HR	Hypersensitive response
HRP	Horseradish peroxidase
KB	Kings broth
MAPK	Mitogen-activated protein kinase
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
PSI	Photosystem I
PSII	Photosystem II
PTI	PAMP-triggered immunity
R-proteins	Resistance-proteins
RBOHD	Respiratory bust oxidase homolog protein D
RK	Receptor Kinase
ROS	Reactive oxygen species
SA	Salicylic acid
SDS	Sodium dodecyl sulfate

1. Introduction

1.1 Plant-pathogen interactions

Due to the sessile nature of plants they are constantly exposed to a wide variety of biotic and abiotic conditions for which they need to be able to adapt and respond accordingly if they are to successfully grow and reproduce. Attack by pathogens such as: fungi; bacteria; viruses and nematodes, is one of these biotic stresses and has led to the development in plants of a complex multi-layered innate immune system that allows plants to perceive and co-ordinate an attack on pathogens. Plants, unlike animals have no adaptive immunity or specialised circulating cells, such as phagocytes and lymphocytes, for the detection of invading pathogens. Instead, pathogen detection is conducted by every cell in the plant autonomously through receptors termed Pattern Recognition Receptors (PRRs) which are able to detect conserved Pathogen-Associated Molecular Patterns (PAMPs) released by pathogens in to the plants' apoplast. PAMPs act as ligands for the transmembrane PRRs enabling the presence of an apolplastic pathogen to be detected and transduced to the internal plant cell environment (Monaghan & Zipfel, 2012). For example, *Arabidopsis thaliana* can recognise the bacterial PAMPs flg22 and elf18 and the fungal PAMP chitin through the PRRs: FLS2 (Flagellin sensing 2) (Gómez-Gómez & Boller, 2000); EFR (EF-Tu receptor) (Zipfel et al., 2006) and CERK1 (Chitin elicitor receptor kinase 1) (Miya et al., 2007) respectively. Activation of these receptors via their PAMP ligands induces a variety of signal transduction pathways that lead to the metabolic and transcriptome reprogramming of the cell, culminating in the activation of plant basal defenses termed PAMP-triggered immunity (PTI).

In addition to release of pathogen derived PAMPs into the plant apoplast, pathogens can also activate plants' PTI responses indirectly through instigating the production of host-derived peptides termed DAMPs (Damage associated molecular patterns). These endogenous epitopes are released into the host

apoplast during the pathogen infection and colonization process and are predicted to act as a marker for pathogen induced cellular damage (Zipfel, 2014). Examples of Arabidopsis DAMPs include oligogalacturonides which are usually embedded in the cell wall matrix but are thought to be released by cell wall degrading enzymes secreted by fungi during the invasion process. DAMPs similarly to PAMPs are recognised by PRRs such as the RK WAK1, which is capable of recognizing oligogalacturonides and consequently instigates a PTI immune response in the plant cell (Brutus et al, 2010). Other examples of DAMPs released by large-scale cellular damage as the result of pathogen attack or wounding include the release of extracellular ATP (eATP) believed to also be able to funcation as a DAMP activating the PRR RK DORN1/LecRK-I.9 (Choi, 2014).

The best-characterized family of DAMPs in Arabidopsis is the AtPEPs. These endogenous DAMP epitopes are predicted to be released through the cleavage of the C-terminal region of PROPEPs, a seven-member multigene family of proteins. Induction of PROPEP genes has been observed to increase upon wounding or activation of PTI (Huffaker et al, 2006). In this way perception of PROPEP cleaved epitopes AtPep1-7 could act as important PTI amplification signals. Atpep1, released from PROPEP1 is perceived independently via two related RKs PEPR1 and PEPR2 with double mutants of these receptors showing complete insensitivity to AtPEP1 as well as AtPEP2 and AtPEP3 released from PROPEP2/3 respectively (Krol et al, 2010). PROPEPs are phylogenetically conserved across plants with orthologs found in many species, for example the maize AtPEP1 ortholog ZmPep1 (Huffaker et al, 2011).

PRR recognition of PAMPs and DAMPs activates convergent mitogen-activated protein kinase (MAPK) pathways as well as calcium dependent protein kinases (CDPKs) which initiate the transient early signalling events of PTI such as an apoplastic ROS burst through the enzyme NADPH oxidase RBOHD (Respiratory bust oxidase homolog protein D) (Miller et al., 2009). These early

events are followed by more persistent changes that include activation of hormone signalling, callose deposition (Brown et al., 1998), ROS metabolic changes and biosynthesis of antimicrobial secondary metabolites (Bednarek & Osbourn, 2009) and collectively these mechanisms contribute to PTI.

Pathogens have evolutionarily responded with a mechanism to suppress plants immune response through the production of effector proteins. Effector proteins work to attenuate the host defenses and are released into the host plant cell via a Type-II or Type III secretion system or exocytosis. For example, *Psedomonas syringae* strain DC3000 delivers approximately 28 effectors and a variety of small molecules into the host plant cell via a type III secretion system via a Hrp Pilus (Cunnac et al., 2009; Roine et al., 1997). These effector proteins are thought to collaborate and target multiple susceptible sites in the plant cell through which plant defense signalling occurs in order to disrupt host basal defenses and thereby aiding the virulence of the pathogen (Macho & Zipfell, 2010).

In the second layer of plants' innate immunity pathogen effector proteins can be detected directly or indirectly in the plant cell via intracellular receptors termed Resistance-proteins (R-proteins) whose activation leads to the induction of Effector-triggered immunity (ETI) (Jones & Dangl, 2006). Initiation of ETI is often associated with the induction of a specific form of programmed cell death, the hypersensitive response (HR). The HR is localised to the site of infection in the plant and is thought to restrict pathogen growth and colonization of further plant tissue (Lam et al., 2001). The ability of pathogens to produce new effector proteins and plants' capability to recognise these new effectors via R-proteins has led to a constant evolutionary arms-race between the plant and pathogen illustrated by the Zig-zag model (Fig. 1) (Jones & Dangl, 2006).

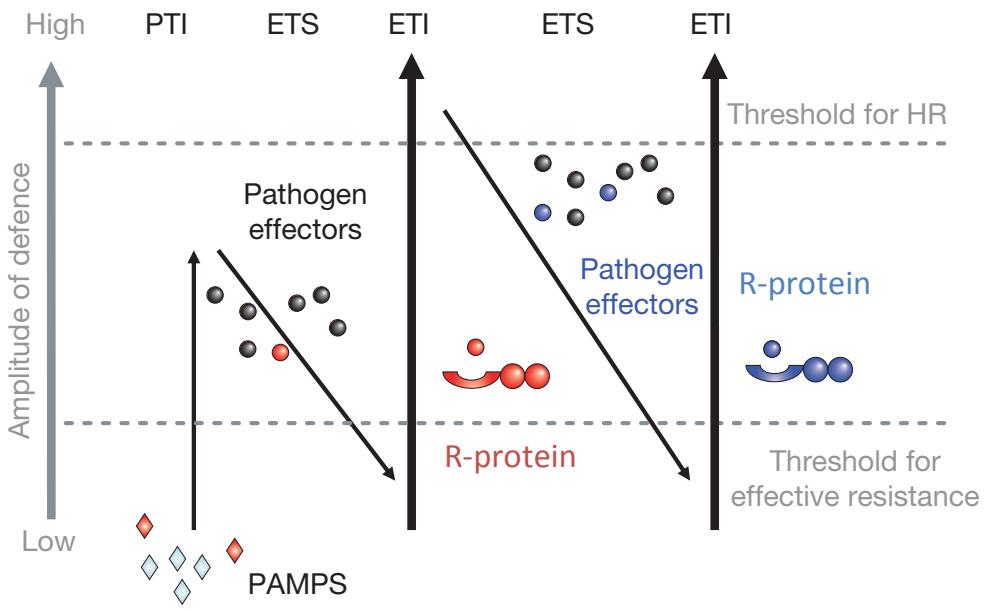


Figure 1) Zig-zag model of pathogen-plant host interaction evolution
(Adapted from Jones & Dangl, 2006)

Our current knowledge of the mechanisms that underlie plant immunity and the mode of action pathogen effectors use to disrupt these processes still remains relatively rudimentary. Understanding how effectors collaborate and manipulate multiple susceptible sites within the plant cell will be crucial for designing targeted intervention strategies to aid plant resistance and attenuate pathogen virulence tactics in the future.

1.2 The Involvement of the chloroplast in plant innate immunity

Chloroplasts are metabolically versatile organelles that play an important role in the plant cell integrating the multiple environmental stimuli plants are subjected to (Shapiguzov et al., 2012). In addition to being the site of the photochemical utilization of light in energy production in the cell, chloroplasts host major hormone biosynthetic pathways and have central roles in redox homeostasis and retrograde signalling (Stael et al., 2015). However, the role the chloroplast plays in plant immune defense responses and how pathogens target the chloroplast,

as part of their virulence strategy is only recently beginning to emerge and still remains largely elusive.

ROS have several functions within plant basal defenses working not only as direct antimicrobial particles but also as important signalling molecules in a variety of downstream signalling pathways. As the site of photosynthesis in the plant cell, chloroplasts are capable of producing ROS in a light dependant manner (Asada, 2006). Chloroplast generated ROS have been implicated in many facets of plant innate immunity and have been long established for their role in ETI in instigating a localised cell death HR response upon effector protein detection (Zurbriggen et al., 2009). Chloroplast derived ROS have also been linked to stomatal immunity in plants with ROS bursts in the chloroplasts of guard cells coinciding with stomatal closure and therefore limitation of pathogen entry to the plant tissue (Vahisalu et al., 2010).

Chloroplasts role in the generation of photoassimilates as well as co-factors NADPH and ATP makes them a key players in the biosynthesis of many plant hormones. The defense hormones Jasmonic acid and Salicylic acid (SA) both involve biosynthetic pathways which occur in the chloroplast further implicating the integral role these plastids play in the plant innate immune response (Li et al., 2014). Similarly, chloroplasts are also responsible for a variety of defensive secondary metabolites that have antimicrobial properties such as alkaloids and terpenoids (Wink, 1985).

As the host of key defense hormone biosynthetic pathways and a major intracellular source of ROS it is crucial that chloroplasts are well integrated into the signalling cascades that makeup a plant immune response. Molecular cross talk between cytoplasmic PAMP detection at the plasma membrane and the chloroplast has recently been demonstrated involving the calcium sensor CAS. Application of the PAMP flg22 to *A. thaliana* leaves was shown to be capable of inducing a specific Ca^{2+} signature in the chloroplast stroma involving the

thylakoid calcium sensor CAS, indicating a direct signalling link between PAMP detection and the chloroplast (Nomura et al., 2012). Detection of these Ca^{2+} signatures through CAS was also shown to be vital for the induction of Salicylic Acid (SA) biosynthesis, the downreguation of photosynthesis related genes and upregulation of defense genes in response to the PAMP flg22 (Nomura et al., 2012).

Evidence of direct physical links between chloroplasts and the nucleus has also recently emerged via dynamic tubular projections termed stromules (Stael et al., 2015) (Fig. 2). These chloroplast projections are thought to aid in the transport of pro-defense signals from the chloroplast to the nucleus in order to instigate an HR response. Stromule growth from chloroplasts could be induced by exogenous application of SA or H_2O_2, both of which are potentially chloroplast derived, and are strongly induced as part of both an *Arabidopsis* and *Nicotiana* immune response. Chloroplast stromule-nuclei connections were seen to be particularly strong during an immune response and preceded the accumulation of chloroplast localized NRIP1-cerulean in the nucleus, suggesting that these tubular extensions may act as a means of transducing pro-defense signals from the chloroplast to the nucleus (Stael et al., 2015). This provides an example of how small signalling molecules may be transferred from the chloroplast to an independent organelle such as the nucleus thereby relaying important innate immune defense signals and demonstrates how chloroplasts may participate in a coordinated cellular immune response.

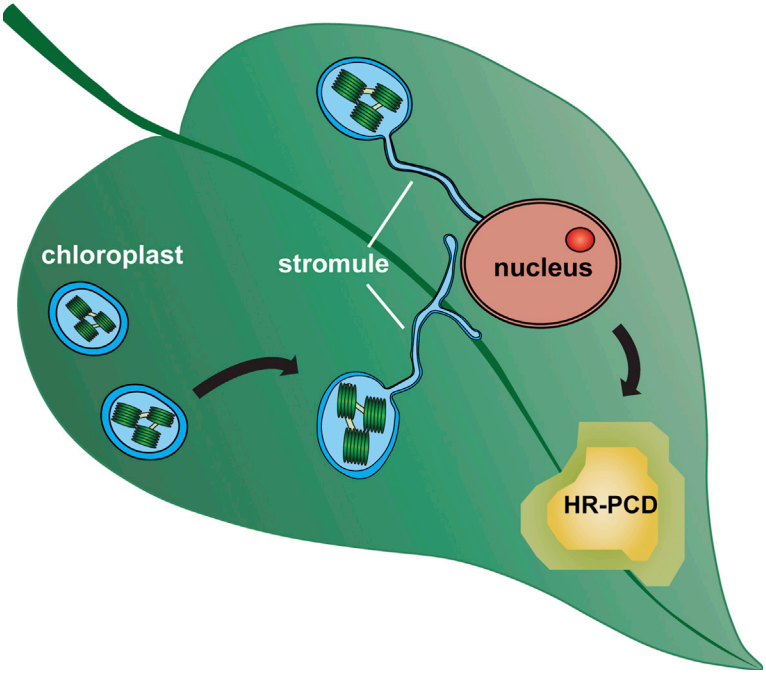


Figure 2) Chloroplast-nucleus stromule formation. Stromules are dynamic, stromal filled tubule extensions of the chloroplast induced during the immune response and hypothesized to allow the exchange of pro-defense signals from the chloroplast to the nucleus (taken from Caplan et al., 2015)

1.3 How pathogen virulence strategies manipulate the chloroplast

As a site of ROS and defense hormone production in addition to being the carbon source for many defense secondary metabolites through carbon fixation, it is to clear to see why the chloroplast may be a prime candidate for targeting by pathogen effector proteins. Suppression of host photosynthesis therefore would provide an excellent mechanism for pathogens to inhibit plant innate immune responses that operate through the chloroplast. Evidence of this has risen from chlorophyll fluorescence studies which have shown that photosynthesis is decreased upon pathogen challenge in a host plant (Berger et al., 2007 & Rodriguez-Moreno et al., 2008). Though it may seem counterintuitive for a pathogen to downregulate photosynthesis as this provides an ideal carbon source through photo-assimilate production for an invading pathogen, (Chen et al., 2010) it is likely the benefits of suppressing the host defense responses that

rely on photosynthesis outweigh these advantages. This explanation is likely to have provided a strong selective force for the evolution of bacterial effector proteins whose purpose is to collaborate and target the chloroplast and its functioning as a means of aiding pathogen virulence.

Evidence of such manipulation and targeting of the chloroplast by bacterial effector proteins is already emerging with a recent protein-protein interaction network (Mukhtar et al., 2011) identifying several *P. syringae* effector proteins which yeast-2-hybrid screens found to interact with chloroplast localised proteins. For example, the effector protein HOPR_1 interacted with chloroplast localised proteins JAI1, LEJ1 and PTF1. The mode of action these effector proteins use to disrupt the chloroplast and host photosynthesis appears to vary with effectors targeting different aspects of the chloroplast immune response. For example, *P.syringae* effector HopI1 suppresses the accumulation of SA through altering the integral structure of the thylakoid membrane (Jelenska et al., 2007). Contrastingly, protease effector HopN1 targets the photosynthesis machinery directly through the cleavage of Photosystem II (PSII) oxygen evolving complex protein PsbQ. This leads to the breakdown of the electron transport from PSII to PSI needed for a functional plant defense response (Rodríguez-Herva et al., 2012).

In addition to targeting the physical protein features of the chloroplast, evidence is emerging that bacterial effectors also target the regulation of nuclear encoded chloroplast targeted genes (NECGs). High-resolution micro-array data has shown a dramatic differential expression of NECGs between *P. syringae* strains DC3000 and DC3000*hrpA*, a strain of *P. syringae* which is unable to deliver the crucial effectors, with ~ 8% of all genes differentially regulated between the two challenges 3hpi. This differentiation in NECG regulation continued to increase peaking later in infection at 37% (de Torres-Zabala et al., 2015). This demonstrates effector proteins can collaborate in many ways to

target the chloroplast not only in the targeting of functioning chloroplast proteins but also through the reconfiguration of NECG transcription.

The *P. syringae* type III effector HopK1 has demonstrated one possible mechanism for effector protein entry in to the chloroplast giving the effector access to disrupt proteins and photosynthetic machinery within the organelle. It has been found that the in planta processing of HopK1 reveals a chloroplast transit peptide on the N-terminus of the protein, allowing its import into the chloroplast organelle itself (Li et al, 2014). The abilities of HopK1 to suppress PTI responses, such as ROS production and callose deposition, were dependent upon this chloroplast transit peptide highlighting the importance of chloroplast import in this effector's method of aiding pathogen virulence. The well-studied effector AvrRps4 shares a very similar sequence homology with the chloroplast transit peptide of HopK1 and similarly localises to the chloroplast post *in planta* processing (Li et al, 2014). This suggests that import into the chloroplast via a transit peptide maybe a common mechanism shared amongst a range of effectors and highlights the important role the chloroplast must play in plant immunity.

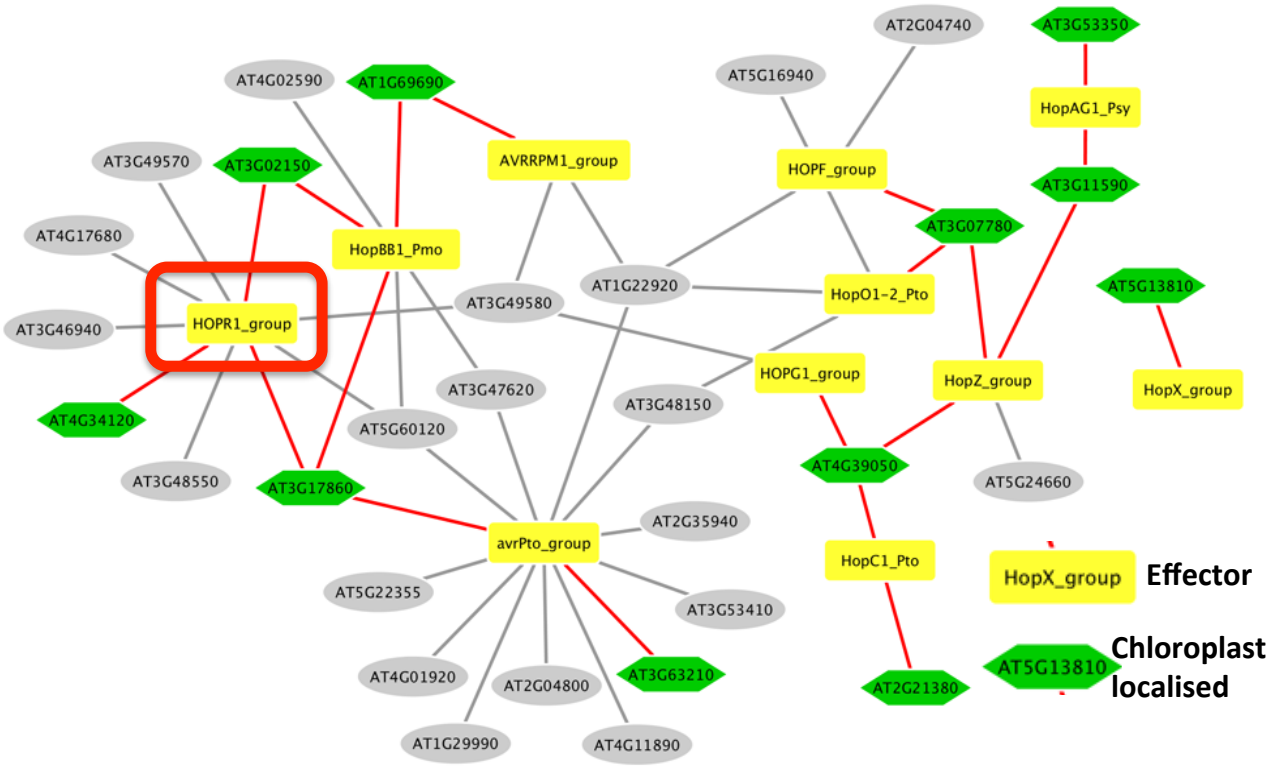


Figure 3) Protein-protein interaction network of *P. syringae's* effectors thought to interact with chloroplast localised proteins. Based of modelling by Mukhatr et al (2011). (Taken from de Torres-Zabala et al., 2015)

1.4 Study aims

This study looks to examine the role the chloroplast plays in *A. thaliana* host defences and how the bacterial pathogen *P. syringae* targets the chloroplast to suppress host photosynthesis as part of the bacteria's virulence strategy. The key hypothesis of the study is that bacterial effector proteins can collaborate and target the chloroplast suppressing host photosynthesis in order to aid the bacteria's virulence by attenuating chloroplast derived immune responses (Fig. 4).

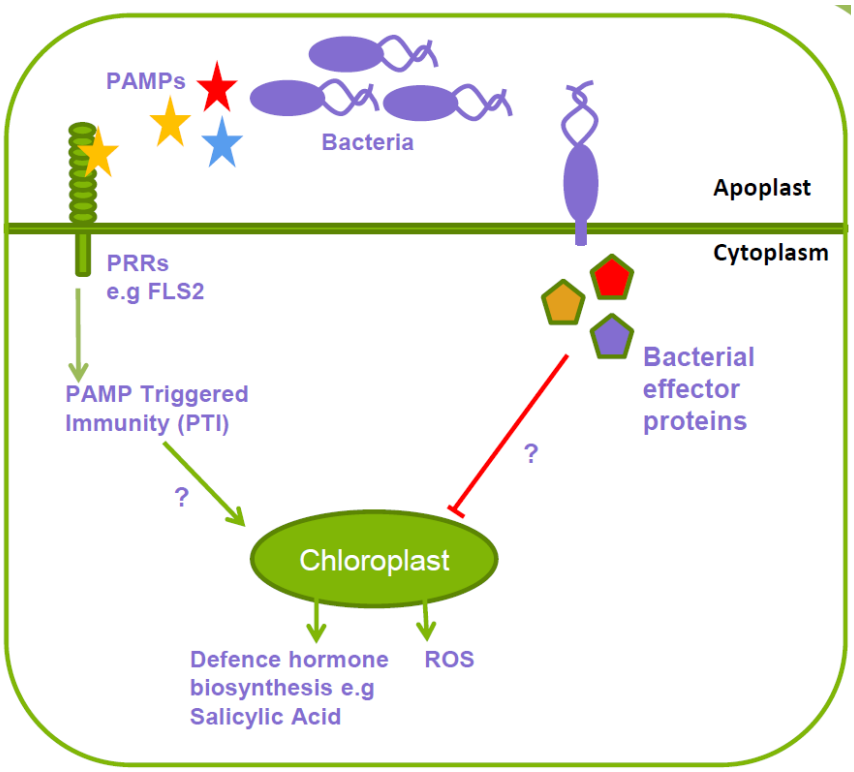


Figure 4) Model of interaction between *A. thaliana* host chloroplast and *P. syringae* bacteria

Using Fluorimagery to analyse rate of photosynthesis through measuring a variety of chlorophyll fluorescence parameters this study looked to examine how photosynthesis is supressed in the host *A. thaliana* post virulent DC3000 *P. syringae* challenge compared to challenge with the mutant DC3000*hrpA* strain of *P. syringae* which is unable to deliver the pathogen's crucial effector proteins. The project looks to understand the different signalling pathways and events that underpin this suppression through examining DC3000 induced photosynthesis suppression in a variety of PTI signalling mutants and confocal imaging of ROS production post *P. syringae* inoculation.

Through examining the effect of a range of PAMP pre-treatments on the suppression of photosynthesis with subsequent DC3000 and DC3000*hrpA* challenge we can begin to understand the relationship between bacterial effector proteins and suppression of host *A. thaliana* photosynthesis in the chloroplast. It

is crucial we gain such knowledge as it is only through understanding the complex interactions that occur between plant host and pathogen that we will be able to engineer effective intervention strategies that will enable us to enhance plants' resistance to the pathogens they face today.

2. Methods and Materials

2.1 Arabidopsis growth conditions

Arabidopsis thaliana wild type and mutant seed were sown in a compost mix 3:1 ratio of Levingtons F2 compost + sand (LEV206) and vermiculite (medium grade). Seed were stratified at 4^{o}C in the dark for 2 days. Plants were grown for approximately 4-5 weeks prior to use in a controlled environment growth chamber under 10 h day (23^{o}C; 120 µmol m^{-2} s^{-}) and 14 h night (20^{o}C) regime with relative humidity set to 65%. All mutants: *fls2 (*Gomez-Gomez & Boller, 2000), *cerk1-2* (Miya et al, 2007), *bak1-5* (Schwessinger et al., 2011), *bkk1-1* (He et al., 2007), *bak1-5 bkk1-1* (Schwessinger et al., 2011), *fin4-3* (Macho et al., 2012), *fin4-1* (Macho et al., 2012) and *rbohD* (Torres et al., 2002) used were in a Columbia (Col-0) background. Col-0 and Col-5 were used as controls where appropriate.

2.2 Bacteria growth and maintenance

Pseudomonas syringae strains were routinely grown on solidified Kings medium B (KB) (King et al., 1954) containing the relevant antibiotics (Table. 1) for each strain and stored at 4^{o}C. Antibiotics were used at the following concentrations: Rifampicin (50µg/ml), Kanamycin (25µg/ml) and Spectinomycin (100µg/ml).

Table 1) *P. syringae* strains and their antibiotic resistance used within this project

Pseudomonas Syringae strain	Antibiotic Resistance
DC3000 pvsp61	Rifampicin & Kanamycin
DC3000*hrpA*	Rifampicin & Kanamycin
DC3000*flic*	Rifampicin
DC3000*avrRpm1*	Rifampicin & Kanamycin
DC3000 477 DBL (*cor*$^{-1}$/*cor*$^{-2}$)	Rifampicin & Kanamycin & Spectinomycin

2.3 Arabidopsis infiltration

For *P. syringae* infiltration of *A. thaliana*, overnight cultures consisting of 10 ml of liquid KB media supplemented with the strain appropriate antibiotics were grown at 28^{o}C on a shaker (200 rpm). Bacteria cells were subsequently harvested by centrifugation at 2800g x 7 min, washed then resuspended in 10 mM $MgCl_2$. Bacteria density was adjusted to OD_{600} = 0.15 (~0.75 x 10^8 colony forming units (cfu) ml^{-1}) for chloroplast isolation, fluorimagery and confocal imaging, or OD_{600} = 0.002 (~1 x 10^5 colony forming units (cfu) ml^{-1}) for low inoculum growth assays. Mock treated leaves were infiltrated with 10mM $MgCl_2$. *A. thaliana* rosette leaves were nicked with a scalpel and infiltrated with a 1ml blunt ended syringe.

2.4 Elicitor Treatment

A. thaliana rosette leaves were treated with water or the appropriate elicitor at the following concentrations: flg22 (1µM), elf18 (1µM), flg22$^{A.tum}$ (1µM) (GeneCust), crude chitin mixture (100µg/ml). The adaxial surfaces of rosette leaves were nicked with a scalpel and the elicitors or water were delivered with a 1ml blunt ended needless syringe 17 h prior to bacterial inoculation. For non-PAMP pre-

treated control leaves a mock challenge of 10mM $MgCl_2$ was infiltrated into leaves as a replacement.

2.5 Chlorophyll fluorescence imaging

Photosystem II chlorophyll fluorescence imaging of *A. thaliana* rosettes was performed with a CF Imager (Technologica Ltd., Colchester, UK) as per de Torres Zabala et al (2015) in order to monitor variations in *A. thaliana* Photosystem II (PSII) photochemistry. Plants were placed in the chamber for 10 min post inoculation and then dark adapted for 20 min and an image of minimum fluorescence when PSII centres are fully oxidised (*Fo*) taken. This was followed by a saturating light pulse (6349 µmol m^{-2} s^{-1} for 0.8 s) to obtain maximum dark-adapted fluorescence (*Fm*). Actinic light (120 µmol m^{-2} s^{-1} – the same as plant growth light intensity) was then applied for 15 min and an image of steady state fluorescence (*F'*) taken, followed by a saturating pulse to obtain maximum light adapted fluorescence (*Fm'*). The plants were then left for a further 24 min in actinic light before returning to the dark for 20 min. At this point the cycle of measurements (59 min duration) was repeated 23 times. *Fm,* F_v and *Fo* were used to calculate chlorophyll fluorescence parameters related to photosystem II photochemistry, primarily *Fv*/*Fm* (maximum dark adapted quantum efficiency), a measurement of the maximum efficiency of PSII, and was analysed using FluorImager V 2.229 software (Technologica Ltd.). Fv/Fm was calculated using the following formula where F_0 is minimal photosynthesis, F_m is maximal fluorescence and F_v is the variable fluorescence as described by Baker (2010).

$$Fv/Fm = (Fv\text{-}Fo)/ Fm$$

The temperature during measurements was 20 °C.

2.6 Bacterial infection assay

DC3000 cells were harvested as per 2.3 and resuspended in 10mM $MgCl_2$ to an OD_{600} = 0.002 prior to infiltration into *A. thaliana* rosette leaves using a needleless syringe. Leaf disc samples were taken from three challenged leaves per plant and six plants per genotype 3 d post-innoculation using a cork borer. Leaf discs were ground in 1 ml 10mM $MgCl_2$ before required dilutions were made and plated on Kings B plates with appropriate antibiotic selection. Plates were incubated for 2 d at 28^{o}C before colonies were counted.

2.7 ROS production confocal imaging

ROS production was monitored in *P. syringae* bacterial challenged *A. thaliana* leaves using the ROS probe 2'7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Enzo). *A. thaliana* rosette leaves were challenged as per 2.3 and 4-5 hpi the challenged rosette leaves were infiltrated with a solution of 10mM $MgCl_2$ containing 10 µM H_2DCF-DA for 40 mins before the leaves were detached and floated, adaxial surface upwards in H_2O for 20 mins prior to imaging. Leaf samples were mounted in perfluorodecalin (Littlejohn et al, 2010) and imaged on a Leica SP8 using a 40X oil emersion lens. Argon laser excitation at 488nm and an emission window of 512-527nm was used to capture the dichlorofluorescein (DCF) signal. Chloroplast fluorescence was measured at 659-679nm.

2.8.1 Isolation of *Arabidopsis thaliana* chloroplasts

Chloroplasts were harvested from *A. thaliana* rosette leaves 16 h post inoculation with DC3000, DC3000*hrpA* (OD_{600} =0.15) or 10 mM $MgCl_2$. Chloroplast were harvested as per Kley et al (2010) by first preparing a 1x chloroplast isolation (Clp) buffer (0.3M Sorbitol, 50mM Hepes/KOH pH 7.5, 5mM EDTA, 5mM EGTA 1mM $MgCl_2$, 10mM $NaHCO_3$ and 0.5mM DTT) at 2x stock.

Prior to chloroplast isolation two 12ml polypropylene tubes (Greiner Bio-one) per *A. thaliana* treatment were prepared with a 50% percoll layer by mixing 2.5ml of percoll (Sigma) and 2.5ml of 2 x Clp buffer in each tube and placed on ice. Approximately 2-3g fresh weight of rosette leaves were then harvested per treatment and blended with 15ml of 1x chloroplast isolation buffer for 4 s at low speed using a Waring commercial blender. The suspension was then gently filtered through two layers of miracloth pre-soaked in the 1x Clp buffer and the homogenate carefully loaded onto the 50% percol layer using a serological pipette. The tubes were then centrifuged (4^{o}C for 10 min at 2000g) in a swing out rotor with acceleration set 5, brakes set 1. The upper layer containing the broken chloroplast fragments was then removed using a serological pipette and the remaining intact chloroplast (4^{o}C for 5 min; 1000g acceleration 9; brake 7) the supernatant was removed and the chloroplast pellet frozen in liquid N_2. Frozen pellets were stored at -80^{o}C. Chloroplast integrity was assessed via imaging on 1X81 motorized inverted microscope (Olympus) for the chloroplasts' natural auto-fluorescence and DAPI staining to check for nucleus contamination.

2.8.2 Chloroplast protein extraction and quantification

Proteins were extracted from previously generated chloroplast pellets (2.8.1) using a chloroplast protein isolation buffer (50mM Hepes KOH pH 7.4, 5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, PIC (protein inhibitor cocktail mix, Sigma), 5mM DTT and 0.1% SDS) adapted from Zrenner et al (1993). The frozen chloroplast pellets were resupsended in 1ml of Chloroplast isolation buffer per 100mg of chloroplast pellet. The suspensions were mixed and vortexed well then incubated on ice for 10 min before being centrifuged at 4^{o}C for 10 min at 13.2 rpm (Eppendorf centrifuge S415R). The subsequent supernatant was divided into 150µl aliquots and stored at -20^{o}C.

The dissolved protein concentration of the aliquots was quantified relative to lysoszyme protein extract adapted from Bradford (1976). 10µl of protein sample was added to 790µl of milliQ water and 200µl of undiluted Bradford

reagent (Bio-rad). Each sample was vortexed and incubated at room temperature for 40 min before the samples absorbance was read at A_{595} and the protein concentration determined through comparison with a standard curve generated using lysoszyme protein standards.

2.9 SDS-PAGE and Western blotting

Sample chloroplast proteins were denatured prior to loading by mixing and heating for 5 min at 95^{o}C in 1x SDS buffer prepared at 5x SDS buffer stock (10%SDS, 250mM Tris 6.8, 1mg/ml bromophenol blue, 0.5M DTT, 50% glycerol). The samples were then centrifuged at 13,000 g for 5 min and the pellet discarded. Chloroplast proteins were separated on a 12% (w/v) SDS-PAGE gradient polyacrylamide gel. Proteins were resolved for 30 min at 100V and then separated for 40 min at 150V. Pre-stained, broad range markers (Bio-Rad) ladders were used for protein molecular mass determination.

Separated proteins were transferred from SDS-PAGE gel electrophoretically to a polyvinylidene difluoride (PVDF) (LifeTechnologies) for 1 h at 4^{o}C. PVDF membranes were stained with Ponceau S to visualise transferred proteins and ensure equal protein loading between samples. Membranes were blocked for 1 h in TBST with 5% dried milk powder before being rinsed and washed for 5 mins in TBST. Membranes were then incubated for 1h with anti-rabbit primary antibody for selected PSII protein: PsbQ and PsbO (diluted 1:10,000 with TBST with 2% milk powder) (Agrisera) or AtpE and HemY (diluted 1:500 with TBST with 2% milk) (Agrisera). The membranes were rinsed, washed three times for 10 min in TBST then incubated for 1 h with 2nd antibody IgG-HRP (Promega) in TBST with 2% milk at a dilution of 1:20,000 for PsbO and PsbQ antibodies and 1:10,000 for AtpE and HemY. The membranes were rinsed and three times again in TBST for 10 min prior to antibody visualisation by incubation with HRP substrate solution.

3. Results

3.1 *Pseudomonas syringae* supresses host *Arabidopsis thaliana* photosynthesis in an effector protein dependant manner

A. thaliana challenge with the *P. syringae* strain DC3000 resulted in a suppression of host maximum dark-adapted quantum efficiency (F*v*/F*m*), a measurement of PSII quantum efficiency, beginning around 6-7 hpi (Fig. 1A) . This bacterial suppression of host photosynthesis was dependant of the delivery of bacterial effector proteins as challenge with DC3000*hrpA* or mock inoculation showed no inhibition of host F*v*/F*m.*

The timing of *P. syringae* suppression of host F*v*/F*m* correlated with the virulence of the pathogen strain in question. For example, DC3000*cor* ⁻ a strain of DC3000 lacking the phytotoxin coronatine known to contribute to pathogen virulence, showed a slower suppression of host F*v*/F*m* than the wild type DC3000 strain at around 8-9 hpi. The difference in the virulence of these strains is clearly demonstrated in growth curves comparing bacterial growth between the strains where DC3000*cor* ⁻ showed significantly less growth than DC3000 3dpi (t-test, $p = 0.0014$) (Fig. 5B).

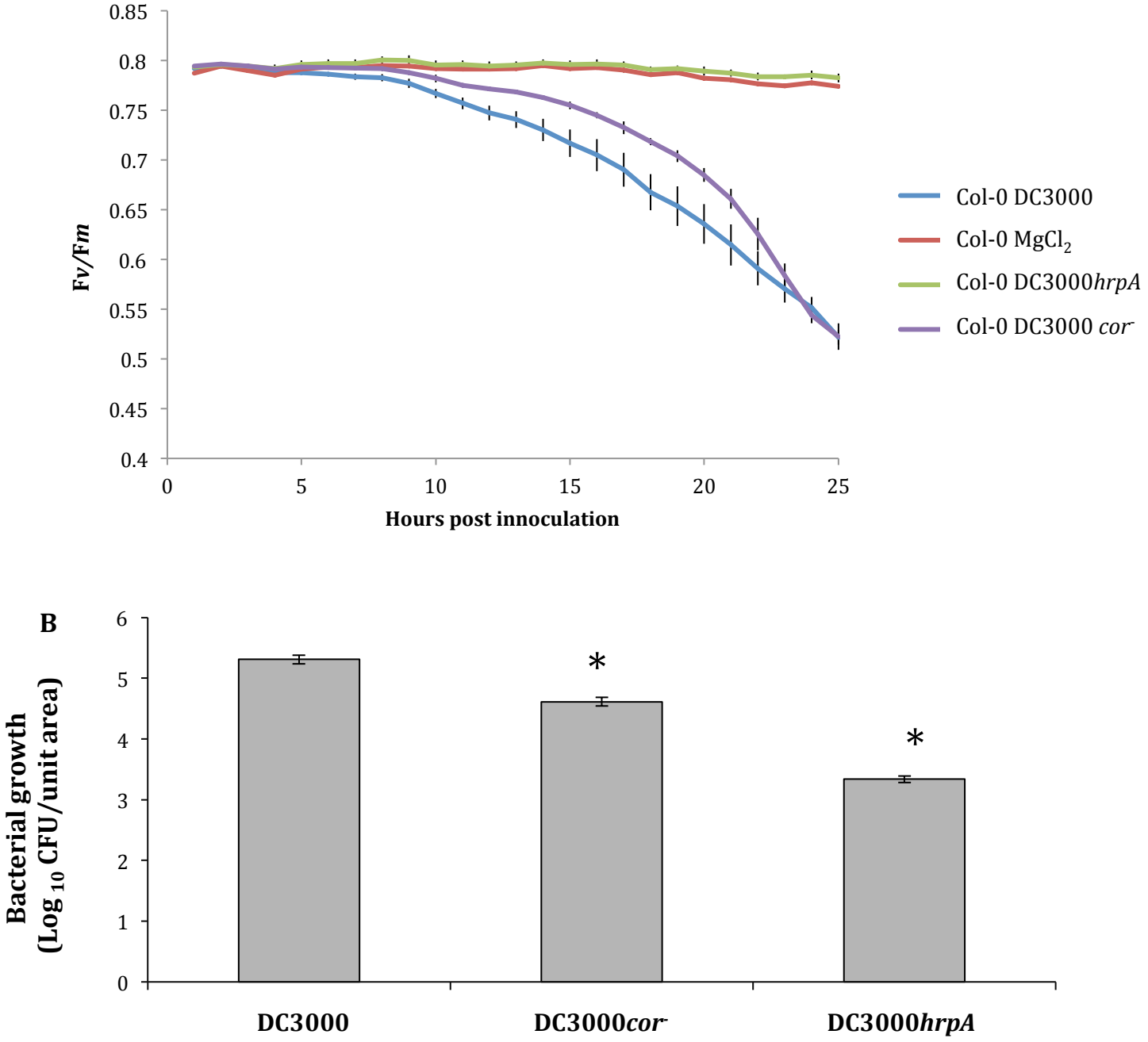


Figure 5) ***Pseudomonas syringae*** **supresses host photosynthesis in an effector protein dependant manner. (A)** Maximum quantum efficiency of PSII (F*v*/F*m*) is decreased by *P. syringae* capable of delivering effector proteins: DC3000, DC3000*cor* - but not DC3000*hrpA* or mock inoculation, error bars represent +/- SE (n=3 or 2) repeated twice **(B)** Inplanta bacterial assays of *P. syringae* DC3000, DC3000*hrpA* and DC3000*cor* - 3dpi (inoculum of ~1 x 10^8 CFU/ml), values are means +/- SE (n=5) Statistical significance compared to DC3000 ($p < 0.01$) is indicated by asterisks, growth curve conducted once.

3.2 PAMP pre-treatment of *A. thaliana* protects against bacterial suppression of host photosynthesis as part of an early PAMP response

Pre-treatment of *A. thaliana* Col-0 leaves with the bacterial PAMPs flg22 and elf18 was capable of inducing protection of the chloroplasts against bacterial manipulation and suppression of host photosynthesis upon subsequent bacterial challenge 17 h later (Fig. 6A). This protection was lost if the corresponding receptor for the PAMP was mutated inhibiting the PAMPs detection. For example, in the f*ls2* mutant no protection of PSII efficiency following 1**µM** flg22 pre-treatment was observed, but it was still protected by the PAMP elf18, as the PAMP receptor for EFR is functional in *fls2* (Fig. 6B). PAMP pre-treatment photosynthesis protection was also lost if the PAMP itself was mutated to be non binding as the *Agrobacterium tumefaciens* flg22^{Tum}, which cannot bind FLS2, was unable to induce protection of the chloroplast against subsequent bacterial challenge in Col-0 (Fig. 6A).

The fungal PAMP chitin was also capable of inducing chloroplast protection and prevented suppression of F*v*/F*m* by DC3000 (Fig. 6C) when given as pre-treatment to Col-0 leaves. This chitin induced protection was lost in the chitin PAMP receptor mutant *cerk1-2* but the mutant still received protection from a 1uM flg22 pre-treatment.

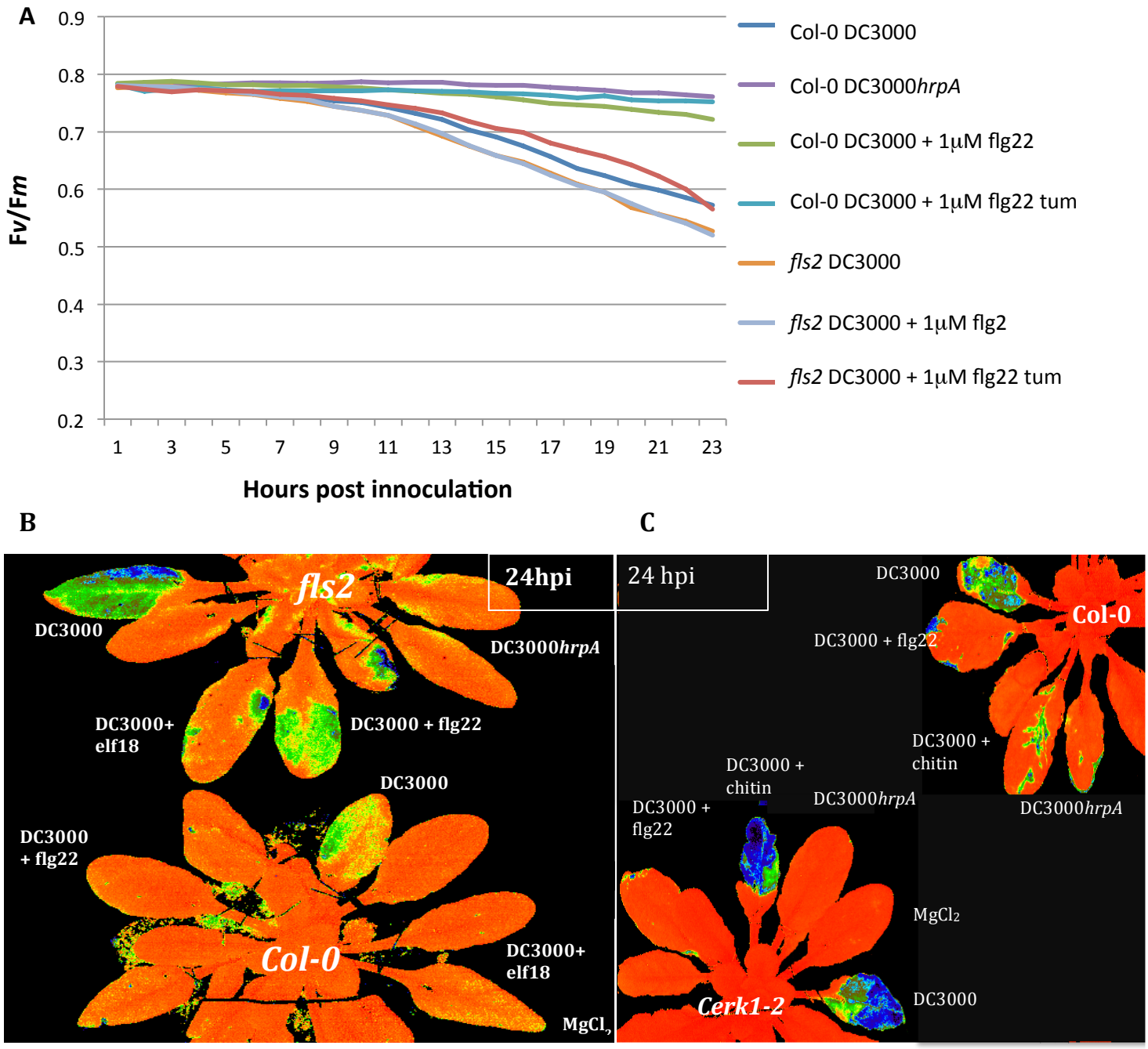


Figure 6) Bacterial and fungal PAMP pre-treatment of *A. thaliana* leaves protects against effector-mediated manipulation of host PSII by DC3000. **(A)** Prior activation of PTI signalling through pre-treatment with bacterial PAMPs flg22 (1μM) 17h prior to bacterial challenge attenuates DC3000 suppression of F*v*/F*m*. The PAMP receptor mutant *fls2* received no protection against DC3000 from a flg22 pre-treatment. Pre-treatment with the *A. tumefaciens* flg22-tu (1μM) or mock (10mM $MgCl_2$) failed to provide protection against DC3000 suppression of F*v*/F*m*. **(B)** Figure shows fluorimager false colour imaging of F*v*/F*m* 24hpi showing a decrease in F*v*/F*m* (green/yellow) in leaves that received mock pre-treatment compared to leaves pre-treated with functioning PAMPs flg22 (1μM) or elf18 (1μM) whose F*v*/F*m* remain at normal levels (orange), flg22 but not elf18 protection was lost in the *fls2* mutant **(C)** PAMPs flg22 (1μM) or chitin (100μg/ml) provide F*v*/F*m* protection against DC3000 suppression, this protection is lost in the *cerk1-2* mutant, figure shows F*v*/F*m* 24hpi. Imaging was repeated at least three times.

The signalling events activated by PAMP pre-treatment of leaves which lead to protection against bacterial photosynthesis manipulation upon subsequent challenge appear to be part of an early PAMP response. Restriction of suppression of F*v*/F*m* by DC3000 was evident within 2 h of PAMP pre-treatment and by 4 h post PAMP pre-treatment full chloroplast protection against bacterial manipulation was activated (Fig. 7).

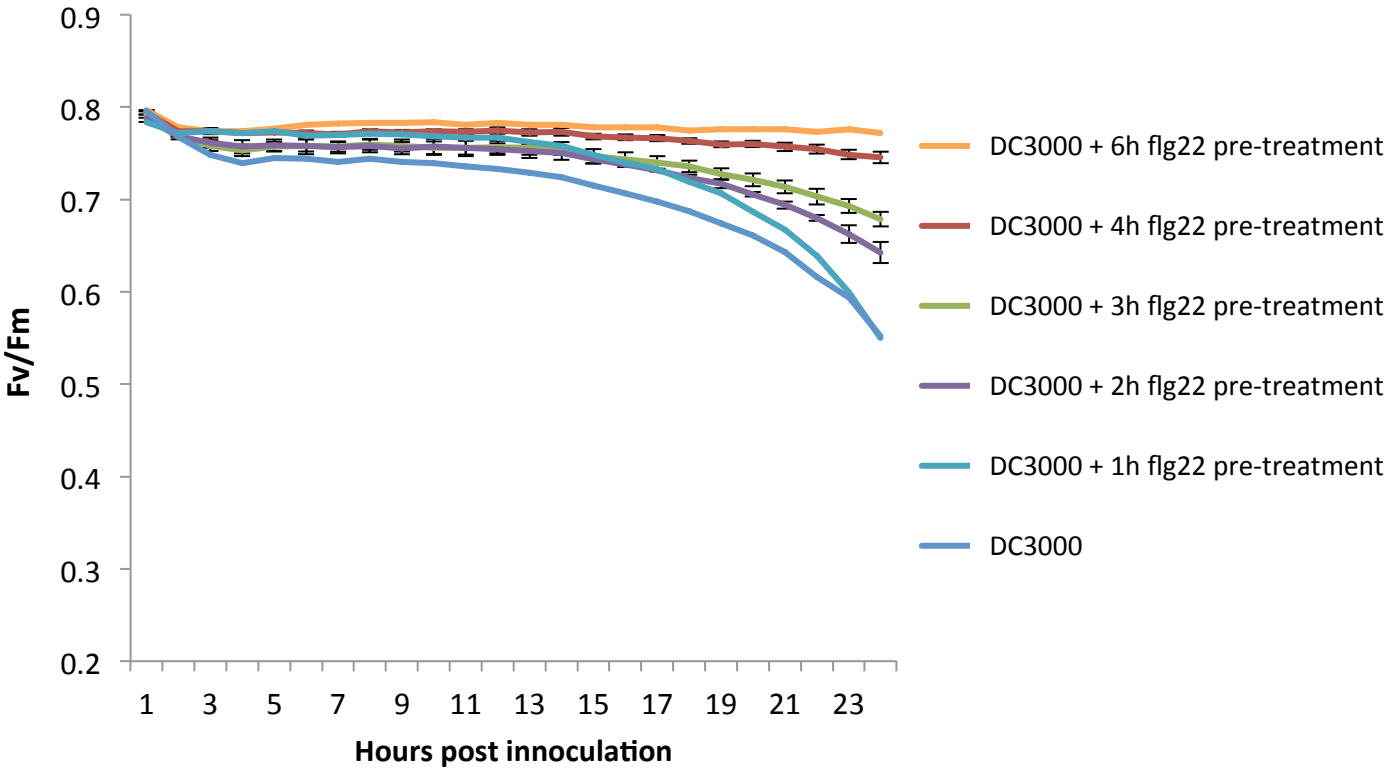


Figure 7) Activation of chloroplast protection mechanism against DC3000 is part of an early PAMP response. flg22 (1μM) induced protection of the chloroplast against DC3000 manipulation starts to show within 2 hours of flg22 pre-treatment with full chloroplast protection reached by 4 hours post flg22 pre-treatment. Values are means +/- SE (n=3 for 4h & 3h, n=2 for 2h & 1h). Experiment repeated 2 times.

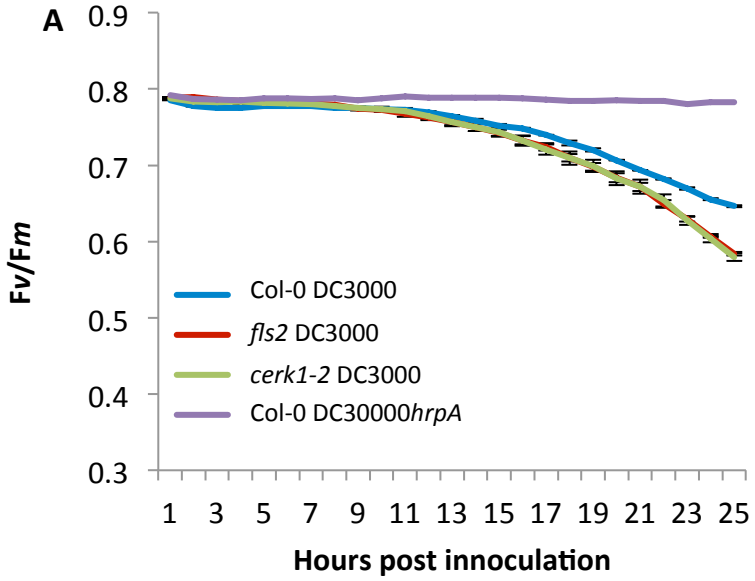
3.3 PTI signalling mutants have increased susceptibility to bacterial inhibition of host photosynthesis

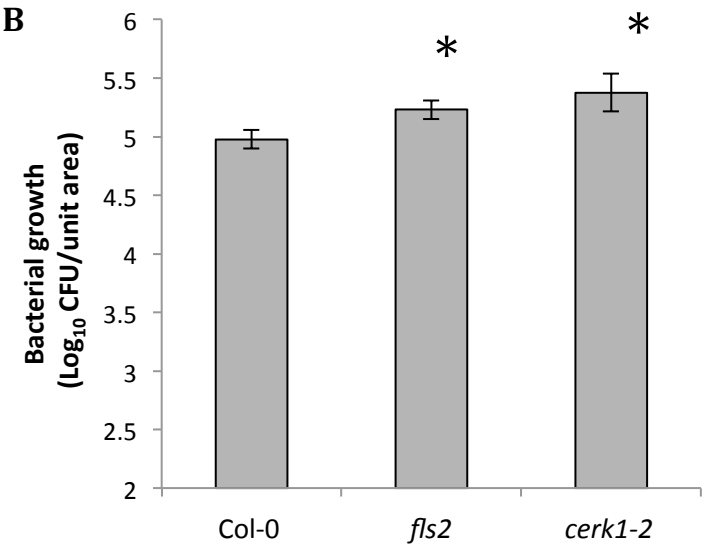
The ability of a PAMP pre-treatment to inhibit suppression of photosynthesis by DC3000 implies that one facet of PTI signalling must involve activation of a chloroplast protection mechanism. To examine this the suppression of host photosynthesis by DC3000, a variety of PTI signalling mutants was analysed.

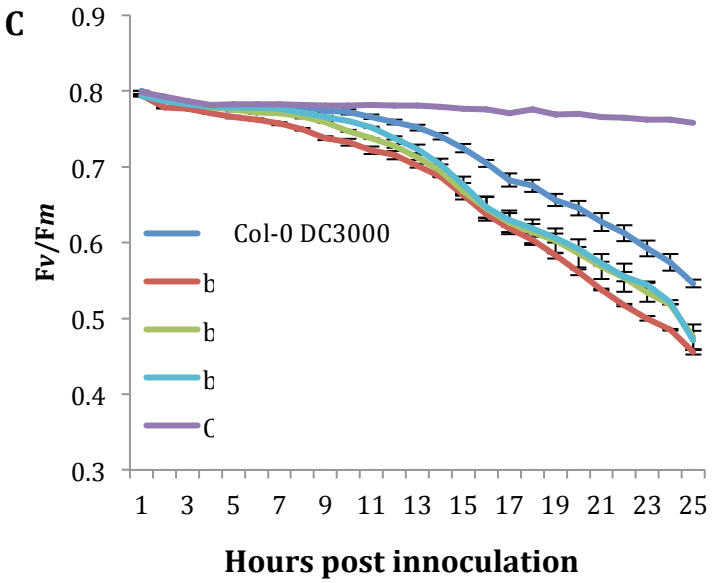
Firstly, the PAMP receptor mutants *fls2* and *cerk1-2* were examined. Bacterial growth curves showed both these mutant lines to be more susceptible to bacterial challenge with significantly more bacterial growth occurring in the mutant leaves 3dpi than Col-0 plants (*fls2*: t-test, p=0.028; *cerk1-2*: t-test, p= 0.024) (Fig. 8B). This increased susceptibility was mirrored in the fluorimager analysis of host photosynthesis suppression. Both PAMP receptor mutants showed increased suppression of F*v*/F*m* compared to Col-0 with F*v*/F*m* value of 0.584 and 0.579 24 hpi for *fls2* and *cerk1-2* compared to Col-0 0.647 F*v*/F*m* (Fig. 8A).

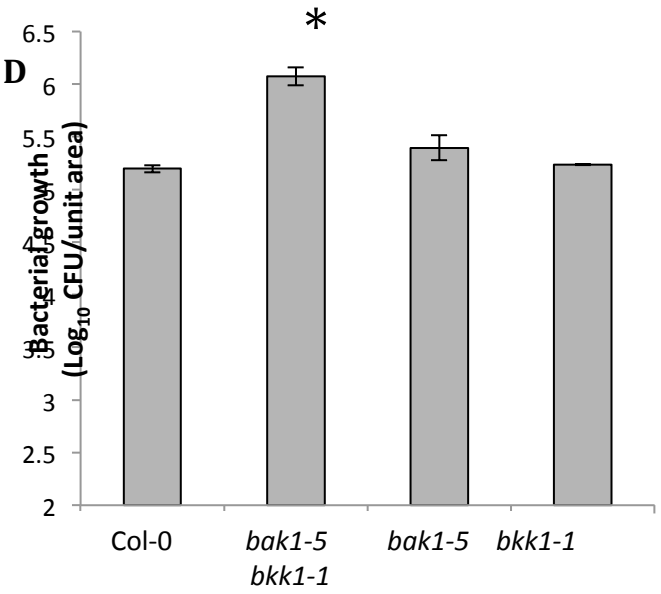
The receptor-like kinases BAK1-5 and BKK1-1 were also examined as both single and double mutants as these proteins are known to heterodimerise with a variety of PAMP receptors including FLS2 (Roux et al, 2011). The single mutants *bak1-5* and *bkk1-1* show increased susceptibility to DC3000 demonstrated in the growth curves with the double mutant *bak1-5 bkk1-1* exhibiting significantly more bacterial growth 3dpi than Col-0 (t-test, p<0.001) (Fig. 8D). A similar pattern in susceptibility was demonstrated in the DC3000 suppression of F*v*/F*m* in challenged leaves. As expected, both the single mutants showed greater suppression of photosynthesis than Col-0. The double mutant *bak1-5 bkk1-1* showed greater suppression of F*v*/F*m* greater than either single mutants and greater than that of the individual PAMP receptors *fls2* and *cerk1-2* (Fig. 8C).

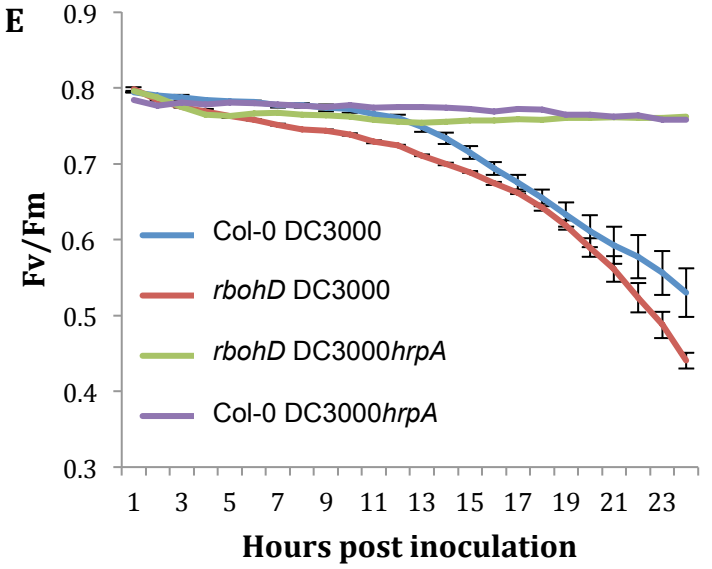
A number of enzymes involved in the immediate early downstream signalling of PTI were analysed to establish if they might be involved in PTI signalling involved in chloroplast protection. Firstly, the plasma membrane localized enzyme NADPH oxidase RBOHD responsible for the PAMP induced apoplastic ROS burst (Torres et al, 2002). Though the *rbohD* mutant showed increased susceptibility to DC3000 in terms of bacterial growth (Fig. 8F) its bacteria induced suppression of leaf F*v*/F*m* only really became significantly apparent from Col-0 very late (22hpi; Fig. 8E). This was in contrast to mutants of the Aspartate Oxidase enzyme FIN4. FIN4 catalyses the first step of *de novo* biosynthesis of NAD and has been linked to the ROS burst mediated through NADPH (Macho et al, 2012). In both this study and previous ones *fin4* mutants have shown increased susceptibility to *P. syringae* (Fig. 8H). Fluorimager analysis of *fin4-1* and *fin4-3* showed the mutants to have dramatically increased suppression of F*v*/F*m* following DC3000 challenge compared to Col-0 (Fig. 8G). F*v*/F*m* values for *fin4-1* and *fin4-3* dropped to 0.397 and 0.401 respectively by 24hpi contrasting to the fall of Col-0 F*v*/F*m* 0.535 in the same time period.

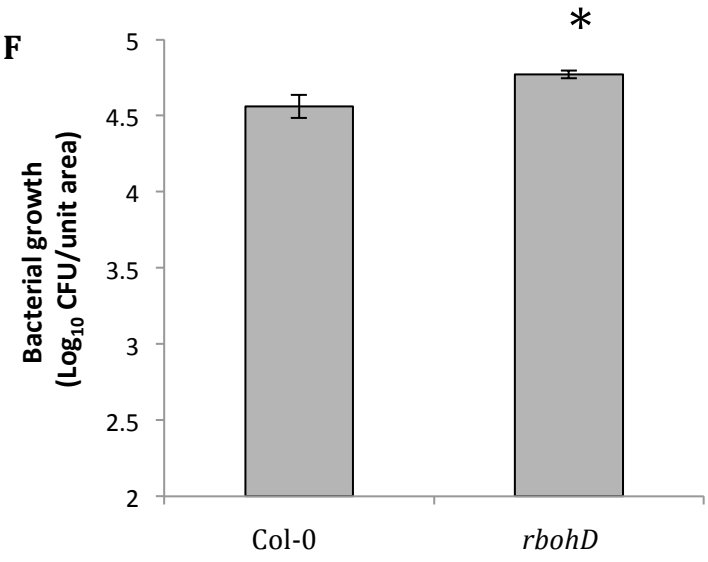

A
Fv/Fm
Hours post innoculation
Col-0 DC3000
fls2 DC3000
cerk1-2 DC3000
Col-0 DC30000hrpA


B
Bacterial growth (Log10 CFU/unit area)
Col-0
fls2
cerk1-2


C
Fv/Fm
Hours post innoculation
Col-0 DC3000


D
Bacterial growth (Log10 CFU/unit area)
Col-0
bak1-5 bkk1-1
bak1-5
bkk1-1


E
Fv/Fm
Hours post inoculation
Col-0 DC3000
rbohD DC3000
rbohD DC3000hrpA
Col-0 DC3000hrpA


F
Bacterial growth (Log10 CFU/unit area)
Col-0
rbohD

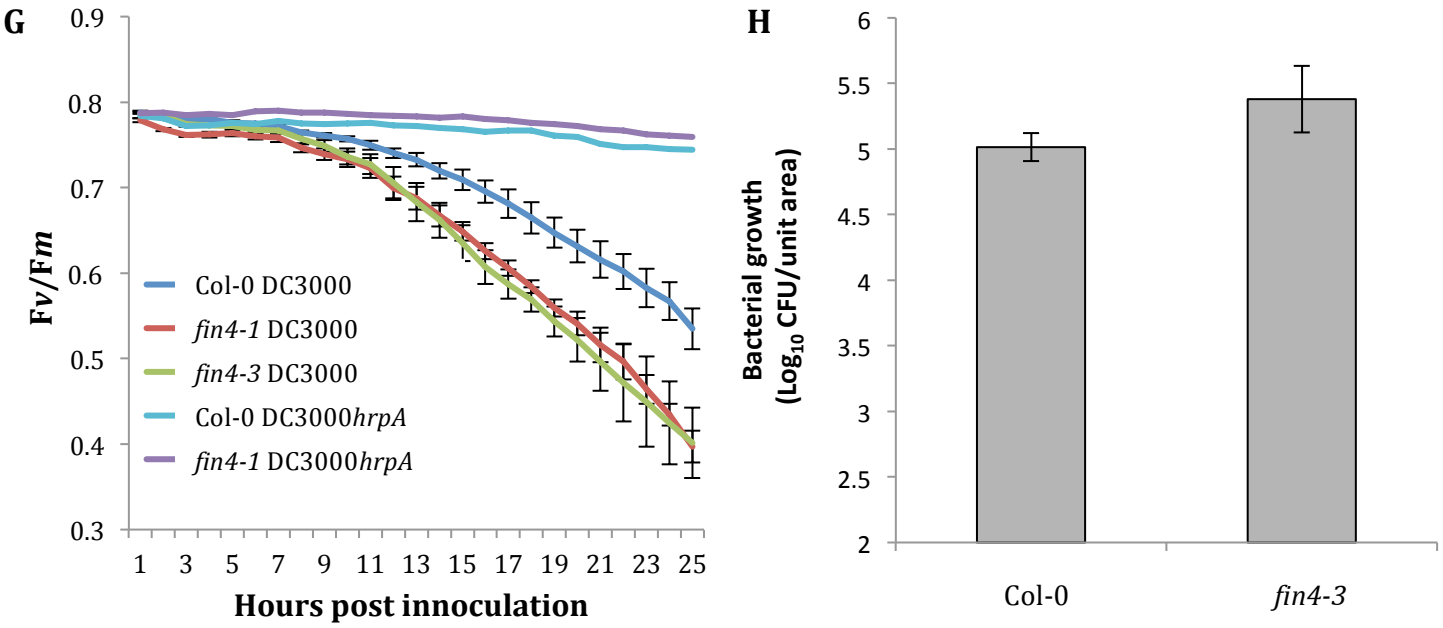


Figure 8) PTI signalling mutants show varying degrees of increased susceptibility to DC3000 suppression of host F*v*/F*m*. (A) PAMP receptor mutants *fls2* and *cerk1-2* showed increased susceptibility to DC3000 suppression of F*v*/F*m*. **(B)** These mutants showed overall significantly increased susceptibility to DC3000 in growth curves (*fls2*: t-test, p=0.028; *cerk1-2*: t-test, p= 0.024). **(C)** Both double and single *bak1-5 bkk1-1* mutants showed significantly increased susceptibility to DC3000 manipulation of photosynthesis with the double *bak1-5 bkk1-1* mutant showing greater susceptibility than the single mutants. **(D)** This patter of susceptibility was mirrored in terms of overall bacterial growth in leaf 3dpi where *bak1-5 bkk1-1* double mutant showed significantly more growth than Col-0 (t-test, p<0.001). **(E)** Though *rbohD* mutants showed increased suppression of F*v*/F*m* by DC3000 but this was only strongly apparent in the late stages of infection. **(F)** The mutants did show significantly increased susceptibility to DC3000 at the whole leaf level (t-test, p = 0.046). **(G)** FIN4 mutants *fin4-1* and *fin4-3* showed very similar dramatic reductions in F*v*/F*m* post DC3000 challenge **(H)** with the *fin4-3* mutant exhibiting increased susceptibility at the whole leaf level as well. All Chlorophyll fluorescence F*v*/F*m* values are mean +/- SE (n=3) for DC3000 inoculated plants. All Bacterial growth curves were conducted 3dpi (inoculum of ~1 x 10^8 CFU/ml) values are mean +/- SE (n=5). Fluorimager repeated at least twice for all mutants, growth curves once for B &H, three times for D and twice for F, Statistical significance for growth curves compared to DC3000 (T-test, p< 0.05) is indicated by asterisks

3.4 Receptor like kinases BAK1-5 and BKK1-1 are required for full PAMP pre-treatment induced protection of host photosynthesis

The ability of PTI signalling mutants to induce chloroplast protection against DC3000 upon flg22 pre-treatment was investigated. The aim of this was to highlight any important components in PTI signalling that are required for activation of the PAMP induced chloroplast protection mechanism.

The double receptor kinase mutant *bak1-5 bkk1-1* lost all ability to confer chloroplast protection against DC3000 with PAMP flg22 pre-treatment (Fig. 9A). Interestingly, the *bkk1-1* single mutant had normal fully functioning flg22 pre-treatment protection against DC3000 photosynthesis manipulation whereas the *bak1-5* single mutant only had partial functioning of PAMP inducible chloroplast protection. *bak1-5* F*v*/F*m* fell to 0.632 24 hpi, almost midway between *bak1-5 bkk1-1* F*v*/F*m* at 0.518 and non-supressed Col-0 at 0.733 24hpi (Fig. 5A).

Both *rbohD* (Fig. 9B) and *fin4* (Fig. 9C) mutants showed normal flg22 pre-treatment induced protection of against DC3000 F*v*/F*m* suppression. This suggests these two enzymes are not crucial for PTI signalling involved in instigating chloroplast protection against DC3000 manipulation.

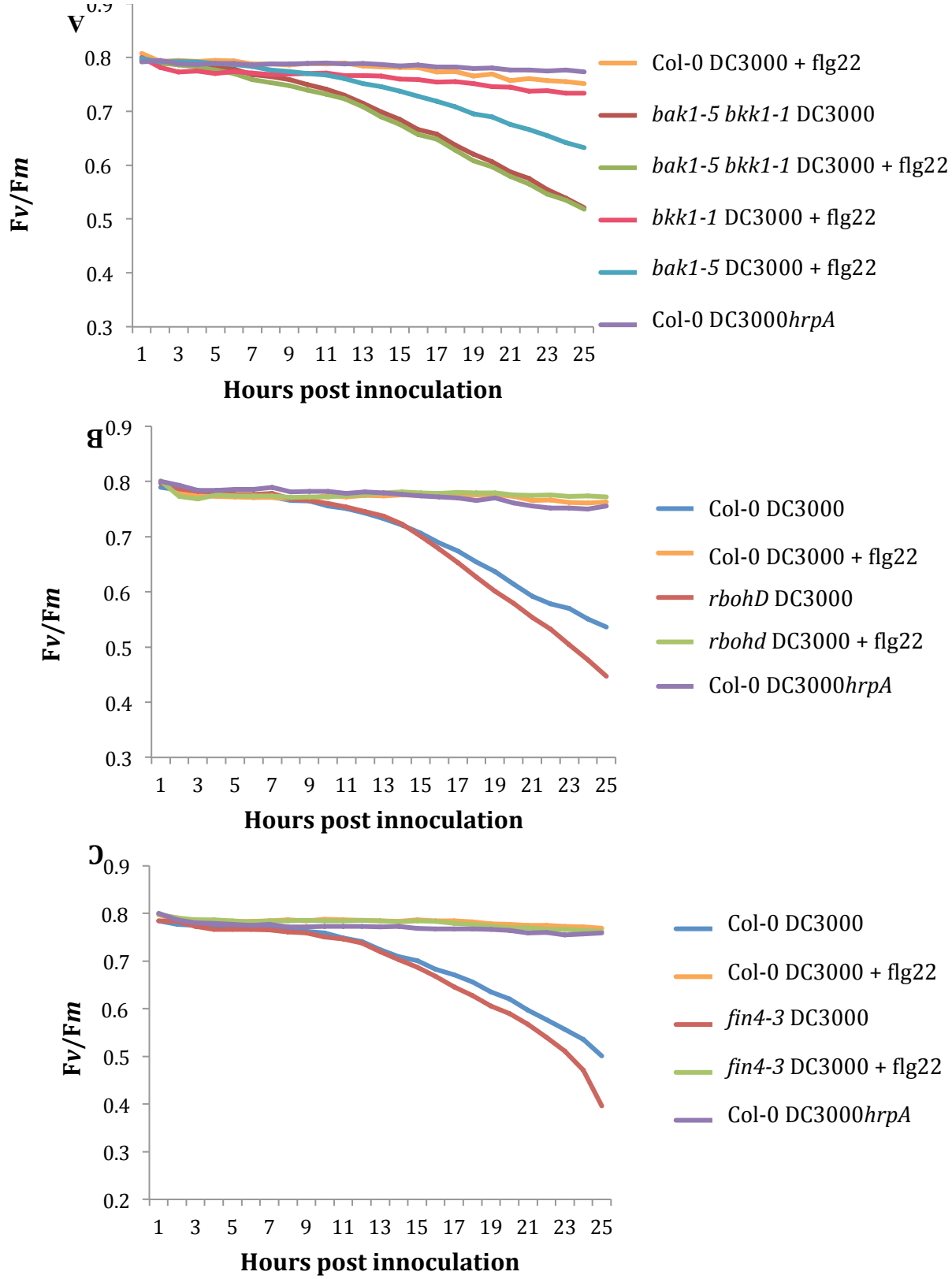


Figure 9) BAK1-5 and BKK1-1 are involved with instigating PAMP induced protection of host photosynthesis. (A) flg22 pre-treatment gave no chloroplast protection to double mutant *bak1-5 bkk1-1.* Single mutant *bkk1-1* received chloroplast protection from the flg22 pre-treatment but *bak1-5* only received partial protection of host photosynthesis, experiment repeated twice. **(B)** *rbohD* had a fully functioning chloroplast protection mechanism instigated upon flg22 pre-treatment single experiment conducted. **(C)** Similarly, *fin4-3* received full flg22 induced protection against bacterial manipulation of host photosynthesis, single experiment conducted. All flg22 (1μM) pre-treatments were infiltrated 17h prior to bacterial challenge.

3.5 DAMP Atpep1 pre-treatment of *Arabidopsis thaliana* protects leaf against bacterial suppression of host photosynthesis

Having observed the capability of bacterial and fungal PAMP pre-treatments to induce protection of the host chloroplast against bacterial manipulation it was examined whether this protection extended to include DAMPs (Damage-associated molecular patterns) as well. Leaves were pre-treated with the DAMPs Atpep1-3 and chlorophyll fluorescence measurement taken following DC3000 challenge 17 hpi later. Pre-treatment with 1uM Atepep1 appeared to produce the same protection of the host chloroplasts as had flg22 and chitin (Fig. 10). However, Atpep2 and Atpep3 were only capable of a partial protection of the host chloroplasts (Fig. 10).

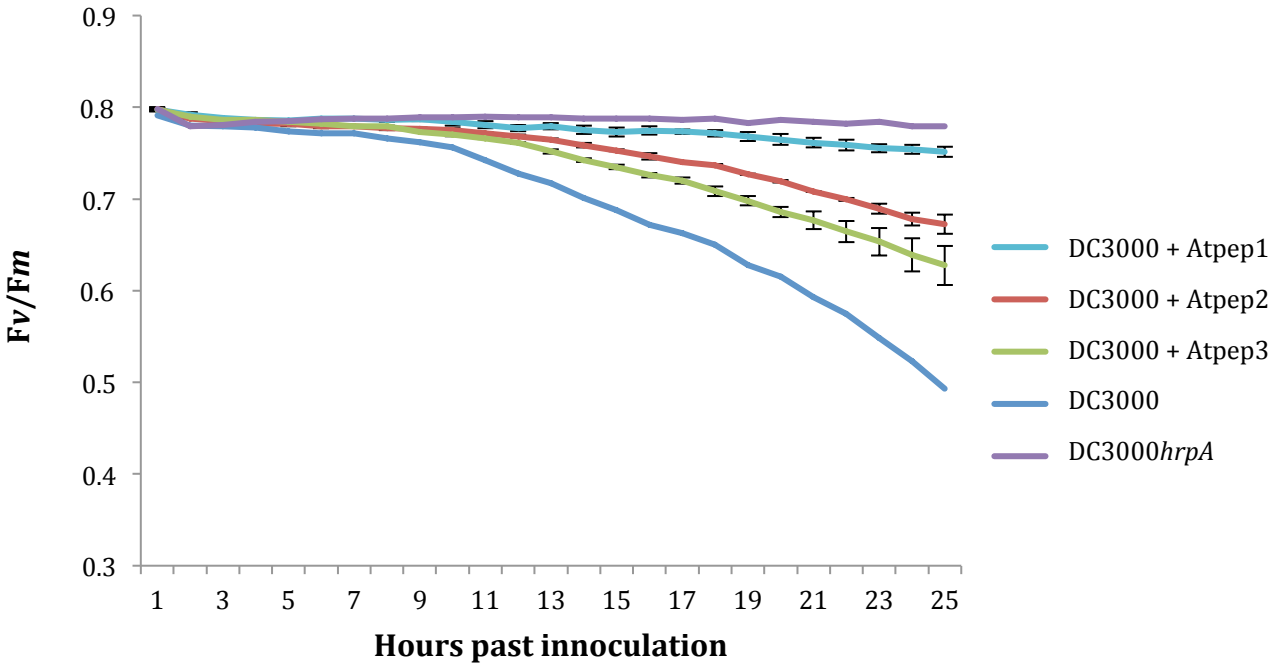


Figure 10) Atpep1 pre-treatment protects host F*v*/F*m* from DC3000 suppression. Pre-treatment with Atpep1 (1μM) 17 h prior to bacterial challenge protect chloroplast from DC3000 manipulation. Pre-treatment with Atpep2 (1μM) and Atpep3 (1μM) gave some level against DC3000 suppression of F*v*/F*m* but not the full protection received from Atpep1. Values represent means +/- SE (n=3) for Atpeptide pre-treated leaves, experiment repeated twice

3.6 PAMP pre-treatment vs PTI signalling

It was noted that the PAMP receptor mutant *fls2* was not protected from DC3000 suppression of F*v*/F*m* by chitin pre-treatment (Fig. 11A). This was unexpected as the *fls2* mutant had the same fully functioning chitin recognition system as a Col-0 plant. Conversely, the *cerk1-2* mutant which could not induce a chitin pre-treatment dependant protection of chloroplast but received full photosynthesis protection from a flg22 pre-treatment (Fig. 11A)

In order to mimic a similar PTI signalling setup in the Col-0 plants as the chitin pre-treated *fls2*, Col-0 leaves were treated with chitin 17h prior to being challenged with a mutant strain of *P. syringae* DC3000 *flic*. The *P. syringae* mutant has a deletion of the FliC gene encoding the flagellar structural filament protein flagellin and consequently does not produce flagellar or the PAMP flg22 (Hu et al, 2001). This meant that Col-0 plants challenged with DC3000 *flic* would not be able to sense flg22 and therefore incapable of activating FLS2 signalling pathways, mimicking an *fls2* mutant. Col-0 leaves that received no chitin pre-treatment but were challenged with DC3000*flic* had a reduction of their F*v*/F*m* greater than that of DC3000 treated and leaves similar to that of *fls2*, as expected (Fig. 11B). However, whilst DC3000 challenged leaves had no reduction in their F*v*/F*m* values when subjected to a chitin pre-treatment the DC3000*flic* leaves did have a reduction of their F*v*/F*m* to that of a similar decline as non-pre-treated DC3000 challenged leaves (Fig. 7B). This mirrored the F*v*/F*m* pattern seen in the *fls2* mutant. If plants were incapable of sensing flg22 at the time of bacterial challenge they did not receive the full chloroplast protection capabilities activated by the fungal PAMP chitin pre-treatment.

This relationship however was not found in the case of the bacterial PAMP flg22. Col-0 plants pre-treated with flg22 before subsequent challenge with DC3000 or DC3000 *flic* received full chloroplast protection from flg22 pre-

treatment with no reduction in leaf F*v*/F*m* in either bacterial challenges (Fig. 11C).

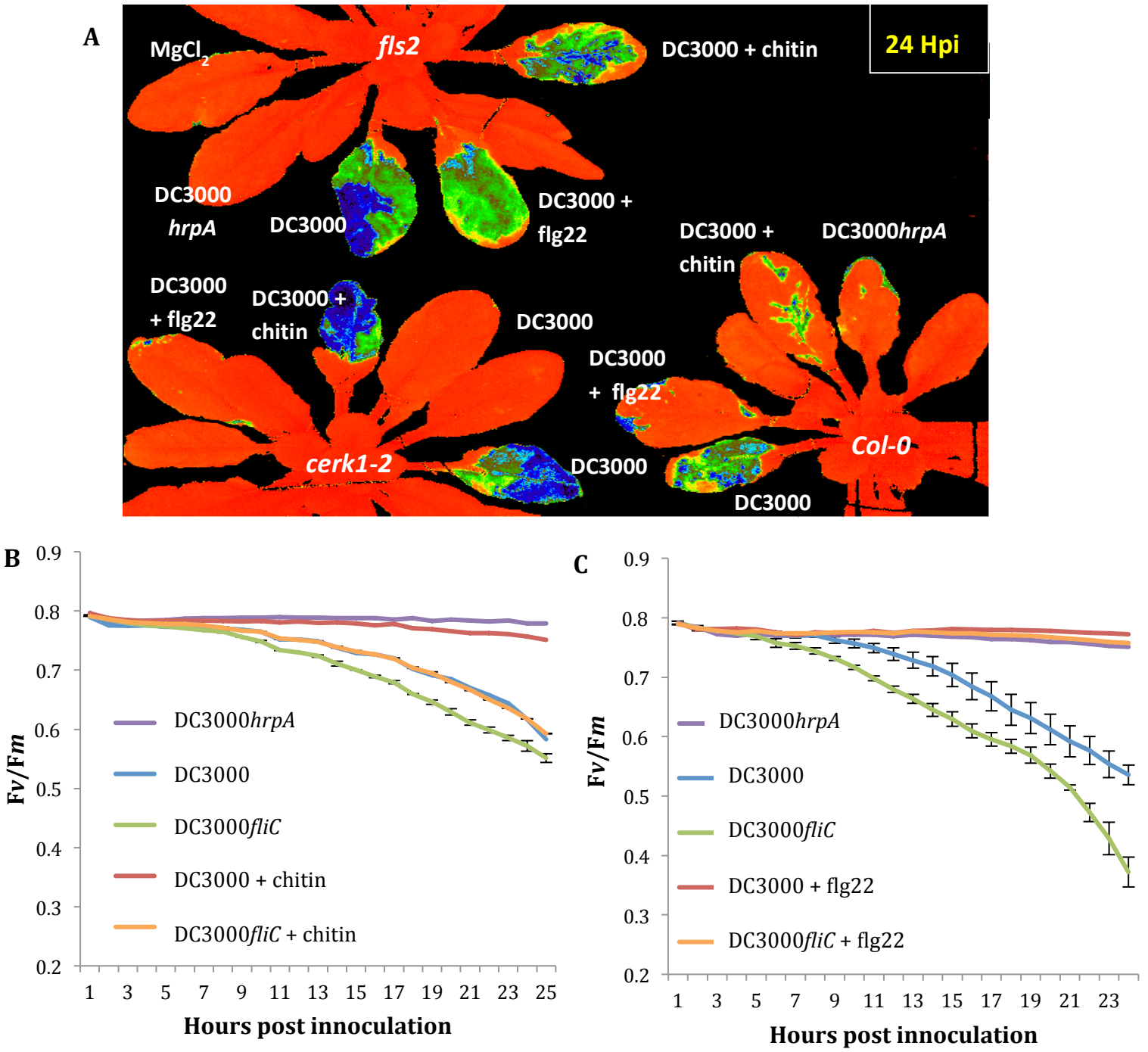


Figure 11) Interplay between PAMP pre-treatment and functioning PTI signalling at time of bacterial challenge. (A) False colour image of F*v*/F*m* 24hpi. As expected fls2 does not receive protection against DC3000 of host F*v*/F*m* but interestingly fls2 does not receive protection from chitin either. In contrast cerk1-2 does not receive protection from chitin, as expected, but does receive chloroplast protection from flg22, experiment repeated 3 times **(B)** The chitin pre-treated Col-0 receives chloroplast protection when challenged with DC3000 bu not DC3000*flic*, experiment repeated 2 times, **(C)** Col-0 does however receive chloroplast protection against DC3000 and DC3000 flic manipulation from a flg22 pre-treatment. Flg22 (1μM) and chitin (100μg/ml) Pre-treatments were infiltrated 17 h prior to bacterial challenge, error bars represent +/- 1SE, experiment conducted 1 time.

3.7 Bacterial effector proteins supress the PTI induced chloroplast derived ROS burst

It was hypothesised that the selective benefits behind *P. syringae* suppression of host photosynthesis may be to attenuate the ability of chloroplasts to produce ROS. Chloroplast ROS production was examined prior to bacterial multiplication 5-6hpi using the ROS probe 2'7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). A remarkably strong ROS burst was seen from individual chloroplasts 5-6hpi with DC3000*hrpA* consistent with the theory that chloroplasts are capable of producing a ROS burst as part of the plant's PTI response (Fig. 12). Strikingly though this chloroplast derived ROS burst was not seen in leaves challenged with DC3000 (Fig. 12). This suggests *P. syringae* can supress the PTI induced chloroplast ROS burst in an effector protein dependant manner.

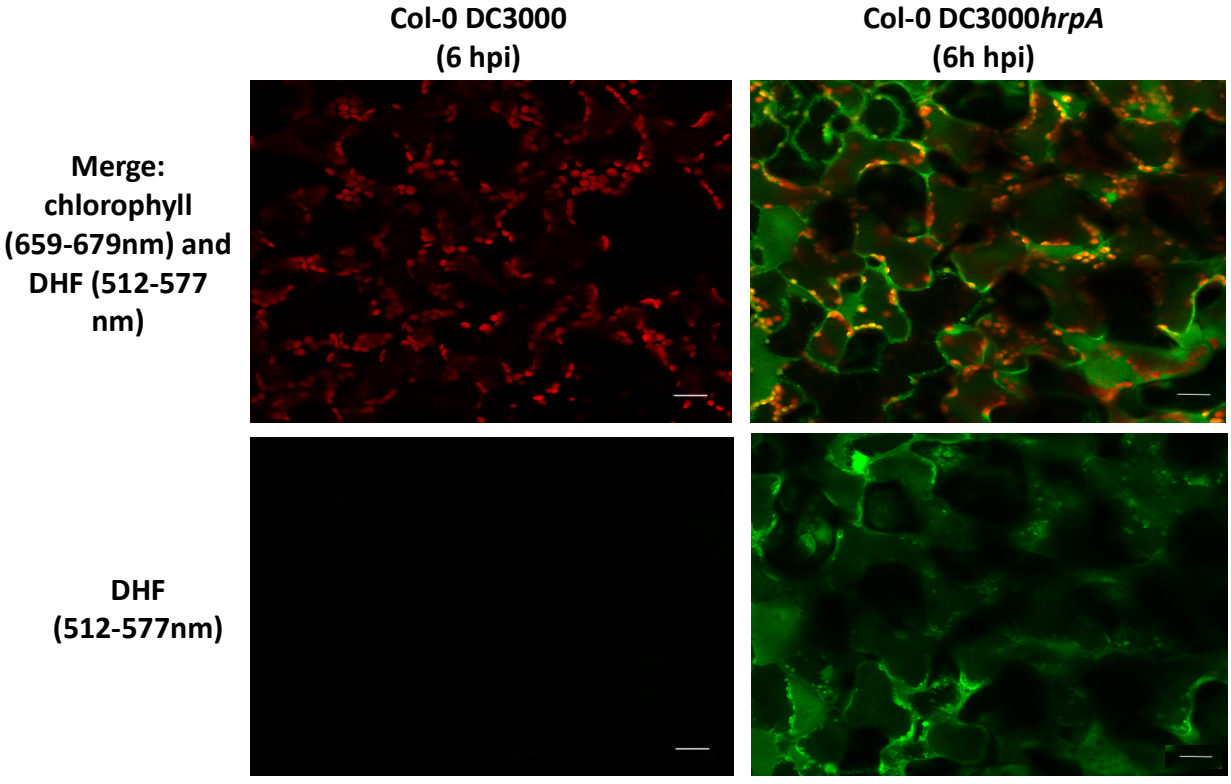


Figure 12) DC3000 supresses PTI associated chloroplast derived ROS burst in an effector dependant manner. ROS were detected using confocal microscopy to monitor the conversion of H_2DCF-DA to fluorescent DHF detected at 512-577nm. DHF emission was merged with chlorophyll emission (659-679nm) to identify ROS production from chloroplasts. ROS generation was only observed in samples challenged with DC3000*hrpA* not DC3000. Samples were imaged 6hpi. Scale bars represent 20μM. Single experiment conducted.

3.8 PTI signalling mutant *bak1-5 bkk1-1* is unable to produce chloroplast derived ROS burst involved Arabidopsis basal defences

The capability of receptor kinase mutants *bak1-5*, *bkk1-1* and double mutant *bak1-5 bkk1-1*to produce a chloroplast derived ROS burst in response to DC3000*hrpA* challenge was examined. This was to identify a direct signalling link between PTI signalling and the production of ROS in the chloroplast. Strikingly, the ability of *bak1-5 bkk1-1* to produce a chloroplast derived ROS burst in response to DC3000*hrpA* was abolished (Fig. 13). The single mutants *bak1-5* and *bkk1-1* retained some level of chloroplast ROS production though much reduced in comparison to that of Col-0 (Fig. 13).

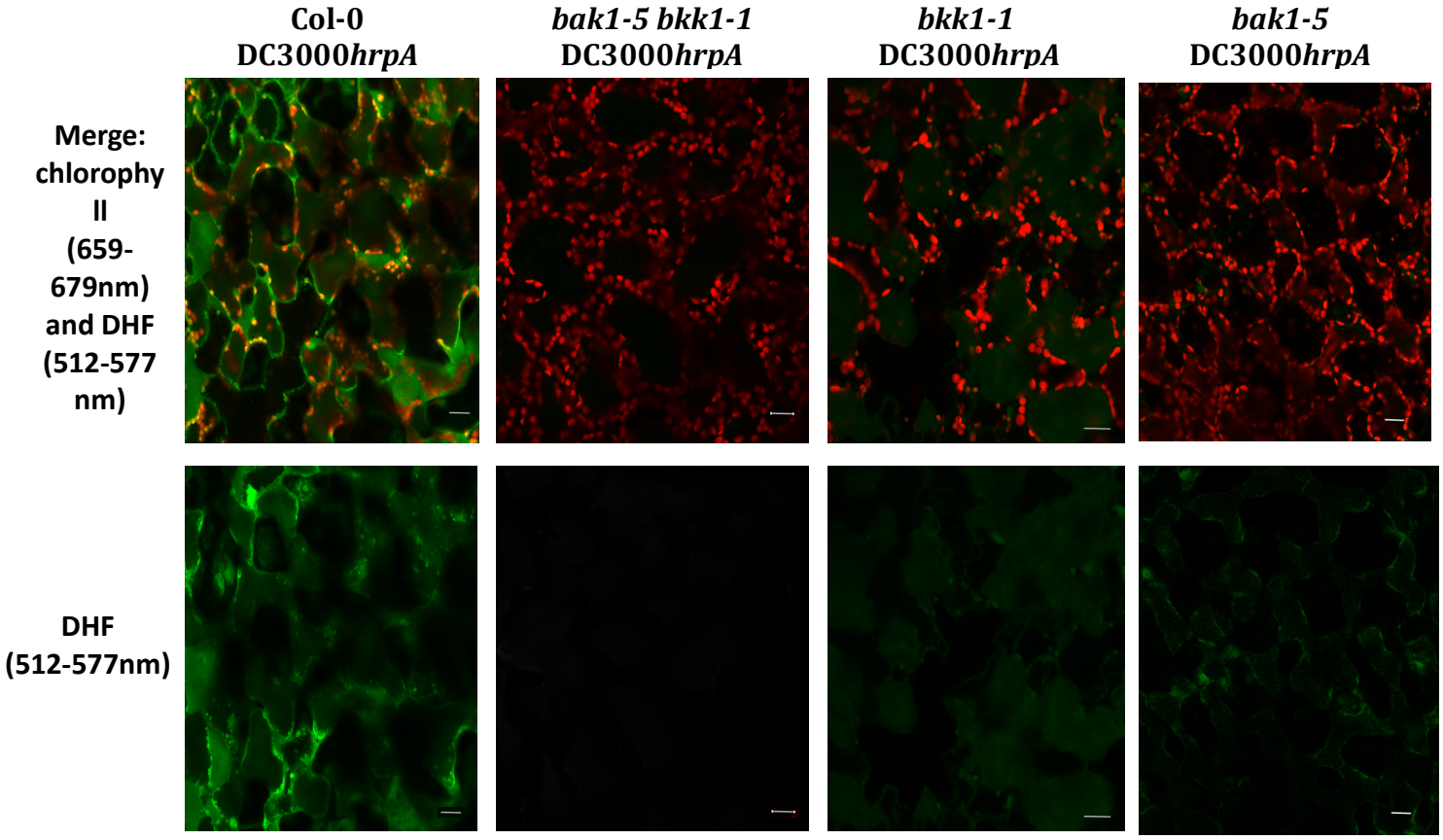


Figure 13) Receptor kinases BAK1-5 and BKK1-1 are required for production of PTI associated chloroplast ROS burst. No ROS DHF emission was detected in *bak1-5 bkk1-1* leaves challenged with DC3000 *hrpA.* Though ROS generation was detected in the single mutants *bak1-5* and *bkk1-1* it was greatly reduced compared to that of col-0. ROS were detected using confocal microscopy to monitor the conversion of H_2DCF-DA to fluorescent DHF detected at 512-577nm. DHF emission was merged with chlorophyll emission (659-679nm) to identify ROS production from chloroplasts. Samples were imaged 6hpi. Scale bars represent 20μM. Single experiment conducted.

3.9 *Pseudomonas syringae* may supress host photosynthesis through degrading PSII chloroplast proteins in an effector dependant manner

It was hypothesised that the mechanism through which bacteria manipulate and supress host photosynthesis may be through the bacterial effector proteins' role of degrading and/or inhibiting the synthesis of PSII proteins. This was examined through the comparison of western blots of the PSII oxygen evolving complex proteins PsbO and PsbQ, 17hpi with DC3000, DC3000*hrpA* or mock treatment. Antibody probing of these westerns gave rudimentary evidence that this may indeed be the case as levels of both these PSII proteins were at notably lower levels in DC3000 inoculated plants compared to those challenged with DC3000*hrpA* or mock treated plants (Fig. 14).

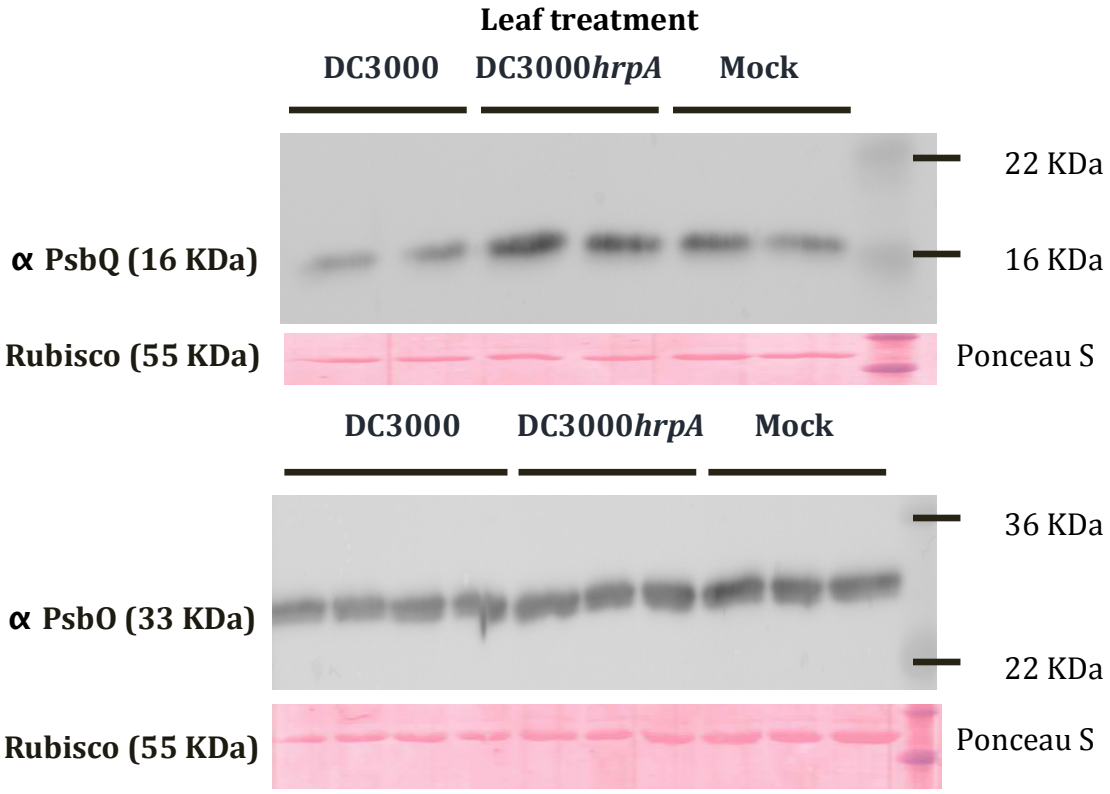


Figure 14) DC3000 reduced quantities of PSII proteins PsbO and PsbQ in the chloroplast 16hpi. Chloroplasts were isolated from challenged leaves 16hpi with DC3000, DC3000*hrpA* or mock treatment (10mM $MgCl_2$). Immunoblot analysis of chloroplast PSII proteins was then conducted using anti-PsbO (A) and anti-PsbQ (B) antibodies respectively (Agrisera). Equal loading of chloroplast proteins was confirmed by Rubisco expression via Ponceau S staining of gel. Only single experiment conducted.

4. Discussion

4.1 Effector protein dependant suppression of photosynthesis: a key bacterial virulence tool?

Suppression of photosynthesis following bacterial challenge has previously been observed in *A. thaliana* though the mode of action employed by bacteria to induce such suppression remained illusive. The work in both this study and that of de Torres-Zabala (2015) has clearly demonstrated the role bacterial effector proteins play in this suppression and that in their absence bacteria loose all apparent chloroplast manipulation capabilities. The results obtained in this study have shown the ability of *P. syringae* strain DC3000 to supress host photosynthesis in *A. thaliana* is reliant upon delivery of the pathogen's effector proteins demonstrated by the inability of DC3000*hrpA* to supress photosynthesis in the host cell. This clearly highlights the critical role bacterial effector proteins play in suppression of host F*v*/F*m* and highlights the chloroplast as an important target of effectors in the plant cell.

The results also indicate that the timing of bacterial suppression of host photosynthesis might correlate to that of the virulence of the strain being examined. This was demonstrated in the case of *P. syringae* by comparing the timing in photosynthesis suppression of the wild type strain DC3000 and the virulence compromised strain DC3000*cor*⁻ (Brooks et al., 2005), which supressed host photosystem II efficiency more slowly than DC3000. This may indicate the importance that suppression of photosynthesis plays in enhancing the virulence of a pathogen with strains capable of supressing host photosynthesis earlier on in their virulence strategy gaining faster colonisation rate of a plant tissue than others.

This study looked at the suppression of Fv/Fm as a surrogate for suppression of photosynthesis, as shown by de Torres Zabala (2015), by *P.*

syringae DC3000 but other studies have also found a similar event occurring in a variety of other pathogenic bacteria. Suppression of host photosynthesis has previously been demonstrated in other stains of Pseudomonas such as *P. syringae pv maculicola M4* as well as other species of bacteria for example *Xanthomonas campestris*. It should be noted however that both these pathogen's suppression of F*v*/F*m* occurred later in infection and was generally weaker reflecting the strains reduced virulence in the host *A. thaliana* (de Torres-Zabala, 2015).

Pathogen induced suppression in PSII quantum yield has also been demonstrated in both biotrophic and nectrophic fungi including *Albugo candida* (Chou et al., 2000) and *Botrytis cinerea* (Berger et al., 2004), though whether this suppression was dependant upon delivery of the pathogens' effector proteins was not established. Suppression of F*v*/F*m* by these fungi also showed different spatio and temporal patterns to that of the photosynthesis suppression observed in this study and therefore may possibly be occurring through a different mechanism than that observed in *P. syringae*.

Evidence that manipulation of the chloroplast via effector proteins may be a strategy not employed exclusively by bacteria is also beginning to emerge. In the case of fungi for example the leaf rust *Melampsora larici-populina* effector CTP1 has recently been shown to target the chloroplasts of its host using a chloroplast transit peptide to translocate into the chloroplast stroma (Petre et al., 2016). This strongly suggests that fungi too may manipulate the chloroplast as part of their virulence strategy. Whether this manipulation involves the suppression of photosynthesis remains to be seen as the exact function of CTP1 has yet to be identified. However, the ability of the fungal PAMP chitin to induce protection of the chloroplast from bacterial manipulation, as demonstrated in this study, may indicate that fungi employ similar virulence strategies to bacteria in order to supress host photosynthesis. This would help to explain the evolution of chitin's ability to induce protection against such manipulation. Nevertheless, this

may be the consequence of downstream PAMP signalling convergence between the different PAMP kingdoms.

It will be interesting to see whether this facet of the *P. syringae* effector protein virulence strategy is one commonly employed across the various kingdoms of plant pathogens or restricted to only that of bacteria. If effector-dependant suppression of host photosynthesis by a plant pathogen was shown to be a common tool for suppressing host defences it would provide a key new target for engineering intervention strategies enhancing crop immune defences.

4.2 PAMP induced chloroplast protection mechanism appear to be a convergent PTI event

The remarkable observation by de Torres-Zabala (2015) that bacterial PAMP pre-treatment of an Arabidopsis leaf is capable of inducing chloroplast protection mechanisms against photosynthesis suppression by subsequent bacterial challenge implicated the importance of PTI signalling and the chloroplast in plant immunity.

This study looked to examine whether chloroplast protection mechanisms were solely linked to bacterial PAMP induced PTI or whether this was a convergent PTI signalling event across the PAMP kingdoms. Having established the ability of the bacterial PAMPS flg22 and elf18 to evoke protection of the chloroplast from bacterial manipulation we found this PAMP protection extends to include the fungal PAMP chitin as well. This suggests that protection of the chloroplast against bacterial manipulation may be a widely employed or convergent PTI signalling event not only associated with bacterial PAMPs, similar to other well described features of PTI such as MAPK cascade activation and defence gene induction (Boller & Felix, 2009). Whether convergent or not, induction of photosynthesis protection was observed to be part of an early PAMP

response in the case of flg22. Evidence of such chloroplast protection was visible within 2h of flg22 PAMP pre-treatment with full chloroplast protection occurring within 4h. It will be interesting to see if this timing is mirrored amongst the other PAMP inducers, particularly the fungal PAMP chitin as this may indicate a similar method of activation of chloroplast protection amongst the PAMP families. Preliminary results also suggest chloroplast protection may not only be restricted to PAMPs but also be induced by DAMPs. DAMP pre-treatment of leaves with Atpep1 was also shown to activate protection of host photosynthesis from bacterial suppression. However this might not be the case of all DAMPs as Atpep2 and Atpep3 only gave partial chloroplast protection.

Though further testing of both PAMPs and DAMPs and their corresponding receptor mutants will be required to give conclusive evidence, our results indicate that induction of a chloroplast protection mechanism by PAMPs may be a convergent event amongst the PAMP kingdoms. This would provide an exciting new facet of PTI signalling events in plant innate immunity and indicates the important role the chloroplast must play in plant defence for a convergent PTI induced mechanism to evolve to protect and maintain full chloroplast function during a pathogen attack.

4.3 The chloroplast and PTI

With the aim of identifying key signalling components linking PTI signalling to protection of the chloroplast this study examined *P. syringae's* manipulation and suppression of host photosynthesis in a variety of Arabidopsis PTI signalling mutants. An increase in the reduction of host F*v*/F*m* was observed in several PTI signalling mutants at a range of levels in the signalling cascade (Fig. 8). As expected, the PAMP receptor mutants *fls2* and *cerk1-2* showed increased suppression of photosynthesis upon bacterial infection indicating that one facet of PTI signalling is involved in implementing protection of the chloroplast from pathogen manipulation. Carrying on downstream in the PTI signalling pathway,

F*v*/F*m* suppression by *P. syringae* was also observed to be greater in the receptor kinase-associated mutants *bak1-5* and *bkk1-1*. As predicted given the redundancy between these two kinases, the greatest photosynthesis suppression was observed in the double mutant of the two kinases *bak1-5 bkk1-1*.

The receptor kinase BAK1 has been identified as the partner of multiple plant ligand binding LRR RKs involved in the signalling pathways of plant immunity, cell death regulation and the growth hormone brassinosteroids (BR). BAK1 regulates the innate immune and brassinosteroid signalling pathways through interactions and heterodimerization with LRR RKs, PRRs e.g. FLS2 and EFR and BRI1 respectively. How BAK1 individually regulates these two different pathways though appears to differ as demonstrated by the BAK1 mutant *bak1-5* (Schwessinger et al, 2011). This mutant disrupts the immune responses induced by the PAMPs flg22 and elf18 but had little to no effect on the capacity of brassinosteroid signalling pathways. The mutant has also been used to demonstrate that BAK1 may regulate its interactions with BR and PTI signalling in different manners through the use of discriminative auto phosphorylation and transphosphoryation of their ligand binding co-receptors. Heterodimerization of FLS2, for example was seen not to be dependant upon the kinase activity of BAK1 unlike the brassinosteroid receptor BRI1 (Schwessinger et al, 2011). It was also hypothesised that BAK1 may play an important role in the trade-off observed between immunity and growth due to the important role the RK plays in PAMP and BR perception (Heese et al, 2007). However this was later shown to not be the case as though BRs were observed to be inhibitors of PTI this was executed in a manner independent to BAK1 (Albrecht et al, 2012). It has been suggested that an asymmetric mechanism may be occurring in the brassinosteroid pathway, independent of BAK1, suggesting different pools of BAK1 are recruited during initiation of PAMP and BR recognition (Albrecht et al, 2012).

The signalling abilities of BAK1 and BKK1 have been shown to be crucial

in instigating both a successful protection of chloroplast upon flg22 pre-treatment induced chloroplast protection and reducing and delaying photosynthesis suppression during bacterial challenge itself. Mutants of these SERK proteins showed a stronger suppression of photosynthesis during DC3000 bacterial infection and an inability to instigate the flg22 pre-treatment chloroplast protection mechanism, highlighting the important role these SERK proteins play in chloroplast-PTI interactions. The exact downstream interactors and signalling components involved in this aspect of PTI have yet to be established though.

The results in this study suggest there may also be a BAK1 independent pathway that can induce chloroplast protection. This was demonstrated by the ability of a chitin leaf pre-treatment to induce chloroplast protection. Recognition of chitin in Arabidopsis is reliant upon the PRR CERK1, which does not require a BAK1 co-receptor to activate PTI. Components of BAK1 dependent and independent PRRs downstream signaling are known to converge, for example induction of defense genes and MAPK activation (Couto and Zipfel, 2016) but chitin has been observed in previous studies to have a weaker induction of several PTI response classically associated with flg22, for example a reduced ROS burst and callose deposition (Gimenez-Ibanez et al, 2009). This suggests that there may be a number of PTI signalling pathways that are specific to different PRRs initiation. This may help explain the inability for example of a chitin pretreatment to protect chloroplast from bacterial manipulation when the FLS2 path is compromised at the point of bacterial challenge, for example in the *fls2* and *bak1-5 bkk1-1* mutants or challenge with DC3000*fliC* bacteria. Chitin may be protecting chloroplast in an independent manner to BAK1 and BKK1 that is not as effective as that instigated with these SERKs especially when combined with a BAK1 BKK1 compromised PTI signalling pathway at the point of bacterial infection.

It may be interesting to test the ‘robustness’ of chitin pretreatment induced protection of the chloroplast, perhaps by increasing the inoculum level of bacterial challenge between chitin and flg22 pre-treatment to observe if there is

threshold that chitin fails to protect the chloroplast that flg22 can still protect at.

The photosynthesis suppression of PTI downstream enzymes was also examined in attempt to narrow down the downstream signalling components involved in PTI's protection of the chloroplast. Interestingly, F*v*/F*m* suppression of the Aspartate oxidase FIN4 mutants *fin4-1* and *fin4-3* was greatly increased relative to Col-0 indicating the enzyme's possible role in the protection of chloroplast from bacterial manipulation (Fig. 8). How FIN4 may contribute to chloroplast protection still remains to be identified. It should be noted however that the enzyme's localisation to the chloroplast and its important role in NAD biosynthesis and previously established links to involvement flg22 induced PTI response (Macho et al., 2012) provide strong evidence for FIN4 to be a possible candidate for linking the chloroplast and photosynthesis into plants' immune defences.

The NADPH oxidase RBOHD, responsible for the apoplastic ROS burst classically associated to PTI, appeared not be involved in PTI protection of host photosynthesis as no large discrepancies in F*v*/F*m* suppression were observed between the mutant *rbohD* and Col-0 until late in the observation period (22 hpi). This indicates that the PTI induced mechanisms protecting photosynthesis suppression are independent of the apoplastic ROS burst and RBOHD, decoupling the mechanism to downstream signalling events such as callose deposition in the cell walls (Luna et al., 2011).

Surprisingly though, it was only the receptor mutants and mutants of the receptor associated kinases *bak1-5* and *bkk1-1* that exhibited inability to induce the PAMP pre-treatment chloroplast protection observed in Col-0. *fin4* mutants, though showing increased susceptibility to DC3000 induced F*v*/F*m* suppression with no PAMP pre-treatment relative to Col-0, exhibited fully functioning ability to induce protection of chloroplast from flg22 pre-treatment. This suggests that there may be multiple pathways and mechanisms linking PTI to protection of the chloroplasts. The evidence in this study supports this hypothesis as

photosynthesis suppression was increased in the early PTI signalling mutants involving the PRR complex both with and without PAMP pre-treatment comparative to Col-0. Conversely, further downstream FIN4 showed increased susceptibility to *P. syringae* suppression of host photosynthesis relative to Col-0 when no pre-treatment was applied to leaves but showed normal functional chloroplast protection mechanisms when leaves were pre-treated with flg222.

The significance of these discrepancies is not yet fully understood but examination of further PTI signalling mutants upstream in the signalling cascade may help to elucidate the mechanisms linking PAMP perception to chloroplast protection. For example it will be interesting to test the involvement of MAPK cascades classically involved with PTI such as MPK3 and MPK6 (Asai et al., 2002) as this will provide crucial information as to the arm of PTI signalling linking PAMP perception to the chloroplast.

There also appears to be some level of temporal interplay in the mechanisms used to protect the chloroplast between protection induced prior to pathogen challenge via PAMP pre-treatment and PTI signalling ability at the time of bacterial inoculation. This was first observed in this study when *fls2* mutant leaves did not receive the chloroplast protection given to Col-0 leaves from chitin pre-treatment. This observation was confirmed through use of the *P. syringae* strain DC3000*fliC*, which lacks flagellin and therefore does not activate host FLS2 receptors thereby mimicking the signalling of an *fls2* mutant in a Col-0 leaf. Col-0 leaves infiltrated with DC3000*fliC* showed greater reduction in leaf F*v*/F*m* values upon bacterial challenge mimicking the pattern shown in the fls2 mutant infiltrated with DC3000. Similarly when Col0-0 leaves pre-treated with chitin were challenged with DC3000*fliC* little chloroplast protection was observed as seen in the DC3000 challenged chitin pre-treated fls2 mutant (Fig 11B).

What was striking however was that Col-0 leaves pre-treated with flg22 prior to challenge with DC3000flic, were protected against effector protein

manipulation of the chloroplast (Fig 15B). It should also be noted that *cerk1-2* mutants received full chloroplast protection from flg22 pre-treatment when challenged with DC3000. This suggests it is not merely the loss of an unspecific single PRR that inhibits PAMP induced full chloroplast protection at the time of bacterial challenge but either a facet of FLS2 specific signalling or the entire family of bacterial PAMP recognising PRR complexes as a whole. One possible hypothesis for this could be that bacterial PAMPs show stronger activation of chloroplast protection mechanisms than fungal PAMPs with this only becoming apparent when PTI at the point of bacterial challenge is compromised. Conclusive results of this will only be able to be drawn when further bacterial and fungal PAMPs are tested. One particular key experiment will be whether elf18 pre-treatment protects Col-0 from DC3000*fliC* induced photosynthesis suppression, as this should help to distinguish whether this phenomenon is specifically related to FLS2 signalling or bacterial PAMP signalling in general.

Another possible explanation could be that separate PTI signalling mechanism are occurring between PAMP pre-treatment 17h prior to bacterial challenge and at time of challenge which are differentially activated between bacterial and fungal PAMPs. This may also help to explain the differences in susceptibility of some PTI mutants to bacterial induced suppression of photosynthesis at time of bacterial challenge and capabilities of the mutants to induce chloroplast protection following flg22 pre-treatment. For example, though *fin4-3* and *fin4-1* mutants showed significantly greater suppression of host F*v*/F*m* when challenged with DC3000 both mutants showed fully functional chloroplast protection mechanisms induced by PAMP flg22 pre-treatment. It may also be considered that full protection of the chloroplast may be reliant upon a combination of active PRRs. As such, the removal of one PRR or associated adaptor complex components may affect the functioning or effectiveness of other PRR's defense signalling and activation of photosynthesis protection mechanisms.

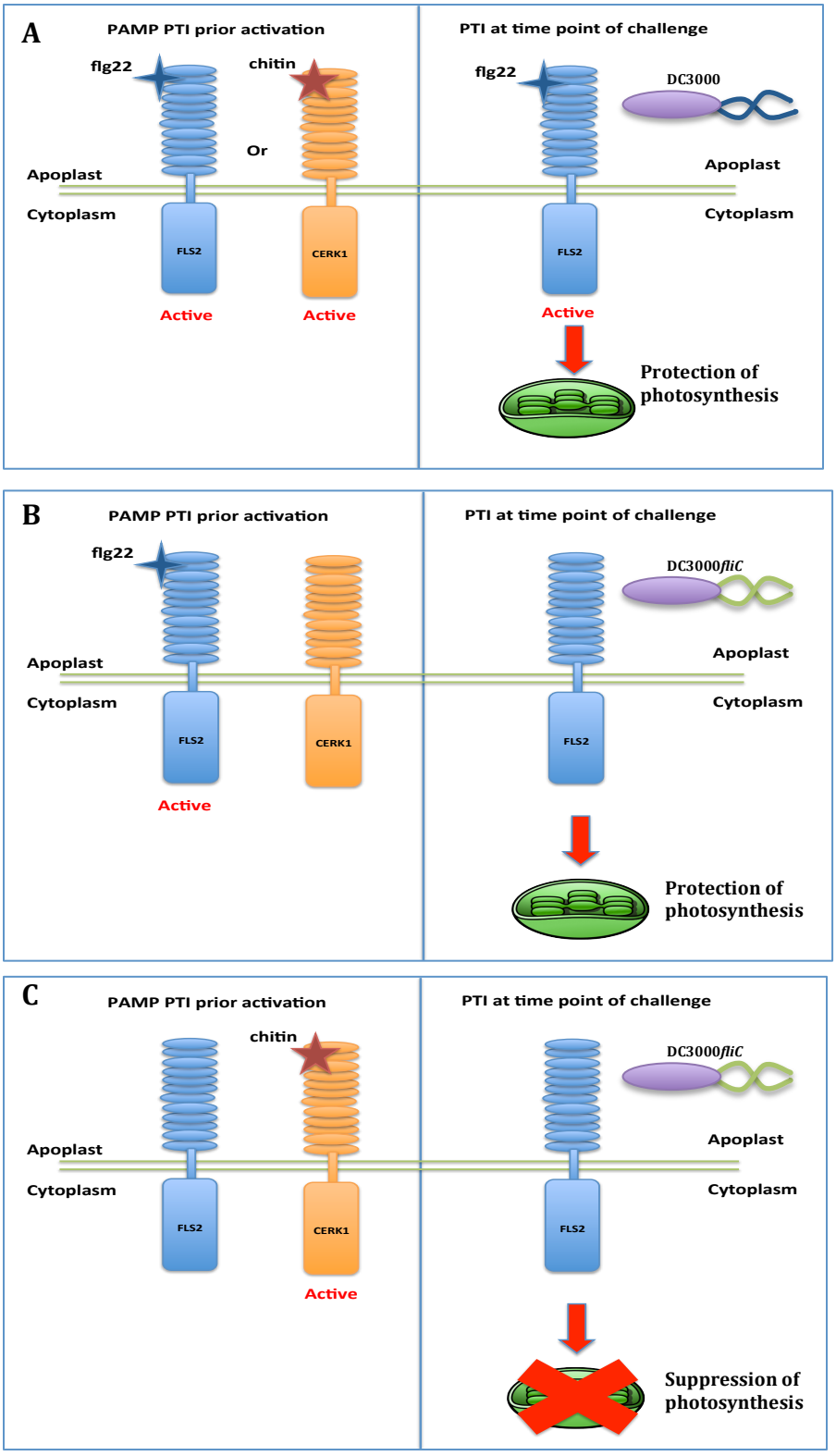


Figure 15) Interplay between PAMP pre-treatment and PTI signalling at time of bacterial challenge. Summary model of signalling occurring when Col-0 leaves are challenged with DC3000 **(A)** or DC3000*fliC* ***(B,C)*** with flg22 or chitin pre-treatment 17 h prior to inoculation and the combined effect on host photosynthesis suppression

4.4 The chloroplast ROS burst: possible pathogen selective benefit behind supressing host photosynthesis

The role the chloroplast plays in a range of plant immune responses and production of pro-defense signals highlights the organelle as a prime candidate for phytopathogen effector targeting. The selection benefits for successful manipulation of the chloroplast by an effector are undoubtedly strong given the wide variety of effectors predicted to target the organelle (Mukhtar et al., 2011). The evolutionary selective benefits of targeting the chloroplast as means of supressing host immunity have been demonstrated for a number of bacterial effectors. For example, the *P. syringae* effector HopI1 has been shown to target the chloroplast and supress plant immune responses through reducing host levels of the defence hormone SA whose biosynthesis involves pathways in the chloroplast (Jelenska et al., 2007, 2010).

As a site of electron transport in the plant cell, chloroplasts are also a possible source of ROS, a widely associated hallmark of PTI which acts as both a direct defence cytotoxic agent and important signalling molecule (Shapiguzov et al., 2012). We hypothesised that one such pathogen selection benefit of photosynthesis suppression by an effector protein would be in the abolishment of ROS production by the chloroplast upon the suppression of F*v*/F*m*.

Here we have shown through use of the ROS probe H_2DCF-DA, that not only is the chloroplast capable of producing a clear ROS burst as part of the plant immune response to challenge with DC3000*hrpA* but that this burst is completely abolished when the leaf is challenged with DC3000 in an effector protein dependant manner. This chloroplast derived ROS burst has been shown by de Torres-Zabala et al (2015) to be dependant upon photosynthesis within the organelle as ROS production was inhibited upon co-infiltration with the PSII electron transport blocker DCMU. This clearly demonstrates the activity of *P. syringae* DC3000 effectors in inhibiting ROS production in the chloroplast

through the manipulation of photosynthesis electron transport chain within the organelle and provides evidence of a possible pathogen selection benefit of targeting the chloroplast in the host-pathogen evolutionary arms race.

Confocal imaging with H_2DCF-DA in this study demonstrates the abilities of individual chloroplast to produce a ROS burst in response to bacterial challenge. The purpose of this ROS production is not entirely understood but given its intracellular nature is most likely acting as a signalling molecule to activate downstream defence pathways. Evidence supporting this role for chloroplast ROS comes from the recent identification of stroma filled tubular extensions connecting the chloroplast and nucleus termed stromules (Caplan et al., 2015). Formation of these stromules between the chloroplast and the nucleus are induced during the plant defence response and correlate with the accumulation of ROS in the nucleus. This suggest there maybe a direct link and transfer of ROS from the chloroplast to the nucleus which may explain the previously described links between chloroplast ROS production and changes in nucleus gene regulation and programmed cell death of HR (Shapiguzov et al., 2012). These suggested links between chloroplast ROS production, nuclear transcription and HR would provide a clear evolutionary benefit to phytopathogen effectors for disrupting photosynthesis and inhibiting the defence-associated chloroplast ROS burst.

Here we have also shown evidence for a link between that production of ROS in the chloroplast and the receptor sensing stage of the PTI response. The ability of DC3000*hrpA* challenge of *A. thaliana* leaves to induce production of ROS in the chloroplast demonstrates a signalling link between PAMP detection and the chloroplast. Such signalling mechanisms have been demonstrated through the calcium sensing protein CAS though whether this protein is involved in inducing ROS production in the chloroplast has not been established (Nomura et al., 2012). Some early PTI signalling links between PRRs and the chloroplast have begun to emerge in this study through the demonstration of the requirement for

the receptor-associated kinases BAK1 and BKK1 in DC3000*hrpA* induction of ROS in the chloroplast. Though these are early signalling components in the PTI signalling cascade the abolishment of ROS in the chloroplast upon DC3000*hrpA* bacterial challenge in the double mutant *bak1-5 bkk1-1* clearly demonstrates that ROS production in the chloroplast is a well-integrated signalling components of a downstream PTI response.

4.5 Possible mechanism for *P. syringae* effector protein suppression of host *A. thaliana*

Extensive work has been conducted in recent years into the modes of action employed by pathogenic effector proteins to promote host colonisation. Several key effectors in both bacteria and fungi (Jelenska et al., 2007; Rodriguez-Herva et al., 2012; Li et al., 2014; Petre et al., 2015) have been identified as chloroplast targeting and many more are hypothesised to do so through *in vitro* protein interaction studies (Mukhatar et al., 2011). The mechanisms employed by effector proteins to disrupt the chloroplast appears to vary amongst effectors though many appear to have the same end goal of disrupting photosynthesis in the host plant. For example, HopN1 is a cysteine protease that cleaves PSII protein PsbQ in order to disrupt electron transport in photosynthesis (Rodriguez-Herva et al., 2012).

Using this well documented effector as a model it was hypothesised that one possible method for photosynthesis suppression by DC3000 effector proteins may be through the degradation of PSII proteins in the thylakoids

This study examined the levels of PSII proteins PsbO and PsbQ post DC3000 and DC3000*hrpA* challenge hypothesising that levels of these proteins would be lower in DC3000 treated plants due to their degradation by DC3000's effector proteins. As hypothesised this appeared to be the case for these two PSII proteins, PsbO and PsbQ, whose levels both seemed reduced in DC3000

challenged leaves comparative to that of DC3000*hrpA*. It should be noted however that Rubisco levels were used as a loading control in this study and what effect DC3000 effectors proteins may have on Rubisco levels has not been studied.

The physiological significance of PSII protein effector protein dependant degradation in terms of suppression of photosynthesis by a pathogen however is still questionable. A direct link between degradation of these PSII proteins and pathogen effector dependant photosynthesis suppression has not yet been clearly demonstrated as *A. thaliana* challenge with DC3000Δ*HopN1* showed no significant change in suppression of host photosynthesis compared to DC3000 nor was the overall virulence of the pathogen effected (de Torres-Zabala, 2015). This suggests that effectors may either employ different mechanism to supress host photosynthesis or they may collaborate with one another hence no significant difference was observed in F*v*/F*m* suppression when only one effector protein was removed from DC3000's armoury due to redundancy amongst effectors.

It should also be noted that pathogenic effectors might disrupt photosynthesis indirectly of targeting the chloroplast through the use of nuclear targeting effectors that target and down regulate the transcription of nuclear encoded chloroplast genes (NECGs). Over the course of plant evolution a significant subset of the chloroplast genome has been transferred to the nucleus via endosymbiotic gene transfer, these genes now being referred to as NECGs. This has resulted in the transcriptional control of a number of important photosynthetic chloroplast proteins residing in the nucleus of the plant cell rather than the chloroplast itself. Previous studies have shown NECGs to be significantly differentially expressed in response to PAMP treatment and induction of a PTI response (de Torres-Zabala, 2015). Further, significant differences in the transcriptional activity of these NECGs has also been observed between DC3000 and DC3000*hrpA* challenged leaves noted within 3hpi,

indicating that transcriptional regulation of NECGs could be susceptible to hijacking and manipulation by bacterial effector proteins (de Torres-Zabala, 2015). This has lead to the hypothesis that DC3000 effector proteins may be targeting chloroplastic proteins as part of their virulence strategy on both the post-transcriptional level, acting directly on translated proteins in the chloroplast but also at the transcriptional level targeting chloroplast proteins encoded in the nucleus by down regulating transcription of NECGs.

It is important that we fully understand how effector proteins manipulate the chloroplast in order to understand the mechanism employed by pathogens as part of their virulence strategies. Only when we are able to fully understand how effectors collaborate to target multiples sites in the plant cell will we be equipped to engineer effective and durable interception methods to target these mechanism and supress pathogen virulence.

5. Conclusion

Conservative estimates currently state that crop pathogens claim 13% of the world's harvest and this is set to increase as pathogen boundaries expand due to climate change (Evans et al., 2008; Fletcher et al., 2006). This fact, combined with further pressures from global population growth, urbanisation and climate change induced weather extremes mean our agricultural and food resources are set to be further stretched then ever before in the near future. It is therefore crucial that we gain knowledge and understanding of how plant pathogens work to supress plant immunity. It is only when we understand the mechanism underlying pathogen virulence and plant immunity will we be able to engineer effective and stable intervention strategies to protect crops in the future and help to increase global food yields to meet the planets growing demands.

Understanding the mechanism behind bacteria's suppression of photosynthesis and the ability of plants' immunity to protect against such suppression will give an

important insight into possible intervention strategies to protect crops. This study has shown the important role the chloroplast plays in plant immunity as a target of bacterial effectors and how bacterial effector proteins can be employed to supress host photosynthesis. We have begun to understand the innate immune signalling pathways plants' use to protect their chloroplasts from bacteria manipulation and proposed new avenues of research to further this learning.

This study has clearly demonstrated that the chloroplast is an important target of bacterial effector proteins that work to manipulate the chloroplast and supress host photosynthesis. We have hypothesised that one possible selection benefit of this suppression is through the abolishment of chloroplast generated ROS burst observed upon PTI activation but there are likely many others given the central role the chloroplast plays is many plant defence mechanisms. We have also provided evidence of a novel PTI mechanism employed by plants to protect chloroplasts from bacterial manipulation. We have shown this PTI induced mechanism to be compromised in a range of PTI signalling mechanisms, particularly in those components associated with early PAMP detection by PRRs but also the potential involvement of the downstream enzyme FIN4. This in itself is interesting, as FIN4 is involved in primary metabolism, as are many biosynthetic pathways in the chloroplast. Though further work is required to decipher more of this novel PTI pathway, this study has provided strong evidence for the link between PAMP perception and activation of PTI signalling and the chloroplast, demonstrating the important role the chloroplast plays in plant-pathogen interactions.

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