The Interaction between Abiotic and Biotic Stress in *Arabidopsis thaliana*

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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 is included for which a degree has been previously approved from this or any other university.

Signature.....

Abstract

Plants are continuously exposed to different abiotic and biotic stresses in their natural environment. Their capacity to survive depends on the capacity to perceive external signal and quality amount a defence response for protection from the stress perceived. The purpose of this project was to study the impact of combined abiotic stress and biotic stress on the outcome of the disease inducing *Arabidopsis thaliana – Pseudomonas syringae* interaction. This study included a focus on the role of ABA in these interactions and also whether 3´-*O*-β D- ribofuranosyl adenosine (hereafter it called '400' compound), a novel adenosine derived compound induced during compatible interactions, was involved. The later involved the targetted disruption of a putative 400 biosynthetic pathway involving analysis of knockout mutants of enzymes; APD-ribose diphosphatase NAD binding / hydrolases of the NUDIX class, glucosyl transferases, ribosyltransferases, a ribose-phosphate pyrophosphokinase3 and galactosyltransferases. Unfortunately, none of these targeted interventions modified the host response to *Pseudomonas* infection, nor altered levels of 400 in challenged leaves.

The primary research investigated the interaction between abiotic and biotic stresses in Arabidopsis plants focussing on the modulation of plant defence against multiple, and possibly antagonistic, stress responses and the role plant hormones play in this process. We showed that high light caused enhanced susceptibility to the already virulent *Pseudomonas syringae* DC3000pvsp61. The pathways contributing to this enhanced susceptibility were largely ABA independent. Subsequent characterization of transgenic lines expressing the soluble Arabidopsis abscisic acid receptors, *PYRABACTIN RESISTANCE1-LIKE4-6* provided compelling evidence for a role for these receptors in DC3000 virulence strategies, but they contribute to a lesser extent to the enhanced susceptibility under high light. This was corroborated genetically by using mutants of the immediately downstream targets of PYLs, the type two protein phosphatase, specifically the triple mutant *hab1-1/abi2-1/abi1-2.* A number of epitope and fluorescent constructs were generated to facilitate future studies of the role of ABA signaling.

Targetted profiling suggested that SA dynamics were altered under DC3000 challenged Arabidopsis grown under high light. Furthermore, differential accumulation of flavonoids suggested these may also play a role in attenuating host defences under high light.
Finally we provide evidence based on comparative analysis of that the photoreceptors phytochrome double mutant *phyA-211/phyB-9* and *cry1/cry2* behave antagonistically in Arabidopsis response to DC3000. Overall our studies support the conclusion that plants abiotic stress (HL) response takes precedence over biotic stress (DC3000) responses and that abiotic stress is detrimental to plant immunity.

The luciferase transgenic *PYL* lines showed high level of expression of ClucP::*PYL5* plant tissues challenged 2hpi of DC3000 (OD_{600}: 0.15) in comparison with C1lucP::*PYL6.* This result opposes to what RT-PCR reported; which was that three *PYLs* genes display similar expression level at 6hpi of *hrpA* or 18hpi of DC3000. The epitope tags of CaMV::HA transgenic plants showed HA-tagged signal with stunted phenotype in a range of *PYL4, 5* and *6* plants but none of the plants displayed any differences in susceptibility to DC3000. Although, RT-PCR assay showed high levels of expression in the three *PYLs,* 6hpi of *hrpA* but no signal was detected in B8eGFP::PYL5 transgenic line either followed the DC3000 and *hrpA* infection or by examined plant seedlings at early stages under confocal microscopy.

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List of abbreviations

ABA: abscisic acid
ABF: ABA response element binding factor
ABI1: ABA insensitive 1
ABREs: ABA-responsive promoter elements
AO: aldehyde oxidase
APS: Ammonium Persulfate
At: Agrobacterium tumefaciens
AtMRK: Arabidopsis thaliana MLK/Raf-related protein kinase
AtMPK: Arabidopsis thaliana mitogen-activated protein kinase
Avr: Avirulence
BABA: β-aminobutyric acid
CaMV: Cauliflower Music Virus
CaM: calcineurin B-like
CBP60g: CaM-binding protein 60-like.g; At5g26920
CD domain: Common docking domain
CFU: Colony forming unit
CIPK: CBL-interacting protein kinase
CKI1: Cytokinin histidine kinase sensor
coi1: coronatine-insensitive 1
COR: *cold-regulated A. thaliana*
CRE1: Cytokinin response 1
CTR1: Constitutive ethylene response mutant
dH2O: distilled Water
DNA: Dinucleic acid
EDS1: ENHANCED DISEASE SUSCEPTIBILITY 1
EDR1: Enhanced disease resistance
EDTA: ethylenediaminetetraacetic acid
ein2: ethylene-resistant mutant
ELIP: Early light-induced proteins
ERM kinase: Elicitor-responsive MAP kinase
ESI: electrospray ionisation
ET: Ethanol
ETR: Relative electron transport rate
ETR1: Ethylene insensitive mutant
Fig: Figure
FLS2: Flagellin sensitive locus; encoding a leucine-rich repeat receptor kinase
GFP: Green Fluorescent Protein

HA: Hemagglutinin
HL: High Light
HR: Hypersensitive response
IBC: Induced by cytokinin
JA: Jasmonic Acid
jar1: Arabidopsis JA response 1
JIN1: Arabidopsis loci reduce sensitivity to JA
JNK: c-Jun amino (NH2)-terminal kinase
Ko: Knock out
LAR: local acquired resistance
LC-MS: Liquid chromatography mass spectrometry
Log: Logarithm
LPS: lipopolysaccharide
LSD1: lesion simulating disease resistance 1
LRR: Leucine-rich repeat
M: Molar
M6PR: mannose-6-phosphate reductase
MAMPs or PAMPs: microbe- or pathogen-associated molecular patterns
MAP3K/MAPKKK: Mitogen- activated protein kinase kinase kinase
MAPK: Mitogen-activated protein kinase
MAPKK: Mitogen-activated protein kinase kinase
Mbp: Mega-base pairs
MeJA: methyl jasmonate
MEK: MAPK/ERK kinase; another name for a MAPKK
MEKK: MAPK/ERK kinase kinase; another name for a MAPKKK
MHK: Mak-homologous kinase
min: Minutes
MKK: Another name for a MAPKK
MKP: MAP kinase phosphatase
mM: mille Molar
MMK: Medicago MAPK
MPK: Mitogen-activated protein kinase
MS: Murashige and Skoog
MSK: Medicago sativa MAP kinase
NBS-LRR: Nucleotide Binding Site-Leucine Rich Repeat.
NL: Normal Light
NO: nitric oxide
NPK1: Nucleus- and phragmoplast-localized protein kinase (renamed from *Nicotiana* protein kinase 1)
NPR1: NONEXPRESSOR OF PR GENES 1

NPQ: Non photochemical quenching
NTF: *Nicotiana tabaccum* MAP kinase homolog
OD: Optical density
OE: Over Expression
O/N: Over Night
PAD4: *PHYTOALEXIN DEFICIENT 4*
PAMP: Pathogen Associated Molecular Patterns
PAR: Photosynthetically active radiation
PCD: Programmed cell death
PCR: Polymerase chain reaction
PGN: peptidoglycan
PIG: Pathogen-induced gene
PP2C: Protein phosphatase 2C
PSII: Photosystem II
PTI: PAMPs triggered immunity
PTP: Protein tyrosine phosphatase
PR1: PATHOGENESIS RELATED 1
PRRs: pathogen recognition receptors
R gene: Resistance gene
RAF: Transforming gene
RCARS: regulatory components of ABA receptor
RD29A: *responsive to dehydration 29A*
ROI: Reactive oxygen intermediate
ROS: Reactive Oxygen species
RNA: Ribo nucleic acid
RPK1: Arabidopsis receptor-like protein kinase 1
RT: Room Temperature
SA: Salicylic Acid
SAMK: Stress-activated MAPK
SAR: Systemic Acquired Resistance
SDS: Sodium dodecyl sulphate
Sec: Second
SID2: SALICYLIC ACID INDUCTION DEFICIENT 2
SNF: sugar non-fermenting
SnRK2: SNF1-telated protein kinases2
SOS: Salt Overly Sensitive
TEMED: N,N,N,N-Tetramethyl Ethyl Enediamino
TIR domain: Toll- and interleukin-1-receptor domain
TRIS-HCL: Trisaminomethane hydrochloride
TTSS: Type III Secretion system

Vol: Volume
WIPK: Wound-induced protein kinase
Wk: week
WRKY: Zinc-Winger-type transcription factor (containing the WRKY domain) and binds to the W-box domain
Xcc: Xanthomonas campestris pv
Xg22: Flagellin elicitor
YFP: Yellow Fluorescent Protein

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Dedication

This is dedicated to; the souls of my father and mother whom I wished so much that they were still alive to share with me my happiness today and to go some way towards repaying a little of what they have endured in my upbringing.

To my brothers: El-Fellani, Abdul-Salam and Mohammed for their support. El-Fellani and Abdul-Salam; they really took the responsibility of looking after me side-by side with my parents. They were truly like both parent and friends.

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My dedication is also to my relatives and cousins for their wishes, prayers for my success and the completion of this endeavour.

Finally, to my friends in my town back home and those who have missed me in my absence.

Chapter 1: Introduction

1.1 *Arabidopsis thaliana* a model of plant molecular biology

.*Arabidopsis* was first discovered in the sixteenth century by Johannes Thal in the Harz Mountains, he called it *Pilosella siliquosa*. Since that time the name has been changed many times but later in 1841 the plant was renamed as *Arabidopsis thaliana* by German botanist Gustav Heynhold in honor of Thal (http://www.*arabidopsis*.org/). The small flowering plant belongs to *Brassicaceae* family that is widely used as a model organism of choice for research in plant biology (Meinke *et al*., 1998). The life cycle of *Arabidopsis* can be completed within six weeks, which is ideal for research purposes (Meinke *et al*., 1998, Chris and Maarten, 2002, Bennett *et al*., 2003). *A. thaliana* has one of the smallest genomes ~157 mega-base pairs (Mbp), containing 25,498 genes encoding proteins from 11,000 families (Meinke *et al*., 1998, Bennett *et al*., 2003, Chris and Maarten, 2002, Koornneef and Meinke, 2010). However, since *Arabidopsis* is self-fertile and has more than 10 000 seed sets in a single plant. These features promote efficient genetic analysis of various aspects of plant growth, development and proliferation (The Arabidopsis Genome, 2000). Thus *Arabidopsis* individual chromosomes do not exhibit the cytological details that often proved useful in cytogenetic studies with crop plants (Koornneef *et al*., 2003). Therefore, combining pachytene chromosomes, which appear longer than mitotic chromosomes and have distinct heterochromatic and euchromatic regions, with sensitive in situ hybridization methods, *Arabidopsis* chromosomes became amenable to cytogenetic analysis (Fransz *et al*., 1998), leading to a number of advances such as the discovery of chromosome inversions in some accessions (Fransz *et al*., 2000). Additional variations in mutant screens were developed over the years, as described by Page and Grossniklaus (2002). Dobritsa et al., (2011) genetic screen in Arabidopsis led to a recovery of mutants with a variety of defects in exine structure, including multiple mutants with novel phenotypes. Recently, it has been shown that Arabidopsis gene identification through genetic screens represented novel mediators of Arabidopsis responses to sulfanilamides suggest that these responses extend beyond the perturbation of folate biosynthesis (Schreiber et al., 2012).

1.2 Plant stresses

1.2.1 Abiotic stress

In General there are multi-abiotic stress factors that have been reported to affect plant growth and development. Thus during the life cycle of vegetative plants (either annual or perennial) they are subject to various environmental challenges such as heat, cold, salinity, drought and high light (HL), which impact on growth and development. These stresses are perceived by the plant and a unique response elicited. The inception of signal transduction pathways associated with such environmental changes initially involves the perception of the stress and then the generation of second messengers, for example; inositol phosphates and reactive oxygen species (ROS) which can subsequently modulate intracellular Ca^{+2} levels (Xiong *et al*., 2002). ROS is known to be produced as by-products of a range of metabolic pathways localized in different cellular compartments (Foyer *et al*., 1994, Apel and Hirt, 2004, Quan *et al*., 2008). In addition, under physiological steady state conditions ROS is scavenged by different antioxidative defense components that are confined to particular components (Alscher *et al*., 1997, Ahmad *et al*., 2010). Generation of ROS is occurred by activating various oxidases and peroxidases that produce ROS in response to certain environmental changes (Allan and Fluhr, 1997, Bolwell *et al*., 2002, Bolwell *et al*., 1998, Schopfer *et al*., 2001, Kotchoni and Gachomo, 2006, Yoshioka and Shinozaki, 2009, Affenzeller *et al*., 2009). In addition, it has been shown that mitogen-activated protein kinase (MAPK) cascade is a universal module of signal transduction from the cell surface to the nucleus. MAPK plays a crucial role in the regulation of biochemical and physiological changes associated with extracellular stimuli. Furthermore, MAPK cascades have been reported to function in various signal transduction pathways in eukaryotes (Madhani and Fink, 1998, Robinson and Cobb, 1997, Cristina Rodriguez et al., 2010). It has also been demonstrated that specific MAPKs are activated in response to environmental stimuli and phytohormones (Brodersen et al., 2006, Fujii et al., 2007, Jammes, 2009, Cristina Rodriguez et al., 2010, Sinha et al., 2011). In several plant species, including Arabidopsis, MAPK cascades have been shown to be involved in signaling pathways activated by abiotic stresses such as cold, salt, touch, wounding, heat, UV, osmotic shock, heavy metals (Sinha et al., 2011). Moreover, the *cis*-acting elements in the promoter regions of *Arabidopsis* drought-, high salinity- and cold-inducible gene responsive to dehydration 29A (*RD29A*), located on the *Arabidopsis* genome and

shown to be induced under conditions of dehydration, low temperature, high salt, or treatment with exogenous abscisic acid (ABA). Furthermore, the *RD29A* has two *cis*-acting elements, one of which involved in the ABA-associated response to dehydration and the other induced by changes in osmotic potential. However, ABA-responsive element (*ABRE*) is involved in ABA-responsive gene expression whereas *DRE/CRT* is involved in osmotic stress- and cold stress-inducible gene expression in conjunction with the corresponding transcription factors that affect the expression of these genes (Yamaguchi-Shinozaki and Shinozaki, 1994, Nakashima *et al*., 2006). In addition, in *Arabidopsis*, 10 genes have been identified to be up regulated by drought, cold, HL and also salt stress. These genes include *RD29A,* Enhanced disease resistance (EDR7 and *EDR10),* late embryogenesis abundant (*LEA14)* and cold responsive (*KIN1* and *COR15a),* most of which are thought to be involved in the protection of cellular components (Kimura *et al*., 2003).

1.2.1.1 High Light (HL) stress

High light is among the major environmental stresses that affect plant growth and crop productivity (Havaux *et al*., 1991, Vass *et al*., 2007). Plants exposure to excess light can lead to many harmful effects on various physiological process and cellular activities. HL causes damage to DNA, proteins, and lipids, including components of the photosynthetic apparatus (Takahashi and Badger, 2011). Among the negative effects of HL is the inhibition of photosynthesis activity and the production of ROS, which are toxic for many of cellular processes and also alters the redox state of photosynthetic components such as the electron carrier, plastoquinone (Niyogi, 1999, Demmig-Adams and Adams, 2006, Vass *et al*., 2007). In other words, light, as an important resource for plant growth processes, affects photosynthetic systems. Photosynthesis is the sole source of energy, derived from photosynthetic reaction centres and thus insufficient limited light causes limited growth. However, exposure to excess light relative to the plants adapted photosynthetic capacity has the potential to damage the cells, in part by generation of ROS by excitation energy and electrons lacking from the photochemical reactions and electron transport system (Kimura *et al*., 2003). However, plants have developed several strategies to protect their cells. These include the development of ROS scavengers such as peroxidases, accumulation of anthocyanins to reduce light intensity within tissues and also the development of systems to dissipate absorbed light energy (Kimura et al., 2003, Niyogi et al., 1998, Baker and

Asada, 2004). In addition to the scavenging enzymes of ROS, the genes involved in biosynthesis of lignins and flavonoids are activated by HL leading to increased accumulation of lignins and flavonoids. Furthermore, the twenty eight HL-responsive genes of *Arabidopsis* were analysed in response to different conditions to determine how their behaviour is affected. *Ascorbate Peroxidase2* (*APX2*) is one of those HL-responsive genes that required photosynthetic electron transport for their expression and were also responsive to ABA (Bechtold et al., 2008, Rossel et al., 2006, Foyer and Noctor, 2011). These two signals are crucial in the expression of HL-responsive genes. The ascorbate peroxidases use ascorbic acid (vitamin C) as substrate to catalyse the conversion of H_2O_2 to H_2O. This occurs when plants exposed to light energy in excess of the photochemical capacity of the photosystems, which can lead to an imbalance between the production and removal of ROS (Rossel *et al*., 2006). On the other hand, early light-induced proteins (ELIPs) belong to the multigenic family of light harvesting complexes, which bind chlorophyll and absorb solar energy in green plants (Hutin et al., 2003, Heddad et al., 2006). The ELIPs accumulate transiently in plants exposed to high light intensities. *A. thaliana* mutant, *chaos*, that is affected in the posttranslational targeting of light-harvesting complex-type proteins to the thylakoids, suppressed the rapid accumulation of ELIPs during HL stress, resulted in leaf bleaching and extensive photooxidative damage (Hutin et al., 2003, Heddad et al., 2006). Furthermore, Tzvetkova-Chevolleau et al., (2007) has been shown that ELIPs work as chlorophyll sensors that modulate chlorophyll synthesis to prevent accumulation of free chlorophyll and hence prevent photooxidative stress (Tzvetkova-Chevolleau et al., 2007).

However, it has been shown that plant hormone signalling pathways play important roles in converting light inputs into outputs that shape plant growth and development. For instance, light-mediated inhibition of hypocotyl elongation is at least in part mediated by the plant hormone gibberellin (GA). Another light-regulated developmental plant response, the shade avoidance syndrome (SAS), is primarily mediated by the plant hormone auxin, but also by other plant hormones such as brassinosteroids, cytokinins, GAs, and ethylene (Hanno and Gerd, 2009). Furthermore, Kazan et al., (2011) also showed that plant hormone JA implicates in a number of light mediated responses, including SAS. Previous study has revealed that an interplay between SAS and JA signalling through growth repressor DELLA proteins. Similarly to PHYB, DELLA proteins inhibit SAS by interacting with Phytochrome Interacting Factors (PIFs) and inhibiting their function (Djakovic-Petrovic et al., 2007). Light induces GA biosynthesis, and GA-mediated

degradation of DELLAs relieves PIF inhibition and promotes SAS and thus DELLA proteins, similarly to PHYB, promote JA-responsive defence gene expression under pathogen challenge (Feng et al., 2008, de Lucas et al., 2008).

Plant photoreceptors function to sense changes in light period, direction, wavelength composition, and intensity. The main types of photoreceptors are the red/ far-red light-absorbing phytochromes and the UV-A/ blue light-sensing phototropins, cryptochromes, and Zeitlupe protein families (de Carbonnel *et al*., 2010; Chen *et al*., 2004; Jiao *et al*., 2007; Demarsy and Fankhauser, 2009). The signalling pathways triggered by these photoreceptors are integrated to fine-tune responses to ever-changing light environments (de Carbonnel *et al*., 2010; Casal, 2000; Franklin and Whitelam, 2004; Iino, 2006). Furthermore, Gribel et al., (2008) reported that the induction of defense responses by either avirulent or virulent *P. syringae* at inoculation sites is relatively robust in leaves of photoreceptor mutants, indicating cross talk between local defense and light signalling. In addition, the blue-light receptor mutant *cry1/cry2* and *phot1/phot2* are both capable of establishing a full SAR response and thus induction of SAR and salicylic-acid-dependent systemic defense reactions, however, are compromised in *phyA/phyB* mutants. Phytochrome regulation of SAR involves the essential SAR component FLAVINDEPENDENT MONOOXYGENASE1 (FMO1). These findings highlight the importance of phytochrome photo-perception during systemic rather than local resistance induction. The phytochrome system seems to accommodate the supply of light energy to the energetically costly increase in whole plant resistance (Griebel and Zeier, 2008, Gordon et al., 2012).

In other respect, it has been shown that Phytochrome Interacting Factors (PIFs), a group of basic helix–loop–helix transcription factors, bind directly to the photoactivated phytochromes and are likely to play important roles in photoperception and signalling (Castillon *et al*., 2007). Wolyn *et al*, measured *Arabidopsis* seedling hypocotyl lengths under blue, red, far-red, and white light, and in darkness. Out of eight quantitative trait loci (QTL), two QTL in blue light are associate with cryptochrome *(CRY1* and *CRY2)*, two in red light are near phytochrome (*PHYB* and *PHYC)*, and one in far-red light localized near *PHYA* (Wolyn *et al*., 2004). Leivar, Monte *et al*. (2008) showed that under prolonged red light, the operation mechanism of PIFs on the PhyB signaling pathway is through maintaining low phyB protein levels. Recently it has been demonstrated that PIF4 regulates levels of auxin and the expression of key auxin biosynthesis genes at high

temperature. The study also demonstrated the expression of the *SMALL AUXIN UP RNA* (*SAUR*) genes family in a *PIF4*-dependent manner to promote elongation growth (Franklin *et al*., 2011). However, previously it has been shown that in cyanobacteria, harvested light energy absorbed by phycobilisomes (PBS) is transferred from the core-membrane linker, LCM, to the chlorophylls of photosystem II (PSII) and photosystem I (PSI) (Misra and Mahajan, 2000, Rakhimberdieva et al., 2001). Thus some excitons are formed in the reaction center (RC) and are then deactivated through three pathways: photochemical reaction, fluorescence, and thermal dissipation or non-photochemical quenching (NPQ) (Rohacek and Bartak, 1999). NPQ is an indispensable pathway of deactivation and plays an important role in protecting PSII from photo-inhibition or photo-damage when exposed to stress conditions (Ralph and Gademann, 2005, Allen et al., 2008). However, NPQ can be de-convoluted into three components based on the different relaxation times in dark periods after exposure to high intensity light (Krause and Weis, 1991, Walters and Horton, 1991). These three components of NPQ were found to be (i) a fast quenching component (NPQ_f), which refers to the ΔpH-dependent process or high-energy state (*q*E); (ii) a medium quenching component (NPQm), related to the quenching of state transition process (*q*T); and (iii), a low quenching component (*q*I), resulting from photo-inhibition of photosynthesis. Whereas, Wilson et al., (2006) showed that in cyanobacteria there is no *q*E dependence on a trans-thylakoid proton gradient, although it is a predominant component of NPQ in higher plants (Champbel et al., 1996). Since *q*E is a fast component of NPQ that appears in a few seconds in higher plants when exposed to high-intensity light (Walters and Horton, 1991), the rapid response of PSII to saturated light might be affected due to *q*E deficiency in cyanobacteria. Additionally, Wang et al., (2012) demonstrated that the rapid light curves (RLCs) and fluorescence induction dynamics of the fast phase showed that excess excitation energy was dissipated by conformational change in the photosynthetic pigment proteins on the thylakoid membrane (PPPTM) and thus the fast NPQ_f was closely related to PPPTM conformational change. Accordingly, Wang hypnotized that NPQ_f induced by PPPTM conformation is an important adaptation mechanism for *Microcystis*, freshwater cyanobacteria, blooms under high-intensity light during summer and autumn (Wang et al., 2012).

1.2.2 Biotic stress

1.2.2.1 Pathogens with different lifestyles

1.2.2.2 *Pseudomonas syringea*

The bacterium, *Pseudomonas,* is identified as a rod shaped, gram-negative with polar flagella. It is a plant pathogen which can infect a wide range of plant species, and exists as over 50 different pathovars. *Pseudomonas spp* same as all biotrophs pathogens derive their nutrients from living host tissues (Pegg, 1990). *P. syringae (P.st)* is often considered a biotroph, occasionally, classified as a necrotroph (Butt et al., 1998, van Kan, 2006) and should probably be considered a hemi-biotroph depending on the conditions of their life cycle and/or nutrient acquisition strategies (Thaler et al., 2004, Rico and Preston, 2008). *P. syringae* pv. *tomato* DC3000 causes bacterial speck in tomato plants (Cuppels, 1986, Gautam, 2008) and it is also a pathogen of the model plant *A. thaliana* (Whalen et al., 1991, Nobuta and Meyers, 2005).

Infection of the pathogen occurs when the bacteria infect the host through wounds and stomata and multiply in the intercellular spaces. In the early stages of compatible infections, host cell death does not occur, but later stages of infection are associated with host tissue chlorosis and necrosis (Bender et al., 1999, Cyril, 2009).

Subsequently, the initial perception of plant pathogens is thought to occur by recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by host plant pattern recognition receptors (Boller and Felix, 2009). PAMPs are molecules that are essential for microbe viability and conserved between diverse genera; thus, they are unlikely to be lost through selection and are an efficient form of pathogen monitoring for the plant. DAMPs are signals generated by the plant in response to pathogen damage. PAMP recognized by corresponding pattern recognition receptor triggers basal defense responses, pattern-triggered immunity (PTI), providing protection against nonhost pathogens and limiting disease caused by virulent pathogens (Jones and Dangl, 2006, Bittel and Robatzek, 2007, Boller and Felix, 2009, Cyril, 2009, Knepper and Day, 2010). The pathogenicity of *P.st* DC3000 resembles that of most animal and plant pathogens in the gamma Proteobacteria, which rely on a type III secretion system (TTSS) (Cornelis and Van Gijsegem, 2000, Alfano and Collmer, 2004). It has been shown that the pathovars of *P. syringae* encode as many as 40 effector proteins

(Alfano and Collmer, 2004, Greenberg and Vinatzer, 2003). However, many strains, including *P.st* DC3000, which infects *Arabidopsis*, produce toxins that contribute to pathogenicity (Bender et al., 1999, Yao et al., 2013). Then dozens of proteins are actively transported into host cells through a specialized system known as type III secretion which several of them have been shown to contribute to virulence in *Arabidopsis* (Alfano and Collmer, 2004, Espinosa and Alfano, 2004, Alto et al., 2006, Lewis et al., 2009) The bacterial effectors have two roles, either to act as suppressors or inducers of resistance, which reflect the evolution of plant–pathogen interactions occurring in the following sequence: (i) Effectors initially evolved within prototype pathogens to overcome basal defences in eukaryotes. (ii) In plants, recognition systems based on R genes allowed specific detection of injected effector proteins, triggering the HR and establishing gene-for-gene interactions. (iii) Loss of the recognized effector has often allowed pathogens to continue parasitizing resistant varieties of their hosts, to break gene-for-gene mediated resistance, because other effectors are able to suppress basal defences (De Torres et al., 2006, Gimenez-Ibanez and Rathjen, 2010). The evidence for such a sequence is, however, based on limited functional analysis of very few effectors. Where enzymatic activities have been demonstrated, for example AvrPphB and AvrRpt2 have been identified as cysteine proteases (Kim et al., 2005, De Torres et al., 2006, Hann et al., 2010) and HopPtoD2 found to possess protein tyrosine phosphatase activity (Espinosa et al., 2003). However, *P. syringae* effector protein AvrB perturbs hormone signalling, as exemplified by up-regulated expression of jasmonic acid response genes, and enhances plant susceptibility (Cui et al., 2010). The AvrB effect requires Arabidopsis MAP kinase 4 (MPK4), HSP90 (heat shock protein 90) chaperone components, and the AvrB-interacting protein, RIN4. AvrB interacts with MPK4 and the HSP90 chaperone, and AvrB induces MPK4activation in a manner promoted by HSP90; RIN4 likely acts downstream of MPK4. These findings link Arabidopsis proteins MPK4, HSP90, and RIN4 into a pathway that *P. syringae* AvrB activates for the benefit of the bacterium, perturbing hormone signaling and enhancing plant susceptibility (Cui et al., 2010). Furthermore, Arabidopsis ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), a positive regulator of basal resistance and of effector-triggered immunity (ETI) specifically mediated by Toll-interleukin-1 receptor-nucleotide binding-leucine-rich repeat (TIR-NB-LRR) resistance proteins, forms protein complexes with the TIR-NB-LRR disease resistance proteins RPS4 and RPS6 and with the negative immune regulator SRFR1 at a cytoplasmic membrane. Moreover, the cognate bacterial

effectors AvrRps4 and HopA1 disrupt these EDS1 complexes. Thus, tight association of EDS1 with TIR-NB-LRR-mediated immunity may therefore derive mainly from being guarded by TIR-NB-LRR proteins, and activation of this branch of ETI directly connect to the basal resistance signalling pathway via EDS1 (Bhattacharjee et al., 2011)

1.2.2.3 *Botrytis cinerea*

Botrytis cinerea is considered as the second most important fungal plant pathogen, after *Magnaporthe oryzae,* (Dean et al., 2012). Its broad host range and ability to cause disease both pre- and postharvest lead to large economic effects. *B. cinerea* is a necrotrophic pathogen, meaning it kills plant tissue prior to feeding, and uses a range of toxic molecules (Williamson et al., 2007) as well as the plant's own defense mechanisms (Govrin et al., 2006) to destroy host cells. Plant defense against *Botrytis cinerea* depends on jasmonic acid (JA) and ethylene signalling and camalexin production. It has been shown that mutations block JA signalling, including coronatine-insensitive 1 (*coi1*) (Thomma *et al*., 1998) and *Arabidopsis* JA response 1 (*jar1)* (Ferrari *et al*., 2003), cause enhanced susceptibility. Moreover, expression of some JA-responsive genes is controlled by the MYC transcription factor *JIN1, Arabidopsis* loci reduce sensitivity to JA, and plants bearing *jin1* mutations are more resistance to *B. cinerea* (Lorenzo *et al*., 2004). In addition, JA signalling leads to expression of at least two classes of genes. The first class is regulated by *JIN1* and the second class is not regulated by *JIN1* but have a net negative effect on *B. cinerea* resistance (Glazebrook, 2005). Recently, It has been reported that Chromatin immunoprecipitation (ChIP)-PCR experiments showed direct binding of WRKY33 to sequences upstream of genes involved in JA signaling (jasmonate ZIM-domain1 [JAZ1] and JAZ5), ET-JA crosstalk ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59), and camalexin biosynthesis (PAD3 and CYP71A13) following *B. cinerea* infection (Birkenbihl et al., 2012). However, the basal defense mechanisms are thought to underlie differences in host susceptibility to necrotrophic pathogens. Multiple PAMPs are involved in the interaction between *B. cinerea* and *Arabidopsis thaliana.* The essential fungal cell wall component, chitin, and its constituent oligosaccharides are fungal PAMPs that activate numerous defense responses (Poinssot B, 2003, Windram et al., 2012).

1.2.2.4 Plant innate immunity

The first line of microbial recognition leading to active defence responses relies on the perception of PAMPs by pattern-recognition receptors (PRRs). This recognition leads to PAMP-triggered immunity (Tsuneaki et al., 2002, Melotto et al., 2006, Zipfel, 2009). Thus plant response to pathogen attack depends on the nature of activation of several defence mechanisms by PAMPs and specifically evolved pathogen effectors. Success depends upon the ability to recognize and respond to particular pathogen molecules/effectors in a measured and timely manner. Often these interactions are of either (i) through PAMP-activated basal resistance, which is effective against general microorganisms or (ii) via gene-for-gene type resistance involving the recognition of a single dominant pathogen gene (avirulence genes) in a plant host carrying single dominant cognate resistance (*R*) gene (McDowell and Dangl, 2000, Belkhadir et al., 2004, Pozo et al., 2004, Yasuda et al., 2008, Boller and Felix, 2009, Cyril, 2009, Davidsson et al., 2013)

An initial response to the recognition of a PAMP is the phosphorylation of defence related transcription factors via a MAPK cascade inducing the transcription of hundreds of gene *A. thaliana* (Asai et al., 2002, Cui et al., 2010); a response known as PAMPs triggered immunity (PTI). PTI produces a number of different outcomes; WRKY 29/22 transcription factors activate a number of genes including a large number of known and suspected receptors that carrying leucine-rich repeat (LRRs) and kinase domains (Gómez-Gómez and Boller, 2000, Tsuneaki et al., 2002, Belkhadir et al., 2004, Chini et al., 2004, Matsushima and Miyashita, 2012). PTI also leads to callose deposition, strengthening the cell and limiting availability of water and nutrients, (Gómez-Gómez et al., 1999, Cyril, 2008, de Torres Zabala et al., 2009) and reactive- oxygen species (ROS) generation (Nürnberger et al., 2004, Maruta et al., 2012). In most cases, plants are resistance to most pathogens due to a complex and rapidly activated basal (or innate) immune system (Glazebrook, 2005). In addition, stomata function as innate immunity gates to actively prevent bacterial entry in *Arabidopsis*, and the stomatal defense against bacterial invasion is an important function of innate immunity in plants (Melotto et al., 2006, Zeng et al., 2011).

1.2.2.5 Type three secretion system (TTSS)

The bacterial TTSS is specific secretion system used by many gram-negative pathogens such as *Pseudomonas* and *Xanthomonas,* which colonize the intercellular spaces (apoplast) of plants to

deliver specific effetor proteins into plant cells. In many cases these effector proteins function as virulence determinants and collaborate to cause disease. In other cases, they are recognized by plant R proteins to activate effector triggered immunity (Jones & Dangl 2006). In such cases these effectors are referred to as avirulence proteins (Cornelis and Van Gijsegem, 2000, Alfano and Collmer, 2004, Block et al., 2008, Tsuda et al., 2008) but not all virulence determinants are effector proteins (Alfano and Collmer, 2004). Successful colonization of *P. syringae* in the apoplastic space eventually results in the development of disease symptoms, which usually include localized tissue necrosis and discoloration (Cintas et al., 2002, Alfano and Collmer, 2004). Phytopathogens can produce small molecules that promote virulence and disease symptom development such as the phytotoxic coronatine (COR) (Alfano and Collmer, 2004, Zeng et al., 2011). Both the TTSS and COR are involved in the suppression of host basal defenses and thus production of the TTSS and COR is co-ordinately regulated in *P. st* DC3000 (Thilmony *et al*., 2006, Fouts *et al*., 2002, Penaloza-Vazquez *et al*., 2000), suggesting that the expression of both TTSS and COR could be affected by mutations in the regulatory *hrp* genes, e.g. *hrpS, hrpL,* and *hrpA* (Thilmony *et al*., 2006). Hauck *et al* (2003) showed that TTSS is involved in biased suppression of *Arabidopsis* genes that encode putatively secreted proteins. The largest group of *R* genes the nucleotide binding site-leucine rich repeat (NBS-LRR) class (Belkhadir *et al*., 2004). In the case of bacterial pathogens, many of the avirulence genes encode type III effectors and presumably function by contributing to virulence in hosts lacking the appropriate *R* genes (Abramovitch and Martin, 2004). *R* gene products interact with avirulence gene products, but usually not directly. Rather R proteins detect alterations in host proteins that are caused by virulence proteins (Van Der Biezen and Jones, 1998). This idea is known as the guard hypothesis model (de Wit, 2002, Marathe and Dinesh-Kumar, 2003). Many of the rapid gene expression changes that occur during gene-for-gene responses also occur during susceptible interactions, but with slower kinetics and reduced amplitude (Tao *et al*., 2003). There are two defense responses that are considered hallmarks of gene-for-gene resistance. One is a rapid production of reactive oxygen intermediates (ROI), a common mediators for Poly(ADP-ribose) polymerase (PARP) cleavage (McGowan *et al*., 1996), called the oxidative burst. These may have direct antimicrobial effects, as well as serving as a signal for activation of other defense responses. The other is a form of programmed cell death known as the hypersensitive response (HR). The HR is thought to act against biotrophic pathogens by restricting pathogen access to

water and nutrients. It also activates SA-dependent signaling (Nimchuk et al., 2003, Glazebrook, 2005, Mur et al., 2008).

1.2.2.6 Systemic acquired resistance (SAR)

The systemic acquired resistance (SAR) is a potent innate immunity system in plants that is effective against a broad range of pathogens. SAR development in dicotyledonous plants, such as tobacco (*Nicotaina tabacum*) and *A. thaliana* is mediated by salicylic acid (SA) (Yasuda *et al*., 2008). It has been shown that signalling pathways mediating SAR induction, which require endogenous accumulation of the plant hormone salicylic acid (SA) and the downstream signalling protein NON EXPRESSOR OF PR1 (NPR1; Durrant and Dong, 2004). NPR1 has also been implicated in the cross talk between SA- and jasmonic acid (JA)-dependent defense pathways, which enables plants to mount an appropriate defense reaction, depending on the nature of the attacker and the stage of infection (Spoel et al., 2003; Koornneef and Pieterse, 2008). However, Yasuda et al (2008) has been reported that there are two type of SAR-inducing chemicals; 1,2-benzisothiazol-3 (2H)-one1,1-dioxide (BIT) and benzo (1,2,3) thiadiazol-7-carbothioic acid S-methyl ester (BTH) which acts upstream of SA in the SAR in signalling pathway and treatment with ABA suppresses the induction of SAR in *Arabidopsis* by inhibiting the pathway both upstream and downstream of SA, independently of the jasmonic acid/ethylene-mediated signalling pathway. Whereas, other studies have revealed that systemic accumulation of SA during the onset of SAR is preceded by a variety of metabolic signals, such jasmonates (Truman et al., 2007) and indole-derived compounds (Truman et al., 2010). Attaran et al (2009) has also been shown that the SAR signal in Arabidopsis post-infection by avirulent *P. syringae* remains complex and has been a matter of debate. Recently, Liu et al (2011b) proposed that SAR is controlled by an interaction between at least two mobile signals, methyl salicylate (MeSA) and a complex formed between the lipid transfer protein DIR1 and glycerolipid or lipid derivatives. Luna et al (2012) has been reported that Arabidopsis produces progeny with enhanced disease resistance, which can be maintained over one stress-free generation. This transgenerational SAR is effective against (hemi) biotrophic pathogens, requires an intact NPR1 protein, and is associated with priming of SA-dependent genes. Furthermore, transgenerational SAR is associated with an NPR1-dependent repression of JA-dependent defense genes and enhanced susceptibility to the necrotrophic fungus *A. brassicicola*. This shift in the cross talk balance

between SA- and JA-dependent defences is associated with permissive and repressive histone H3 modifications at SA- and JA-inducible gene promoters, respectively.

1.2.2.7 Effector triggered immunity (ETI)

Gram-negative bacteria pathogens rely on the TTSS and the type III secreted effectors (TTSEs) to cause diseases in animals and plants. TTSEs are essential virulence proteins delivered directly from bacteria into the host cytoplasm (Grant and Lamb, 2006) Guo et al., 2009). During a typical infection, *P. syringae* delivers about 32 effector proteins inside its host (Lindeberg et al., 2009). Cysteine protease, AvrRpt2, is one of the best characterized effector. Their catalytic activity sets into motion a series of defense signalling responses which have become hallmark tenants for the Gene-for-Gene and Guard Hypotheses, Are pathogen effector proteins the magic bullets that must be stopped? (Knepper and Day, 2010). However, aside from AvrRpt2's well-established role in avirulence, studies investigating the manipulation of host physiology, and more specifically hormone signaling, have revealed an intimate link between pathogen effector action and hormone signaling (Knepper and Day, 2010). The bacterial effectors function to manipulate host cell processes for the purpose of enhancing infection and pathogen proliferation. It is reported that the complex genetic and biochemical interactions between pathogen effectors and their cognate host proteins evoke specific responses, that when recognized, elicit disease resistance, or when evaded, promote susceptibility (Knepper and Day, 2010). However, *Avr* gene products are commonly known as effectors in plants of a susceptible genetic background (i.e. lacking the necessary *R* genes) effectors function to attenuate host defence mechanisms (PTI), known as effector triggered suppression (ETS), or reprogram host biochemistry to favour the pathogen (Jones and Dangl, 2006). ETI is triggered when a plant possess a cognate R gene, and results in the hypersensitive response, unless further pathogen effectors have evolved to prevent this (Jones and Dangl, 2006). The association of the Arabidopsis R protein RPS5 (Resistant to *P. syringae 5*), with a protein kinase avrPphB susceptible1 (PBS1), fulfilled all of the requirements of the Guard Hypothesis. This mechanism requires that: RPS5 associates/interacts with PBS1 (Ade et al., 2007); the *P. syringae* effector AvrPphB, a cysteine protease, cleaves PBS1 (Shao et al., 2003); and, following cleavage of PBS1, the RPS5- PBS1 association is disrupted, leading to a (likely) conformational change in RPS5 and activation of ETI (Ade et al., 2007). Thus, the

detection of the pathogen relies on the disruption, or perturbation, of a protein-protein surveillance mechanism by the action of the pathogen effector protein (Knepper and Day, 2010).

1.2.3 Small molecules in plant stress

1.2.3.1 Plant phytohormones

Plant phytohormones, in general, play an important role in plant development, in plant adaptation and in plant resistance to/against both biotic and abiotic stresses. The hormones, ABA, GA, ET, IAA, CK and BR, are extremely important for the regulation of seed dormancy, germination, plant development and the influence of the environmental changes (Koornneef *et al*., 2002, Finkelstein *et al*., 2002). One of the response ways to pathogen attack is the JA-dependent signalling which proceeds through increased JA synthesis and that lead to increase in expression of defense effector genes such as *PDF1.2.* JA signalling has also an important role in responses to wounding, insect attack and in fertility. *PDF1.2*, JA-regulated genes, induces expression requires both JA and ET, but in another case, ET is not required for expression of the JA-inducible gene *VSP1* (Norman-Setterblad *et al*., 2000, Guo and Ecker, 2004, Farmer *et al*., 2003). Ellis *et al*., (2002) suggest that cellulose synthases in the plant cell wall is involved in regulation of JA levels, where the cellulose synthase mutant *cev1* shows high level of JA and JA-dependent gene expression.

1.2.3.1.1 Jasmonic acid (JA)

Jasmonic acid (JA) is derived from linolenic acid (a fatty acid). It is one of the jasmonate class members of plant hormones with a major function of JA and its various metabolites to regulate plant responses to abiotic, biotic stresses, plant growth and development. JA is synthesized from linolenic acid by the octadecanoid pathway (Delker *et al*., 2006). It has an important role in response to plant wounding and systemic acquired resistance. In response to wounding (e.g by insect herbivory), JA is released by the plants and the expression of protease inhibitors activity (Zavala *et al*., 2004). However, the F-box protein coronatine insensitive 1 (COI1), a component of the SCF E3 ubiquitin ligase, has been identified as a principal component of a receptor of JA in Arabidopsis and other plants (Fonseca et al., 2009, Katsir et al., 2008a), and the JA ZIM-domain (JAZ) family proteins are key regulators of JA signaling that repress transcription of JA-

responsive genes through interaction with transcription factors, such as MYC2 (Yan et al., 2007, Bryan et al., 2007, Chini et al., 2007). This transcriptional repression requires novel interactor of JAZ (NINJA) and TOPLESS corepressor proteins (Laurens et al., 2010). The activation of JA defense signalling is known to severely restrict plant growth, representing a prominent example of growth–defense tradeoff in plants. Kazan et al., (2011) reviewed several reports of crosstalk between JA and GA signalling pathways in Arabidopsis, mostly documenting the antagonistic effect of GA on JA signalling and therefore, a quadruple della mutant (which lacks four of the five Arabidopsis DELLA proteins) was shown to be partially insensitive to gene induction by JA, whereas the constitutively active dominant DELLA mutant *gai* was found to be sensitized for JA-responsive gene induction, implicating DELLAs in JA signalling and/or perception (Navarro et al., 2008). Cheng et al., (2009) has been found that GA promotes JA biosynthesis, thereby inducing the expression of MYB21, MYB24, and MYB57 to promote stamen filament elongation. Recently, it has been shown that DELLA repressors promote JA signaling through physically interacting with JAZ1 (Hou et al., 2010), suggesting a mechanism for GA-mediated down-regulation of JA defense responses. Most recently, and through analysis of rice and Arabidopsis, Yang and his colleagues have elucidated a molecular cascade by which JA antagonizes GA signalling that explains how monocot and dicot plants prioritize JA defense over growth (Yang et al., 2012). However, Pathogen attack triggers complex signalling cascades have shown to be regulated by signalling molecules such as SA, JA, and ET, resulted in expression of defense-related genes such as those encoding pathogenesis-related (PR) proteins (Van Loon, 1997, Glazebrook, 2001, Sels et al., 2008), and the production of antimicrobial secondary metabolites (Glazebrook, 2001, Wallace, 2004, Reichling, 2010, Sadrati et al., 2013). The SA- and JA/ET-signalling pathways are often considered to be effective against biotrophs and necrotrophs (Glazebrook, 2005, Beckers and Spoel, 2006). Resistance against the necrotrophs pathogens, such as *Botrytis cinerea*, is compromised in the JA-signalling mutant *coronatine insensitive 1* (*coi1*) (Thomma et al., 1998). The *coi1* plants were more resistant to necrotroph pathogen *Fusarium. oxysporum* compared to Col-0 (Thatcher *et al*., 2009). The JA signal is perceived by the F-box protein COI1, which plays a key role in JA signalling (Katsir et al., 2008a). Thus the coronatine produced by *P. syringae* strains is a mimic of the bioactive jasmonate JA-isoleucine (Fonseca *et al*., 2009).

1.2.3.1.2 Salicylic acid (SA)

Salicylic acid (SA) plays an important role in plant growth and development, photosynthesis, transpiration, ion uptake and transport. SA also found to induce specific changes in chloroplast structure, and it is involved in endogenous signalling, mediating in plant defense against pathogens and or with important regulatory role in multiple physiological processes including plant immune response (Hayat and Ahmad, 2007, An and Mou, 2011). Furthermore, SA also plays a role in resistance to biotrophic pathogens by inducing the production of pathogenesis-related proteins (PR) (Van Huijsduijnen et al., 1986, Pasquer et al., 2005, Makandar et al., 2006). However, it has been shown that, Nonexpressor of pathogenesis-related genes 1 (NPR1) is a master regulator controlling multiple immune responses including SAR. It represents a key node in signalling downstream from SA (Dong, 2004, Durrant and Dong, 2004, Pieterse and Van Loon, 2004). Previous, research group have been reported that *Arabidopsis npr1* mutants were unable to activate the expression of *PR* genes or disease resistance (Cao et al. 1994; Delaney et al. 1995; Shah et al. 1997). Furthermore, the promoter region of the *NPR1* gene contains W-box sequences, which are binding sites of WRKY family protein. Thus mutation in the W-box sequences of the *NPR1* gene promoter adversely affects its expression, suggesting that WRKY transcription factor plays an important role in mediating signaling between SA and NPR1 (Yu et al. 2001). SA is involved in the SAR in which a pathogenic attack on one part of the plant induces resistance in other parts. Moreover, the signal can also effect nearby plants by SA being converted to the volatile ester, methyl salicylate (Taiz and Zeiger, 2002). Wildermuth *et al*., (2001) suggested that the main route of defense-associated SA production in *Arabidopsis* involves chloroplast-localized *isochorismate synthase1* (*ICS1*) encoded by *ICS1*/*EDS16/SID2*. The shikimate pathway (Fig1.1) provides the SA substrate chorismate, SA made by this pathway is required for local and systemic acquired resistance (LAR and SAR respectively) responses (Wildermuth et al., 2001, An and Mou, 2011). Transport of SA from plastids to the cytoplasm may be facilitated by a putatively chloroplast localized *trans*-membrane protein encoded by *EDS5*/*SID* (Jean-Pierre, 2002, Straus et al., 2010). However, De Torres *et al* (2009) demonstrated that at the early stage of bacterial attack levels of SA and ABA play an important role in the infection where SA is required to the full innate immune responses while the ABA biosynthesis is activated rapidly by the action of bacterial effectors. Increased ABA appears to

down-regulate SA biosynthesis and SA-mediated defense, possibly by ABA induced suppression of inducible innate immune responses.

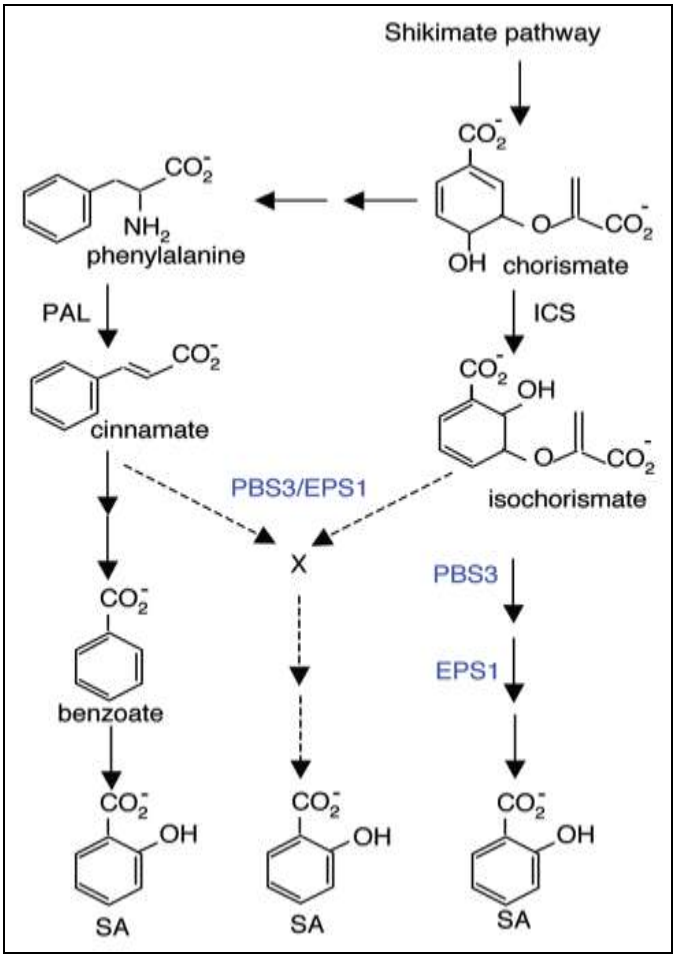

Figure 1.1 Potential pathways of SA biosynthesis in plants, It was initially suggested that plants synthesize SA from cinnamate produced by PAL, however it has since been shown that the bulk of pathogen induced SA is produced from isochorismate. *PBS3* and *EPS1* have been identified as important for pathogen-induced SA production and may encode enzymes catalyzing related, and possibly sequential, reactions in the synthesis of an important precursor or regulatory molecule for SA biosynthesis adapted from (Chen et al., 2009b).

1.2.3.1.3 Auxin (IAA)

The phytohormone auxin [indole-3-acetic acid (IAA)] regulates a range of plant developments including the tropic responses (Davies, 1995). It has been shown that some microorganisms also produce auxin (Costacurta and Vanderleyden, 1995, Patten and Glick, 1996). Interaction between plants and these microorganisms, such as *Agrobacterium spp*. and *Pseudomonas savastanoi pv. savastanoi*, caused unbalanced level of plant auxin (Jameson, 2000, Mole *et al*., 2007). Navarro showed that plant auxin signalling is part of *Arabidopsis* defense responses against leaf pathogen in which auxin signalling in *Arabidopsis* is down-regulated, thus decreasing susceptibility for the pathogen (Navarro *et al*., 2006). Previous studies demonstrated an important role for host auxin signalling in a particular aspect, effector-triggered susceptibility, pathogen associated molecular PTI and ETS, respectively, of the four phased model of the plant immune system (Jones and Dangl, 2006, Navarro *et al*., 2006, Chen *et al*., 2007). However, An et al., (2011), showned that plants have evolved mechanisms to repress auxin signaling during pathogenesis. Plants overproducing the defense signal molecule SA frequently have morphological phenotypes that are reminiscent of auxin-deficient or auxin-insensitive mutants, suggesting that SA might interfere with the auxin response (Wang et al., 2007). SA application causes global repression of auxin-related genes, resulted in stabilization of the Aux/IAA repressor proteins and inhibition of auxin responses (Wang et al., 2007). Similarly, it was found that the majority of the auxin inducible genes are also repressed in systemic tissues after induction of SAR, indicating that SAR response involves downregulation of auxin responsive genes (Wang et al., 2007). In contrast, exogenous application of auxin has been shown to promote disease (Yamada, 1993, Navarro et al., 2006, Chen et al., 2007) and blocking auxin responses led to increased resistance (Wang et al., 2007). The finding that enzymes involved in auxin amino acid conjugation, and thus inactivation, affect SA-mediated defenses indicates another possible level of crosstalk between SA and auxin (Park, 2007). GH3.5 conjugates both SA and indole acetic acid, and altered expression of this enzyme affects disease resistance (Zhang, 2007). The loss-of function mutant of the auxin response factors (ARFs), *arf6 arf8*, displays reduced expression of genes involved in JA biosynthesis and low JA levels, suggesting that activation of JA signalling may play an important role during the interaction of SA and auxin (Tiryaki and Staswick, 2002, Bari and Jones, 2009b, Robert-Seilaniantz et al., 2011).

1.2.3.1.4 Abscisic Acid (ABA)

The isoprenoid derived phytohormone ABA has been known to serve as an endogenous messenger in biotic and abiotic stress responses of plants (Galvez-Valdivieso et al., 2009, Ton et al., 2009). Abiotic stress factors such as drought and high salinity resulted in strong increases of foliar ABA levels, accompanied by a major change in gene expression and in adaptive physiological responses (Priest et al., 2006, Zeller et al., 2009, Raghavendra et al., 2010).

ABA is synthesized by the plastidal 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Eisenreich et al., 2004, Nambara and Marion-Poll, 2005, Zhao et al., 2013). ABA is produced by the conversion of zeaxanthin to violaxanthin by zeaxanthin epoxidase (ZEP) via the intermediate of antheraxanthin. cis-isomers of violaxanthin and neoxanthin are prodiced via neoxanthin synthase (NSY) and an isomerise (Fig.1.2). Cleavage of *cis*-xanthophylls is catalysed by a family of 9-cis-epoxycarotenoiddioxygenases (NCED). (ABA2) converted xanthoxin into abscisic aldehyde, which is oxidized into ABA by an abscisic aldehyde oxidase (AAO3) (Nambara and Marion-Poll, 2005). In *Arabidopsis*, there are nine *NCED*-related genes (*AtNCED*s) on the genome database. Only four out of six gene products exhibit NCED activity when tested. Among them, *AtNCED3*, whose expression is up-regulated by dehydration, is involved in ABA biosynthesis *in vivo* (Iuchi et al., 2001, Tan et al., 2003). *AtNCED1* the first *NCED* identified in *Arabidopsis*, lacks the targeting signal peptide to plastids and in most instances the recombinant protein cleaves a variety of carotenoids symmetrically to produce a C_{14}-dialdehyde and two C_{13} products (Schwartz *et al*., 2001). In *Arabidopsis*, at least two types of *NCED* exist; one, such as *PvNCED1*, a paralogue of maize VP14, which localizes only in the thylakoid membrane, and another, which exits as two distinct forms (Tan *et al*., 2001). Aldehyde oxidase (AO) isoforms have been reported in several plant species (Sagi *et al*., 1999, Seo *et al*., 2000a, Koshiba *et al*., 1996, Omarov *et al*., 1999, Zdunek and Lips, 2001) and the gene family has been found in *Arabidopsis* (Sekimoto *et al*., 1998), maize (Sekimoto *et al*., 1997) and tomato (Min *et al*., 2000). In general, plant AO consists of two similar subunits. Four of AO isoforms are generated in *Arabidopsis* as homo- and hetero-dimers by three *AAO* gene products; AOα, AOβ, AOγ and AOδ. The isoforms exhibit different substrate preferences, and are distributed differently in the organs or tissues, depending upon the expression of the corresponding genes (Seo *et al*., 2000b, Akaba *et al*., 1999).

Although stress signal dependent, ABA is induced primarily in vascular tissues and ABA is exported from the site of biosynthesis and uptake is stimulated into other cells by specific ATP-dependent transporters. This mechanism allows the rapid distribution of ABA into neighbouring tissues (Kang et al., 2010, Kuromori et al., 2010) and thus, ABA is not only a stress signal but is also required to fine-tune growth and development under non-stress conditions. The physiological processes controlled under these conditions include the regulation of growth, stomatal aperture and hydraulic conductivity, as well as seed dormancy (Finkelstein et al., 2002b). In addition, stomatal closing is mediated by ABA-triggered changes of ion fluxes in guard cells (Siegel et al., 2009). Recent work has shown that stomatal closure upon perception of MAMPs/bacteria is an important preinvasive innate immune response involving ABA signaling components to restrict bacterial entry (Melotto et al., 2006). However, at post-invasive stages, ABA biosynthesis and signalling pathways may be targeted by *P. syringae* TTSS effectors to suppress the plant defense response ((de Torres-Zabala et al., 2007).

ABA also acts together with other phytohormones such as brassinosteroids, gibberellic acid and auxin in regulating plant growth and development (De Smet et al., 2003, Jammes, 2009).

Antagonist interaction are often observed between ABA and the prominent defence phytohormones SA, JA/ET suggesting that abiotic stress responses can take precedence over biotic stress responses and abiotic stress may be detrimental to plant immunity (Mauch-Mani and Mauch, 2005, Robert-Seilaniantz *et al*., 2007, Yasuda *et al*., 2008, Anderson *et al*., 2004). Consistent with this, it has been demonstrated that a number of abiotic stresses such as increases in temperature and humidity as well as drought and salinity, have a negative effect on resistance to biotic stress (Mohr and Cahill, 2003, Koga *et al*., 2004). Infection of *Arabidopsis* plants under drought condition with avirulent *P.st 1065* resulted in susceptible phenotypes including necrosis and chlorosis, as well as enhanced bacteria growth (Mohr and Cahill, 2003, Moeder and Yoshioka, 2008, Koga *et al*., 2004, Yoshioka and Shinozaki, 2009)

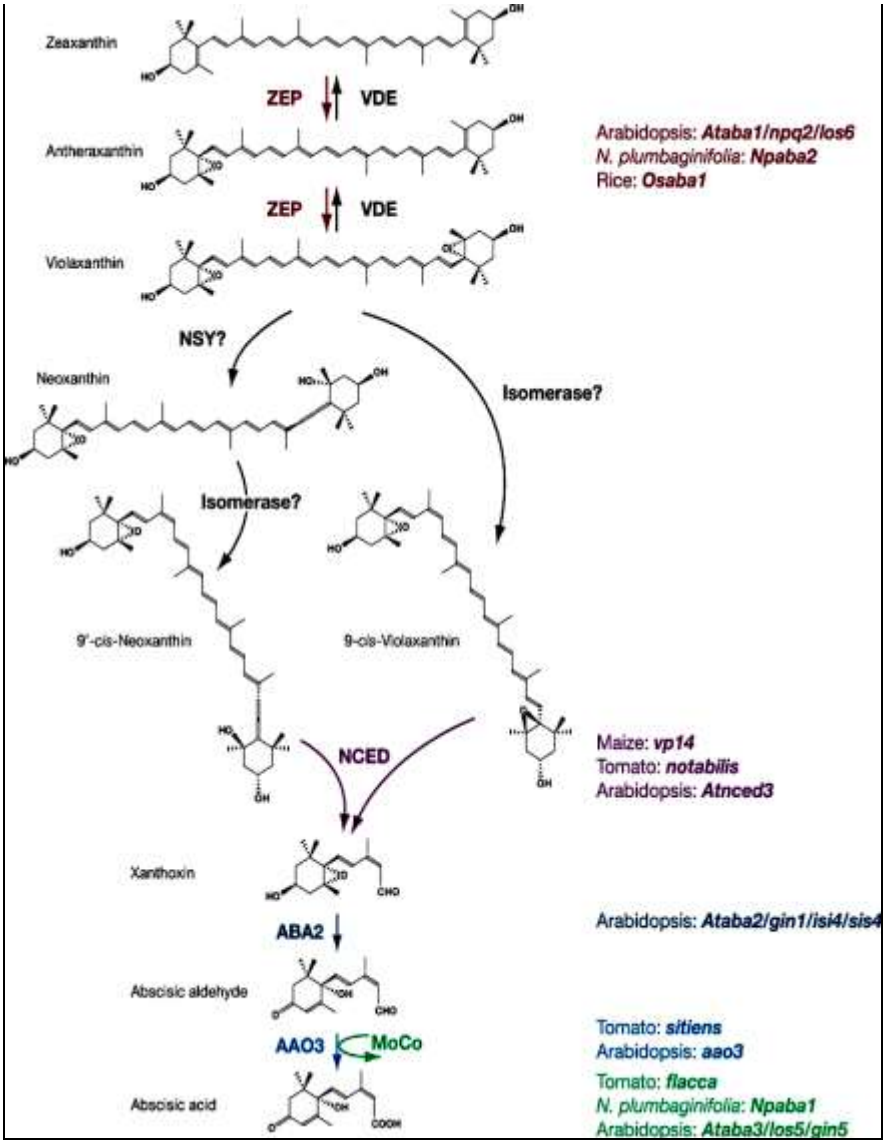

Figure 1.2 ABA biosynthetic pathway; in this pathway zeaxanthin converted to antheraxanthin by zeaxanthin epoxidase (ZEP). A reverse reaction occurs in chloroplasts in high light conditions catalysed by violaxanthin de-epoxidase (VDE). Then two enzymes may require for the formation of *cis*-isomers of violaxanthin and neoxanthin, a neoxanthin synthase (NSY) and an isomerase. Cleavage of *cis*-xanthophylls is catalysed by a family of 9-*cis*-epoxycarotenoid dioxygenases (NCED). Then the anthoxin is converted by a short-chain alcohol dehydrogenase (ABA2) into abscisic aldehyde, which is oxidized into ABA by an abscisic aldehyde oxidase (AAO3). Adapted from; (Nambara and Marion-Poll, 2005)

1.2.3.1.5 Role of ABA in plant stress response

In *Arabidopsis* the main abiotic factor leading of formation of ABA and thus triggering of signalling events is any form of limited cellular water availability. High ABA level leads to preservation of seed dormancy (Finkelstein *et al*., 2008), inhibition of germination and lateral root formation (Xiong *et al*., 2006) and reduction of water transpiration through stomatal pores (Hetherington, 2001, Kim *et al*., 2010). Abiotic stresse arises from factors that contribute to an unfavourable environment, including temperature changes, water and nutrient deficiency and salinity stress. Whereas, the biotic stresses are those imposed by other organisms and include pathogenic interactions with microbes, fungi, oomycetes, animals, as well as other plants (Mauch-Mani and Mauch, 2005, Robert-Seilaniantz et al., 2011). Thus, ABA mediates responses and tolerance against unfavourable environmental conditions and also affects the outcome of biotic stress. ABA therefore represents a critical player in the interrelationship between abiotic and biotic stress signalling that will be crucial for engineering and breeding crop species with improved abiotic stress tolerance and pathogen resistance (Cao et al., 2011). However, it has become apparent that responses to abiotic and biotic stresses heavily influence one another, and that there is important crosstalk between their respective signalling pathways (Fujita et al., 2006, Robert-Seilaniantz et al., 2011).

ABA can also be synthesized by plant-associated bacteria, plant pathogenic fungi, certain cyanobacteria, algae, linchens, protozoa and sponges (Nagamune *et al*., 2008, Hartung, 2010). ABA makes plants more resistant in the early stages of infection by closing the stomata, but makes them more susceptible in later stages where it acts to suppress SA synthesis and other defences (Ton *et al*., 2009). ABA suppresses the synthesis of SA by attenuating the expression of the *isochromate synthase* (*ICS1*) (an enzyme in the SA biosynthesis pathway), and suppresses PAMP induced gene expression (de Torres Zabala et al., 2009). Furthermore, recent study by Fan et al. (2009) reported that on the *constitutive disease susceptibility2-1D* (*cds2-1D*) mutant activation of ABA biosynthesis weakened several plant defense systems against bacterial infection. The significantly enhanced growth of both virulent (*P.st* and *P.sm*, *P. syringae* pv. *maculicola*) and non-pathogenic (*hrcC*, mutant defective in TTSS) *P. syringae* strains on *cds2-1D* in comparison to wild-type Col-0 indicates an ABA effect on suppression of the nonspecific

basal resistance against bacterial infection, which is consistent with the observation that suppression of *P.st* growth by treatment with bacterial MAMP (flg22 peptide) was attenuated in the *cds2-D* mutant (Fan et al., 2009). Previous studies showed that treatment of Arabidopsis plants with flg22 peptide or the nonpathogenic *hrcC* strain of *P.st* may lead to callose-associated cell wall modification (Gómez-Gómez et al., 1999, Hauck et al., 2003), and this extracellular defense response is suppressed by wild-type pathogenic bacteria or overexpression in planta of bacterial TTSS effectors (Hauck et al., 2003, Kim et al., 2005). Other recent studies have been shown that showed that wild-type *P.st* enhances callose deposition in Arabidopsis mutants impaired in ABA biosynthesis or signaling, and exogenous ABA suppresses flg22 peptide induced callose deposition in wild-type Arabidopsis seedlings (de Torres-Zabala et al., 2007, Clay et al., 2009, de Torres Zabala et al., 2009), indicating a negative role of ABA in activation of callose deposition. Both flg22- and Harpin-triggered defence share early signalling components, but differ in perception, oxidative burst, and integration into a qualitatively different output, such that for flg22 a basal PTI is elicited in both cell lines, while Harpin induces cell death mimicking an ETI-like pattern of defence (Chang and Nick, 2012).

Microarray data suggests that bacterial effectors up-regulate the transcription of genes involved in ABA biosynthesis and in the ABA signalling pathway (de Torres-Zabala et al., 2007). In other words, ABA has been shown to play early and important roles in disease susceptibility, resistance to pathogen infection, and interaction with other hormone-mediated biotic stress response (Mauch-Mani and Mauch, 2005, Melotto et al., 2006, de Torres-Zabala et al., 2007, de Torres Zabala et al., 2009). In addition, other studies have also shown that pathogen effectors (in particular *AvrPtoB*) stimulate the production of ABA in order to render the plant susceptible to invasion (de Torres-Zabala et al., 2007, de Torres Zabala et al., 2009, Ton et al., 2009, Fan et al., 2009).

1.2.3.1.6 Abscisic acid receptors proteins

Abscisic acid (ABA) is an important phytohormone that involved in adaptation to both biotic and abiotic stresses and in plant development. Recently it has been discovered a fourteen-member family of intracellular ABA receptor, named *PYRABACTIN RESISTANCE 1* and/or *PYRABACTIN RESISTANCE1-LIKE1-13* (PYR1 and PYL1-13) (Park *et al*., 2009, Ma *et al*., 2009, Santiago *et al*., 2009a), they also have been named as regulatory components of ABA receptor (RCARs) (Ma *et al*., 2009). Pyrabactin has been identified as a synthetic growth inhibitor that function as a selective ABA agonist and it determined by genetic analysis the necessity of *PYRABACTIN RESISTANCE 1* (PYR1) for the *in vivo* action of pyrabactin (Park *et al*., 2009, Ma *et al*., 2009, Santiago *et al*., 2009b, Melcher *et al*., 2010). Activation of ABA receptors by ABA led to inhibition of type 2C protein phosphatases (PP2Cs), such as ABA-insensitive1 (ABI1) (Melcher *et al*., 2010).

However, there are three major components to the ABA perception complex; PYR/PYL/RCAR, PP2C; a negative regulator and SNF1-related protein kinase 2 (SnRK2; a positive regulator), and they represent a double negative regulatory system, (*PYR/PYL/RCAR-*, *PP2C-*, *SnRK2*) (Umezawa *et al*., 2010). *REGULATORY COMPONENT OF ABA RECEPTOR 1* (RCAR1/PYL9) overexpression line showed hypersensitivity to ABA-promoted guard cell closure (Ma *et al*., 2009), whereas, overexpression of *PYR1-LIKE 5* (*PYL5*) confers drought tolerance on transgenic *Arabidopsis* plants (Santiago *et al*., 2009a) suggested that the new receptor family is confirmed as a target for manipulating abiotic stress tolerance (Weiner *et al*., 2010).

The *PYR1* and its 4 closest relatives (*PYL1 – PYL4*), regulated by ABA, showed a physical interactions with PP2Cs (ABI1, ABI2 and HAB1). Upon binding to ABA, PYL proteins associate with PP2Cs such as ABI1 and ABI2, inhibiting their phosphatase activity (Park *et al*., 2009, Yin *et al*., 2009). In the presence of ABA PYL8 and PYL9 proteins inactivate certain PP2Cs such as ABI1, ABI2 and HAB1 (Raghavendra et al., 2010). Recently Soon et al (2012) showed that in the absence of ABA, the kinase activity of SnRK2 is abrogated by PP2Cs through physical interaction and dephosphorylation. However, PYR1 and PYL2 are dimers either in the presence or in the absence of ABA (Nishimura et al., 2009, Santiago et al., 2009b, Yin et al.,

2009). While, upon binding to PP2C, ligand-bound PYL dimers disassociate and form a PYL/PP2C heterodimer via the newly formed surface. Therefore, the heterodimer PYL/PP2C occludes the catalytic site of PP2Cs and releases PP2C-mediated inhibition of proteins such as SnRK2 (Fujii et al., 2009). Whereas, Yin et al (2009) has been shown that the apo-PYLs exist as a homodimer, with each protomer containing a ligand-binding pocket guarded by four conserved loops CL1–CL4.

In response to environmental or developmental cues, ABA promotes the interaction of PYR/PYL/RCAR and PP2C, resulting in PP2C inhibition and derepression of SnRK2s. This signalling complex can work in both the nucleus and cytosol, as it has been shown that SnRK2 can phosphorylate both basic-domain leucine zipper (bZIP) transcription factors and membrane proteins. A number of structural analyses of PYR/PYL/RCAR showed two mechanisms; ABA binding of receptors and a 'gate–latch–lock' mechanism leading to interaction with PP2C in inhibiting activity (Umezawa *et al*., 2010). Recently it has been proposed that productive and nonproductive modes in ligand binding to PYL family members exist (Peterson et al., 2010) in which the gate closure responding to ligand exclusively underlies the capacity of PYL inhibiting PP2C (Melcher et al., 2009, Miyazono et al., 2009, Yin et al., 2009). Zhang et al (2012) reported that PYL3-pyrabactin complex displayed nonproductive binding mode in which the gate closes too tightly to bind to PP2C. The PYL3-pyrabactin structure with an excessive closure of L4 is incompatible for interaction with HAB1, and thus inhibits PP2C very weakly. Therefore, only the appropriate gate closure can induce PP2C binding. It was found that pyrabactin was an agonist for PYR1 and PYL1 by gate closure, an antagonist for PYL2 by gate open (Hao et al., 2010, Melcher et al., 2010, Peterson et al., 2010, Yuan et al., 2010).

Mutants defective in ABA biosynthesis have been used to analyze ABA precursor, conjugate, and catabolite effects on hormone signalling in *A. thaliana.* The results showed a physiological activity associated with ABA precursors derives predominantly from their bioconversion to ABA with a weak ABA-like activity in ABA glucose ester conjugation, the assays were in germination and in triggering ABA signalling in protoplasts, moreover, negligible activity has been shown in ABA conjugate and precursors for the regulatory ligand of the ABI2/RCAR receptor complexes (Kepka *et al*., 2011). The apo-PYLs exist as a homodimer, with each protomer containing a ligand-binding pocket guarded by four conserved loops CL1–CL4 (9)

However, ABA receptor quadruple mutant *(pyr1/pyl1/pyl2/pyl4)* identified by Park *et al*., (2009) displayed defects in several ABA responses, including ABA-induced gene expression, ABA-mediated SNF1-telated protein kinases2 (SnRK2) kinase activation and ABA-promoted guard cell closure (Nishimura *et al*., 2010). In addition, investigation in biological relevance to the PYR/PYLs analyzed the *pyr1/pyl1/pyl2/pyl4* quadruple mutant plants and found strong insensitivities in ABA-induced stomatal closure and ABA-inhibition of stomatal opening. These findings demonstrated that ABI1 and PP2C can interact with several PYR/PYL/ RCAR family members in *Arabidopsis*, that PYR1–ABI1 interaction is rapidly stimulated by ABA in *Arabidopsis* and indicate new SnRK2 kinase-PYR/PYL/RCAR interactions in an emerging model for PYR/PYL/RCAR mediated ABA signalling (Fig.1.3) (Nishimura *et al*., 2010).

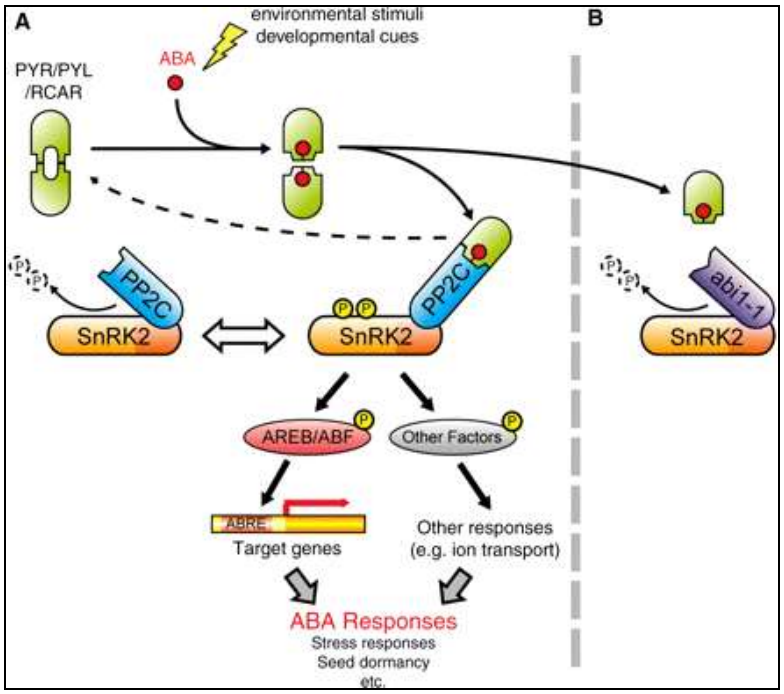

Figure 1.3 ABA signalling pathway (A) Interaction and dephosphorylation of SnRK2 under normal conditions and abiotic stresses and (B) Binding of abi1-1 to PYLs adapted from (Umezawa *et al*., 2010)

Recently, Lackman (2010) identified a tobacco gene, *NtPYL4*, that codes for a protein belonging to the PYR/PYL/RCAR protein family of ABA receptors from *Arabidopsis* (Lackman *et al*., 2010). Thus functional analysis of *PYL4* and *PYL5 Arabidopsis* homologs of *NtPYL4* indicated that also in *Arabidopsis* altered *PYL* expression affected the JA responses. These findings point toward the existence of a conserved mechanism for ABA perception and signalling in plants (Lackman *et al*., 2010).

Arabidopsis Genome Uncoupled 5 (GUN5) (Mochizuki *et al*., 2001) was found to bind ABA with high affinity and mediate all major aspect of ABA responses including seed germination, early seedling development and stomatal closure (Shen *et al*., 2006). GUN5 was initially reported as ABA receptors localized in the nucleus, chloroplast and plasma membrane, respectively (Razem *et al*., 2006, Shen *et al*., 2006, Liu *et al*., 2007). Furthermore, G protein-coupled receptor2 (GCR2) was identified as an ABA receptor and found to interact with *Arabidopsis* heterotrimeric Gα subunit (Liu *et al*., 2007). Liu also showed that GCR2 is involved in all aspects of ABA-regulated physiological response including seed germination, stomatal closure and the expression of ABA-responsive genes (Liu *et al*., 2007).

Thus there exist a varety of ABA receptors with different cellular addresses.

1.2.3.2 Flavanones in *Arabidopsis*

Plants respond to environmental changes by implementing a number of physiological, metabolic, and developmental changes. Flavonoids are a class of phenolic compounds with a low-molecular-weight and are widely distributed in the plant kingdom. These water-soluble compounds serve as pigments in plants with several activities *in vitro*, such as involvement in many biological possible beneficial influence on human health, and their possess antiviral, antibacterial, antifungal or anti-allergenic properties (Hertog *et al*., 1995, Holiman *et al*., 1996, Peterson and Dwyer, 1998, Ross and Kasum, 2002, Bohm, 1998, Seigler, 1998). However, in *Arabidopsis thaliana* production of anthocyanin is a clear visible marker of plant response to unfavourable growth conditions (Chalker-Scott, 1999). Furthermore, Anthocyanins, have been shown to be the most conspicuous class of flavonoids, they are important plant pigments responsible for most of the red, pink, purple, and blue colours in plants (Grotewold, 2006). They protect plant cells from UV irradiation, and act as antimicrobial agents and feeding deterrents against pathogens and herbivores (Harborne and Williams, 2000; Winkel-Shirley, 2001). In other respect, anthocyanin accumulation in various tissues has been shown to be associated with low availability of nutrients such as nitrogen and/or phosphorus, wounding, pathogen infection, jasmonate treatment, drought, and ultraviolet, visible, and far-red radiation (Hipskind et al., 1996, Nicole et al., 2010). In addition, the induction of anthocyanin synthesis has been suggested to result from carbohydrate “overflow” during the active recycling of photosynthetic proteins (Matile, 2000), the induction of anthocyanin synthesis by HL in tissues that are unlikely to have

an excess of carbon reserves, such as germinating seedlings, is inconsistent with the carbon overflow hypothesis (Christie *et al*., 1994; Yanovsky *et al*., 1998). Anthocyanin synthesis in autumn leaves often precedes chlorophyll breakdown and the colour intensity of red-senescing leaves is increased by HL, cool (but not freezing) temperatures, and mild drought (Chalker-Scott, 1999, Kozlowski and Pallardy, 2002). Anthocyanins are synthesized by a branch of the flavonoid pathway, which is composed of a sequence of enzymatic steps, including chalcone synthase (CHS) for the synthesis of naringenin chalcone, chalcone isomerase (CHI) for the conversion of naringenin chalcone to naringenin, flavanone 3-hydroxylase (F3H) and flavonoid 3′-hydroxylase (F3′H) for the subsequent hydroxylations of naringenin, NADPH-dependent dihydroflavonol reductase (DFR) for the production of leucoanthocyanidins, leucoanthocyanidin dioxygenase (LDOX) for the conversion of leucoanthocyanidins to anthocyanidins, and UDP-Glc:flavonoid 3-Oglucosyltransferase (UF3GT) for the generation of glycosylated anthocyanidins (Grotewold, 2006). Furthermore, in Arabidopsis, the ectopic expression of Myb transcription factors PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1) and PAP2 results in an enhanced expression of the anthocyanin biosynthetic genes PAL, CHS, and DFR, but the increases in expression of the 'early' structural genes are more modest compared with the 'late' genes (Tohge et al., 2005). Meanwhile, reduced Myb expression results in a reduction of the 'late' genes expression from the beginning with F3'H in Arabidopsis (Gonzalez et al., 2008). Thus PAP1 and PAP2 mainly regulate the 'late' steps of anthocyanin biosynthesis. The bHLH anthocyanin biosynthetic regulators glabra3 (GL3) and enhancer of glabra3 (EGL3) also control the expression of the 'late' flavonoid pathway genes in Arabidopsis (Zhang et al., 2003; Gonzalez et al., 2008). The expression of the 'late' anthocyanin biosynthetic genes such as dihydroflavonol 4-reductase (DFR) and leucoanthocyanidin dioxygenase (LDOX) is nearly off or undetectable in *transparent testa glabra 1* (*ttg1*) mutants, whereas the expression of the 'early' anthocyanin biosynthetic genes including CHS, CHI, and F3H is not affected in the same mutant (Gonzalez et al., 2008). However, it has been found that the F-box protein COI1 was essential for JA-induction of transcription factors PAP1, PAP2, and GL3. It is speculated that COI1 regulates the expression of the transcription factors, including PAP1, PAP2, and GL3, which mediates the 'late' anthocyanin biosynthetic genes DFR, LDOX, and UF3GT, thereby modulating JA-induced anthocyanin biosynthesis in Arabidopsis (Wang et al., 2005, Shan et al., 2009).

Flavonoids are synthesized by the phenylpropanoid metabolic pathway where phenylalanine is used as a substrate to produce 4-coumaroyl-CoA. The 4-coumaroyl-CoA is combined with malonyl-CoA to yield a group of compounds called chalcones (the backbone of flavonoids), which contain two phenyl rings. Conjugate ring-closure of chalcones results in the familiar form of flavonoids, the three-ringed structure of a flavone. The metabolic pathway continues through a series of enzymatic modifications to yield flavanones, dihydroflavonols and then anthocyanins (Ververidis *et al*., 2007). Flavonoids serve as UV protectants (Schmelzer *et al*., 1988), signal molecules in plant-microbe interactions (Sharon R, 1989) and antibiotics in plant defense responses (Dixon, 1986, Lamb *et al*., 1989). In some plant species synthesis of flavonoid compounds are induced by UV-B, which possibly due to an adaptive response (Li *et al*., 1993). Thus it has been shown that plants grown under such condition were more tolerance to UV irradiation (Murali and Teramura, 1985). Li at el, (1993) also showed that nutrient limitation or/and high white light pre-treatment was used to induce flavonoid synthesis and resulted in multiple physiological and developmental changes with no primary role for flavonoids in UV-B protection. However, PreuB et al., (2009) examined the activities of putative flavonol synthase (FLS) genes which have been shown to be responsible for the formation of flavonols in the leucoanthocyanidin dioxygenase/flavonol synthase (*ldox/fls1-2*) double mutant. Recombinant FLSs and LDOX proteins led to the identification of FLS3 as a second active FLS. These results suggested that the FLS activity of LDOX might contribute to the formation of flavonols in the Arabidopsis *fls1-2* mutant, which is supported by the additional drop of flavonol contents observed in the *ldox/ fls1-2* double mutant and thus the double mutant demonstrated that LDOX is capable of catalyzing the in planta formation of flavonols (PreuB et al., 2009)

Arabidopsis plants exposed to HL accumulate anthocyanins comprising glycosides of phenolic aglycons with a flavan C6-C3-C6 skeleton. Anthocyanins, end products of the flavonoid pathway (Fig.1.4), are produced in the cytoplasm and then transported into the vacuole (Marrs et al., 1995, Shirley, 1996). It has been reported in previous literature, that most of the >6000 flavonoids, described in higher plants (Harborne and Williams, 2000), are glycosides of a relatively small number of flavonoid aglycons, non-sugar compound, which are generally water-soluble and accumulate in plant vacuoles cells (Bohm, 1998, Seigler, 1998, Jaakola, 2003). While Solfanelli et al., (2006) has been shown that the whole-genome transcript profiling reveals that the flavonoid and anthocyanin biosynthetic pathways are strongly up-regulated following

sucrose (Suc) treatment. In addition, Suc affects both flavonoid and anthocyanin contents and thus the investigation the effects of sugars (Suc, glucose, and fructose) on genes coding for flavonoid and anthocyanin biosynthetic enzymes in Arabidopsis revealed that the sugar-dependent up-regulation of the anthocyanin synthesis pathway is Suc specific. An altered induction of several anthocyanin biosynthetic genes (Teng et al., 2005, Solfanelli et al., 2006)

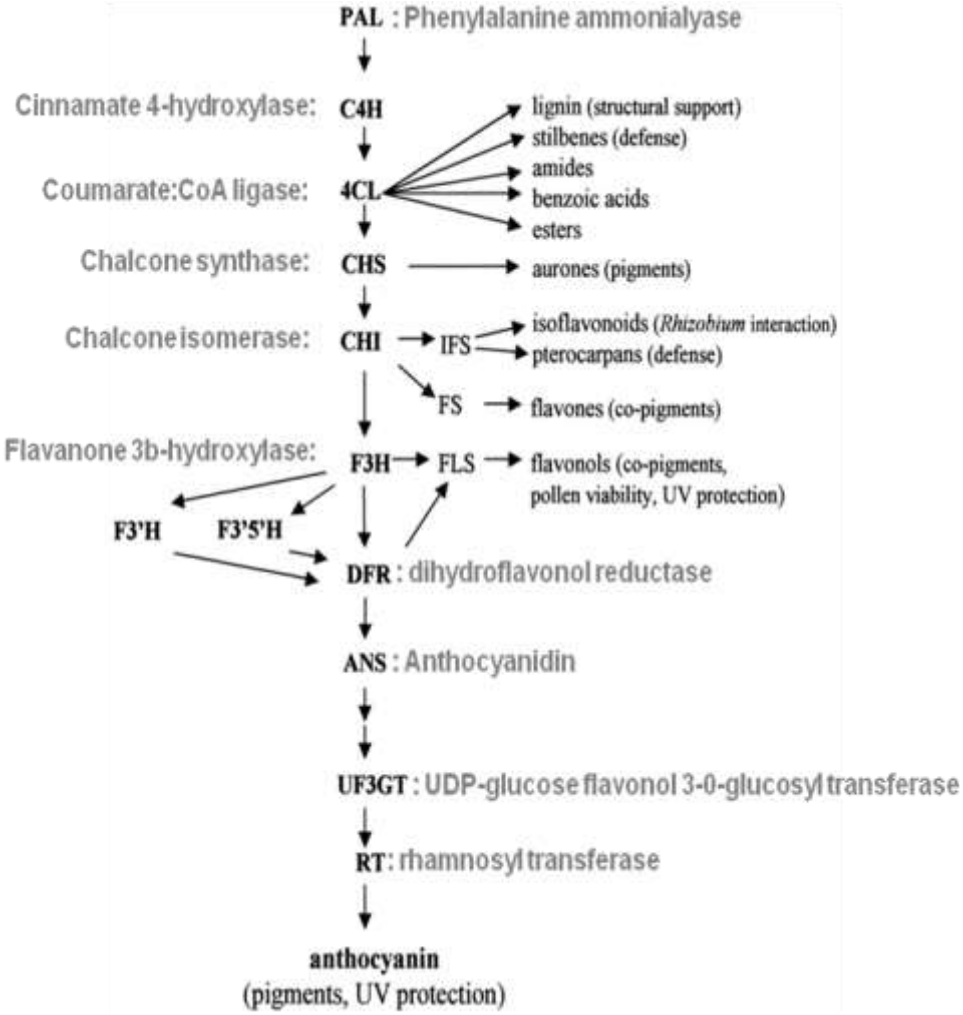

Figure 1.4 Flavonoid biosynthetic pathway adapted from (Clegg and Durbin, 2000)

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Recently, Page at el., (2010) examined the level of anthocyanin and flavonols in ascorbate-deficient mutants (*vtc1, vtc2* and *vtc3*) in comparison to Col-0 wild type plants during HL acclimation. Page's investigation has led to a positive correlation between ascorbate and anthocyanin accumulation among the *vtc1, vtc2* and *vtc3* mutants exposed to HL (Page *et al*., 2011). Furthermore, Shi at el., (2010) used the *Production of anthocyanin pigmentation1-Dominant* (*pap1-D*) and wild-type *Arabidopsis* to investigate anthocyanin biosynthesis in rosette leaves. Plants were grown in nine growth conditions, three media derived from MS medium (medium-1, 2 and 3) and three different light intensities (low light, middle light and high light). The result showed that anthocyanin and pigmentation patterns levels were differentially affected in rosette leaves. The combined growth conditions of high light and either medium-2 or medium-1 (full strength of basal MS and ½ strength of NH4ON3 and KNO3 in MS respectively) of *pap1-D* plants revealed that *pap1-D* rosette leaves produced much higher levels and more diverse molecular profiling of cyanins than those of WT plants (Shi and Xie, 2010). In addition Shi and Xie have been shown that there are 14 genes involved in the biosynthesis pathways of anthocyanin. The expression levels of these genes were significantly higher in the red cells compared with the WT cells. Furthermore, most of the genes with significant differential expression levels in the red cells versus the WT cells were characterized with diverse biochemical functions, many of which were mapped to different metabolic pathways (e.g., photosynthesis and plant secondary metabolisms). This findings suggested that the difference in gene expression profiles between the two cell lines likely results from cell types, the overexpression of *PAP1*, and the high metabolic flux toward anthocyanins (Shi and Xie, 2011).

1.2.3.3 Disease discriminatory metabolites

The bacterial plant pathogen, such as *P. syringae* and *Xanthomonas* spp., use a variety of strategies to colonize plants and derive nutrients from their hosts. These strategies rely on living plant cells for nutrient acquisition of infection. The lifestyle, of such plant pathogens, is largely

dependent on their ability to avoid and suppress plant defense responses most notably by secretion of effector proteins enabling them to obtain nutrients and multiply within living plant tissue (Gohre and Robatzek, 2008, Collmer et al., 2009, Kay and Bonas, 2009). Previously, Alfano and Collmer have been shown that bacterial effectors are secreted mainly through the type III secretion system (T3SS) which is multi-protein injection machinery capable of translocating proteins directly from the bacterial cytosol into the host cell. Thus different effector proteins target specific components of plant defense and are effective only against a particular plant species and therefore, strains of (hemi) biotrophic bacteria often show a high degree of host specificity (Niks and Marcel, 2009, Lindeberg et al., 2012). Plant immune systems include preformed defenses and infection-induced basal and *R* gene-mediated defenses (Jones and Dangl, 2006, Bent and Mackey, 2007, McDowell and Simon, 2008). Basal immune responses are mediated by receptors that recognize ubiquitously expressed, highly conserved PAMPS such as bacterial flagellin or EF-Tu proteins or fungal chitin. Many pathogens express effector proteins that suppress basal host immune responses, but *R* gene-mediated defenses can be activated when host R proteins recognize the presence or activity of specific pathogen effectors. Both basal and R gene-mediated defenses can engage protein phosphorylation, ion fluxes, reactive oxygen species (ROS) production, and production of defense signaling compounds such as salicylic acid (SA), nitric oxide, ethylene, and jasmonic acid (Feys and Parker, 2000, Hammond-Kosack and Parker, 2003). Previously, it has been found that poly(ADP-Rib) glycohydrolase (PARG2) and a Nudix hydrolase active on ADP-Rib and NADH (NUDT7) among a small group of less than 40 genes significantly up-regulated in multiple *R/avr* interactions between *A. thaliana* and *P. st*DC3000 (Adams-Phillips et al., 2008, Adams-Phillips et al., 2010), *nudt7* plants were more resistant to virulent and avirulent *P.st* DC3000 (Bartsch et al., 2006, Jambunathan and Mahalingam, 2006, Ge et al., 2007, Adams-Phillips et al., 2008, Adams-Phillips et al., 2010) and also displayed a greatly reduced hypersensitive response to avirulent *P.st* DC3000 (Adams-Phillips et al., 2008). In addition, Adams-Phillips et al., (2010) has been also found that pharmacological inhibition of poly(ADP-Rib) polymerase (PARP) blocked the formation of callose-containing cell wall depositions induced by the MAMPs flg22 and elf18 (Adams-Phillips et al., 2008). This suggested a role for poly (ADP-ribosyl)ation in the pathways that regulate pathogen-elicited callose deposition and plant innate immune responses (Adams-Phillips et al., 2010).

Previously Bednarek *et al.*, (2004) reported a strong accumulation of 3´-*O*-β-D- ribofuranosyl adenosine (3´RA) during compatible interactions in *Arabidopsis* leaves infected with virulent or avirulent strains of *P. syringae* pathovar tomato. The accumulation of these purine derived compounds in *A. thaliana* plant leaves occurred after infection with virulent or avirulent strains of *P.st*. 3´RA was undetectable in incompatible interactions of *A. thaliana* leaves with an avirulent *P.st* strain, as well as in uninfected control leaves.

However, Poly (ADP-ribosyl)ation is an important posttranslational modification in many eukaryotes (Otto et al., 2005, Hassa and Hottiger, 2008). It is biochemically and functionally distinct from mono-ADP-ribosylation. Poly(ADP-ribosyl)ation is carried out by PARPs, which use NAD+ as a substrate to catalyze both the attachment and elongation of ADP-Rib polymers on acceptor proteins. Automodified PARP and other poly (ADP-ribosyl)ated nuclear proteins (Huletsky et al., 1989) can affect chromatin structure, transcription, replication, and DNA repair processes through PARP-mediated recruitment of other proteins (Masson et al., 1998, Simbulan-Rosenthal et al., 1999, Ahel et al., 2009). Therefore, PARP can act as a DNA damage sensor (Petrucco, 2003, Schreiber et al., 2006, Roldan-Arjona and Ariza, 2009). In addition, PARP and poly (ADP-ribosyl)ation can regulate cellular processes by modulating cellular levels of NAD+. Strong PARP activation can cause massive consumption of NAD^+, which can alter cellular reduction/oxidation states, impact nicotinamide levels, and induce ATP depletion (Hassa and Hottiger, 2008, Hashida et al., 2009, Tao et al., 2009, Dea et al., 2011, Briggs and Bent, 2011). Furthermore, it has been shown that PARP-1 functions at the center of cellular stress responses, where it processes diverse signals and, in response, directs cells to specific fates (e.g., DNA repair vs. cell death) based on the type and strength of the stress stimulus. PARP-1 functions are intimately tied to nuclear NAD^+ metabolism and the broader metabolic profile of the cell and thus this findings demonstrated a role of PARP-1 in stress responses (Luo and Kraus, 2012). In addition, recent study has been shown that Poly(ADP-ribosyl)ation, with the massive charge accumulation that it confers, can break up chromatin structure by interfering with protein– DNA interactions, as well as alter the surface properties of substrate proteins. Mono-ADP-ribosylation can alter the chemistry of specific protein side chains, provide a handle for the binding of a specific recognition or recruiting domain, or be a destruction mark on a protein substrate. Mono-ADP-ribosylation has been also shown to emerge as an important regulatory mechanism (Koch-Nolte and Ziegler, 2013).

1.3 Objective:

This project is consists of three parts, firstly; we aimed to investigate the hypothetical biosynthesis pathway of the 3´-*O*-β D- ribofuranosyl adenosine (hereafter referred to as "400" compound) that determined by Nicolas Smirnoff. The primary objective was to study the increase of this compound following infection with DC3000 and determine what role it may play. The reason this is interesting that it implicates an important role for nucleotides in defense responses. One approach is to stop synthesis of 400 by analysis knockout (KO) mutants of enzymes that are presumably involved in its synthesis; APD-ribos diphosphotase NAD binding / hydrolase (*NUDIX6-1*), Nudix hydrolase8 (*NUDIX8-1*), glucosyl transferase (*UDP-GTA*), ribosyltransferase (*SRO-14* and *SRO-2*), ribose-phosphate pyrophosphokinase3 (*PRS3*), galactosyltransferase (*GLYCOSIL-T*), glucosyl transferase 75B1 (*UGT1*), (*UGT85A-1*) further more we generated double mutants by crossing the single KO that belongs to the same family of Nudix hydrolase and ribosyltransferase (*NUDIX6-1* X *NUDIX8-1*and *SRO-14* X *SRO-2)*.

The major aim of this project was to study the interaction between abiotic and biotic stress. Specifically we are interested in commonalities between biotic and abiotic interactions and how a plant priorities its response (output) when subjected to multiple, and possibly antagonistic, stress responses. In this part of study we used *Arabidopsis* ecotype Col-5, Col-0, Landsberg erecta, two hormone synthesis mutants (*sid2-1* SA biosynthetic and *aao3* ABA biosynthetic), and *P. st* DC3000 to investigate the susceptibility of *Arabidopsis*following abiotic stresses.
In addition we studied the role of abscisic acid (ABA) and salicylic acid (SA) as phytohormones that regulate plant stress responses. Additionally, we use a range of *Arabidopsis* genotypes such as *sid2-1/aao3* double mutant (de Torres Zabala et al., 2009), *enhanced disease susceptibility1* (*eds1*), *phytoalexin deficient 4* (*pad4*) (Glazebrook *et al*., 1996), overexpression of pyrabactin resistance1 like5 (*pyl5.OE*) (Santiago et al., 2009a), type two protein phosphatase, triple mutant, (*hab1-1/abi2-1/abi1-2*) (Rubio *et al*., 2009) and the phytochrome interacting factor (PIF) double mutants *phyA/phyB* and *cry1/cry2* (Leeds University)

The third major objective was to investigate three of PYR-PYL-RCAR protein family (PYL4, 5 & 6) that were identified (Yue Ma, *et al*. 2009; Sang-Youl Park, *et al*. 2009) as ABA receptors and associates with type 2C protein phosphatases (PP2Cs). We also used *pyl4/5* double mutant and *PYL5* over- expression kindly derived from Pedro Rodriguez laboratory-Spain and we generated *pyl5/6* double mutant. We wanted to address the question whether *PYR*/*PYL* (4, 5 and 6) are involved in plant pathogen interactions, whether *PYL5* over- expression line are more susceptible to *P. st* DC3000 under a combination of abiotic and biotic conditions, and whether the DC3000 can dismantle photosynthesis as quickly in *PYL5* over- expression line as in the type 2C protein phosphatases, triple mutant, (*hab1-1/abi2-1/abi1-2*) compared with wild- type (Col.0). To achieve this we sought to generate luciferase transgenic lines for *PYL4*, *5* and *6* under cooled charge device (CCD) camera for determining expression levels and localisation. In addition we sought to express *PYL4, 5* and *6* under a strong (CaMV v35S) promoter with HA and MYC tags. Finally, we tried to express GFP or YFP tagged the *PYLs* and use confocal microscopy to examine histochemical localisation of the *PYL4*, *5* and *6* following pathogen challenge and other stresses.

Chapter 2: Material and Methods

2.1 Plant growth conditions

Arabidopsis thaliana genotypes were sown in Levington F2 compost with sand and stratified for 2 days at 4ºC. Plants were grown under short days in a controlled environment chamber (10 h light, 100-120 µmol/$m^{-2}sec^{-1}$ intensity, at 22ºC day, 20ºC night and 65% relative humidity) for 5 weeks before use. The *Arabidopsis* genotypes studied in this work were Col-0, Col-5, Landsberg erecta, *nudix6-1*(At2g04450), *nudix8-1*(At5g47240), *udp-gta* (At3g466670), *sro-14* (At5g62520), *sro-2* (At1g23550), *prs3* (At1g10700), *glycosil-t* (At5g57500), *ugt1* (At1g05560), *ugt85a-1*(At1g22400) (NCAS), *aao3* (At2G27150) (De Torres-Zabala *et al*., 2007), *sid2-1* (At1G74710) (Wildermuth *et al*., 2001), *aao3/sid2-1* (De Torres Zabala *et al*., 2009), *eds1*(At3G48090), *pad4* (At3G52430) (Glazebrook *et al*., 1996), *hab1-1/abi2-1/abi1-2* (Rubio *et al*., 2009), *ply4* (At2g38310), *pyl5* (At5g05440), *pyl6* (At2g40330) (NCAS), *pyl4/pyl5*, *pyl5*-OE (Santiago *et al*., 2009b), *pif.i* (*phyA-211/phyB-9*) and *pif.h* (*cry1/cry2*) (Edinburgh University) and the double mutants *nudix6-1*/*nudix8-1*, *sro-14*/*sro-2* and *pyl5/pyl6*. All the mutations used in this study are in Col.0 background except the *eds1* mutant which is in the Landsberg *erecta* background.

2.1.1 Seed sterilization and selection

Arabidopsis seeds were surface sterilised in Laminar Flow Cabinet. The seeds, approximately 100 µl (about 1000-2000 seed), were placed in 1.5 ml centrifuge tube before 200-300 µl of 70% ethanol was added, briefly vortexed and incubated for 2 min at room temperature (RT). The tubes containing the seeds were centrifuged for 1 min X 13000g. Supernatant were removed before 400-500 µl of 5% bleach + 1% sodium dodecyl sulphate (SDS) were added and incubated at RT for 10-15 min. Then the seeds were rinsed in sterilised distilled water (dH_2O), vortexed and centrifuged for 1 min at 13000 g. Seeds rinses were repeated three times before water was totally removed and the seeds were resuspended in 2-3 ml of 0.1% agarose before being poured on Murashige and Skoog (MS) plates containing the appropriate antibiotics. The seeds were

spread by tilting the plates before being left to dry in Laminar Flow Cabinet for 30-60 min, covered and cold treated in the fridge for 2 days.

In the case of selection in BASTA, soil trays were drenched with BASTA solution (0.25g/l - Bayer) before about 0.5 ml seeds (approximatlly10000-20000) were distributed evenly in the trays and cold treated in the fridge for 2 days before moved to growth room. Seven to ten days from germination seedlings were pricked out for further investigation.

2.1.2 Plant media

Arabidopsis seeds were germinated in 0.5xMS /0.8% agar plates where, for 2 L media, 4.8 g of MS salts (Sigma, USA) dissolved in 1 L of dH_2O and the pH was adjusted to 5.7 with potassium hydroxide (KOH). Volume was completed to 2 L before the mixture distributed into 500 ml Duran bottles (400 ml media per bottle). Then 3.2 g agar was added to each bottle before autoclaving at 121ºC for 15 min. When bottles cooled down (around 60ºC) 1000x filter-sterilized vitamin B5 (400µl/bottle) and appropriate antibiotic (Kanamycin 50 µg/ml final- Melford, UK; Hygromycin 30 µg/ml final- Melford, UK) were added before the media poured in the plates.

2.2 Plant nucleic acid analysis

2.2.1 Isolation of genomic DNA

Arabidopsis plant leaves were removed using clean scissors and transferred to a clean micro centrifuge tube. Leaves were totally crushed with a plastic pestle in 500 µl of Shorty buffer (0.2 M Tris-HCL- pH9, 0.4 M LiCl, 25 mM EDTA, 1% SDS) or using a tungsten ball (3 mm) in tissue lyser (Qiagen) for 2 min, frequency 25/S. Subsequently, supernatants were transferred to a fresh 1.5 ml micro centrifuge tube and 500 µl of phenol chloroform was added. The tubes were briefly vortexed and centrifuged at maximum speed for 5 min at RT. Upper (aqueous) phase (~450 µl) was carefully transferred by pipetting into a fresh 1.5 ml micro centrifuge tube. Next, 1 volume (Vol.) of isopropanol (450 µl) was added and the mixture was mixed by inversion and centrifuged at maximum speed for 10 min. The supernatant was decanted and tubes containing nucleic acid pellets left to dry on paper towel at RT. The precipitated pellets were washed with 200 µl of 70% ethanol, vortexed briefly and centrifuged as before. Then all liquid was decanted

and the tubes left to dry at RT. The resultant DNA pellet was resuspended in 100 µl of 10 mM sterile Tris pH 8.0 or distilled H_2O by vortexing.

2.2.2 Polymerase chain reaction (PCR) from genomic DNA

To identify homozygous T-DNA knock out (KO) lines, the standard PCR conditions of 30 µl final vol. were used in this work as follows; a master mix of 3 µl of 10x PCR buffer [670 mM Tris-HCl, 160 mM $(NH_4)_2SO_4$, 0.1% Tween 20 filter-sterilised], 0.9 µl of 50 mM $MgSO_4$ (for 20 ml; 264 mg $MgSO_4$-$7H_2O$ and 120 mg $MgSO_4$ anhydrous filter-sterilised), 2.4 µl of 2.5 mM dNTP, 1.5 µl of 10 mM forward primer, 1.5 µl of 10 mM reverse primer, 18.45 µl of dH_2O, 0.25 µl of Taq polymerase. The mixture was mixed well before 2 µl of genomic DNA added and remixed. For PCR made from vector DNA, 1 µl vector was diluted in 500 µl dH_2O and PCR set as before. Standard PCR protocol;

Denaturation 94ºC X 2 min
Final denaturation 94ºC x 30 sec
Annealing 55ºC x 30 sec
Extension 72ºC X x min
Final extension 72ºC x 10min
4-15ºC for ever
Annealing temperature should be adjusted between 0-5ºC below Tm and extension time is about 1min/Kb of the expected implication.

2.2.3 Plant RNA extraction

Arabidopsis frozen tissue was ground to a fine powder before 600µl of Z6 buffer [8 M guanidine hydrochloride, 20 mM MES; pH 7.0, 20mM ethylene diaminetetraacetic acid (EDTA- Ferments, UK), pH 7.0] was added and mixed thoroughly with pestle. Then the mixture was passed into 1.5 ml eppendorf tube and 1 Vol. of phenol/chloroform (Sigma-UK) was added to denature DNA and protein. The mixture was centrifuged at 4ºC for 5 min at 13000g before the aqueous phase was passed into a clean 1.5 ml eppendorf tube. To precipitate RNA, 1/20 Vol. of 1 M acetic acid and 0.7 Vol. of 100% ethanol were added, vortexed and kept on ice for 30 min before being centrifuged for 20 min at 4ºC X 13000. Then liquid was decanted on blue towel papers before 500 µl of 70% ethanol was added, vortexed briefly to wash RNA pellet and then centrifuged for

5 min X 13000. Liquid was removed from the tube and left to dry at RT before RNA pellet was resuspended in 100 µl of nuclease-free water by vortexing. The tube containing RNA was incubated for 10 min at 65ºC and vortexed till the pellet was completely resuspended.

2.2.4 Precipitation of nucleic acids

DNA and RNA were precipitated from solution by adding 0.1% vol of 3M sodium acetate (pH 5.2) and one volume of 100% (v/v) of isopropanol. The mixture was vortexed briefly before being incubated on ice for 30 min. Then the mixture was centrifuged for 30 min at 4ºC x 13000g. Pellets were washed with 70% ethanol by brief vortexing, centrifuged for 1 min at 13000g then the liquid was decanted and the tubes left to dry at RT. The pellets were resuspended in 50 µl dH_2O.

2.2.5 cDNA synthesis

For cDNA synthesis, 1.5 µg of RNA was diluted in 4 µl dH_2O before added to 6 µl of mixture1 (1 µl 10X DNAse buffer, 3.5 µl dH_2O and 1.5 µl DNAse). The mixture was incubated at 37ºC for 30 min before 1 µl of RQ1 stop solution (Progema, UK) was added with other incubation at 65ºC for 10 min to stop the reaction. Then 7 µl from the previous mixture was transferred to clean 1.5 ml tube before 6 µl of [0.5 µl of 100 µM Oligo dT, 4 µl of 2.5 mM dNTP (BioLabs, UK) and 1.5 µl of dH_2O was added]. The mixture was incubated at 65ºC for 5 min before the reaction was stopped on ice for 5 min. Finally, 7 µl of [4µl 5X RT buffer, 1 µl dH_2O, 1 µl of 100 mM DTT (Progema, UK) and 1 µl of Superscript III (Invitrogen, USA)] before being incubated at 50ºC for 60 min. The reaction was stopped by incubation at 70ºC for 15 min, transferred to ice and subsequently stored in the freezer.

2.3 Pathogen challenges:

P. st DC3000 cultures were grown at 28ºC shaker overnight in 10 ml of Kings B solution (King *et al*., 1954) supplemented with appropriate antibiotics. Plants were mock-challenged with (10 mM $MgCl_2$) or inoculated with DC3000 or *hrpA*. The antibiotics rifampicin (50 mg ml^{-1}) and kanamycin (25 mg ml^{-1}) were used for selection. Overnight cultures were washed once in 10 mM $MgCl_2$ resuspended and final cell densities were adjusted to approximately 0.2 at 600 nm (approximately 2 x 10^8 cfu ml^{-1}) in 10 mM $MgCl_2$. Serial dilutions of (0.02, 0.002 & 0.0002)

were prepared. The latter two dilutions were used for phenotype and growth curve respectively. Leaves were infiltrated on the abaxial surface with a needleless 1-ml syringe. Three to four leaves of six plants (three replicates, two plants per replicate) were infiltrated before being sampled for bacterial growth, hormones and flavonoid extraction. Harvested tissues were either examined for bacterial growth or immediately frozen in liquid nitrogen (N_2) for hormones and flavonoid measurements.

2.4 Bacterial media

For Kings B agar plates, 10 g peptone meat, 10 g N-Z casein (Oxoid, UK), 1.5 g $MgSO_4$-$7H_2O$, 1.5 g K_2HPO_4 and 10 ml glycerol (Fisher-UK) were dissolved in 800 ml dH_2O before the pH was adjusted to 7.2 with NaOH, the volume made up to 1 L and distributed into 500 ml Duran bottles (400 ml media per bottle). Six grams of agar (OXOID) were added/400 ml before autoclaved at 121ºC for 15 min. When the media had cooled down (~60ºC) rifampicin (50 mg ml^{-1}) and kanamycin (25 mg ml^{-1}) antibiotics were added for bacterium selection. In the case of bacterial liquid media (LB), 10 g of bacto-Tryptone (SLS), 5 g bacto-yeast extract (Oxoid) and 10 g sodium chloride ($NaCl_2$) were dissolved in 800 ml of dH_2O before the pH adjusted to 7.0-7.5 with NaOH and volume completed to one litter and autoclaved. For LB agar, the one litre media was distributed into 500 ml Duran bottles (400 ml media per bottle). Six grams of agar were added to 400 ml before autoclaved at 121ºC for 15 min.

2.5 Bacterial growth assays

This assay was performed four days post infection of *Arabidopsis* plants with DC3000 as described in Section 2.5. The three inoculated leaves/plant were sampled by disc borer before discs were pooled and homogenized in a 2 ml micro-centrifuge tube containing 1 ml of 10 mM $MgCl_2$ using a tungsten ball (3 mm) in the tissue lyser for 2 min. A set of 3 to 6 10x serial dilutions (depend on each genotype susceptibility and infection conditions) were performed by transferring 100 µl of each dilution into a clean 1.5 ml micro-centrifuge tube containing 900 µl of 10mM $MgCl_2$. Tubes were vigorously vortexed. For each dilution, six 10 µl aliquots were plated in KB agar plates with 50 mg/ml^{-1} rifampicin and 25 mg/ml^{-1} kanamycin before plates

were left to dry-out beside a Bunsen burner. Then the plates were incubated at 28^{o}C incubator for 1-2 days before bacterial colonies were counted under a light microscope.

2.6 Statistical analysis

All experiments in the result chapters were repeated at least twice and data from the representative experiment are shown in each chapter. For bacterial growth analyses mean samples were calculated from six biological replicas where, from each ecotype plant, three inoculated leaves per plant were sampled by disc borer as described in section 2.5. While for hormones and flavonoids, the six biological replicates were distributed into three technical replicates two plants per replicate using the same inoculated leaves for bacterial growth. Errors bars represent one standard deviation. Labels "a, b etc." represents $P < 0.05 = 95\%$ (Student's t-test for pair wise comparison of non-treated and treated plants) All experiments were repeated at least twice.

Chapter 3: The role of the '400' compound in Arabidopsis *Pseudomonas* interactions

3.1 Introduction

In classical gene-for-gene responses plant resistance to pathogen infection is associated with development of a hypersensitive response (HR) that results in programmed cell death to restrict pathogen spread (Mur *et al*., 2008). The biosynthetic pathways of secondary metabolites are strongly induced during the HR (Dixon, 2001). A typical HR occurs on *Arabidopsis* leaves infected with the avirulent bacteria *Pseudomonas syringae pv*. *tomato* DC3000 carrying the *AvrRpm1* gene (*Pst-AvrRpm1*) This results in stimulation of the indolic pathway - one of the major secondary metabolic alterations in the host (Hagemeier *et al*., 2001). In the interactions between *A. thaliana* and avirulent/virulent *Pst* (DC3000) strains, secondary metabolites (SMs), such as 3´-*O*-β D- ribofuranosyl adenosine and glucosinolates, have been proposed to play both positive and negative roles in protection of *Arabidopsis* against particular pathogens and insects (Bednarek *et al*., 2004, Bednarek *et al.*, 2009, Tierens *et al.*, 2001, Hahlbrock *et al*., 2003). Prior to the report by Bednarek *et al.* (2004) 3´-*O*-β D- ribofuranosyl adenosine had been described as *O*-β-ribosyl-(1″→2′) adenosine-5″-phosphate by Keith *et al* (1990) who investigated the chemical structure for Ar(p) (previously referred to the modified nucleotide A•) at the position 64 in yeast initiator $tRNA^{Met}$. Nudix hydrolases are widely prevalent among eukaryotes, bacteria, archaea, and viruses and they consist mainly of pyrophosphohydrolases that act upon substrates of general structure **nu**cleoside **di**phosphate linked to some moiety **X** to yield nucleoside monophosphate plus phosphate-X (McLennan, 2006; Xu et al., 2006). *Arabidopsis* plants contain 29 Nudix hydrolases (Kraszewska, 2008). The enzymes are characterized by the conserved Nudix motif, $GX5EX_7REVXEEXGU$, where U represents a bulky hydrophobic amino acid such as isoleucine (lIle), leucine (Leu), or valine (Val) and X is any amino acid. The enzyme's substrates include (deoxy) ribonucleoside diphosphates and triphosphates, nucleotide sugars, coenzymes, dinucleoside polyphosphates, and RNA cap structures (Ogawa 2008).

Arabidopsis Nudix hydrolase1-11 (AtNUDX1-11) is a subcellular protein and localized in the cytosol (Ogawa *et al*., 2005, Yoshimura *et al*., 2007). Adams-Phillips *et al*. (2008) has shown that the *Arabidopsis nudix7*-knockout mutant allow less growth of a virulent pathogen and exhibit a reduced HR phenotype. Bartsch *et al*. (2006) reported that AtNUDX7 exhibits a

negative regulatory effect on ENHANCED DISEASE SUSCEPTIBILITY1 signaling, which controls defense activation and programmed cell death conditioned by intracellular Toll-related immune receptors that recognize specific pathogen effectors. Furthermore, AtNUDX7 hydrolyzes (NADH) to ADP-Rib as a physiological substrate and functions in modulation of the defense response to prevent excessive stimulation during both biotic and abiotic stresses (Ge *et al*., 2007). Adams-Phillips *et al*. (2010) reported that the cellular levels of ADP-Rib polymer increased after infection with avirulent *P.st* DC3000 *avrRpt2* with the observation of pathogen-dependent changes in the poly(ADPribosyl)ation of discrete proteins. This indicates that poly (ADP-ribosyl)ation is a functional component in plant responses to biotic stress. Recently, Olejnik et al., (2011) found that AtNUDT7 interacts with the regulatory protein RACK1A, AtNUDT7-interactor, both *in vitro* and *in vivo*. Furthermore, in plant protoplasts, both RACK1A and At- NUDT7 associate with AGG1 and AGG2, the gamma subunits of the heterotrimeric G protein signalling complex.

A hypothetical biosynthesis pathway has been proposed by N. Smirnoff (unpublished; Fig. 3.1). Putative genes in this pathway that were significantly differentially regulated by DC3000 compared to the DC3000*hrp* are potential targets to knockout to see if 400 synthesis is perturbed.

This chapter focuses on attempts to understand the role of 3'-O-β-ribosyl adenosine (monophosphate) biosynthetic pathway, in particular their role during *Arabidopsis*/DC3000 infection and plant defense responses. To this end Smirnoff's hypothetical biosynthetic pathway was investigated by using T-DNA insertions informed by gene expression of *Pst* DC3000.

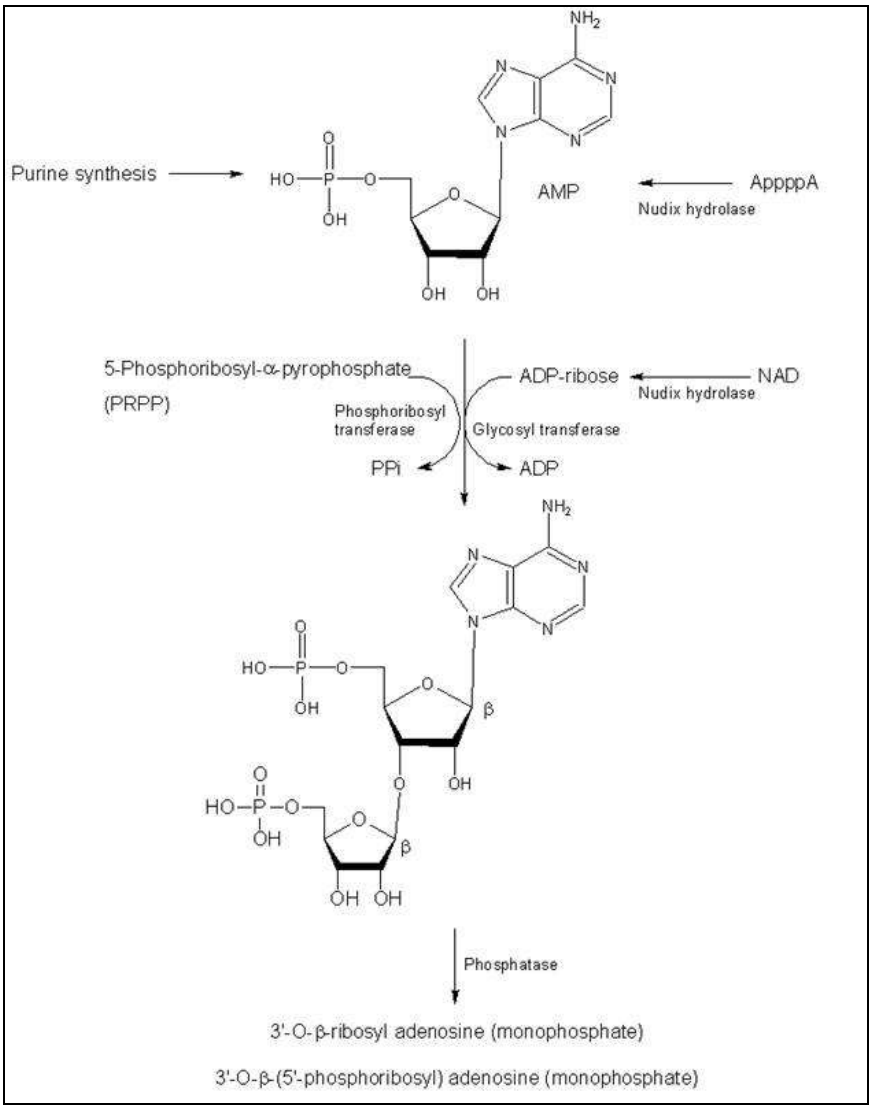


Figure3.1 Hypothetical biosynthesis pathway of 3'-O-β-ribosyl adenosine (monophosphate); the pathway proposed by Nicholas Smirnoff (unpublished data) suggests that this compound is the product of secondary metabolites which play important role in the host-microbe interactions

3.2 Material and methods

3.2.1 Polymerase chain reaction (PCR)

A PCR for knock out genes predicted to be involved in "400" compound biosynthesis (Table3.1) was carried out as described in Chapter 2.2.2.

Table 3.1 List of the knockout genes, presumably involved in "400" biosynthesis, with origin, gene function and the designed primers

Seeds Name	Origin	Gene	Gene function	Primers
Nudix6.1	GK-158B10.01	At2g04450	APD-ribos diphos-phatase NAD binding / hydrolase	**FP:** ACGACATTCGATCATTCCTTG **RP:**TTTATCAAGCCATTGGGATTG
Nudix8.1	GK-344B12.01	At5g47240	Nudix hydrolase 8 (NUDT8)	**FP:**AAACCCTTATGCTTTCGCTTC **RP:**TGCTACAATTGGAGTGGGAAG
GT1	GABI_485D08	At4g15280	UDP-glucosyl transferase 71B5	**FP:**AAAAATCCAGTTTGGTTATTCACC **RP:**ACATCGATCATCGAGGAACAG
SRO14	GK-325B05.07	At5g62520	NAD+ADP ribosyltransferase	**FP:** TTTTCTTCAGCTCAAAGCCAG **RP:** GCTCTGTTTCGAAACATGAGC
UDP-GT.D	SALK_139804C	At2g28080	Glycosyltransferase family protein	**FP:**GCATGTGAATAATGTCAGGGG **RP:**TCATCTCCGAAGAAGATCCAC
UDP-GT.A	SALK_143394	At3g46670	UDP-glucoronosyl /UDP-glucosyl transferase	**FP:** ATGGTGTTCGATGAAATGCTC **RP:** CTTACACTCTTTGGCTGCAGC
UGT85A1.a	SALK_985899C	At1g22400	UDPglycosyltransferase/ transferase	**FP:**ACCTCCGGTTTATTCAGTTGG **RP:**AAGATCGGACTGGAAATTTGC
UGT1	SALK_059989	At1g05560	UDP-glucosyl transferase 75B1	**FP:**GACACGAGCTTGAAGAGGTTG **RP:**CTCAGTGGTTCATTGGATTCC
Glycosil .T	SALK_136059CI	At5g57500	Galactosyl-transferase family protein	**FP:**AATCGAAGCAAACACAAATGG **RP:**CTCAAAGACTCGGAAATTCCC
PRS3.A	SALK_142152C	At1g10700	ribose-phosphate pyrophosphokinase3	**FP:**AGATTCACACTTCAGTTGGCG **RP:**TGATGCAGCGATTACTGACTG
SRO2	SALK_030045AA	At1g23550	NAD+ ADP-ribosyltransferase	**FP:**TCAAACTTTCCTTTTCCCAGG **RP:**AATCGTAACGATCGTCGTGTC
Left Border (LB)- GABI Kat		5`-ATATTGACCATCATACTCATTGC-3` + Reverse primer		
Left Border (LB1.3)-SALK		5`-ATTTTGCCGATTTCGGAAC-3` + Forward primer		

3.2.2 Reciprocal crosses for generating mutant combinations

A single *Arabidopsis* mutant belonging to the same family, Nudix hydrolase and the Ribosyltransferase (*nudix6.1*, *nudix8.1* and *sro14*, *sro2* respectively), were reciprocally crossed to generate double homozygous plants as described in Chapter 2.1.3. The double cross was confirmed by PCR (Chapter 2.2.2) prior to further study.

3.3 Results

To prepare the bacterial growth samples in this study a tissue-Lyser (Qiagen) was used (as described in Chapter2.5) instead of a mortar and pestle. This extraction method raised the question; does the tissue-lyser affect the number of bacteria in plant tissue extractions? To answer this question, 5 week-old Col-5 plants were inoculated, 6 plants, three leaves per plant, with DC3000 (OD_{600} 0.0002). Then 4 dpi plant tissues were extracted in two ways; mortar and pestle or using a tissue-Lyser for 2 minutes, 30 seconds and 10 seconds time intervals (Fig.3.2)

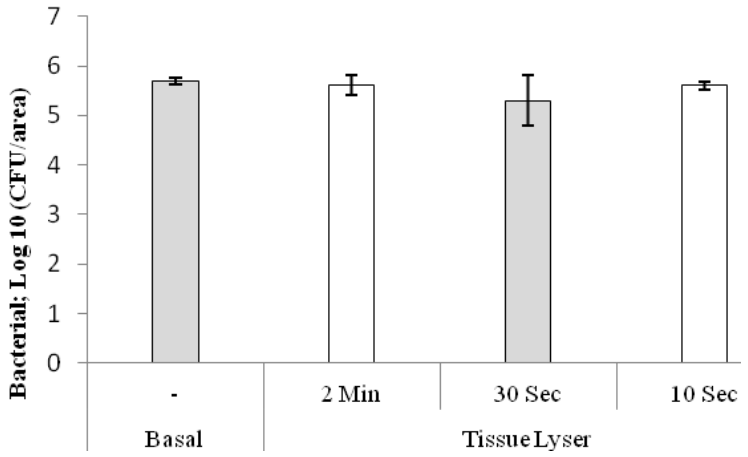


Figure1.2. Determination of DC3000 bacterial number in Arabidopsis challenged tissues. Two methods of extraction were used, mortar and pestle and tissue-lyser (2 min, 30 sec and 10 sec). Plants were challenged with DC3000 wild-type bacterial suspensions (OD_{600}: 0.0002 = 1 X 10^5 cfu ml^{-1}). Bacterial multiplication was determined at 4 dpi. Bars represent the mean of six biological replicates. Errors bars represent one standard deviation. The experiment was repeated twice.

As the statistical analysis shows no significant differences between both methods of bacterial growth analysis (Fig.3.2) reported. Thus since the tissue-Lyser machine saves time and effort this method was utilized in all experiments relating to bacterial growth determination analysis in this study.

Previous studies in the laboratory had identified 3´-*O*-β-D- ribofuranosyl adenosine (hereafter referred to as "400" compound) as the major discriminatory molecule induced very early in *Arabidopsis* leaves challenged with virulent *P.st* DC3000 (Unpublished data). Accordingly, further investigations in the role of the "400" compound biosynthetic pathway during *Arabidopsis*/DC3000 infection and plant defense responses were undertaken.

In this study, single homozygous plants of Nudix hydrolase (*nudix8.1* and *nudix6.1*), ribose-phosphate pyrophosphokinase3 (*PRS3.a*), ribosyltransferase *(sro.14* and *sro.2),* glucosyltransferase (*ugt85a1.a, udp-gt.a, udp-gt.d* and *gt1*) and galactosyltransferase (*glycosil.t*) were identified (as described in Chapter 2.2.2) and examined for any alteration in the phenotype and bacterial growth analysis using DC3000*pVSP61* [at the inoculum of OD_{600}: 0.002 (1 X 10^6 cfu ml^{-1}) and OD_{600}: 0.0002 (1 X 10^5 cfu ml^{-1}) respectively] to investigate whether any KO plants exhibited an increase or decrease in susceptibility. Three 5-6 week-old homozygous plants were challenged with DC3000 (0.002) for phenotype tests. The result revealed no significant differences in phenotype 4dpi was observed in any of the single mutants compared to Col-0. Additionally, bacterial growth analysis of 6 plants per genotype was challenged with DC3000 (0.0002) to determine the susceptibility and bacterial enumeration 4dpi of the single mutants (Table 3.1).

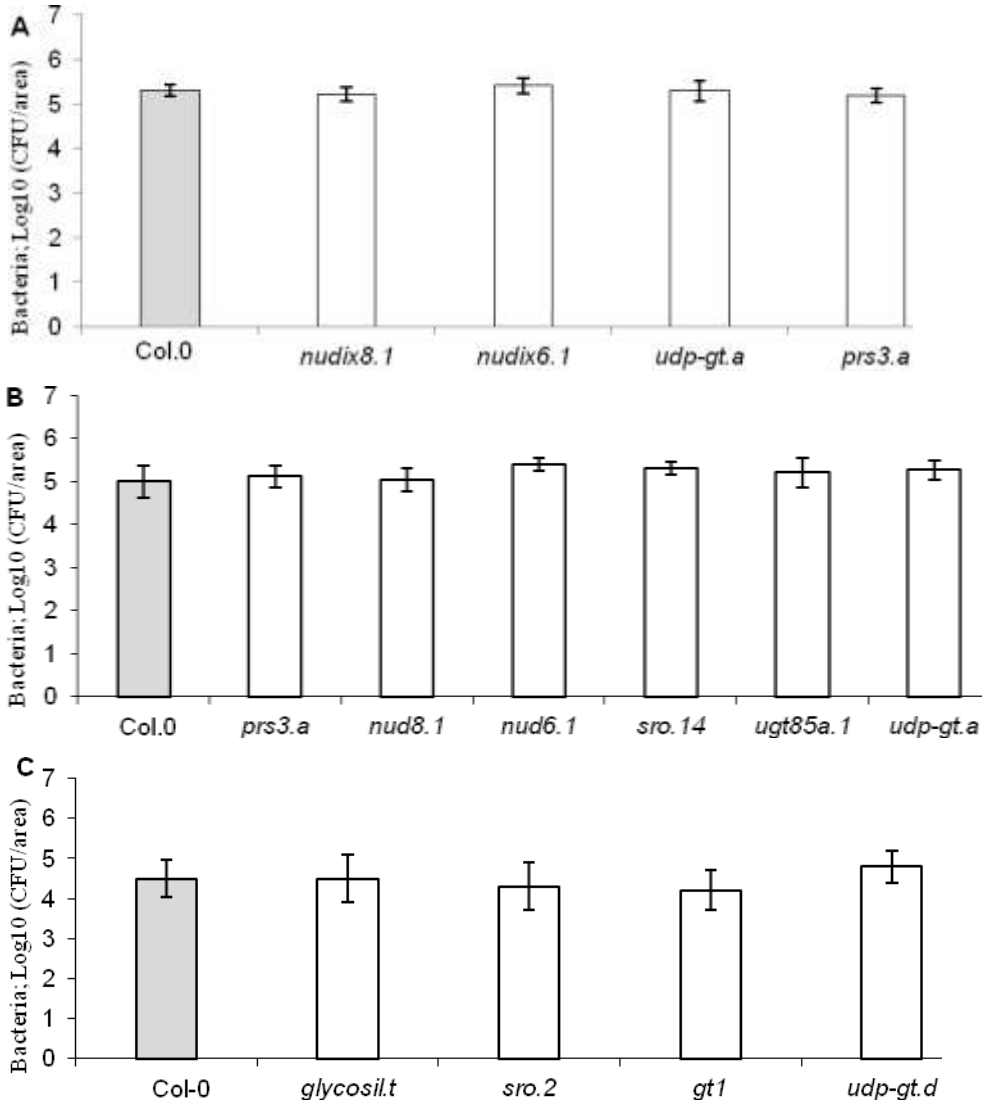


Figure 3.3 Bacterial growth of single knock outs of genes predicted to code for putative biosynthetic enzymes in the "400" biosynthetic pathway. (A) Col-0, Nudix hydrolase8 (*nudix8-1*), APD-ribos diphosphotase NAD binding / hydrolase (*nudix6-1*), glucosyl transferase (*udp-gta*) and ribose-phosphate pyrophosphokinase3 (*PRS3*). (B) Col-0, ribose-phosphate pyrophosphokinase3 (*prs3.a*), Nudix hydrolase8 (*nudix8-1*), APD-ribos diphosphotase NAD binding / hydrolase (*nudix6-1*), ribosyltransferase (*sr0-14)*, UDP glycosyltransferase/transferase (*ugt85a1a*), and glucosyl transferase (*udp-gta*). (C) Col-0, galactosyltransferase (*glycosil-t*), ribosyltransferase *(sro.2)*, UDP-glucosyl transferase 71B5 (*gt1*), glycosyltransferase *(udp-gt.d)*. Plants were challenged with DC3000 wild-type bacterial suspensions (1 X 10^5 cfu ml^{-1}). Bacterial multiplication was determined at 4 dpi. Bars represent the mean of six biological replicates. Errors bars represent one standard deviation. Experiments were repeated twice.

The bacterial growth analysis of the single mutants versus Col-0 4dpi did not show any statistical significant differences. The student t-test of bacterial growth assays of single mutants versus Col-0 are as follows (Fig.3.3.A-; 0.78519, 0.14115, 0.69487 & 0.99265, B-; 0.74313, 0.90665, 0.12024, 0.23542, 0.59732 & 0.26476 and C-; 0.85383, 0.50444, 0.14840 & 0.15873 respectively).

Therefore, further investigation in the role of the "400" compound biosynthetic pathway was carried out. Double mutant plants, *nudix6.1/ nudix8.1* and *sro14/sro2* were generated and confirmed for homozygousity by PCR (as described in Chapter 2.2.1-2). Then, the double mutant lines, *nudix6.1/ nudix8.1* and *sro14/sro2* were challenged with DC3000 to investigate phenotype and bacterial growth (OD_{600}: 0.002 and 0.0002 respectively). Five to six weeks post germination three plants of each double mutant were challenged with DC3000 (0.002) for phenotype development in comparison with Col-0 wild type plants. *nudix6.1/nudix8.1* and *sro14/sro2* did not display any significant differences in phenotype 4dpi with DC3000 (OD_{600}: 0.002) as previously shown by the single mutants of the nudix hydrolase and the ribosyltransferase. Additionally, alongside the phenotype, bacterial growth was also determined whereby *nudix6.1/nudix8.1* and *sro14/sro2* were inoculated with low inoculum of DC3000 (OD_{600}: 0.0002), this was conducted on 6 plants per genotype 4dpi.

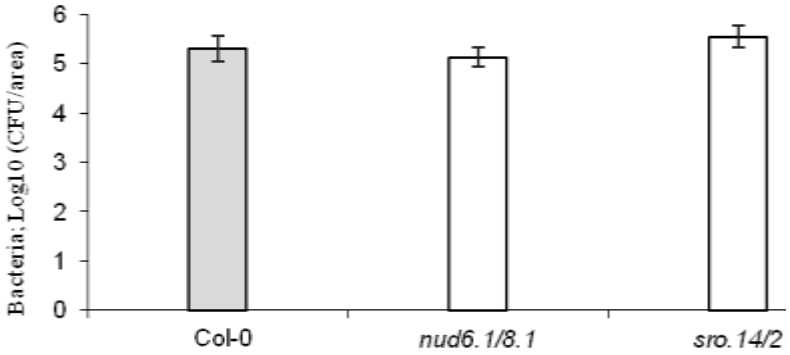


Figure 3.4 Determination of bacterial growth on *nudix6-1/nudix8-1* and *sro.14/sro.2* double mutants. The plants were challenged with DC3000 wild-type bacterial suspensions (OD_{600}:0.0002; ~1 X 10^5 cfu ml^{-1}). Bacterial multiplication was determined at 4dpi. Histograms represent the mean of six biological replicates. Errors bars represent one standard deviation. Experiments were repeated twice with the same results.

The bacterial growth analysis of *nudix6.1/nudix8.1* and *sro14/sro2* mutants revealed that there was no statistical significant differences observed between the double mutants and Col-0 [student t.test of Col-0 vs. *nudix6.1/ nudix8.1* and *sro14/sro2* are 0.30882 and 0.14883 respectively (Fig. 3.4)].

3.4 Discussion:

In this part of study, the role of 3'-O-β-ribosyl adenosine biosynthetic pathway during *Arabidopsis*/DC3000 infection and plant defense responses was investigated. Previous studies have shown that essential changes associated with DC3000 challenge, such as the study conducted by Truman *et al*., (2006), occur in host transcriptional between 4 and 12 h post-infection (hpi). Truman also interrogated the existing microarray databases reporting DC3000 infection compared to DC3000*hrp* to identify genes – often members of a gene family - that encoded predicted components of 400 synthesis. Truman found that these components are differentially expressed – induced – between DC3000 and DC3000 *hrp* challenges. Moreover, clear differences in the metabolome, particularly in phenolic and indolic compounds were reported by Ward et al. (2010) in *A. thaliana* leaves infected with virulent *P. syringae* 8hpi. Rapid changes in the abundance of amino acids and other nitrogenous compounds were also identified. Additionally, Ward and colleagues have also illustrated that pathogens reconfigured host metabolism to provide the sustenance required to support exponentially growing populations of apoplectically localised bacteria.

3.4.1 Role of 3'-O-β-ribosyl adenosine during *Arabidopsis*/DC3000 infection

The investigation of the predicted "400" compound pathway revealed that the single mutants of components predicted to be involved in the synthesis of 400, *nud6.1*, *nud8.1*, *sr0.14*, *sro.2*, *prs3.a*, *glycosil.t*, *ugt1*, *ugt85a1.a*, *udp-gt.a*, *udp-gt.d* and *gt1* (Table3.1) and the double mutants *nud6.1/nud8.1* and *sr0.14/ sro.2* , were challenged with the virulent DC3000 4dpi. Neither single nor double mutants showed any changes in the host response, as evidenced by no significant differences being detected in leaf phenotype and bacterial growth in single, double mutants or Col-0.

These results from *nud6.1*, *nud8.1*, *sr0.14*, *sro.2*, *prs3.a*, *glycosil.t*, *ugt1*, *ugt85a1.a*, *udp-gt.a*, *udp-gt.d* and *gt1* and the double *nud6.1/nud8.1* and *sr0.14/ sro.2* mutants contrast with a recent

study observation where Adams-Phillips (2010) demonstrated that poly (ADP-Rib) glycohydrolase (PARG2) and a Nudix hydrolase active on ADP-Rib and NADH (NUDT7) were among a small group of less than 40 genes significantly up-regulated in multiple R/avr interactions between *A. thaliana* and *P.st* DC3000; in which the *nudt7* mutant plants were more resistant to virulent and avirulent *P.st* DC3000 (Bartsch et al., 2006; Jambunathan and Mahalingam, 2006; Ge et al., 2007; Adams-Phillips et al., 2008). Furthermore, *nudt7* plants also displayed a greatly reduced hypersensitive response to avirulent *P.st* DC3000 (Adams-Phillips et al., 2008). The ADP-Rib, generated by PARG, is rapidly degraded to AMP by certain nudix hydrolase (NUDT) enzymes, including *Arabidopsis* NUDT2 and NUDT7 (Ogawa et al., 2005). Adams-Phillips *et al*. (2010) observed an increase in cellular levels of ADP-Rib polymer increase after infection with avirulent DC3000 *Ps.t avrRpt2*, and pathogen-dependent changes in the poly (ADPribosyl)ation of discrete proteins suggests that poly(ADP-ribosyl)ation is a functional component in plant responses to biotic stress.

Thus, ADP-Rib-specific nudix hydrolases are thought to have multiple roles: they (1) reduce the high levels of toxic free ADP-Rib, (2) re-establish energy levels by supplying a source for ATP, and (3) contribute to NAD+ maintenance (Rossi et al., 2002; Ogawa et al., 2005, 2009; Ishikawa et al., 2009). As highlighted above, multiple groups have identified the impacts of *Arabidopsis nudt7* mutants on responses to pathogen (Bartsch et al., 2006; Jambunathan and Mahalingam, 2006; Ge et al., 2007; Adams-Phillips et al., 2008).

Poly (ADP-ribose) polymerase (PARP) cleavage has direct antimicrobial effects and can activate other plant defense responses. Moreover, PARPs use the coenzyme Nicotinamide adenine dinucleotide (NAD^+) as a substrate to catalyze ADP-Rib polymers on acceptor proteins (Hashida *et al*., 2009). Both, automodified PARP and poly (ADP-ribosyl)ated nuclear proteins affect chromatin structure, transcription, replication, and DNA repair processes through PARP-mediated recruitment of other proteins (Masson *et al*., 1998, Simbulan-Rosenthal *et al*., 1999, Ahel *et al*., 2009). Thus PARP has an important role in plants where it acts as a DNA damage sensor (Petrucco, 2003, Roldan-Arjona and Ariza, 2009).

From the overall results, it can be concluded that the predicted pathway for "400" compound proposed by N. Smirnoff in which the knocked out mutants (*nud6.1*, *nud8.1*, *sr0.14*, *sro.2*, *prs3.a*, *glycosil.t*, *ugt1*, *ugt85a1.a*, *udp-gt.a*, *udp-gt.d* and *gt1* (Table3.1) and the double mutants *nud6.1/nud8.1* and *sr0.14/sro.2*) in *Arabidopsis*/DC3000 infection and plant defense responses

suggests that (i) either an alternative biosynthetic pathway exists - which, consequently, is possibly responsible for the 3'-O-β-ribosyl adenosine biosynthesis and thus further investigation is required or (ii) an additional family redundancy exists among the components of the pathway for the synthesis of 3'-O-β-ribosyl adenosine "400 compound". However, the role of the '400' compound requires further investigation givens its rapid induction dynamics and the magnitude of the increase. Given "400" compound must be derived from energy precursors and its potential signalling capacity and impact pn the energy balance in the cell, its differential increase would be expected to have consequences for the host.

Chapter 4: The role of high light stress in plant pathogen interactions

4.1 Introduction

Environmental conditions change dramatically during the plants life cycle. These changes impact on germination, growth and plant development. Abiotic stresses include salinity, drought and high light each will be perceived by the plant and unique stress specific responses elicited (Hasegawa *et al*., 2000). Numerous studies have been shown that a large number of genes respond to drought, cold and high salinity stresses at the transcriptional level (Lee *et al*., 2005) meaning it is challenging to gain a mechanistic understanding of the contribution of these components to the specific stress. Umezawa *et al* (2006) analyzed the function of stress-inducible genes to understand the plants molecular mechanisms of stress tolerance and responses and used this knowledge to attempt gene manipulation to improve crops for stress tolerance. The processes of plant growth and development are dependent on their response to optimal environmental factors one of which is the light environment. Plants respond to light by increasing their ability to capture light (Ballaré, 1999). During daylight, plant leaves are partly absorbing and transmitting incident light (Campbell and Norman, 1998). Moreover, plant responses to light are designed to maximise light availability by mediating a host of leaf photoreceptors including phytochromes (red/far-red-light–absorbing) and cryptochromes (blue/UV A light–absorbing) through morphological rearrangements such as stem and petiole extension (Bailey *et al*., 2001, Quail *et al*., 1995, Neff *et al*., 2000, Briggs and Olney, 2001).

4.1.1 Plant photoreceptors:

In the plants three classes of photoreceptors perform characteristic photosensory and/or physiological functions (Quail, 2002, Goh, 2009). These photoreceptors involved in light signalling include UV-B photoreceptors, cryptochromes 1 (CRY1), phytochromes (PHY) and phototropins (phot). Wade *et al* (2001) has shown that the *Arabidopsis* transcription factors LONG HYPOCOTYL 5 (HY5) and phytochrome interacting factor 3 (PIF3) positively regulate anthocyanin biosynthesis by directly binding to the promoters of the anthocyanin genes such as *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone-3-hydroxylase* (*F3H*), *dihydroflavonol reductase* (*DFR*), *flavanone-3`-hydroxylase* (*F3`H*) and *leucoanthocyanidin dioxygenase* (*LDOX*). In addition, it has been shown that phototropins (phot) are required for

chloroplast gene expression in rice plants; phot1 and phot2 mediated chloroplast movement to blue light (BL). Mutation of rice phot1a displayed light-induced responses resulting in H_2O_2-mediated damage to chloroplast photosystems, suggesting that phot-regulated responses are associated with cryptochrome (CRY) blue light (BL) receptor (Goh, 2009). Boccalandro *et al* (2009) showed that *phyA* and *phyB* mutant backgrounds caused significant reductions of non-guard epidermal cell density. However, while, *Arabidopsis phyA* mutants display a WT adult phenotype in white and red, *phyB* deficient mutant displayed a reduction in stomatal index (SI) at higher photon irradiances of both white and red (Boccalandro *et al*., 2009, Casson *et al*., 2009) and also showed significantly elongated petioles, reduced leaf area, and increased apical dominance (Franklin and Quail, 2010).

4.1.2 Abiotic stress and Plant phytohormones

Plant hormones play important roles in many developmental processes, mediating abiotic and biotic stress responses, and also have essential roles in plant immunity (Adie et al., 2007, Robert-Seilaniantz et al., 2007, Robert-Seilaniantz et al., 2011). These signalling molecules are present at very low concentrations under physiological conditions. Often plants are able to survive extreme environmental fluctuations through the expression of diverse stress-responsive genes. Many of these genes also affect growth rate or reproductive success, so they are generally inactive until required.

4.1.2.1 The role of ABA in plant response

ABA is a ubiquitous hormone that exists in both lower and higher plants and is synthesized from carotenoids. As a consequence, a wide range of genes encoding enzymes involved in the biosynthesis pathway has been characterized (Nambara and Marion-Poll, 2005, Millar *et al*., 2006). The zeaxanthin is gradually converted to violaxanthin in the plastid and the final steps of ABA biosynthesis occur in the cytosol (Fig. 4.1) (Xiong and Zhu, 2003)

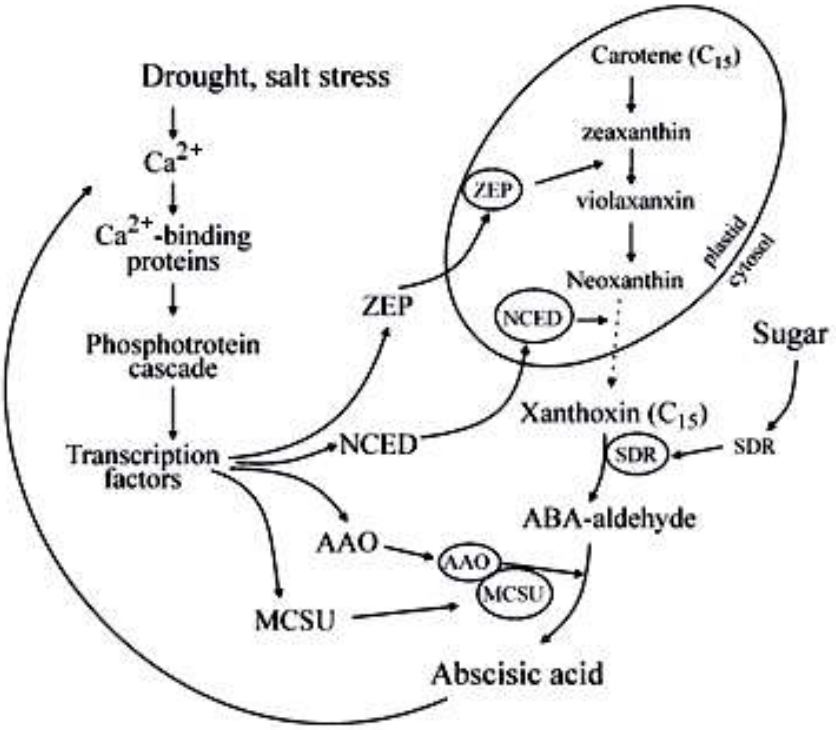


Figure 4.1 ABA biosynthesis regulation. Dashed line indicates the *9-cis* epoxycarotenoid dioxygenase (NCED) step which probably limits ABA biosynthesis in leaves. Zeaxanthin epoxidase (ZEP), *9-cis* epoxycarotenoid dioxygenase (NCED); ABA-aldehyde oxidase (AAO); MoCo sulfurase (MCSU); short-chain alcohol dehydrogenase/reductase (SDR). Adapted from Xiong and Zhu (2003)

ABA is known to be involved in regulation of many processes in plants such as seed germination, embryo maturation, stomatal aperture, leaf senescence and adaptation to environmental stresses (Wasilewska *et al*., 2008). In addition, ABA regulates many genes that preferentially expressed during drought stress in stomatal guard cells (Seki *et al*., 2002). ABA

has a major role in adaptation to environment change conditions through its actions in retaining water by closing stomata during drought - and other abiotic stresses (Endo *et al*., 2008). ABA signalling positively impacts on abiotic stress tolerance by inducing the expression of genes encoding proteins in order to protect the cells of plant seeds or vegetative tissues from damage under dehydration conditions (Bright *et al*., 2006).

However, de Torres *et al* (2007) have been shown that virulent *Pseudomonas syringae* hijack ABA signalling to promote virulence and *in planta* expression of the *P. syringae* effector *AvrPto* induces ABA synthesis and promotes bacterial virulence. Galvez-Valdivieso (2009) showed that in high light, ABA synthesized in the vascular parenchyma activates a signalling network in BSCs (Bundle sheet cells) leading to a redox-retrograde signal emerging from the BSC chloroplasts. Thus ABA is required for adaptation of levels to fluctuating HL conditions, linking to photochemical quenching – the mechanism to dissipate excess excitation energy.

A range of ABA receptors have been identified such as flowering time control protein A (FCA) (Razem *et al*., 2006), ABA receptor (ABAR) similar to H subunit of Mg-chelatase (CHLH) (Shen *et al*., 2006), G protein-coupled receptor (GCR2) (Liu *et al*., 2007) and ABA receptors, intracellular proteins, referred to as PYR, PYL, and RCARs [(**Py**rabactin **r**esistance, **py**rabactin resistance-**l**ike and **r**egulatory **c**omponent of **A**BA **r**eceptor) (Ma *et al*., 2009, Park *et al*., 2009)]. Overall, ABA has been shown to be act as a negative regulator in plant defence against a wide range of biotrophic and necrotrophic pathogens (Bari and Jones, 2009a), although in some cases it can be act as a positive regulator of plant defense so the outcome can be quite pathogen specific (Robert-Seilaniantz et al., 2011)

4.1.3 Interaction between Clade A PP2C's and the PYL family of ABA receptors

Recently, Rubio *et al* (2009) generated two triple mutant protein phosphatases type 2C lines, *hab1-1abi1-2abi2-2*, and *hab1-1abi1-2pp2ca-1*. Both mutants showed strong response to exogenous ABA, emaciated growth and an incomplete constitutive response to endogenous ABA. Microarray studies revealed a partial up-regulation/down-regulation of a subset of ABA-responsive genes in both triple mutants in the absence of exogenous ABA (Rubio *et al*., 2009). Similarly, Santiago *et al* (2009) simultaneously over-expressed HAB1 (a clade A protein phosphatases type 2C) and PYL5 (pyrabactin resistance 1-like 5). Transgenic lines of PYL5 showed an enhanced response to ABA demonstrating that PYL5 antagonizes HAB1 function

suggesting that PYL5 and their protein family inhibited HAB1, ABI1 and ABI2 phosphatase activity in an ABA-dependent manner (Santiago *et al*., 2009).

4.1.3.1 The role of SA in plant responses

In *Arabidopsis* plants the accumulation of SA is light-dependent (Genoud *et al*., 2002, Karpinski *et al*., 2003, Zeier *et al*., 2004). Similarly, other studies on the SA-mediated induction of the hypersensitive response (HR) showed that light and a chloroplastic factor are required to generate a ROS signal (Genoud *et al*., 2002, Mateo *et al*., 2004). Therefore, conditions that enhance an increase in excitation energy may be part of a signal that controls SA accumulation under several abiotic stress conditions (Borsani *et al*., 2001, Munné-Bosch and Peñuelas, 2003, Chini *et al*., 2004, Scott *et al*., 2004, Clarke *et al*., 2004). One of these factors could be the impact that increases in excess excitation energy (EEE) may have on the cellular glutathione pool and thus its redox state, which in turn might impact on the functioning of NON-EXPRESSOR OF RELATED PROTEIN 1 (NPR1). NPR1 has been shown to be redox sensitive *in vivo* and *in vitro* (Mou *et al*., 2003). Congruent with these observations, plant phytochrome signalling is involved in the SA-mediated induction of defence gene expression and HR spread (Genoud *et al*., 2002). In addition, Bechtold *et al* (2005) showed that, while light was a requisite for induction of defence genes, functional chloroplasts were not, but rather functional chloroplasts were essential for the transmission of HR lesions. The requirement for functional chloroplasts clarified that there are two levels of control over the active SA pathway that involve the light environment of the leaf (Genoud *et al*., 2002, Mateo *et al*., 2004, Bechtold *et al*., 2005). In the first of these two levels, light was an absolute requirement for the induction of the defence genes; whereas functional chloroplasts were not. The second level, in contrast, functioning chloroplasts were required for the propagation of HR lesions (Genoud et al., 2002, Mateo et al., 2004, Bechtold et al., 2005).

In plant defence responses SA, JA and ET are three phytohormones known to play fundamental roles in regulation of plant defence responses against plant pathogens organisms, pests and abiotic stresses such as wounding (Glazebrook, 2005, Lorenzo & Solano, 2005, Broekaert *et al*., 2006, Loake & Grant, 2007, Balbi & Devoto, 2008). However, and although, there is mutually antagonistic between SA and JA/ET defence pathways, evidence of synergistic interactions have

also been reported (Schenk *et al*., 2000, Kunkel and Brooks, 2002, Beckers and Spoel, 2006, Mur *et al*., 2006 (Leon-Reyes et al., 2010, Mur et al., 2012).

4.1.3.2 Mediating of SA signalling in plant

ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and its interacting partner, PHYTOALEXIN DEFICIENT 4 (PAD4), compose a regulatory core that is important for basal resistance to the invasion of biotrophic and hemi-biotrophic pathogens (Rusterucci et al., 2001, Wiermer et al., 2005). EDS1 and PAD4 are also conscripted by Toll-Interleukin-1 receptor (TIR)-type nucleotide binding-leucine rich repeat (NB-LRR) proteins to signal isolate-specific pathogen recognition (Wiermer et al., 2005). EDS1 and PAD4 were essential components in the entire *LESION SIMULATING DISEASE 1* (*lsd1*) photooxidative-stress phenotypes, including stomatal closure. (Kunkel and Brooks, 2002, Dong, 2004, Mateo *et al*., 2004, Wiermer *et al*., 2005). Notably, the *lsd1* mutants cannot acclimate to excess EEE created by photosynthesis in high light, causing ROI overload and eventually cell death due to photooxidative stress. The *lsd1* mutants showed some defects, notably reduced stomatal conductance and reduced peroxisomal catalase activity that both lead to increased ROI (Kunkel and Brooks, 2002, Wiermer *et al*., 2005). Previously EDS1 was shown to function in an early stage of plant defence independently of PAD4, whereas, PAD4 is involved in the amplification of defenses by direct interaction with EDS1 (Feys *et al*., 2001). Furthermore, it has been shown that *Arabidopsis* EDS1 has another related protein, Senescence Associated Gene101 (SAG101). The interaction between EDS1 and their two related proteins PAD4 and SAG101 resulted in combined activities that are necessary for defense signalling. Interestingly, in *Arabidopsis* leaf tissues there are differences in size and intracellular distributions of EDS1–PAD4 and EDS1–SAG101 complexes but they don't show any redundant functions (Feys *et al*., 2005, Rietz *et al*., 2011).

4.1.4 Photosynthesis and high light stress:

High light (HL) is one of the major environmental factors impacting upon the life of plants and it is an essential component that determines plant survival (McNellis and Deng, 1995). Changes in the intensity of incident light or spectral quality will frequently lead to imbalanced excitation of photosystem I (PSI) and photosystem II (PSII). This can occur either under shade or light limiting conditions (Dietzel *et al*., 2008). Notably, the ABA biosynthetic pathway in plants such

as tomato, maize or *Arabidopsis* exposed to high light display a reverse reaction (as shown in Chapter 1, Fig. 1.2) in chloroplasts where violaxanthin is converted back to antheraxathin by violaxanthin de-epoxidase (VDE) (Nambara and Marion-Poll, 2005). Furthermore, Maruta *et al* (2012) showed that the chloroplast has synergistic and antagonistic roles on its H_2O_2 in HL response due to the negative effect of thylakoid membrane-bound ascorbate peroxidase (tAPX) silencing on the expression of ROS-responsive genes under HL. Thus, Maruta provide new insight into the role of H_2O_2-triggered retrograde signaling from chloroplasts in the response to such stress plants.

In addition, in higher plants, the early light-induced proteins (ELIPs), nuclear-encoded, light stress-induced proteins located in thylakoid membranes, have been shown to be related to light-harvesting chlorophyll (LHC) *a/b*-binding proteins. The ELIPs showed a photoprotective function in plants. The expression of ELIP1 and ELIP2 transcripts in *A. thaliana* was differentially regulated in green leaves exposed to high light. Furthermore, the accumulation of ELIP1 transcripts and proteins increased almost linearly with increasing light intensities and correlated with the degree of photo-inactivation and photo-damage of PSII reaction centres (Heddad *et al*., 2006). However, plants under abiotic and/or biotic stresses resulted in accumulation of flavonoids as a hallmark of plant stress responses (Pietta, 2000, Borges et al., 2010).

4.1.5 Flavonoids:

Flavonoids are secondary metabolites that play important roles in signalling and plant adaptation to unfavourable environmental conditions and participate in plant defence by acting as antioxidants or screening out UV-B (Einbond *et al*., 2004, Beekwilder *et al*., 2005, Bieza and Lois, 2001). In plant flavonoids are accumulate in response to many types of oxidative stresses such as UV radiation, high light, environmental pollutants, pathogen infections (Shirley, 1998, Shirley *et al*., 1995, Borges *et al*., 2010, Pietta, 2000). Anthocyanins and flavonols are synthesized from phenylalanine and malonyl-CoA through one of two pathways, the malonate or shikimate pathways (Winkel-Shirley, 2002). Of the flavonoid compounds, 3-O-glycosides are common and are important free radicals scavengers (Delazar *et al*., 2010) and they contribute to various pigmentation in plants (Welch *et al*., 2008, Vanderauwera *et al*., 2005, Nomura *et al*., 2009, Kim *et al*., 2008). Anthocyanin is the most well known member of flavonoid. It's largely

present in flowers and fruits, with particularly high levels found in berries and grapes (Borges *et al*., 2010). There are interrelationships between developmental, environmental, and metabolic signal transduction pathways control the production of flavonoids. Often anthocyanin biosynthesis is observed in plants germinated or grown on a sugar containing medium. For instance Arabidopsis plants grown on Suc-containing medium showed high levels of anthocyanins (Tsukaya et al., 1991; Ohto et al., 2001, Baier et al., 2004). The chalcones synthase (*CHS*) gene derived from petunia (*Petunia hybrida*) petals in transgenic *Arabidopsis* leaves was induced by sugars (Tsukaya et al., 1991), whereas petunia corollas cultured *in vitro* without sucrose (Suc) do not show any pigmentation (Weiss, 2000). Petunia and Arabidopsis *CHS* genes are characterized by the presence of Suc boxes in the 5`-flanking regions such as those found in the Suc-inducible sporamin and amylase genes (Tsukaya et al., 1991). Recently, it has been shown that ethylene production is stimulated by Suc and light in Arabidopsis and thus Suc appears to regulate ethylene production as well as anthocyanin biosynthesis, since a mutation in the SUC1 transporter resulted in a partial reduction in both processes. Therefore, Sugar signalling seems to interfere with the ethylene suppression of anthocyanin accumulation, as observed with Glc repression of ethylene signalling, which mediates seed germination and seedling development (Zhou et al., 1998, Cho et al., 2010).

In plants, it has been reported that anthocyanins are glycosylated at different positions or conjugated with malonate, coumarate or sinapate. Their accumulation is induced by various factors some of which are high light (Giacomelli *et al*., 2006, Page *et al*., 2011) or hormones such as ABA and JA (Loreti *et al*., 2008, Shan *et al*., 2009). During photosynthesis, anthocyanin plays an important role during periods of excessive light by effective protection of plants from photoinhibition by reducing absorbed light (Markham *et al*., 2000, Giacomelli *et al*., 2006). Anthocyanin also plays a role in plant defense, notably anti-herbivore defence and plant protection from UV-B radiation (Li *et al*., 1993, Karageorgou and Manetas, 2006). As a consequence, recent studies showed strong evidence that anthocyanin accumulation in leaves protects against photoinhibitory damage caused by high irradiance (Zeng *et al*., 2010, Gould *et al*., 2010).

In preliminary stress response experiments we found that HL increase Col-5 plants susceptibility to virulent DC3000. This was despite the fact that HL stress also caused a strong accumulation of anthocyanin causing the plant leaves to become dark red (purplish), brittle and inwardly curled.

These responses are often associated with enhanced defensive metabolites. We therefore wished to address whether abscisic acid (ABA) and salicylic acid (SA) were involved in the biotic-abiotic stress interaction by exposing ABA and SA hormone mutants to high light and measuring hormone levels and the pathogenic response phenotype to the virulent DC3000.

De Torres *et al* (2009) demonstrated that under normal conditions of ABA-deficient (*aao3*) and SA deficient (*sid2.1*) plants challenged with *P. syringae* the levels of SA and ABA, but not JA, play important early roles in determining, the outcome of the infection process. Based upon our observations under high light, we chose to examine the effect of relevant phytohormone mutants involved in plant defence responses under high light.

Table 4.4.1 List of flavonoid compounds identified in *Arabidopsis* by mass spectrometry (MS); (A) flavonol group. (B) anthocyanin group adapted from (Page *et al*., 2011).

Group A : Flavonols	
Flav. 1.	**Kaempferol** 3-O-rhamnoside
Flav. 2.	**Kaempferol** (pentoside)-rhamnoside
Flav. 3.	**Kaempferol** 3-O-rhamnoside 7-O-rhamnoside
Flav. 4.	**Kaempferol** 3-O-glucoside 7-O-rhamnoside.
Flav. 5.	**Kaempferol** 3-O-[6”-O-(rhamnosyl) glucoside] 7-O-rhamnoside
Group B : Anthocyanin	
Antho.1.	**Cyanidin** 3-O-[2”-O-(xylosyl) 6”-O-(p-coumaroyl) glucoside]5-O-glucoside.
Antho.2.	**Cyanidin** 3-O-[2”-O- (2”′-O-(sinapoyl) xylosyl) glucoside] 5-O- glucoside.
Antho.3.	**Cyanidin** 3-O-[2”-O-(xylosyl) 6”-O-(p-coumaroyl) glucoside]5-O-malonylglucoside.
Antho.4.	**Cyanidin** 3-O-[2”-O-(xylosyl) 6”-O-(p-O-(glucosyl)-p-coumaroyl) glucoside]5-O- glucoside.
Antho.5.	**Cyanidin** 3-O-[2”-O- (2”′-O-(sinapoyl) xylosyl) 6”-O- (p-coumaroyl) glucoside] 5-O- glucoside.
Antho.6.	**Cyanidin** 3-O-[2”-O-(xylosyl) 6”-O-(p-O-(glucosyl)-p-coumaroyl) glucoside]5-O-[6”′-O-(malonyl) glucoside]
Antho.7.	**Cyanidin** 3-O-[2”-O- (2”′-O-(sinapoyl) xylosyl) 6”-O- (p-O-coumaroyl)glucoside] 5-O-[6””-O-(malonyl) glucoside].
Antho.8.	**Cyanidin** 3-O-[2”-O- (2”′-O-(sinapoyl) xylosyl) 6”-O- (p-O- (p-O-(glucosyl)p-coumaroyl) glucoside] 5-O- glucoside.
Antho.9.	**Cyanidin** 3-O-[2”-O- (6”′-O-(sinapoyl) xylosyl) 6”-O- (p-O- (p-O-(glucosyl) -p-coumaroyl) glucoside] 5-O-(6””-O-malonyl) glucoside.

In this part of the study ABA-deficient; Arabidopsis aldehyde oxidase 3 *aao3* (de Torres-Zabala *et al*., 2007), the SA-deficient, Salicylic Acid Induction-Deficient/isochorismate synthase1 *sid2-1* (Wildermuth *et al*., 2001), the *aao3 sid2.1* double mutant (De Torres Zabala *et al*., 2009), a line over-expressing the ABA receptor *PYL5* (*PYL5-OE*; Santiago *et al*., 2009) and a triple mutant of the Clade A protein phosphatase 2C's "*hab1-1/abi1-2/abi2-1*" (Rubio *et al*., 2009) were selected to investigate their impact following the dual stress of high light (abiotic stress) and virulent DC3000 (biotic stress) challenges. As experiments progressed we also undertook preliminary experiments on other mutants we felt may be relevant affecting the high light signal; the phytochrome interacting factor (*PIF*) (*pif.i= phyA-211 phyB-9* and *pif.h= cry1 cry2),* and *enhanced disease susceptibility 1* (*eds1*) and its interacting partner, *phytoalexin deficient 4* (*pad4*) (Glazebrook *et al*., 1996)

4.2 Material and methods

The general material and methods used in this chapter for plant conditions, bacterial growth and bacterial infection are described in Chapter2 sections 2.1, 2.3-2.5

4.2.1 High light stress

Arabidopsis thaliana plants were grown under 23ºC, 65% relative humidity and light intensity 120µmol/m²sec^{-1} (NL; normal light), before being moved to high light (HL) cabinet. Two experiments were designed, the first was to subject 6 plants to HL (600 µmol/m²sec^{-1}) for 5 days before three leaves per plant were inoculated with DC3000 (OD_{600}: 0.0002) 4dpi. Then bacterial growth was determined under 9dHL/DC3000 compared to the control (9dNL/DC3000). The second experiment was to subject 12 plants to 5dNL pre-treatment and 12 plants to 5dHL pre-treatment. After inoculation; six plants were reciprocally swapped from HL pre-treatment to normal light (HL/NL), six plants were reciprocally swapped from pre-treatment NL to HL (NL/HL), six plants from HL pre-treatment were returned back to HL (HL/HL) and six plants from NL pre-treatment were returned back to NL (NL/NL). Plants trays were rotated every 24 hours to eliminate potential differences in total light intensity exposure and watered as needed.

4.2.2 Hormone and Flavonoid extractions

For LC-MS/MS analysis of hormones and flavonoid plant tissues were extracted from the same tissues that used for bacterial growth assay. From three replicates, two plants per replicate and three leaves per plant were harvested. The harvested tissues were wrapped in foil and frozen immediately in liquid nitrogen before being dried in freeze dryer (Heto PowerDry LL3000) at -60ºC for 2-3 days. The dried samples were ground in a 2 ml micro-centrifuge tube using a tungsten ball (3 mm) in a tissue lyser (Qiagen) for 2 min, frequency 25Hz/sec, before 10 mg of each sample was extracted in 400µl of 10% methanol 1% acetic acid containing internal standards; 1 µl $^{2}H_4$ SA (14.2 ng), 2 µl $^{2}H_2$ JA (10 ng), 4 µl (2 ng) $^{2}H_6$ ABA and 10 µl (14.4 µg/sample) unlabeled Umbelliferone-Sigma, UK (stock; H24003-10G, 3.6 mg/ml pH: 3.4). The samples were vortexed properly to mix all the powder before being kept on ice for 30 min during which they were vortexed 2-3 times. Then the samples were centrifuged at 4°C (13000 rpm) for 10 min and supernatants were collected in a separate clean eppendorf tube. Pellets were re-

suspended in 400µl extraction buffer (no internal standards added) and the procedures of extraction were repeated as in the first step. Supernatants from both the extractions were pooled, then filtered with a 4 mm PVDF syringe filter 0.45 µm (www.chromacol.com) prior to transfer to vials (03-FISV- C907 Chromacol Ltd).

4.2.3 Flavonoid and Hormones measurement

The protocol followed the extraction steps of Hormones and Flavonoid mentioned in section 5.2.2. The Hormones and flavonoid analyses were performed using an Agilent 6420B triple quadruple (QQQ) mass spectrometer (Technologies, Palo Alto, USA) as described by Page *et al* (2011). Ten microlitres of sample extract was loaded onto a Zorbax StableBond C18 1.8 mm, 2.1 x 100 mm reverse-phase analytical column (Agilent Technologies). Mobile phase A comprised 5% acetonitrile with 0.1% formic acid in water and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. The data were extracted using MassHunter software (Agilent Technologies). Theoretical *m*/*z* values, isotope abundances and product ion *m*/*z* values for the identified compounds are in brackets. MS/MS spectra were compared with ESI–QToF–MS/MS spectra of known compounds from the MassBank database (Horai *et al*. 2010; Page *et al*., 2011)

4.3 Results

In a controlled environment growth cabinet (Microclima 1000E; Snijders, Tilburg, the Netherlands), plants were exposed to long day periods (16 h light, 8 h dark) of high light intensity (600 µmol/$m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD). The control plant condition was placed in the same growth cabinet with a PPFD of 120 µmol/$m^{-2}s^{-1}$ for 5 days.

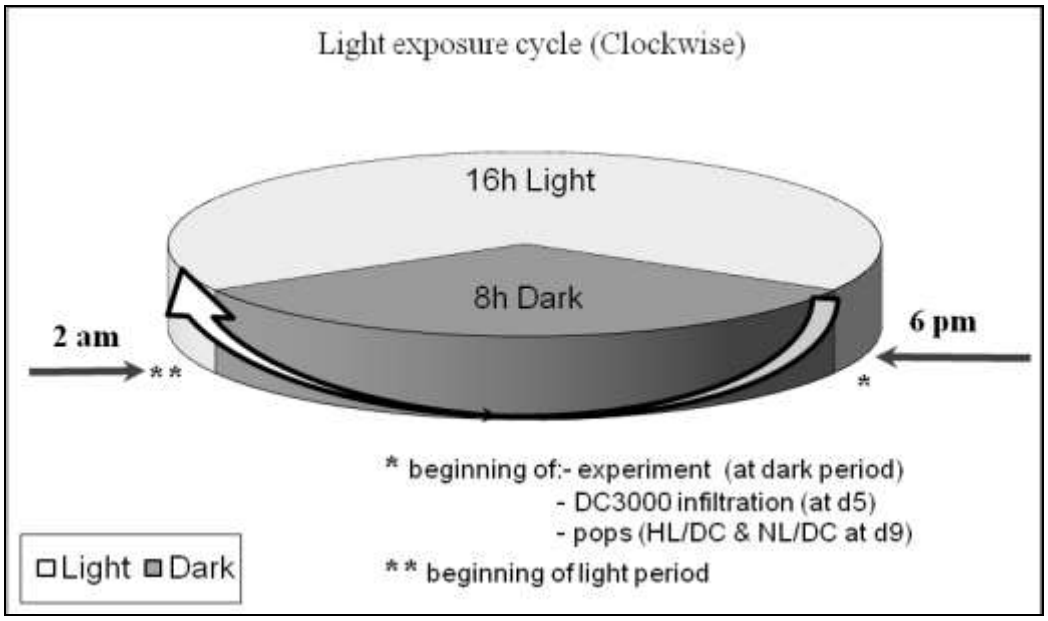


Figure 4.2 A high light exposure cycle. The exposure cycle shown clockwise was set up in a HL- cabinet. The experiment was initiated at the beginning of 8 h dark cycle, corresponding to 6 pm local time. DC3000 challenge was at the end of day five light cycle and bacterial growth determination was at the end of day nine light cycle, equating to 5d HL + 4d HL/DC3000.

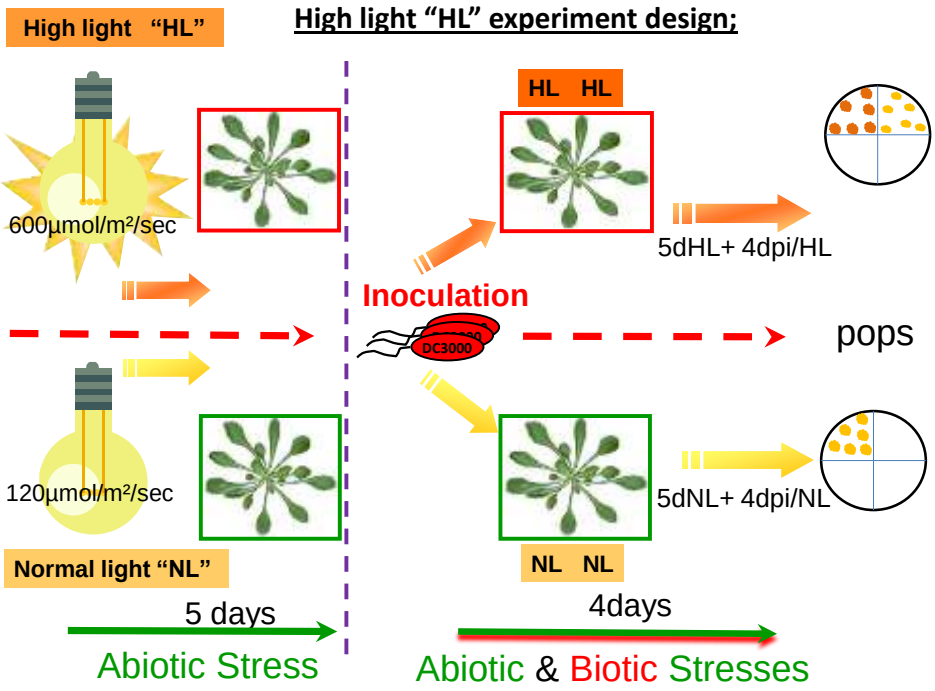


Figure 4.3 High light experiment design comprising two different treatments. From top left, plants under HL (600 µmol/$m^{-2}s^{-1}$) and bottom left under NL (120 µmol/$m^{-2}s^{-1}$) grown at 23°C and 65% humidity for 5 days followed by 4d-DC3000 infection/HL (top right) and 4d-DC3000 infection/NL (bottom right).

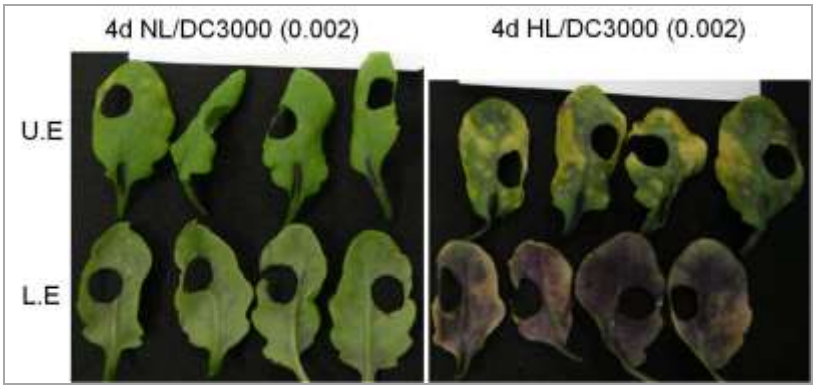


Figure 4.4 HL effect on Col-5 plant leaves. Plants after inoculated with DC3000 (OD_{600}: 0.002) 4dpi for phenotype. The photo shows the effect of high light on Upper Epidermis (U.E) and Lower Epidermis (L.E).

Visual differences between plants subjected to HL were obvious from day five under the intensity of 600 µmol m^{-2} s^{-1}, resulting in accumulation of anthocyanin compared to the cognate control plants under 120 µmol m^{-2} s^{-1}. The accumulation of anthocyanin was first initiated on the lower epidermis (L.E) and by the extended of HL period. The anthocyanin was diffused across to the upper epidermis (U.E) in parallel with development of chlorotic symptoms (Fig. 4.4).

4.3.1 Impact of high light on DC3000 infection in Col-5 phenotype and growth curve

Based on this experiment, samples from non-inoculated plants at day zero (on the day of transfer from growth room to HL cabinet), day 5 NL and HL, day 9 NL and HL alongside with d9NL- and d9HL- DC3000 (d9; 5d pre-treatment of NL or HL + 4d post-treatment of NL- or HL-DC3000). This experiment was primarily carried out to determine the response of Arabidopsis Col-5 to HL/DC3000 challenged. The obtained result showed that HL stress induces strong synthesis of anthocyanin causing the leaves become dark red (purplish) and brittle with a leathery texture. These symptoms are classically associated with accumulation of defensive metabolites. However, bacterial growth determination in 6 replicates plants/treatment showed that HL increased bacterial growth by $\log_{10}$ compared to plants grown under low light.

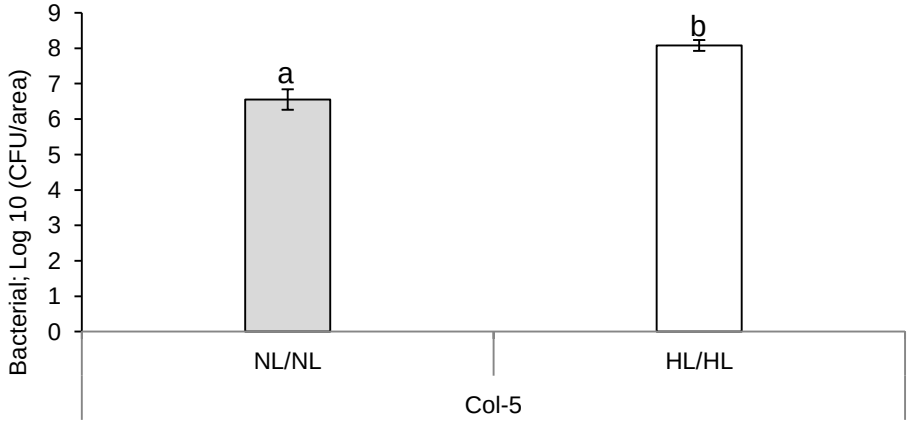


Figure 4.5 Effect of combined light stress and DC3000 infection on *Arabidopsis* Col-5 plants. HL plants were first subjected to 600µmol/m^{-2} s^{-1} (HL) for 5 days, then challenged with DC3000 (OD_{600}: 0.0002) then returned to HL for 4 days. HL plants supported approximately 1.5 $\log^{10}$ more bacterial growth than the infected NL (120 µmol m^{-2} s^{-1}) control plants. Bacterial multiplication was determined at 4dpi. Bars represent the mean of six biological replicates, three leaves per replicate. Errors bars represent one standard deviation. Labels "a, b etc." above the columns discriminate the differences at a significance $p<$ 0.05 (Student's t-test for pairwise comparison of non-treated and treated plants) Experiments were repeated at least three times with similar results.

This result raised a question to further investigate. That is "*Is the change in phenotype due to pre- or post-treatment of light?*" Therefore, we designed another experiment (see schematic in Fig. 4.6) involving reciprocal swaps of pre-treated plants after inoculation. a) 12 plants, 6 plants in group A and 6 plants in B, were subjected to 5d HL then challenged with DC3000 (OD_{600}:0.0002). Then plants in group A were returned to HL (HL/HL) and plants in group B were placed under "normal" conditions (HL/NL). b) 12 plants grown for 5 days under normal condition (120 µmol m^{-2} s^{-1}) (6 plants in group 1 and 6 plants in 2) were inoculated with DC3000 (OD: 0.0002) then 6 plants were transferred to HL and 6 returned to NL (Fig. 4.6).

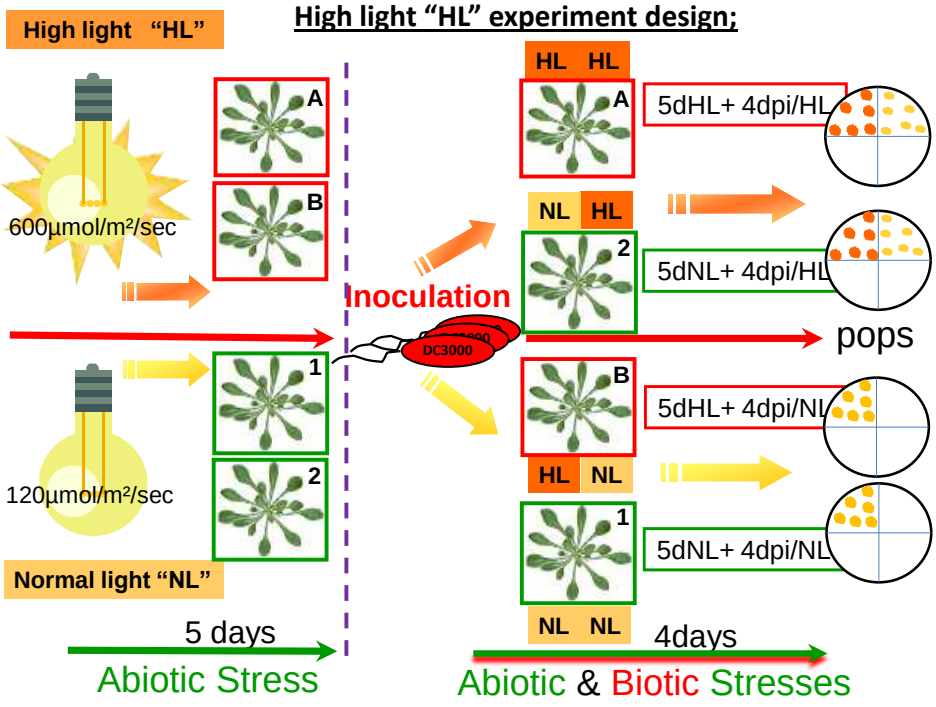


Figure 4.6 HL experimental design comprising four different treatments. Top left: 12 plants were grown under 600 µmol/m^{-2}/s^{-1} PPFD and 12 plants were grown under 120 µmol/m^{-2}/s^{-1} (NL) at 23°C and 65% relative humidity. Plants were inoculated five days later with DC3000 (OD_{600}: 0.0002). After inoculation six plants (A & B under HL; 1 & 2 under NL) were reciprocally swapped resulting in HL containing plants A from pre-treatment HL and plants 2 from pre-treatment NL. In NL plants 1 were from pre-treatment NL and plants B from pre-treatment HL. Plants trays were rotated every 24 h to eliminate potential differences in total light intensity exposure and watered as needed. Bacterial growth was determined 4dpi.

We verified that plants under continuous periods of HL (HL/HL) and plants initially grown in NL then subjected to post treatment of HL (NL/HL) displayed the same phenotype at the conclusion of the experiment; brittle leaves dark red (purplish) in colour, inward rolling and with a leathery texture (Fig. 4.7.A).

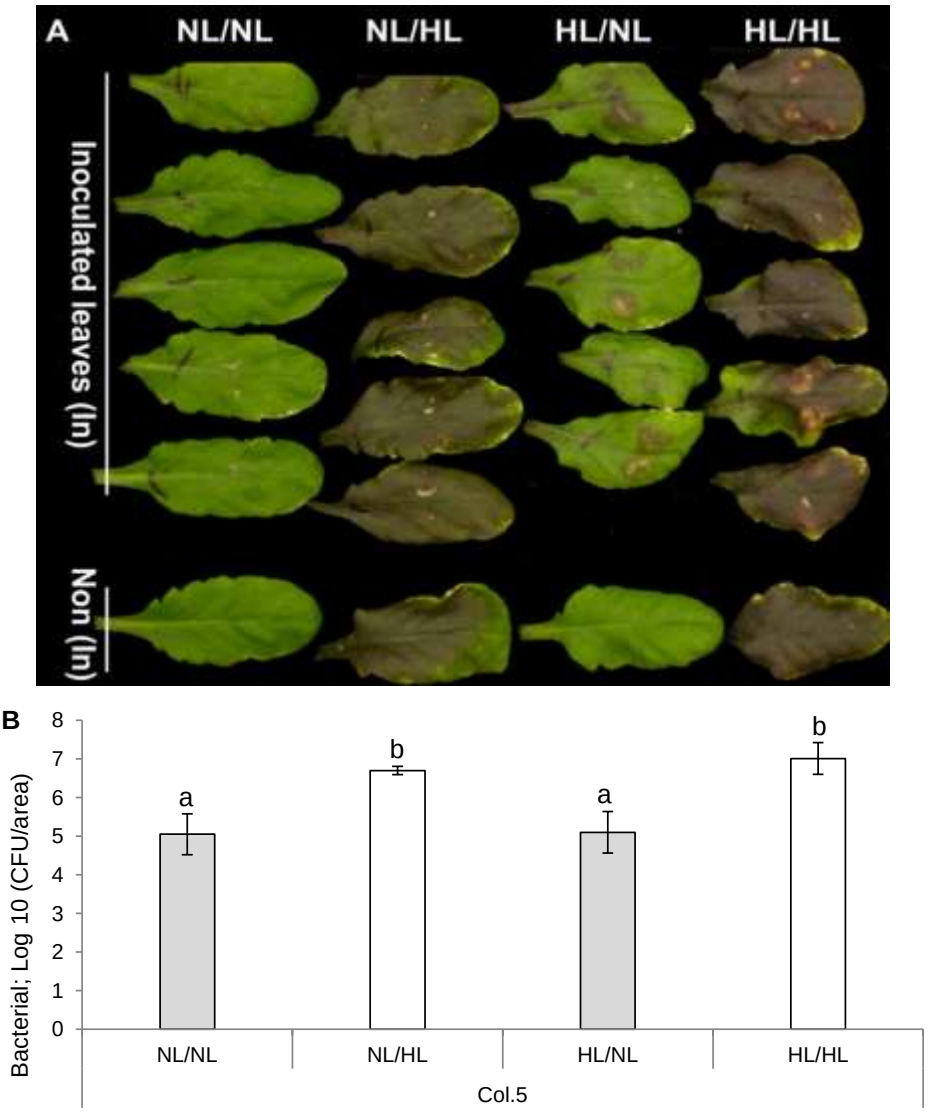


Figure 4.7 Effect of combined light stress and DC3000 infection on *Arabidopsis* Col-5 plants: Plants subjected to HL or NL for 5 days followed by 4dpi of DC3000 low inoculum (OD_{600}: 0.0002). (A) Effect of light treatment on Col-5 plant leaves subjected to either 5d- NL or -HL post-treatment (NL/HL or HL/NL) or 5d continuous HL period (HL/HL) with control continuous NL (NL/NL) followed by 4d of DC3000 infection. (B) DC3000 multiplication Col-5in challenged leaves 4dpi with DC3000. Each treatment represents the mean of six biological replicates. Labels "a, b etc." above the columns discriminate the differences at a significance $p < 0.05$ (Student's t-test for pair wise comparison of non-treated and treated plants). Errors bars represent one standard deviation. Experiments were repeated at least three times.

We found that Col-5 plants grown under continuous HL-challenged (HL/HL) or post-treatment HL-challenged (NL/HL) showed statistically significant increases in DC3000 bacterial growth (t.test; 0.00009, 0.39529, 0.00019 respectively) compared with the control NL-challenged (NL/NL) or plants pre-adapted to HL (HL/NL) (Fig. 4.7.B).

4.3.2 The effect of high light on DC3000 challenged-*Arabidopsis* phytohormone mutants

We next investigated the impact of phytohormone mutants on the response to HL. Here the phytohormone mutants, *aao3* and *sid2.1* plants were examined in response to HL and DC3000 to investigate the interaction between abiotic and biotic stress respectively. The plants were growing as described (Chapter 2.3.3). Five weeks after pricking plants were transferred to the high light cabinet using the same experimental layout as shown in Figure. 4.3. By the end of the fifth day light cycle, 6 plants of each genotype from both conditions (light treatment and control) were challenged with virulent DC3000 (OD_{600}: 0.0002).

As reported by de Torres Zabala *et al* (2009), we validated that the *aao3* mutant was more resistance to DC3000 whilst *sid2.1* was more susceptible compared to wild type Col-0. By contrast, *aao3* HL–DC3000 challenged plants become more susceptible with accumulation of anthocyanin in old leaves (Fig. 4.8.B). In the case of *sid2.1*, no significant differences were seen between *sid2.1*-NL/DC and *sid2.1*-HL/DC3000 (Fig. 4.9.A).

Notably, however, the *aao3/sid2* double mutant HL-challenged leaves displayed stronger symptom development compared to *sid2*.1, whereas the double mutant was reported to exhibit fewer symptoms than *sid2.1* under normal growth condition (de Torres Zabala *et al*., 2009). Despite this observation there were no differences in bacterial growth between *sid2.1* and the double under both conditions NL- and HL-DC3000 (Fig. 4.9.B), as was the case previously (de Torres Zabala *et al*., 2009).

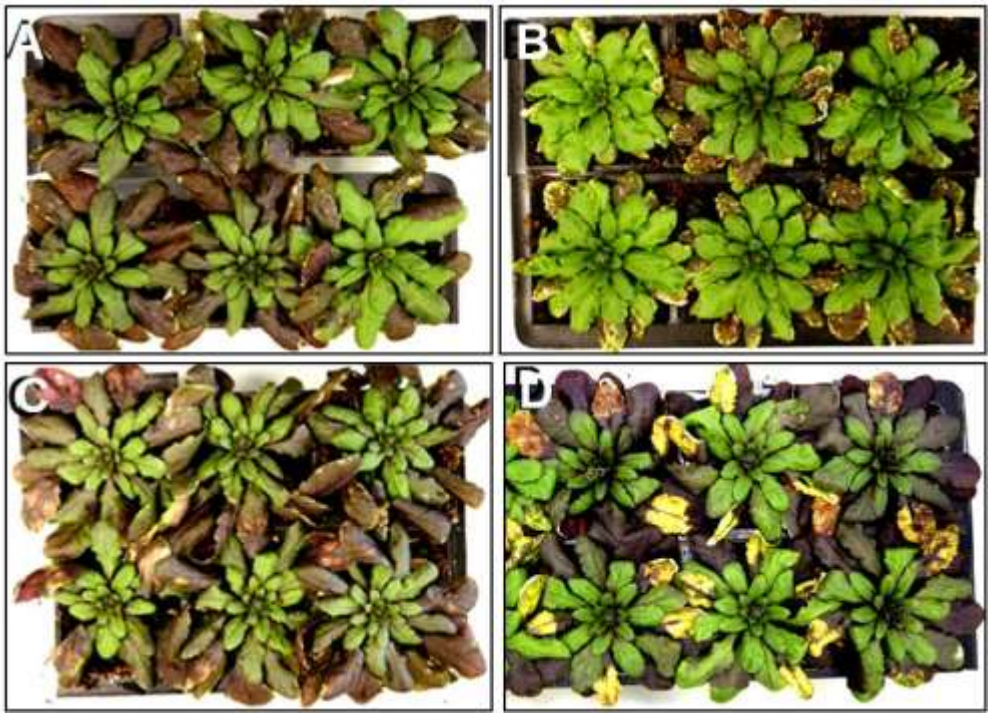


Figure 4.8 Combined effect of light stress and DC3000 infection on hormone biosynthetic mutants: (A) Col-0, (B) *Arabidopsis aldehyde oxidase3- aao3* (C) *Isochorismate synthase 1-sid2.1* and (D) the double mutant *aao3/sid2-1*. (A-D) Plants were grown under the intensity of 600µmol/m²s^{-1} HL at 23°C and 65% relative humidity (RH) for 5 days, control plants were grown under 120µmol/m²/s^{-1} at 23°C and 65% RH, followed by 4dpi of DC3000.

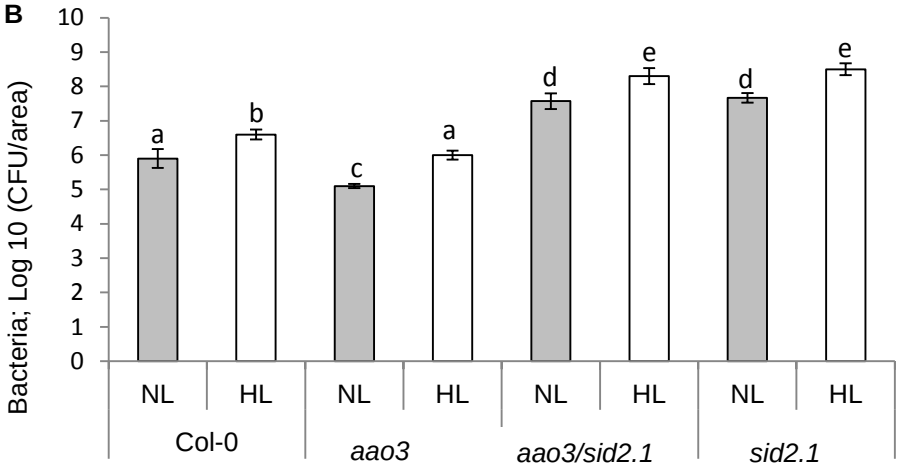


Figure 4.9 Effect of combined light stress and DC3000 infection on hormone biosynthetic mutants. (A) DC3000 bacterial growth for *aao3* and *sid2.1* mutants compared to Col-0 under NL and HL. (B) DC3000 bacterial growth for *aao3/sid2.1* double mutant by comparison with Col-0, *sid2.1* and *aao3*. Bacterial multiplication was determined 4 dpi. Bars represent the mean of six biological replicates, three leaves per replicate. Numbers represent bacterial growth 4dpi. Labels "a, b, c, etc." above the columns discriminate the differences at a significance $p < 0.05$ (Student's t-test for pair wise comparison of non-treated and treated plants) Errors bars represent one standard deviation. Experiments were repeated three or more times.

By contrast, when *sid2.1* plants were inoculated with DC3000 (0.0002 inoculum), HL does not further increase significantly *sid2.1* susceptibility except in the one case as shown in Figure 4.9B. Furthermore, the double mutant *aao3/sid2.1* is also as susceptible to DC3000 infection as *sid2.1* either under NL or HL (Fig.4.9B)

To investigate the response of *sid2.1* to DC3000/HL challenged in more detail, due to insignificant differences between HL- and NL-challenged tissues (Fig. 4.9A), two experiments were performed using two different inoculums of bacterial suspension; OD_{600}: 0.00002 and 0.000002 (1 X 10^4 cfu ml^{-1} and 1 X 10^3 cfu ml^{-1} respectively) which are lower than the usual inoculum (0.0002) used for bacterial population as in Figure 4.9A. The result shows a statistical significant increase in DC3000 in both experiments (student t.test; 0.00111 and 0.00119) for *sid2.1* NL/DC vs HL/DC respectively (Fig. 4.10A and 10B). Although, fluctuations on DC3000 growth were noted between the two inoculums, OD_{600}: 0.00002 and 0.000002, in *sid2-1* infected tissues under NL conditions, but, the growth of DC3000 in HL/challenged tissues with the inoculum 0.000**02** (1 X 10^4 cfu ml^{-1}) was significantly higher (approximately one fold) than the 0.000**002** (1 X 10^3 cfu ml^{-1}) inoculum (Fig. 4.10A and 10B).

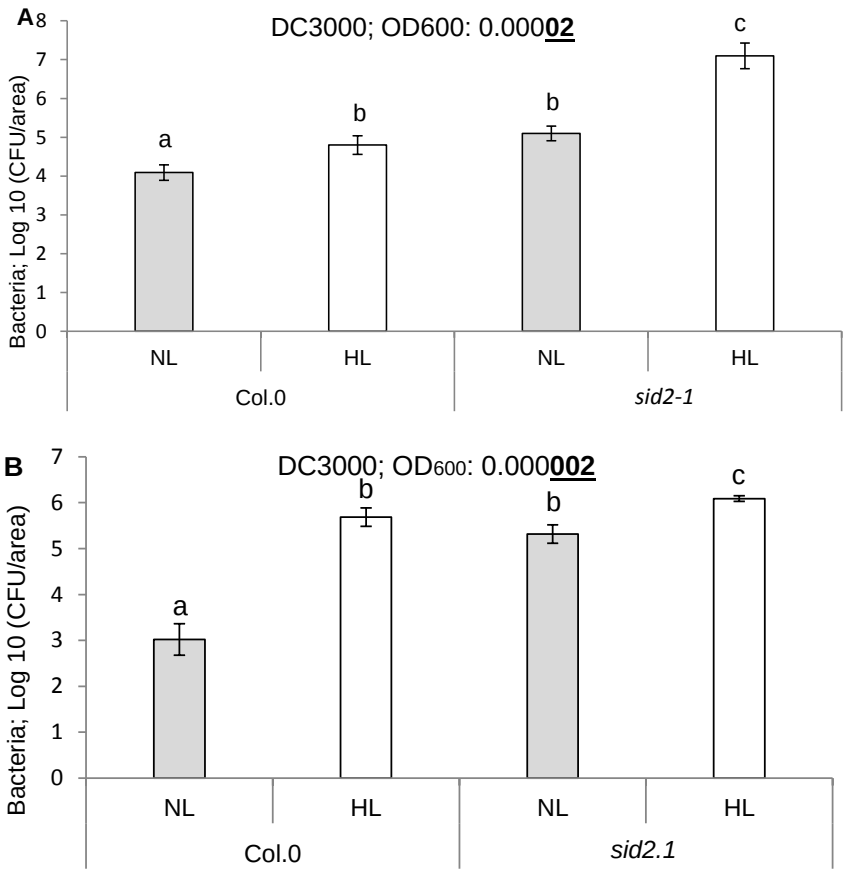


Figure 4.10 Effect of light stress and DC3000 infection in hormone biosynthetic mutant *sid2.1*.The plants were inoculated with very low inoculum of DC3000 (A) Plants were infected with 0.000**02** (1 X 10^4 cfu ml^{-1}) and (B) plants infected with 0.000**002** (1 X 10^3 cfu ml^{-1}). Bacterial multiplication was determined at 4dpi. Bars represent the mean of six biological replicates, three leaves per replicate. Errors bars represent one standard deviation. Labels "a, b etc." above the columns discriminate the differences at a significance $p< 0.05$ (Student's t-test for pairwise comparison of non-treated and treated plants). Experiments were repeated twice.

4.3.3 The relationship between phytohormone levels, high light and bacterial growth

Hormone levels were measured over the key time points (day0, d5NL, d9NL, d9NL/DC3000 with d5HL, d9HL and d9HL/DC3000) using the same conditions for plant growth and bacterial preparation as described in chapter 2 section 2.1 and 2.3-2.5. The levels of three phytohormones (ABA, SA and JA) were quantified in Col-0 and *aao3* as described in section 4.2.2.

Our observation revealed that under NL ABA levels in Col-0 tissues (time point; d0, d5, d9 and d9/DC), remain almost steady with no significant differences but ABA levels were significantly different at d9 HL/DC (Fig. 4.11A). The increase in ABA 9dHL/DC comes from *de novo* ABA

synthesis as it is not seen in *aao3* (Fig. 4.11A). De Torres has also shown that at late stages of infection under normal conditions, ABA increases in *sid2.1*-challenged tissues is significantly attenuated and resulted in promotion in DC3000 growth. However, SA levels in response to HL/DC3000 decreased significantly in Col-0 about 2.5 fold less than in *aao3* under HL (Fig. 4.11B). While *aao3* mutant responds to HL/DC3000 challenged was as susceptible as Col-0 (Fig. 4.9A and 9B).

In addition, the level of ABA in Col-0 increased 2 – 3 fold under 5d/HL and 9d HL/DC3000 but at 9d HL the level of ABA declined to the same level under NL conditions (Fig. 4.11A). By contrast, the levels of ABA in Col-0 were 3.5- 4 fold higher at 9d HL/DC3000 compared to 9 d NL/DC3000 (Fig. 4.11A). ABA levels in *aao3* tissue showed no significant differences in both conditions and were, as expected, significantly lower than Col-0 (Fig. 4.11A)

By contrast, SA was higher in *aao3* than that in Col-0 (Fig. 4.11B). The extended period of HL (9dHL) caused an increase in the levels of JA (3 and 4 fold in *aao3* under HL and NL respectively) but this increase was attenuated following DC3000 infection (Fig. 4.11C). Overall, in Col-0 HL/DC3000-challenged tissues, HL increased ABA levels resulted in significant promotion in DC3000 growth alongside with reduction of SA and JA levels compared to Col-0 NL/DC3000-challenged tissue (Fig. 4.11A-11C). Although the growth of DC3000 in *aao3* HL-challenged tissues significantly increased, the level of foliar ABA did not show any differences neither in infected- nor uninfected-tissues under both conditions.

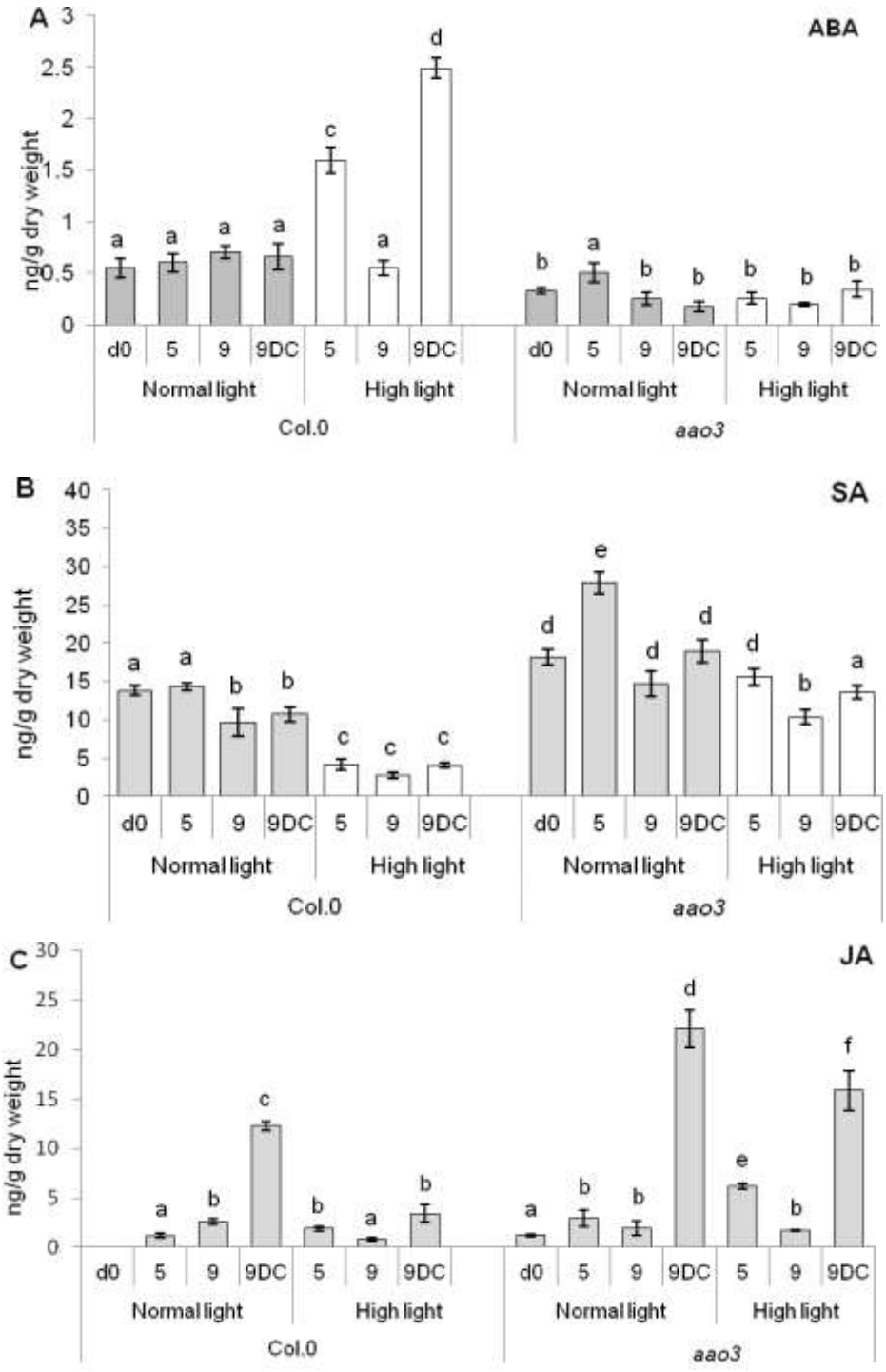
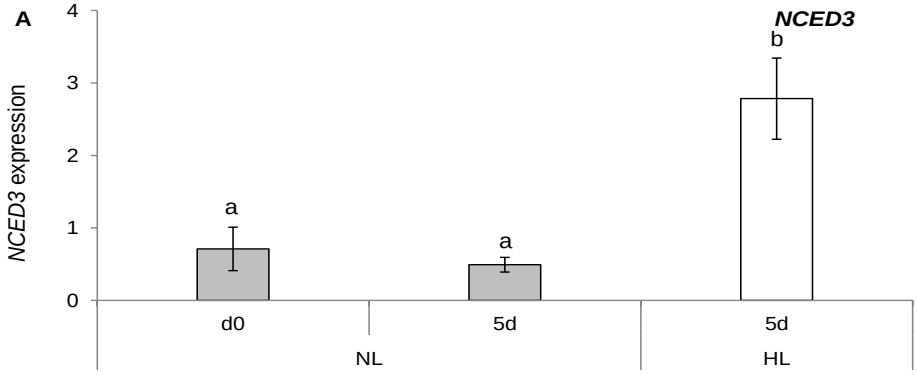


Figure 4.11 Determination of phytohormones in DC3000 challenged -*aao3* and -Col-0 under HL and NL. ABA, SA and JA measured in DC3000 infected Col-0 and *aao3* under both conditions. Samples were harvested at day 0, 5dNL, 9dNL and 9dNL/DC with the same time course under HL. ABA, SA and JA levels were determined by LC-MS as described in Materials & Methods, A-C respectively. The bars represent the mean of three biological replicates comprising two plants per replicate. Labels "a, b etc." above the columns discriminate the differences at a significance $p< 0.05$ (Student's t-test for pairwise comparison of non-treated and treated plants). Error bars represent one standard deviation.

4.3.4 Regulation of ABA and SA synthesis under HL

To investigate expression of the key ABA and SA biosynthetic genes, Col-5 plant tissues were harvested from 6 plants, three replicate each comprising two plants and three leaves were collected per plant. Samples were collected on day zero, 5dNL and 5dHL. RNA was extracted and cDNA was synthesized (as described in Chapter 2.2.3 and 2.2.5) to determine the steady-state mRNA levels for *9-cis-epoxycarotenoid dioxygenase 3* (*NCED3*) and *isochorismate synthase1* (*ICS1/ SID2*) by RT-PCR. The *NCED3* and *ICS1* genes encode key regulatory enzymes of ABA and SA biosynthesis respectively. *NCED3* mRNA levels increased rapidly (~ 5 fold) in Col-5 plant tissues exposed to 5dHL compared to 5dNL tissues (Fig. 4.12A). Figure 4.12B shows that *ICS1* mRNA levels in the samples harvested after 5dHL are significantly lower than 5dNL (~ 2 fold). In the other hand, de Torres Zabala *et al*., (2009) showed that *ICS1* steady-state mRNA levels elevated rapidly after 3 hpi in DC3000-challenged Col-0 leaves, and peaked at 12 h.



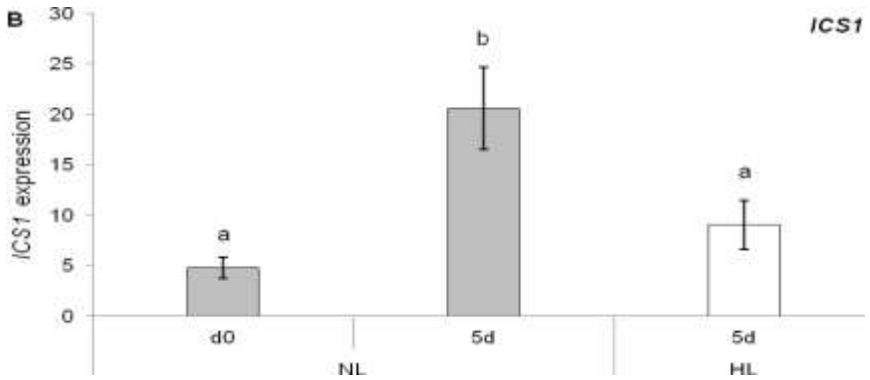


Figure 4.12 Expression levels of the key ABA and SA biosynthetic genes in *Arabidopsis* wild type plants. Steady-state levels of transcript encoding either (A) the ABA biosynthetic enzyme *9-cis-epoxycarotenoid dioxygenase 3* (*NCED3*) or (B) the SA biosynthetic enzyme *isochorismate synthase1* (*ICS1*; *SID2*) following 5 day high light treatment of Col-5 in comparison with d0 and 5d NL .The Expression levels were determined at the indicated times by RT-PCR. Bars represent the mean of three replicates three leaves for each replicate. Labels "a, b, etc" above the columns discriminate the differences at a significance $p< 0.05$ (Student's t-test for pair wise comparison of non-treated and treated plants). Errors bars represent one standard deviation.

4.3.5 The effect of high light on the accumulation of Flavonoids in *Arabidopsis thaliana*

Given the accumulation of flavonoids in HL/DC3000 challenged tissues (day zero "d0", 5dNL, 9dNL, 9dNL/DC3000 "4dNL+5d post-NL/DC3000" in parallel to the same treatment under HL), flavonol and anthocyanin levels were determined. Samples were harvested from plant tissues that used for bacterial growth and in parallel to hormone assays (see section 4.2.2). In this study nine anthocyanins based on cyanidin and five flavonol (kaempferol) glycosides (Tohge *et al*., 2005, Page *et al*., 2011) were investigated (Table 4.1).

Flavonoids were determined by LC-MS as described in Section 4.2.3. The resulting data indicated two different patterns of flavonoid accumulation (Table 4.1 and Fig. 4.13); (i) a group of flavonols enhanced by HL but suppressed by DC3000 infection as in Col-0 and *aao3* plants (Fig. 4.14A-14E). Whilst, *sid2.1*-HL and -HL/DC does not shows any significant difference in flavonol accumulation except in one case as in Figure 4.14D.

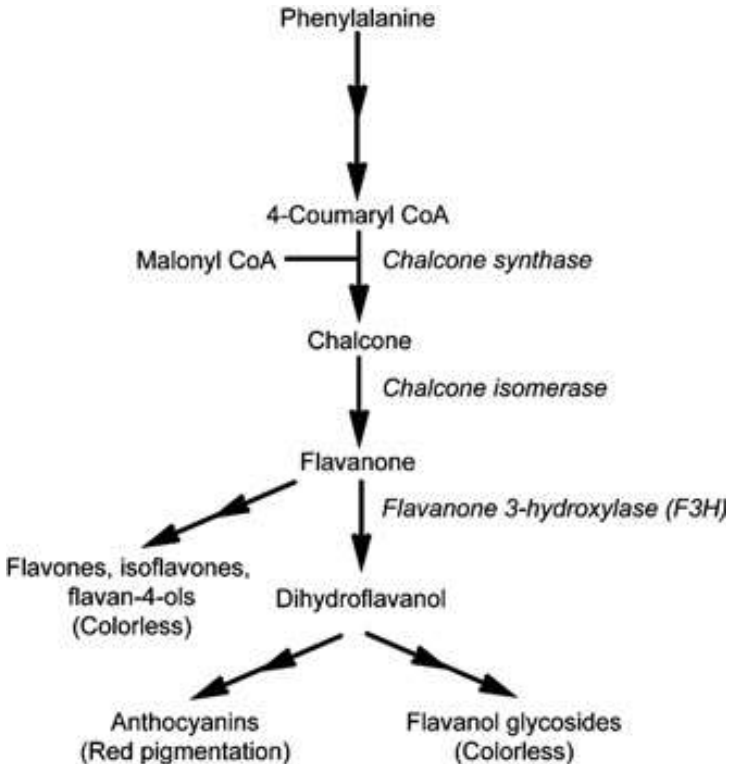
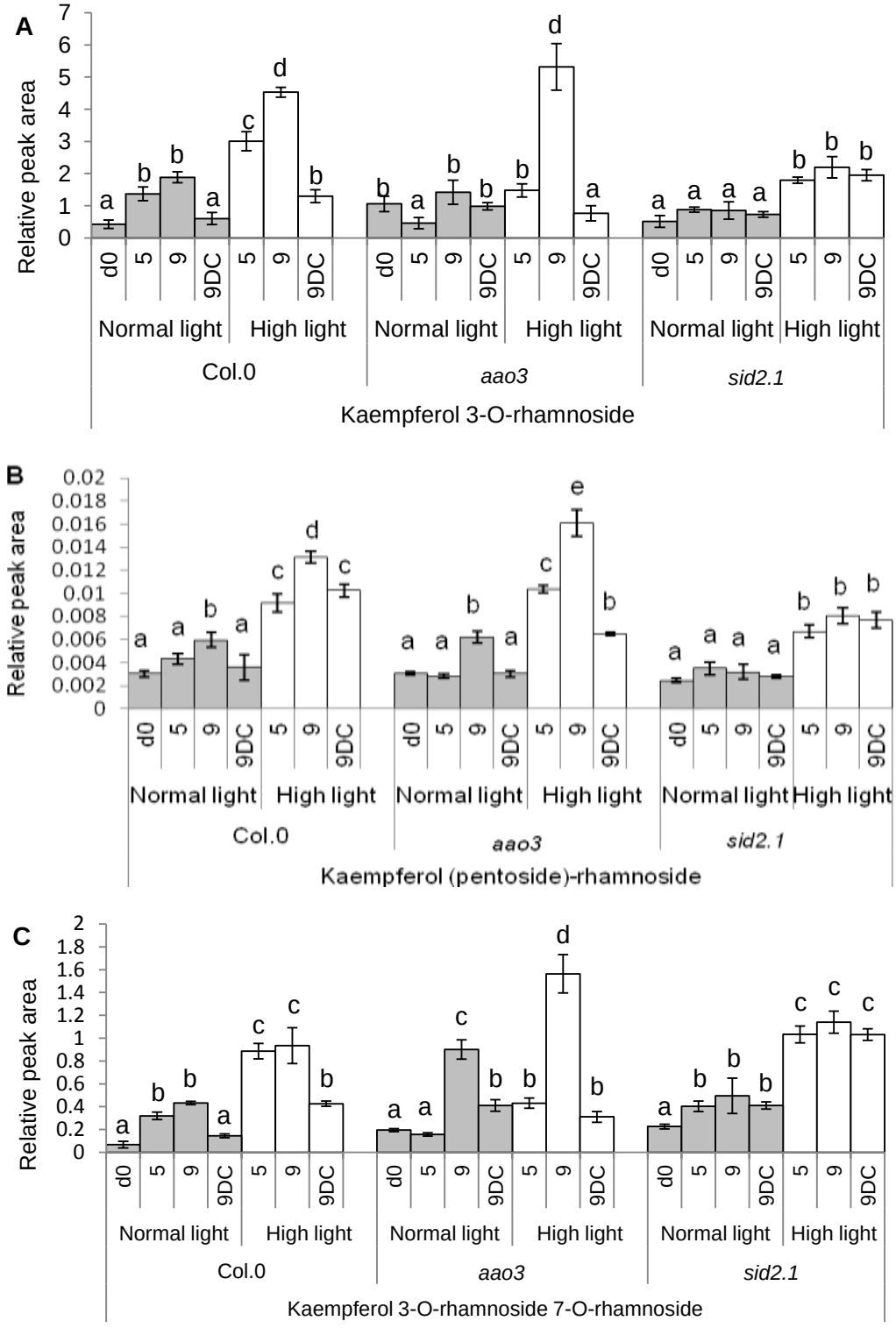


Figure 4.13 Synthetic pathway of both classes of flavonoids**,** the Scheme shows the activity of flavanones 3-hydroxylase (F3H) in the synthesis of flavanol glycosides and anthocyanins (adapted from Rangarajan *et al.* 2004)

The five kaempferol glycosides showed an identical pattern of accumulation in *aoo3* mutant under HL and HL/DC3000 (Fig. 4.14A-14E). Each compound accumulated in Col-0 and *sid2-1* and all were significantly lower in the *aao3* mutants after 9dpi of DC3000 ($P < 0.05$).



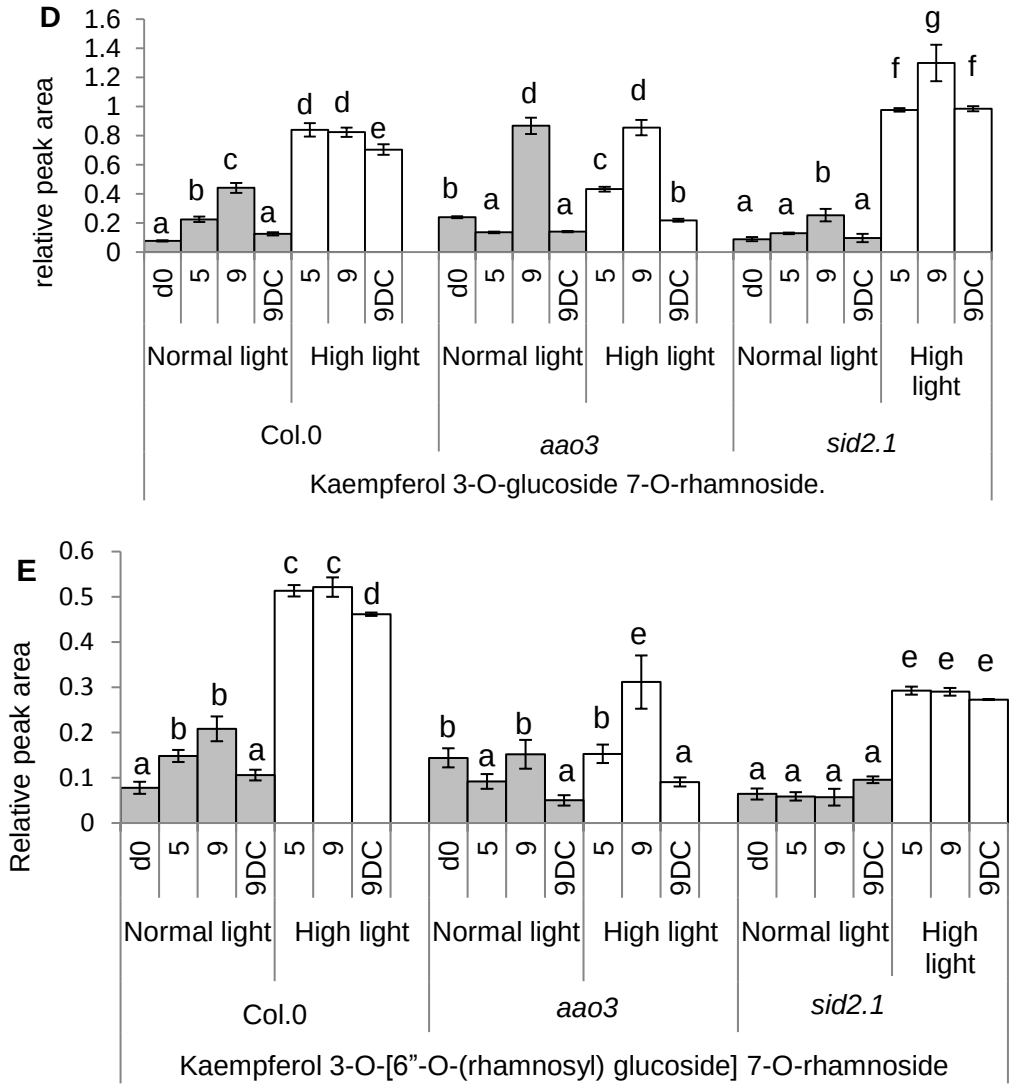
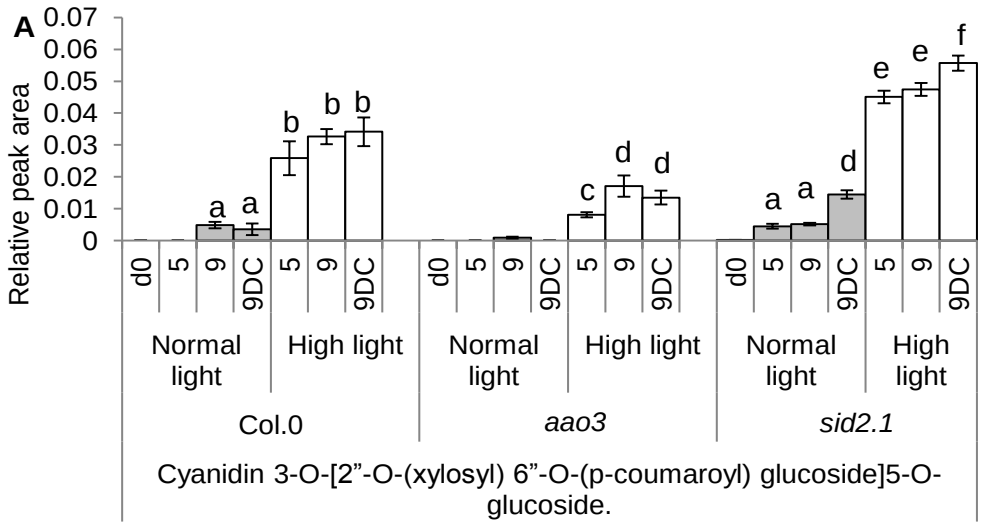
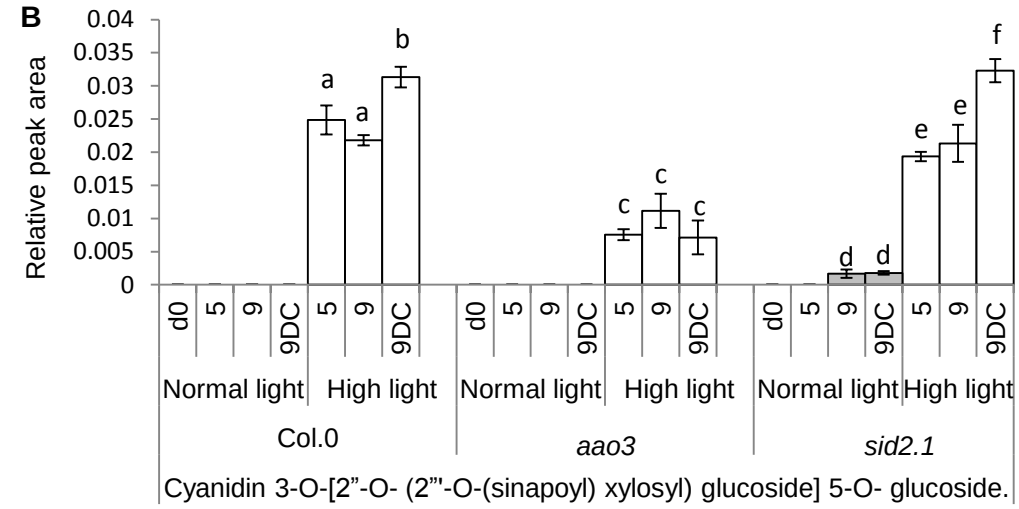


Figure 4.14 HL effect on the accumulation of flavonoid in *aao3*, *sid2.1*and Col-0 (A-E). Time course of flavonoids (subfamily flavonols) accumulation was set up under Normal and High light conditions; d0 (day zero), 5 (5 day NL), 9 (9 day NL), 9DC (5dNL + 4d DC3000 infection) respectively with the same treatments under HL. DC refers to plants inoculated with a low inoculum of DC3000 (OD_{600}: 0.0002; 1 X 10^5 cfu ml^-). Flavonols were determined by LC-MS, A-E respectively. Bars represent the mean of three biological replicates comprising two plants per replicate. Labels "a, b, c etc." above the columns discriminate differences at a significance of $P < 0.05$ (Student's t-test for pairwise comparison of non-treated and treated plants). Errors bars represent one standard deviation.

(ii) A group of anthocyanins enhanced by HL/ DC3000 infection. A low level of anthocyanin accumulations also identified in two Cyanidins out of the nine anthocyanins members measured in Col-0 and *aao3* HL-challenged tissues; (Cyanidin 3-*O*-[2″-*O*-(xylosyl) 6″-*O*-(*p*-*O*-(glucosyl) *p*-coumaroyl) glucoside] 5-*O*-[6-*O*-(malonyl) glucoside] and Cyanidin 3-*O*-[2″-*O*-(6-*O*-(sinapoyl) xylosyl) 6″-*O*-(*p*-*O*-(glucosyl)-*p*-coumaroyl) glucoside] 5-*O*-(6‴-*O*-malonyl) glucoside) (Fig. 4.15F and 15I).

Overal, anthocyanin accumulation was complex. Whilst, all anthocyanins were enhanced by HL/DC3000 challenged in *sid2.1* (Fig. 4.15A-D and F-I) one of the anthocyanins did not change in uninfected nor infected *sid2.1* HL-exposed tissues (Fig. 4.15E). By contrast, generally, anthocyanins are not affected under normal light especially in *aao3*. Although, an accumulation of anthocyanin was displayed under NL/DC3000 challenged in some cases for *sid2.1* (Fig. 4.15A, 15C, 15D, 15G and 15E) and Col-0 plant tissues (Fig. 4.15A, 15C and 15D) generally anthocyanin levels remained substantially lower in plant tissues under NL conditions compared with HL.

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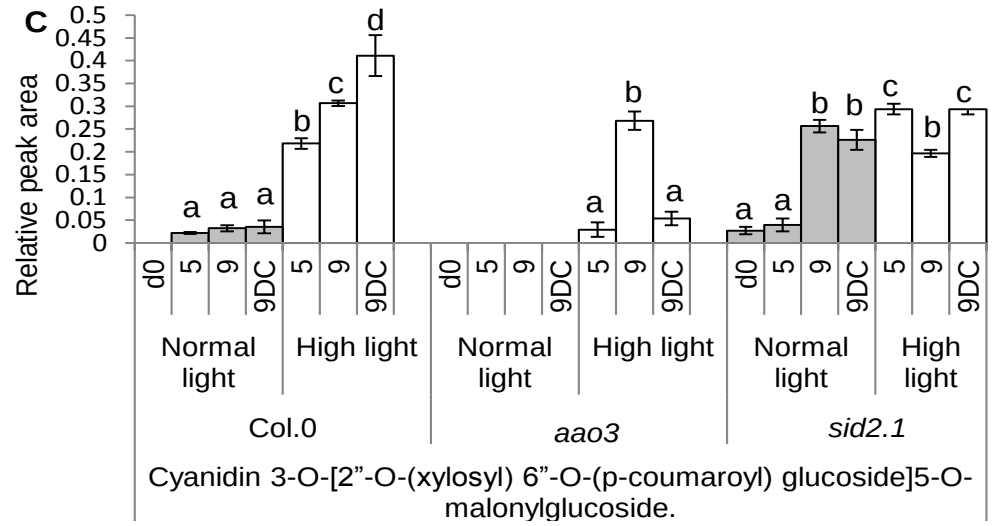
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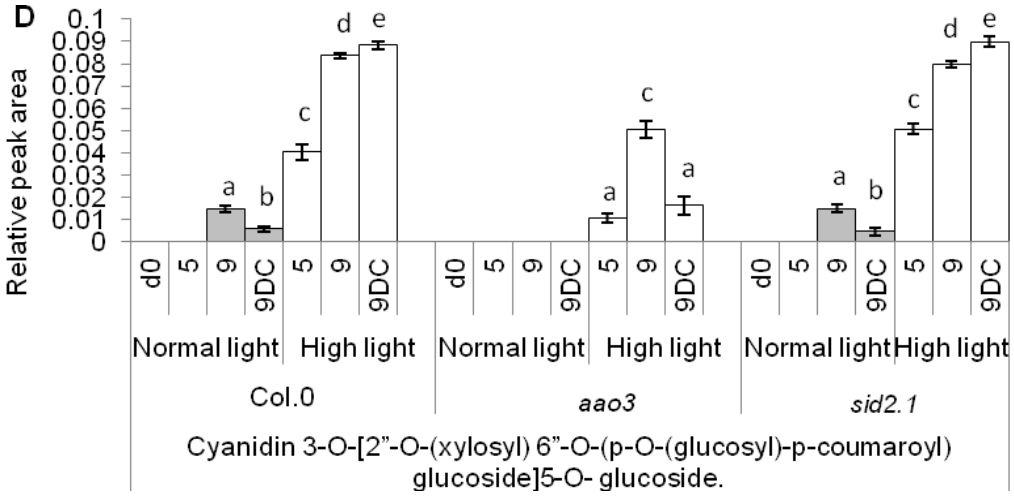
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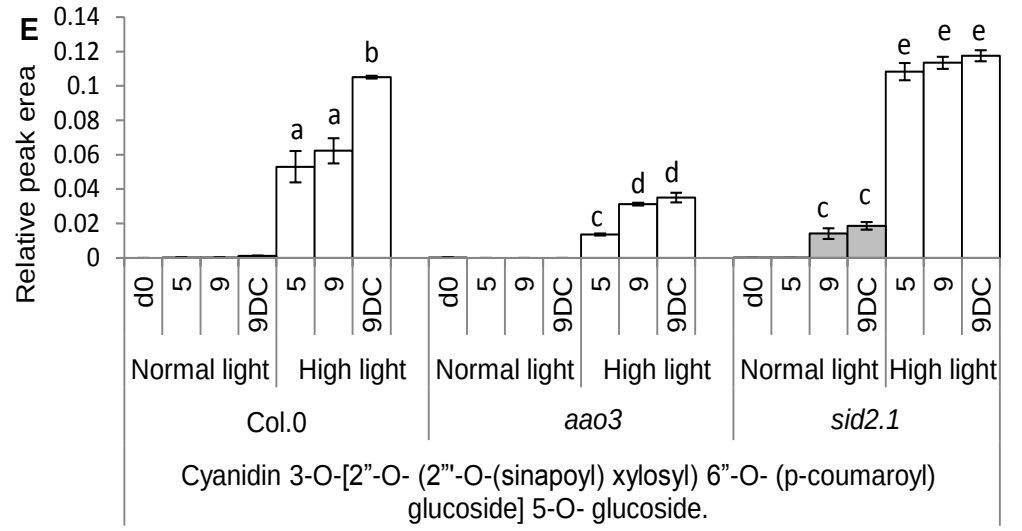
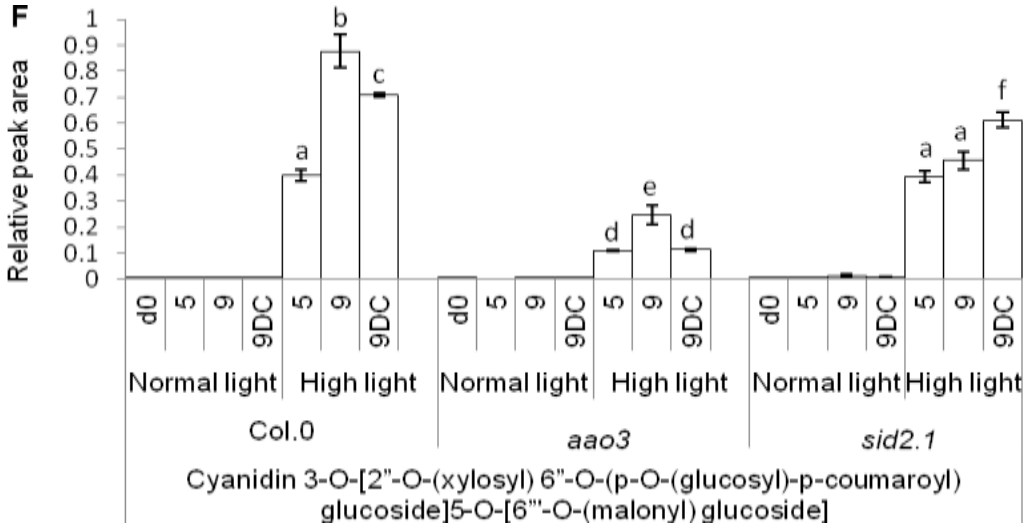
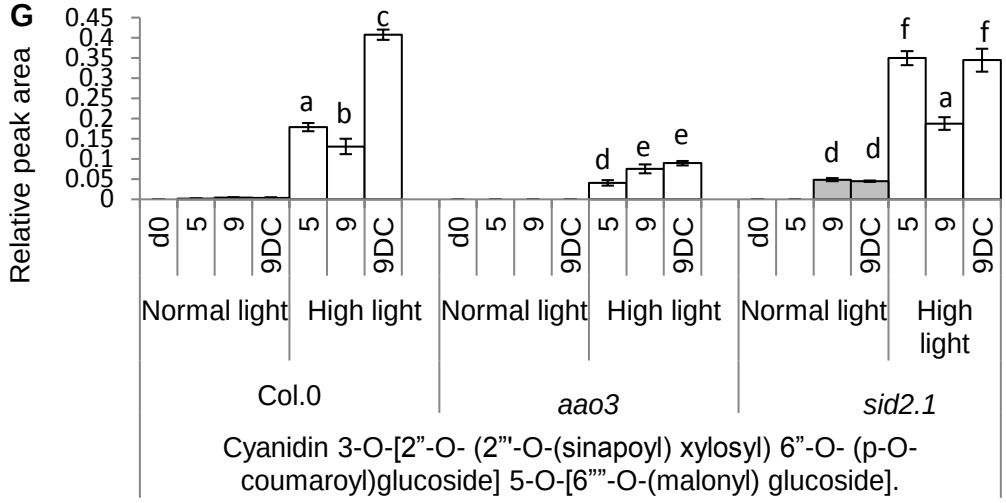
aao3
sid2.1
Cyanidin 3-O-[2”-O-(xylosyl) 6”-O-(p-O-(glucosyl)-p-coumaroyl) glucoside]5-O- glucoside.


E
Relative peak area
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
d0
5
9
9DC
Normal light
High light
Col.0
aao3
sid2.1
Cyanidin 3-O-[2”-O- (2”'-O-(sinapoyl) xylosyl) 6”-O- (p-coumaroyl) glucoside] 5-O- glucoside.


F
Relative peak area
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Normal light
High light
Col.0
aao3
sid2.1
Cyanidin 3-O-[2"-O-(xylosyl) 6"-O-(p-O-(glucosyl)-p-coumaroyl) glucoside]5-O-[6"'-O-(malonyl) glucoside]


G
Relative peak area
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Normal light
High light
Col.0
aao3
sid2.1
Cyanidin 3-O-[2”-O- (2”'-O-(sinapoyl) xylosyl) 6”-O- (p-O-coumaroyl)glucoside] 5-O-[6””-O-(malonyl) glucoside].

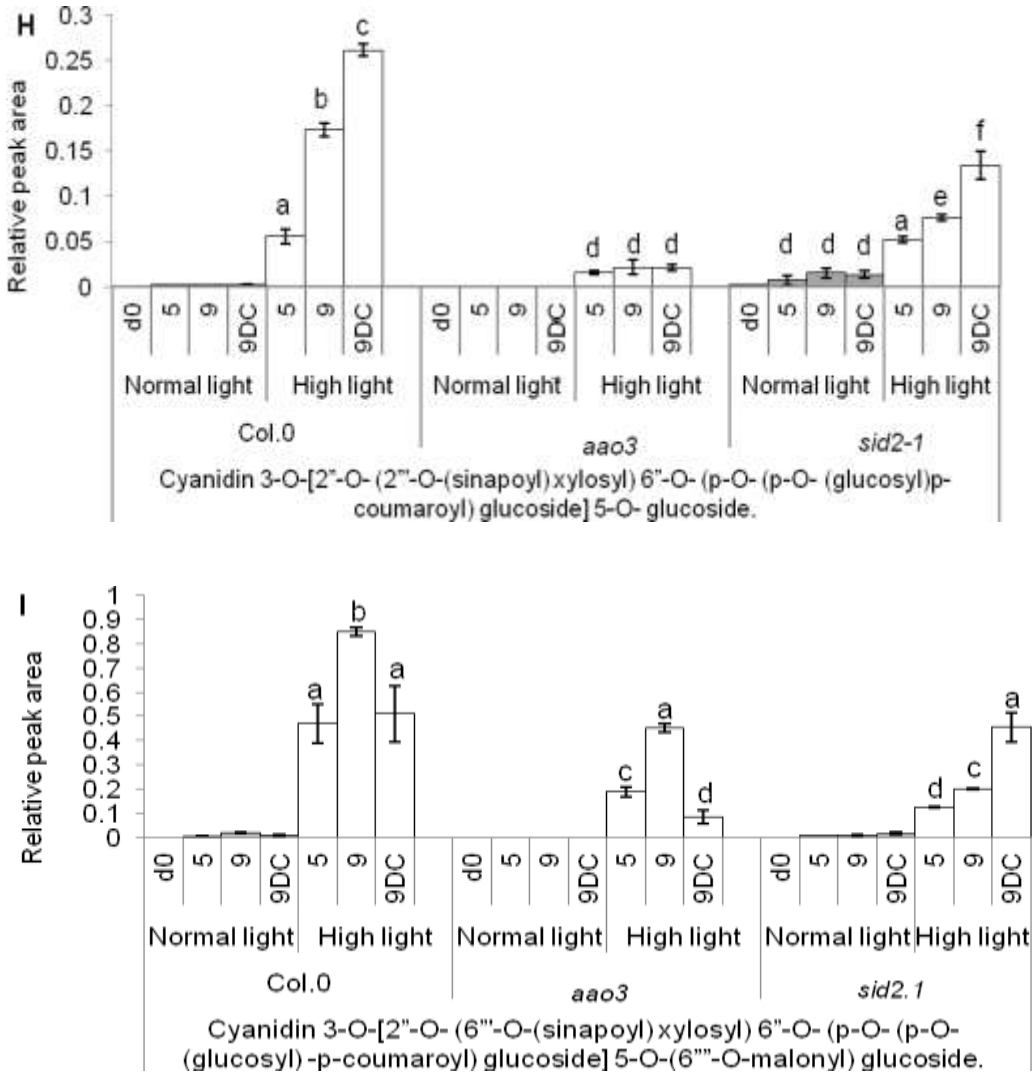


Figure 4.15 HL effect in accumulation levels of anthocyanin on *aao3*, *sid2.1* and Col-0. Anthocyanin was determined by LC-MS, A-I respectively with the same notations used in Hormones and flavonols. Bars represent the mean of three biological replicates comprising two plants per replicate. Labels "a, b, c etc." above the columns discriminate differences at a significance of $P < 0.05$ (Student's t-test for pairwise comparison of non-treated and treated plants) Errors bars represent one standard deviation.

The levels of anthocyanin were very low and were barely detected in the plant tissues in uninfected- and infected-NL leaves except in three cases of *sid2.1* and Col-0 plants where the Cyanidin 3-O-[2``-O-(xylosyl) 6``-O-(p-coumaroyl) glucoside]5-O-glucoside, Cyanidin 3-O-[2”-O-(xylosyl) *6*”-O-(p-coumaroyl) glucoside]5-O-malonylglucoside and Cyanidin 3-O-[2”-O-(xylosyl) *6*”-O-(p-O-(glucosyl)-p-coumaroyl) glucoside]5-O- glucoside (Fig. 4.15A, 15C and 15D). These three Cyanidins were more abundant than the other members of anthocyanin group. However, the level of Cyanidin 3-O-[2”-O-(xylosyl) *6*”-O-(p-coumaroyl) glucoside]5-O-malonylglucoside in *sid2-1* NL and NL/DC3000 (Fig. 4.15C) was almost equivalent to their level in HL and HL/DC3000. Moreover this Cyanidin level was also significantly higher in *sid2-1* than Col-0 in NL and NL/DC3000 tissues.

In general, anthocyanin levels were significantly higher in HL and HL/DC3000 challenged tissues of Col-0 and *sid2.1* than in *aao3* HL and HL/DC3000 challenged tissues (Fig. 4.15A-15I). Whereas HL/DC3000 increased anthocyanin accumulation in Col-0 plant tissues as can be seen in Figure 4.15B, 15C, 15D, 15E, 15G and 15H, the accumulation of anthocyanin was reduced in Figure 4.15F and 15I, while DC3000 infection did not affect the accumulation of anthocyanin in Col-0 under HL as in Figure 4.15A.

In *sid2.1* HL reduced anthocyanin levels (Fig. 4.15C and 15G) but they were increased by HL/DC3000 infection (Fig. 4.15A, 15B, 15D, 15F, 15H and 15I) but remain steady to the same level in *sid2-1* tissues under HL and HL/DC3000 (Fig. 4.15E). By contrast, *aao3* plant tissues generally displayed lower levels of anthocyanin accumulation in comparison to Col-0 and *sid2.1* (Fig. 14.15A-15I).

Overall, the flavonols showed an identical pattern of accumulation across *aao3*, *sid2.1* mutants and Col-0 under NL conditions while *aao3* showed a reduction of flavonols after DC3000 infection under HL conditions compared to Col-0 and *sid2.1* where both genotypes displayed significant increase of flavonols under HL/DC3000 infection. In the case of anthocyanins, all the nine anthocyanins also showed an identical pattern of accumulation across the *sid2.1* mutant and Col-0 under HL and HL/DC3000 challenged tissues, whereas *aao3* mutant showed less accumulation of anthocyanin specifically in HL/DC3000 infected tissues.

4.3.6 Effect of HL on ABA hypersensitive mutants:

We further investigated the role of ABA in *Arabidopsis* plants under HL/*Pseudomonas* interaction by looking at components of the ABA perception pathway. The PP2C protein phosphatases, triple mutant, *hab1.1/abi1.2/abi2.1* and PYL5 ABA receptor overexpressor, *pyl5.OE* were exposed to 5d HL pre-treatment followed by 4dHL/DC3000 infection. Previous determination of DC3000 in *hab1.1/abi1.2/abi2.1* and *pyl5.OE* under normal condition showed that both *hab1.1/abi1.2/abi2.1* and *pyl5.OE* were more susceptible to DC3000 than Col-0. Here, under HL stress, infection with DC3000 (OD_{600}: 0.0002; 1 X 10^5 cfu ml^{-1}) of *pyl5.OE* leaves visually displayed more chlorosis than *hab1.1/abi1.2/abi2.1* although both lines showed the same level of bacterial growth (~2 fold) in comparison to Col-0 (Fig. 4.16A-C and Fig. 4.17). In other words, both lines *pyl5.OE* and *hab1.1/abi1.2/abi2.1* significantly enhanced the bacterial growth under HL condition (students t-test, $p<0.05$).

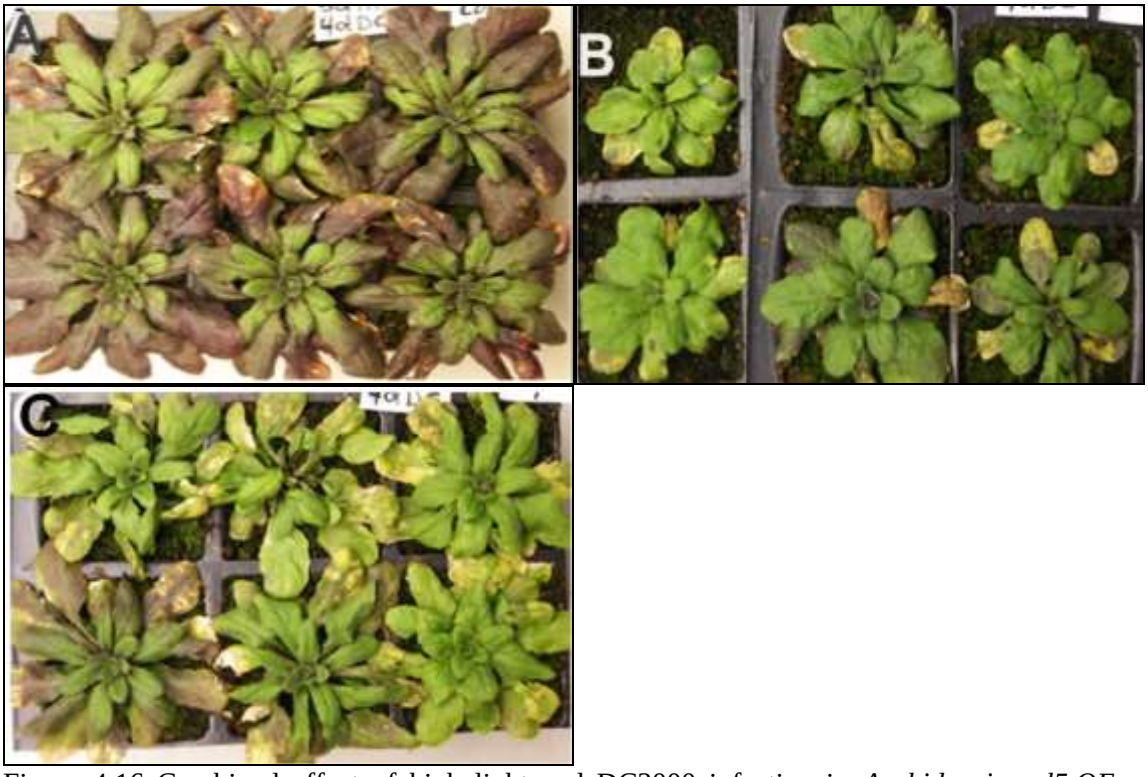


Figure 4.16 Combined effect of high light and DC3000 infection in *Arabidopsis pyl5.OE* and *hab1-1/abi1-2/abi2-1* mutants**.** Photographs show phenotypes associated with 9dHL-DC3000 (5dHL pre-treatment + 4dHL/DC3000 post-treatment), (A); Col-0, (B); *pyl5.OE* and (C); *hab1.1/abi1.2/abi2.1*.

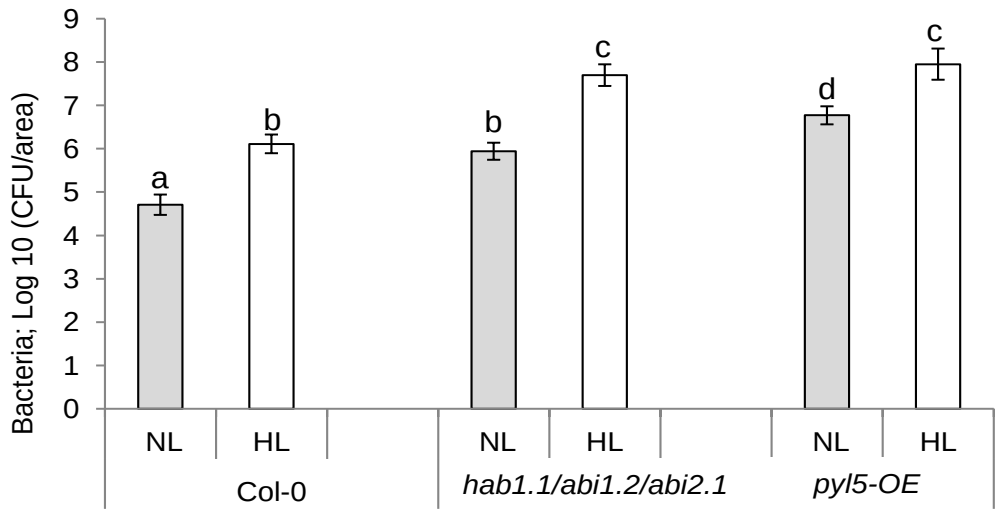
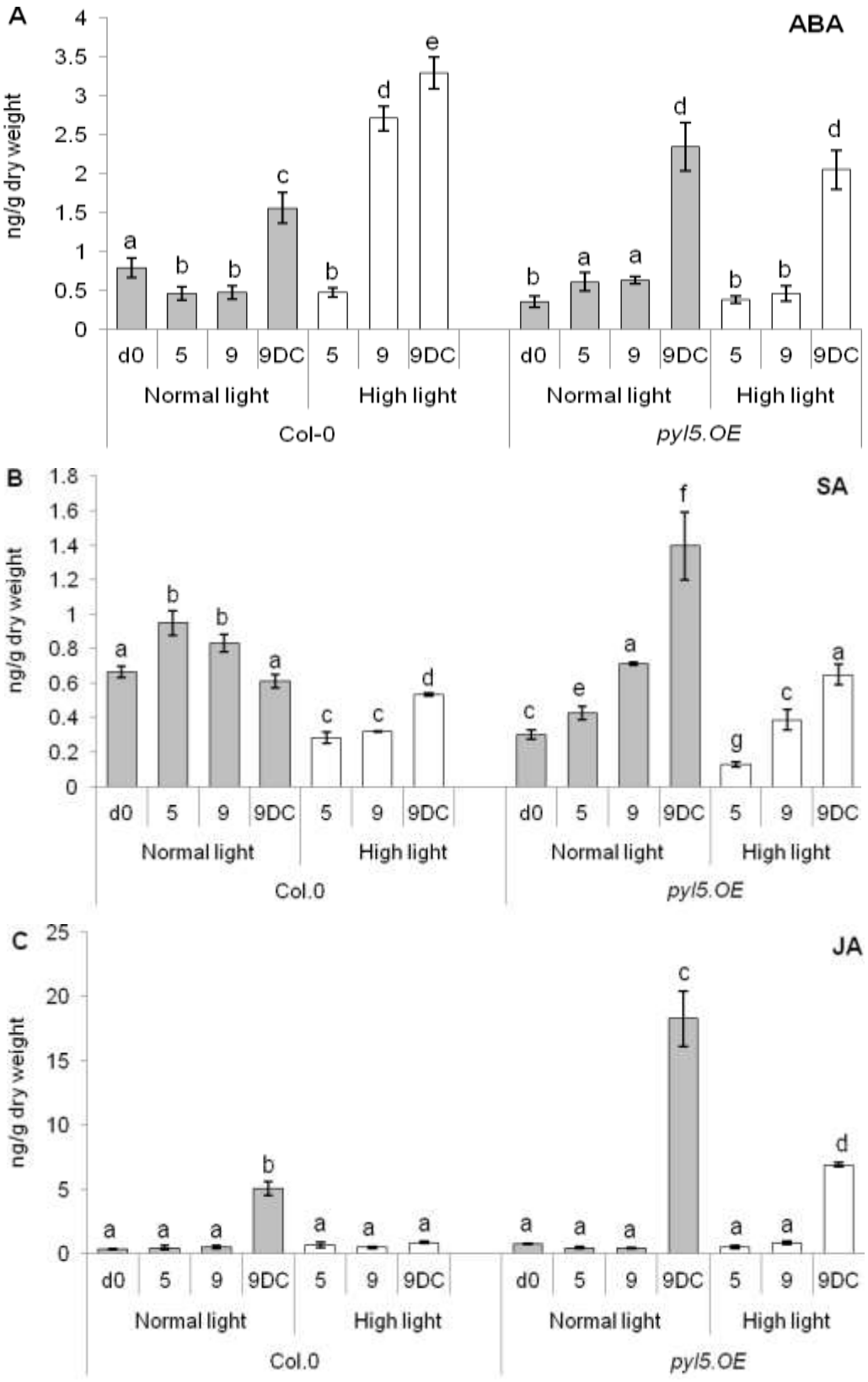


Figure 4.17 Effect of HL and DC3000 infection in ABA signalling mutants. The effect of HL on bacterial multiplication in the ABA perception mutants *hab1.1/abi1.2/abi2.1* and *pyl5.OE* was compared with Col-0. Bacterial multiplication was determined at 4dpi. Bars represent the mean of six plants three biological replicates, two plants per replicate, three leaves per plant. Labels "a, b, c etc." above the columns discriminate differences at a significance of $P < 0.05$ (Student's t-test for pairwise comparison of non-treated and treated plants) Errors bars represent one standard deviation. Experiments were repeated three times.

In addition to the investigation of phenotype and bacterial growth under HL stress, the levels of phytohormones were also analysed as described in Section 4.2.2.

In Col-0, ABA levels increased two fold in HL-challenged leaves compared to NL-challenged leaves. NL- and HL- DC3000 infection (9 dpi) elevated ABA levels in *pyl5.OE* four fold over uninfected leaves (Fig. 4.18A) whereas SA levels in *pyl5.OE* leaves under HL were suppressed ~50 times that measured in NL plants (Fig. 4.18B). By comparison the level of JA was very low in uninfected leaves of Col-0 and *pyl5.OE* under both NL and HL. JA levels increased significantly in Col-0 at d9NL/DC3000 (students t-test, $p<0.05$) but it was suppressed in *pyl5.OE* infected leaves under HL (Fig. 4.18C).


A
ABA
ng/g dry weight
4
3.5
3
2.5
2
1.5
1
0.5
0
a b b c b d e
b a a d b b d
d0 5 9 9DC 5 9 9DC
d0 5 9 9DC 5 9 9DC
Normal light
High light
Normal light
High light
Col-0
pyl5.OE
B
SA
ng/g dry weight
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a b b a c c d
c e a f g c a
d0 5 9 9DC 5 9 9DC
d0 5 9 9DC 5 9 9DC
Normal light
High light
Normal light
High light
Col.0
pyl5.OE
C
JA
ng/g dry weight
25
20
15
10
5
0
a a a b a a a
a a a c a a d
d0 5 9 9DC 5 9 9DC
d0 5 9 9DC 5 9 9DC
Normal light
High light
Normal light
High light
Col.0
pyl5.OE

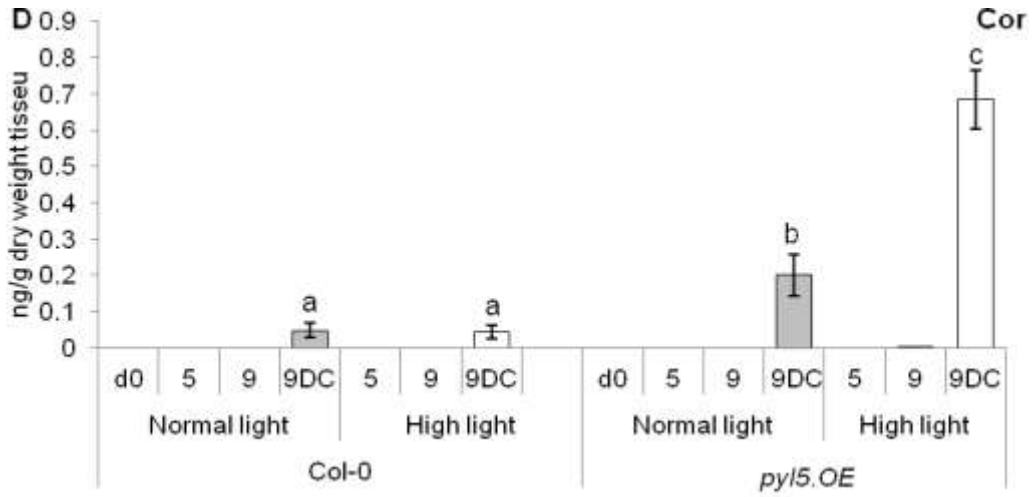


Figure 4.18 Impact of HL on key discriminatory metabolites of DC3000 infection in the *pyl5.OE* and Col-0 plants. Level of analyzed hormones (A) ABA, (B) SA, (C) JA and (D) Cor "coronatine" that were measured at time point; day0, d5, d9 and d9/DC under NL and HL. Bars represent the mean of six plants three biological replicates three leaves per plant Labels "a, b, c etc." above the columns discriminate differences at a significance of $P < 0.05$ (Student's t-test for pairwise comparison of non-treated and treated plants) Errors bars represent one standard deviation.

As expected, in Figure 4.18D the phytohormone mimic, coronatine, which contribute to pathogen virulence (Glazebrook, 2005, López et al., 2008, de Torres Zabala et al., 2009) , was accumulated and detected only in the infected plant tissues under the conditions of NL/DC3000 and HL/DC3000 of Col-0 and *pyl5.OE*. In comparison to Col-0, coronatine level was significantly higher in *pyl5.OE* DC3000 challenged tissues under both NL and HL conditions (students t-test, $p<0.05$) and consistent with enhanced virulence. Subsequently, the coronatine levels are dramatically increased significantly in *pyl5.OE* HL/DC3000 leaves compared to *pyl5.OE* NL/DC3000 and Col-0 HL/DC3000.

4.3.7 Effect of HL on EDS1 and its interacting partner, PAD4:

We expanded the investigation of the effect of HL on *Arabidopsis* mutants to include the *enhanced disease resistance 1* (*EDS1*), and the *phytoalexin deficient 4* (*PAD4*) mutants, representing a classical central regulatory node in plant immunity (Muhlenbock *et al*., 2008). The SA-deficient mutant "*sid2.1"* was included as a positive control alongside with the wild types Ler, the back ground for *eds1*, and Col-0, for *pad4* and *sid2.1*, as controls. The HL experiment was carried out at the same time frame in section 4.3.1 using the same challenge inoculum of DC3000.

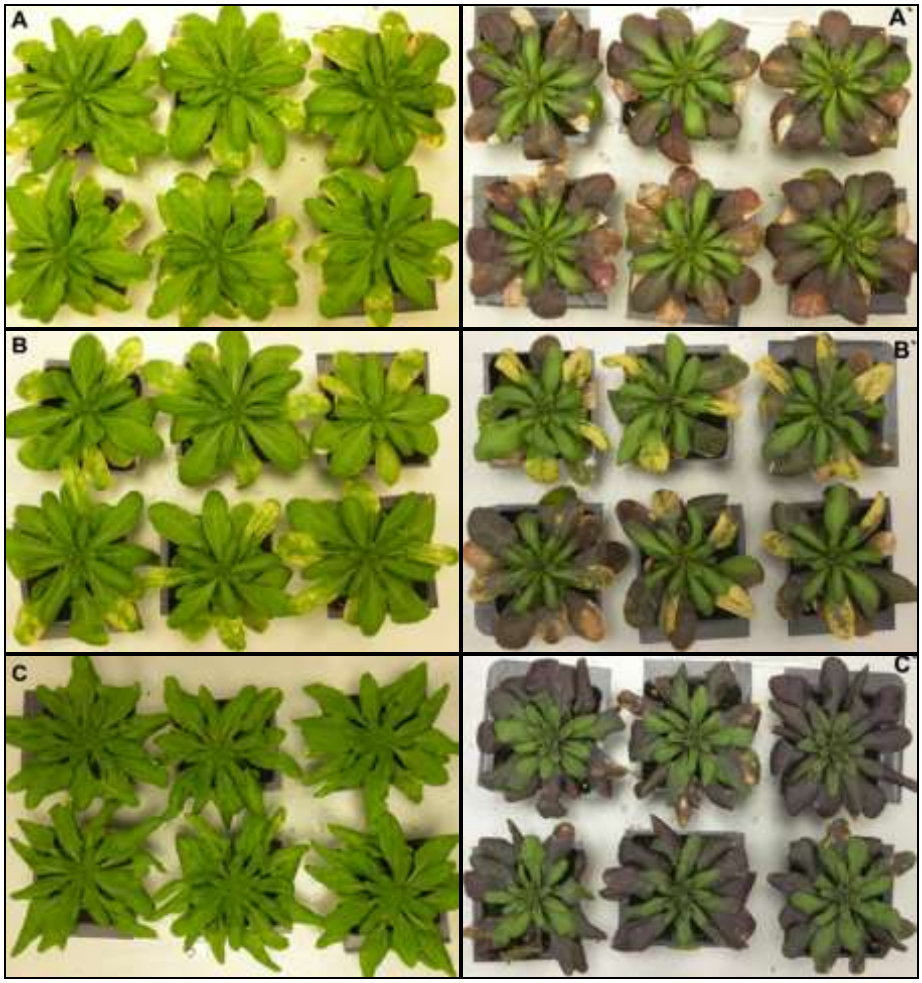


Figure 4.19 Phenotype of *eds1*, *pad4* and *sid2* under HL/DC infection compared with the control, Col-0 and Ler. (A-E) represent plants under normal light/DC3000 and (A`-E`) represent plants under HL/DC3000condition. (A) Landsberg erecta, (B) *eds1*, (C) Col-0, (D) *pad4* and (E) *sid2.1* respectively. Six plants per genotype and three leaves per plant were infected with a low inoculum of DC3000 (0.0002). Experiments were repeated twice.

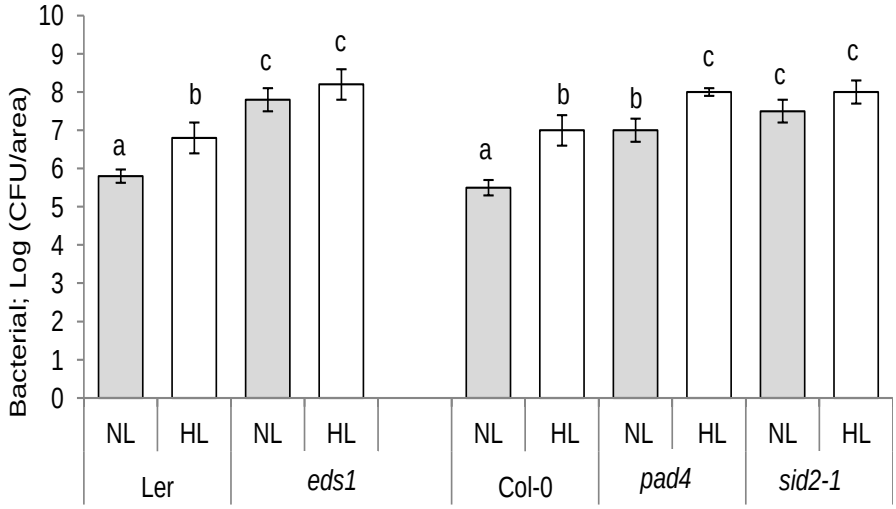


Figure 4.20 Effect of combined high light and DC3000 infection on *eds1 and pad4*. Plants were exposed to 5d HL post-treatment then inoculated with DC3000 (OD_{600}: 0.0002; 1 X 10^5 cfu ml^{-1}). Bacterial growth was determined 4dpi. Bars represent the mean of six biological replicates. Errors bars represent one standard deviation Labels "a, b, c etc." above the columns discriminate differences at a significance of $P < 0.05$ (Student's t-test for pairwise comparison of non-treated and treated plants) Experiments were repeated twice.

The dissociated forms of EDS1 and PAD4 are fully competent in signalling receptor triggered localized cell death at infection foci. While complex formation between these proteins are necessary for basal resistance involving transcriptional up-regulation of PAD4 itself and mobilization of salicylic acid defences (Rietz et al., 2011). Thus infected leaves of *eds1* mutant plants challenged with HL/DC3000 4dpi displayed a strong chlorotic phenotype with visible anthocyanin accumulation in old leaves similar to the observed phenotype in *sid2.1* under both condition (Fig. 4.19B, 19E and 4.19B`, 19E`), while *pad4* displayed accumulation of anthocyanin all over the plant leaves as reported in the wild type plants, Col-0 and Ler (Fig. 4.19A`, 19C` and 19D`).

This observation revealed similar results as the previously detected response of *Arabidopsis* HL/DC3000 in which DC3000 growth significantly increased and promoted under HL. Thus the *pad4* mutants displayed significant differences between HL/DC3000- and NL/DC3000-challenged plants (student t-test, $P<0.05$). In addition, the data collected from *eds1* plant tissues inoculated with 0.0002 inoculum of DC3000 under HL did not show any significant differences compared with *eds1*-NL/DC3000 (student t-test, 0.18). This observation is also similar to our result from *sid2.1* mutant challenged with DC3000 under both conditions (Fig. 4.9A and Fig. 4.20).

4.3.8 Effect of HL/DC3000 infection on phytochrome interacting factor mutants:

Further investigation on the response of *Arabidopsis* plants to HL/DC3000 was carried out using phytochrome interacting factor mutations, two of which were the *Arabidopsis* phytochrome *pif.i (phyA/phyB)* and cryptochrome *pif.h (cry1/cry2).* The PHYA-PHYE act as photoreceptors where the plants use these receptors to perceive and respond to ambient light signals, whereas, CRY1 positively regulates R protein-mediated resistance to avirulent *P. syringae*.

Using the same experimental parameters we demonstrated that the *phyA/phyB* mutant displayed more chlorosis and was more susceptible under normal conditions compared to Col-0 [(students t-test, $P<0.05$) (Fig. 4.21B, 21A, 21C respectively)]. Conversely, *cry1/cry2* was more resistant to DC3000 under NL compared to Col-0 [(students t-test, $p<0.05$) (Fig. 4.21C and 21A)]. Under HL *phyA/phyB* plants seem to accumulate less flavonoid in old leaves compared with Col-0 and exhibit more rosette chlorosis with apparent bleaching in young leaves (Fig. 4.21B`). Most dramatically, HL caused de-elongation in *phyA/phyB* leaf petioles where it becomes shorter than

in *phyA/phyB* plants under NL (Fig. 4.21B and B`) in comparison to Col-0 under both conditions (Fig. 4.20A and 20A`). The *phyA/phyB* - HL/DC3000 challenged plants showed a significant increase in DC3000 growth compared with *phyA/phyB* -NL/DC3000 [(students t-test, $P<0.05$) (Fig. 4.22)] and also Col-0 NL/DC3000-challenged leaves (two fold higher than Col-0) (Fig. 4.22). In addition, HL caused leaf bleaching in *cry1/cry2*-HL/DC3000-challenged tissues and the mutant showed increased suscpetibility as in Col-0 HL/DC3000 challenged tissues, despite being more resistant under normal conditions (Fig. 4.21C` and Fig. 4.22 respectively).

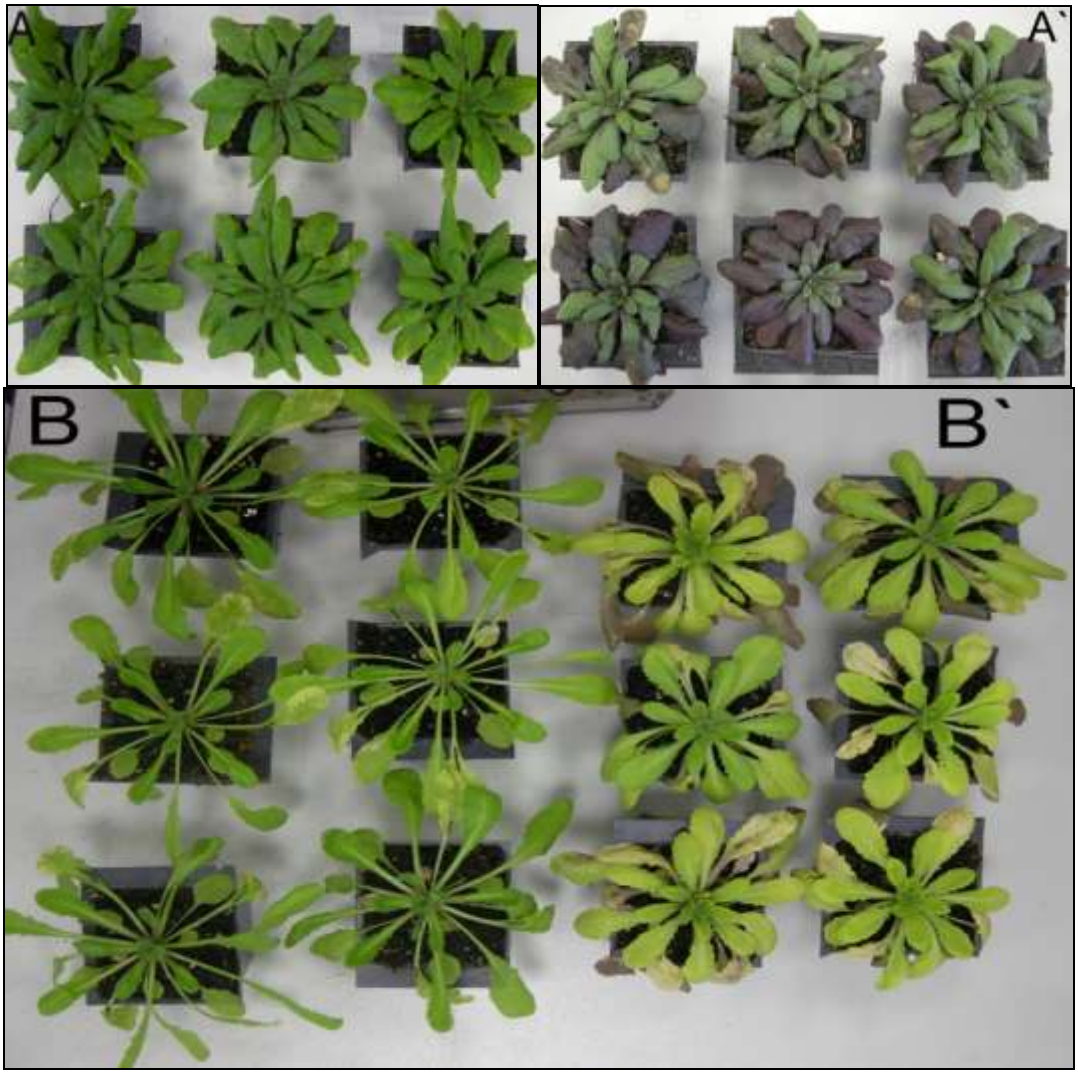


Figure 4.21 Effect of HL/DC3000 on phytochrome interacting factor mutants. (A) Col-0, (B) *pif.i "phyA/phyB"*, (C) *pif.h "cry1/cry2"*. Plants labelled (A to C) are the control and were challenged with low inoculum (0.0002; 1 X 10^5 cfu ml^{-1}) and plants labelled (A` to C`) were exposed to HL/DC3000 with same the inoculum.

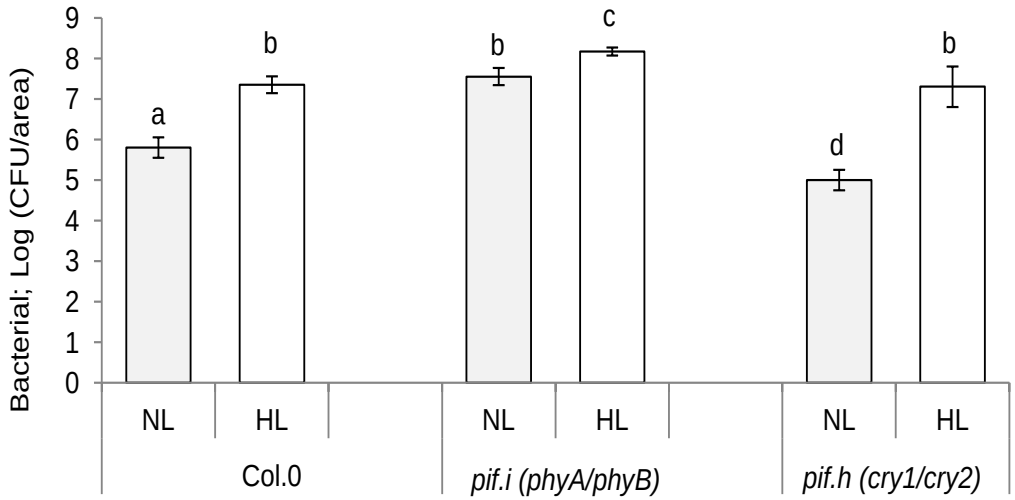


Figure 4.22 Response of phytochrome interacting factor mutants combined HL and DC3000 treatments**.** The *pif.i* and *pif.h* plants were exposed for 5days pre-treatments of NL and HL followed with 4dpi-NL and –HL post-treatments. Bacterial multiplication was determined at 4dpi. Bars represent the mean of six biological replicates, three leaves per replicate. Errors bars represent one standard deviation. Experiments were repeated more than two times.

4.4 Discussion:

4.4.1 HL-induced DC3000 growth and anthocyanin accumulation on *Arabidopsis* Col-5 W.T plants

In this Chapter of the study, initially the response of *Arabidopsis* Col-5 wild type plants to HL/DC3000 infection was examined. The design of highlight experiment was based on the exposure of *Arabidopsis* plants for 16 h continuous high light followed by 8 h dark for 9 days (4 days pre-treatment plus 5days post-treatment of HL/DC3000). The combination of HL exposure and DC3000-challenge induced chlorosis in the upper epidermis (3dpi) with initial anthocyanin synthesis in both epidermises (Fig. 4.4 and Fig. 4.23).

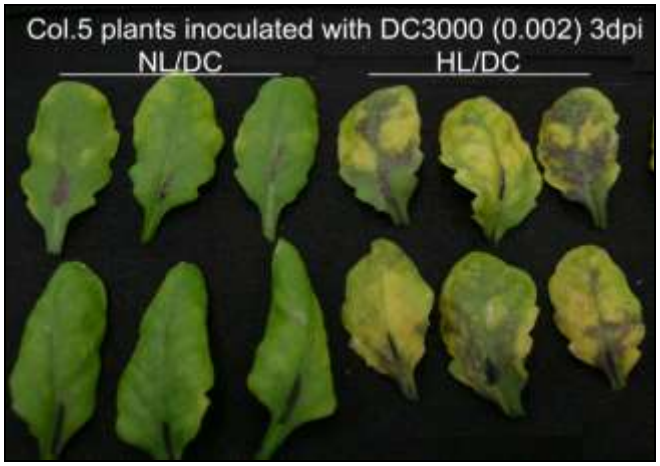


Figure 4.23 Phenotype of Col-5 plants following 3d post-treatment of HL/DC3000 challenged. Three plants were used and three leaves per plant were inoculated with bacterial suspension (OD_{600}: 0.002; 1 X 10^5 cfu ml^{-1}) in comparison to three Col.5 plants under NL/DC3000 conditions.

We demonstrated an increase in susceptibility to DC3000 in Col-5 plants exposed to continuous HL stress [(5d HL pre-treatment followed by 4dpi of DC3000/HL post-treatment "HL/HL") (Fig. 4.3)] with strong HL phenotypes resulting in leaves with dark red purplish, wrinkled, brittle, rolling and leathery in texture compared to Col-5 plants under continuous normal light (NL/NL) (Fig. 4.7A).

Secondly, to answer the original question *"Is the change in phenotype and bacterial growth of/in Col-5 due to pre- or post-treatment of HL?"* The experimental setup mentioned in Section 4.2.1

and Figure 4.6 revealed that there were no significant differences in bacterial growth in Col-5 plants under HL/NL compared with plants grown under continuous normal light "NL/NL" (Fig. 4.7B). Moreover, plants under HL/NL conditions also displayed recovery from anthocyanin accumulation when they post-treated with NL (Fig. 4.7A). The increase in susceptibility in Col-5 plants under NL/HL parallels that in plants under HL/HL with statistically significant differences to the control NL/NL treated plants (students t-test, $p<0.05$) (Fig. 4.7A and 7B). These data suggest that bacterial growth is enhanced by HL treatment and thus plants may develop strategies to avoid simultaneously producing proteins that are involved in abiotic stress and disease resistance responses (Anderson *et al*., 2004). In addition, the long day exposure of Arabidopsis wild type Col-5 to HL/DC3000 (18h exposure of 600µmol for 5days) seem to agree with Bhardwaj et al (2011) findings' that Col-0 plants display greatest resistance to DC3000 in the morning, and greatest susceptibility in the evening under constant light conditions. The bacterial titres observed in the morning in Col-0 plants indicating that the increase in resistance to *P.st* DC3000 observed in wild-type plants in the morning requires a functional circadian clock and results from clock-mediated modulation of pathogen associated molecular pattern (PAMP)-triggered immunity.

4.4.2 Bacterial growth and accumulation of phytohormones on HL-exposed leaves of phytohormone mutants

In response to HL/DC3000 infection the hormone biosynthetic *aao3* and *sid2.1* mutants were examined by comparing them with Col-0 wild type plants. As for Col-5 ecotype HL causes strong synthesis of anthocyanin in Col-0 (Fig.4.8A). Interestingly *aao3* plants had striking chlorotic phenotype and additionally small lesions (Fig. 4.7B). We also demonstrated that HL increased *aao3* susceptibility to the same extent as in Col-0 despite the fact that *aao3* is normally more resistant to DC3000 than the wild type Col-0 (De Torres Zabala *et al*., 2009) (Fig. 4.9A and 9B), whereas Col-0 and *sid2.1* displayed strong synthesis of anthocyanin (Fig. 4.8A and 8C respectively). It is noted that *sid2-1*, inoculation with 0.0002 (1×10^5 cfu ml^{-1}) bacterial suspension for 4dpi, HL/DC3000, showed no significant differences in one case out of two experiments, between *sid2.1* plants under NL- and HL-DC3000 (Fig. 4.9A). These data are consistent with the hypothesis that the inability of *sid2.1* to replenish the initial rapid depletion of the nutritional resources that are essential to sustain such a large bacterial population (De Torres

Zabala *et al*., 2009). However, when *sid2.1* plants were inoculated with 10 - 100 times less dilutions of DC3000 [OD_{600}: 0.00002 (1 X 10^4 cfu ml^{-1}) and 0.000002 (1 X 10^3 cfu ml^{-1})] we demonstrated statistical significant increase in susceptibility [student t-test: 0.00111 and 0.00119 respectively (Fig.4.10A and B)]. Thus the result in Figure 4.9A that showed no significant differences in *sid2.1* infected tissues under both conditions supports our hypthoesis that there were insufficient nutrient resources to support the large bacterial population that grows in *sid2.1* leaves. The evidence that (i) that HL stress reduces SA level (Fig.4.11B) together with (ii) the low expression level of *ICS1* in the *sid2.1* mutant (De Torres Zabala *et al*., 2009) as well as (iii) the reduction of *ICS1* expression level in Col-5 plant tissue subjected to 5d/HL (Fig.4.112B) implicates an important role for modulation of SA underpinning enhanced susceptibility under HL. Furthermore, it has been reported that SA positively activates its synthesis by a feed-forward mechanism (de Torres Zabala et al., 2009). In addition, de Torres hypothesized that pathogen-induced ABA levels locally antagonize PAMP and SA-induced defences.

Our observation suggests that HL promote the enhancement of the antagonism between SA and ABA seen by de Torres under normal conditions (de Torres Zabala *et al*,. 2009), as SA synthesis is suppressed in plant tissues under HL (Fig.4.11B and 4.12B).

Arabidopsis plants produce active photoprotective components while the antioxidative anthocyanin accumulates under HL stress. A proposed mechanism for anthocyanin synthesis, in plants under HL, as reported by Matile (2000), is due to carbohydrate "overflow" during the active recycling of photosynthetic proteins. We conclude that HL-reduced SA alongside an increase in anthocyanin, together with the abundance of carbohydrate, resulted in high susceptibility to DC3000 in *Arabidopsis* HL-challenged tissues as compared with plants under NL.

Previous studies have shown that SA and ABA have antagonistic functions in the *Pseudomonas Arabidopsis* interactions. Analysis of *sid2.1* and *aao3* mutants showed that SA is required to contain growth of the virulent strain DC3000 whereas ABA is required for full DC3000 virulence growth. A concomitant reduction in *NCED3* mRNA levels under HL in *aao3* compared to wild-type plants leads to restriction in bacteria growth in *aao3* indicative of ABA feedback-regulating its own synthesis as demonstrated by De Torres Zabala *et al* (2009). In addition, in Col-0, the increase of ABA under HL/DC3000 (3 fold more than NL/DC3000; Fig.4.11A) seem to promoted the positive effects of ABA on callose formation alongside with the blockage of SA-

inducible defense responses (De Torres Zabala *et al*., 2009) with the reduction of SA levels under HL/DC3000 (2 fold less than NL/DC3000; Fig.4.11B) collectively appear to promote the increase of DC3000 growth under HL conditions.

The strong increase of ABA level in Col-0 HL -challenged leaves (Fig. 4.11) resulted in an increase of bacterial growth (~1.5 fold) compared with NL (Fig. 4.5 and 4.7B) In the *aao3/sid2-1* double mutant, enhanced susceptibility under normal condition, due to loss of SA-mediated basal resistance is epistatically dominant over acquired resistance as a consequence of ABA deficiency, suggests that ABA-mediated negative regulation is essentially through its antagonistic impact on SA-mediated defences (De Torres Zabala *et al*., 2009). These observations agreed with our result that HL/challenged-*aao3/sid2.1* leaves are as susceptible to DC3000 as in *sid2.1* leaves compared with NL conditions (Fig.4.9B)

Additional investigation on ABA receptor overexpression mutant, *pyl5.OE* and protein phosphatase PP2Cs triple mutant, *hab1.1/abi1.2/abi2.1* in response to HL/DC3000 was carried out. Robiu et al (2009) showed that PP2Cs play a major role in regulating ABA signaling both under stress as well as normal growth conditions. Additionally, the phenotype of triple *PP2*C mutants serves to illustrate the importance of ABA in stress responses as well as growth regulation.

Santigo et al (2009a) showed that PYL5 and other members of its protein family inhibited HAB1, ABI1 and ABI2 phosphatase activity in an ABA-dependent manner. Analysis of *hab1.1/abi1.2/abi2.1* and *pyl5.OE* HL/DC3000 challenged plants revealed that HL significantly increased the growth of DC3000 in both lines but ultimately bacterial populations don't exceed ~8 $\log_{10}$ growth, probably due to insufficient nutrient availability. However, inactivation of the three major PP2Cs (HAB1, ABI1 and ABI2) as well as the overexpression of *PYL5* in response to HL/DC3000 contributed in the promotion of DC3000 growth under HL stress, possibly due to the increase of ABA and COR levels in *pyl5.OE* tissues along side the reduction in SA and JA levels compared to Col-0. Robiu et al (2009) showed that inactivation of HAB1/ABI1, ABI1/ABI2, ABI1/PP2CA and HAB1/PP2CA in *Arabidopsis* generated drought-tolerant plants. Additionally, the multiplicity of PP2Cs that regulate ABA signalling appear to have diverse mechanisms to effectively control ABA response both in the absence or presence of stress. Merlot et al (2001) showed that *PP2C*s are strongly induced by ABA through a negative-feedback regulatory mechanism. On the other hand, some members of the *Arabidopsis* family

that includes HAB1- interacting partners were markedly down-regulated by ABA. Therefore, an opposite effect of ABA on gene expression is observed for clade A *PP2C*s and some *PYR/PYL* genes, which encode inhibitors of PP2C activity (Santigo et al., 2009a)

4.4.3 High light effect of flavonoids accumulation on Arabidopsis plants

The investigation of flavonoids accumulation during HL exposure revealed that, the level of flavonols in *aao3, sid2.1* and Col-0 increased under HL treated tissues compared with the plant tissue under NL. The level of kaempferol increased in HL but were suppressed by HL/DC3000 infection. However, anthocyanins accumulated significantly more in Col-0 and *sid2.1* than in *aao3* under HL but remained substantially lower in all plant tissues under NL, suggesting that anthocyanin, glycoside of anthocyanidins, as HL-induced compounds, seem to have a decisive role in response to HL and in susceptibility to *P. syringae*. In other words, our interpretation is that HL contributes directly to *Arabidopsis* susceptibility to *P. syringae*. This seem to agree with Solfanelli *et al* (2006) who found that the augmentation of soluble sugars in Arabidopsis plants as a result of HL exposure provides more nutrient resource to promote the bacterial growth.

Kangasjarvi *et al* (2012) showed that anthocyanin accumulation affects the role of photoreceptor-mediated light signalling, circadian rhythms, and day length in verifying or harmonizing the outcome of defence responses. Such effects could contribute differentially in plant susceptibility to disease and other stresses. In addition photoreceptor pathways are important in determining the day length dependence of responses to intracellular H_2O_2, however light modulation of oxidative stress responses could be dependent on chloroplast pathways (Kangasjarvi *et al*., 2012).

It has been demonstrated that decreased amounts of antioxidative enzymes (APX and catalase) in *Arabidopsis* plants was clearly associated with oxidative stress, although these plants failed to display maintained increases in detectable ROS (Chaouch and Noctor, 2010, Chaouch *et al*., 2011). Maruta *et al.* (2012) showed that thylakoid membrane-bound ascorbate peroxidase (tAPX) plays a role in the regulation of H_2O_2 levels and tAPX silencing enhanced the levels of SA and the response to SA. The tAPX silencing also has a negative effect on expression of ROS responsive genes under HL suggest an antagonistic roles of chloroplastic H_2O_2 in HL responses.

Page *et al.* (2011) showed that ascorbate-deficient mutants (*vtc1* and *vtc2*) were impaired in HL induction of transcripts of anthocyanin biosynthesis enzymes, and the transcription factors PAP1,

GL3 and EGL3 (production of anthocyanin pigment 1, glabra 3 and enhancer of glabra 3 respectively) that activate the pathway of anthocyanin. In addition, HL induction of anthocyanin synthesis involves a redox-sensitive process upstream of the known transcription factors.
Anthocyanins accumulate in preference to kaempferol glycosides and sinapoyl malate in HL. Solfanelli *et al* (2006) showed HL stimulated anthocyanin biosynthesis in *phosphoglucomutase Arabidopsis* mutant (*pgm*), which ws related to augmentation of soluble sugars in Arabidopsis plants which appeared to provide more nutrient resource to promote the bacterial growth under HL conditions. These findings explain and agree with our observation that HL exposure (18h exposure under 600µmol intensity) increased bacterial growth and augmented anthocyanin accumulation in Arabidopsis plants.

4.4.4 Effect of HL/DC3000 on enhanced disease susceptibility 1 (*eds1*) and phytoalexin deficient 4 (*pad4*) mutants

HL/DC3000 interactions in EDS1 and its interacting partner PAD4 were undertaken to determine the defence signalling crosstalk in *Arabidopsis* plants in response to abiotic and biotic stresses. EDS1 protein is necessary for SA- accumulation and has also been shown to be involved in releasing polyunsaturated fatty acids followed by formation of various oxylipins. (Ochsenbein *et al*., 2006). Furthermore, Muhlenbock *et al* (2008) have been shown that EDS1 and PAD4 operate upstream of ethylene and ROS production in the EEE response suggesting that the balanced activities EDS1 and PAD4 regulate signalling of programmed cell death, light acclimation, and holistic defense responses (Muhlenbock et al., 2008)

Recently it has been shown that for successful resistance to host-adapted pathogens and for a balanced response to photo-oxidative stress a combination between EDS1-regulated, SA-antagonized and SA promoted processes, is required and necessary (Straus *et al*., 2010). By contrast, it has been shown that during photo-oxidative stress, initiation of cell death is promoted by the apoplastic ROS-producing enzyme NADPH oxidase (a family of enzymes that generates ROS) and respiratory burst oxidase homolog D (RbohD) (Straus *et al*., 2010, Drummond *et al*., 2011)

The results from *eds1*- and *pad4*-HL/DC3000 challenged plants were; a) a chlorosis phenotype in *eds1*-challenged leaves (Fig. 4.19B`) and a statistically significant increase in DC3000 growth in challenged leaves, b) a visual anthocyanin accumulation in *pad4* HL-challenged leaves (Fig. 4.19D` & 19C` respectively) with increased bacterial growth in HL/DC3000-challenged leaves compared with *pad4* NL/DC3000-and Col-0 NL/DC3000-challenged leaves. Under normal conditions *eds1* is more susceptible than *pad4* and both displayed high level of bacterial growth, than Col-0. One of the possible interpretations is that, as HL reduced SA level in *Arabidopsis* plants, through modulating both EDS1and its co-regulator PAD4. EDS1 and PAD4 also transduce photo-oxidative stress signals leading to cell death and the slowing of plant growth (Mateo et al., 2004, Ochsenbein et al., 2006, Muhlenbock et al., 2008) as well as being required for the defence pathway involving ROI-derived signals in plant cells (Kotchoni and Gachomo, 2006) resulting from abiotic, xenobiotic or biotic environmental stresses (Singh *et al*., 2012). An interesting future experiment would be to look at whether HL accumulates high levels of H_2O_2 which are involved in triggering cell death ROI-derived signals in plants (Cheng *et al*., 2009, Garcia *et al*., 2010, Singh *et al*., 2012).

4.4.5 Effect of HL/DC3000 on phytochrome interacting factor

Analysis of *pif.i (phyA/phyB)* and *pif.h (cry1/cry2)* double mutant plants revealed interesting results. HL/DC3000 treatment resulted in more chlorosis in *pif.i* with visible bleaching in young leaves alongside shortened petioles and increased susceptibility of inoculated leaves. As in *pif.i*, HL caused l bleaching in *pif.h*-HL/DC3000-challenged plants, but despite this *pif.h* was more susceptible compared to its ressitance phenotpye under normal conditions. Thus HL seems to play a role in photobleaching in these mutants, possibly linked to the reduction in SA levels. Shin at el (2009) showed that a single mutant of *pif3* showed severe bleaching compared with the *gun5* (*genomes uncoupled 5*), and double mutants of *pif3/gun5*. Interestingly, PIF3 negatively regulates chlorophyll biosynthesis and photosynthetic genes (Shin *et al*., 2009).

Unexpectedly, under HL/DC3000 challenge *phyA/phyB* plants displayed de-elongated petioles, possibly through hormonal perturbations induced under HL/DC3000 challenge. It is known that auxin (IAA) and brassinosteroids (BR) play an important role in the regulation of enhanced hypocotyl elongation of *Arabidopsis* seedlings in response to blue light depletion (Keuskamp *et*

al., 2011). Furthermore, auxin has been shown to be increased upon exposure to low R:FR and low blue light, and auxin inhibitors abolish the elongation responses to these light signals (Pierik *et al*., 2009a). Moreover, ethylene has also been shown to be an essential component for low R:FR mediated elongation of petioles in *Arabidopsis* (Pierik *et al*., 2009b) and this growth response was abolished by ethylene insensitivity (Pierik *et al*., 2009a)

To conclude, in this chapter,we investigated the interaction between abiotic and biotic stresses in which *Arabidopsis* wild type and mutant plants were exposed to high light stress before being challenged with DC3000. All *Arabidopsis thaliana* ecotypes examined under HL/DC3000 infection exhibited increased susceptibility to the virulent bacterial pathogen *Pseudomonas syringae* under HL. The increase of DC3000 growth in *Arabidopsis*, Col-5, leaves under HL is reflected by a strong induction of *NCED3* expression (Fig. 4.12A) and a concomitant reduction in SA under HL (Fig. 4.12B). Reduction of SA levels correlated with an induction of antimicrobial pathogenesis-related (PR) proteins. In addition, HL suppressed the stimulation of SA synthesis but enhanced accumulation of the light-attenuator anthocyanin. Furthermore, plants infected with DC3000 under HL that showed more bacterial growth with apparent accumulation of anthocyanin tended to have high levels of ABA with exception of ABA deficient mutant (*aao3*) in which the increased growith of DC3000 seemed to be achieved through an ABA independent mechanism. A negative correlation between SA and anthocyanin accumulation under HL was observed. RT-PCR analysis showed that HL impaired the induction of the *ICS1* transcript. In contrast, Bhardwaj *et al* (2011) showed that *Arabidopsis* wild type plants inoculated with *Pst* DC3000*hrpA* showed significantly higher PAMP-triggered callose deposition in the subjective morning than in evening, suggesting that PAMP-triggered immune responses are modulated by the circadian clock and that temporal regulation allows plants to anticipate and respond more effectively to pathogen challenges in the daytime.

Future work;

Future research studies are recommended to shed light on the crosstalk between biotic and abiotic stresses by analysing the level of phytohormones that are involved in plant development and defence responses, such as ethylene, auxin and gibberellin, during the infection of *Pseudomonas syringea* "DC3000". This may shed light on how alterations in hormonal levels

during compatible interaction may facilitate microorganism invasion under specific environmental conditions, but restrict invasion under a different set of conditions.

With respect to photoreceptors (CRY1 and PHY) in light signalling, additional analyses should expand the response of *pif.i* (*phyA/phyB*) and *pif.h* (*cry1/cry2*) particularly to investigate the role of HL effect on petiole de-elongation of *phyA/phyB*under HL stress alongside with the regulation of *phyA/phyB* and *cry1/cry2* on chlorophyll biosynthesis, ROS and photosynthetic gene expression.

It is recommended to continue the work in *hab1-1/abi1-2/abi2-1* by measuring the phytohormones and additionally flavonoids to confirm the very low level of flavonoids present. We have also noted a similar phenotype on *pyl5.OE, hab1-1/abi1-2/abi2-1*and *cry1/cry2* caused by 5 day HL pre-treatment (Fig. 4.24) suggesting that more investigating should be done to shed more light on interaction between high light and ABA receptor alongside with protein phosphatase and phytochrome proteins in *Arabidopsis* plants.

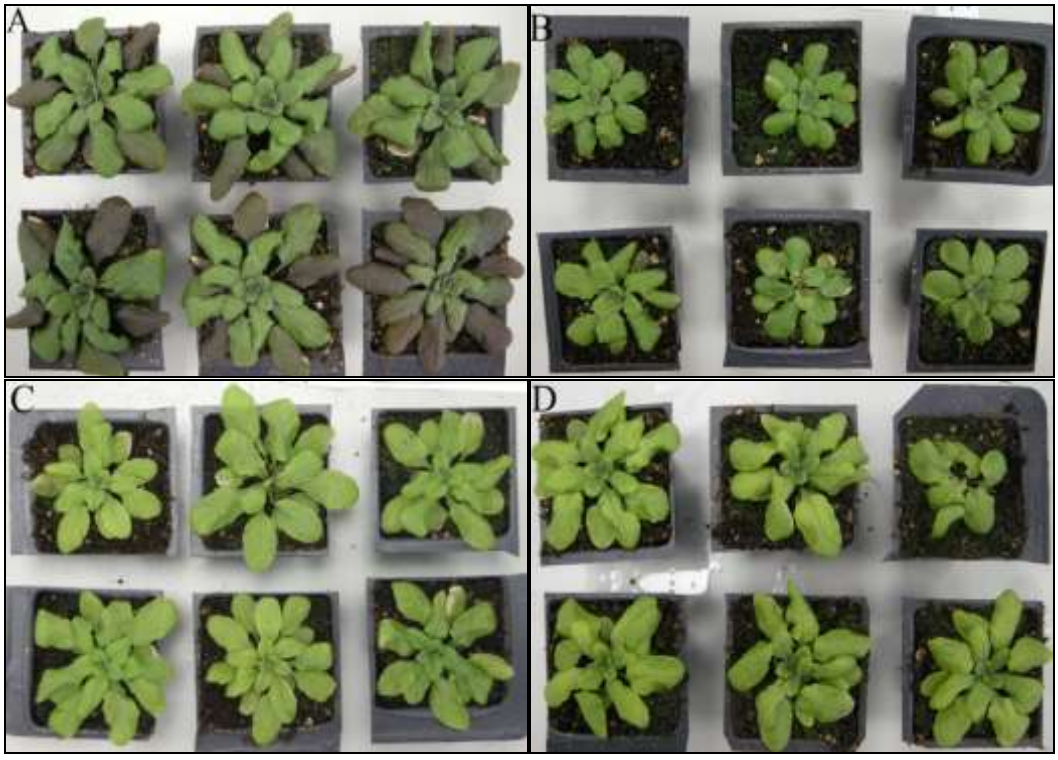


Figure 4.24 Effect of 5 day HL exposure in some *Arabidopsis* mutant plants compared with wild-type. (A) Col-0, (B) ABA PYL5 receptor mutant *pyl5.OE,* (C) protein phosphatases triple mutant *hab1-1/abi1-2/abi2-1,* and (D) phytochrome interacting factor *pif.h* (*cry1 cry2*)

Additionally, and to follow the anthocyanin and flavonol pathways, expression levels of the biosynthetic genes *chalcone synthase* (*CHS), flavonol synthase (FLS)* and *leucoanthocyanidin dioxygenase* (*LDOX*) and signalling genes *enhancer of glabra 3 like* (*EGL3), glabra 3* (*GL3),* production of *anthocyanin pigment 1* (*PAP1)* and *transparent testa glabra 1* (*TTG1*) should be determined under HL and HL/DC3000 infection conditions specifically in *aao3, sid2.1* mutants.

A full understanding of the mechanism of *Arabidopsis* plant–pathogen interactions and their signalling networks under HL/DC3000 challenge will shed new light on how plants can respond to environmental perturbations and help develop more resilient plants. Molecular, biochemical and physiological analyses of pathogen responses in mutants that are impaired in light sensing can now take advantage of rapidly developing genomics tools and bioinformatics techniques.

Chapter 5: Characterization of transgenic lines expressing the PYL cytosolic ABA receptors

5.1 Introduction

The phytohormone abscisic acid (ABA) regulates several developmental processes and adaptive stress responses in plants. In other words, ABA controls vital abiotic stress-induced and developmental responses including seed dormancy, germination, development, growth regulation and stomatal closure (Finkelstein *et al*., 2002b, Cutler et al., 2010, Nishimura *et al*., 2010) ABA signalling is mediated through protein phosphorylation/dephosphorylation events involving several well characterised protein kinases and phosphatases. For instance, the orthologous proteins open stomata 1/ SNF1-related protein kinase 2.6 (OST1/SnRK2.6) regulate ABA-induced stomatal closure (Li et al., 2000; Mustilli et al., 2002; Yoshida et al., 2002). The Arabidopsis genome contains 10 SnRK2s; among them, SnRK2.2, SnRK2.3, and SnRK2.6/OST1 are key regulators of ABA signalling. Both SnRK2.2 and SnRK2.3 regulate ABA responses in germination, growth development, and gene expression, while SnRK2.6/OST1 specifically regulates stomatal aperture (Mustilli et al., 2002; Fujii et al., 2007).
Additionally, Fujii *et al* (2007) showed that SNRK's phosphorylate and activate ABA response element binding factor (ABF) transcription factors. Subsequently, ABF transcription factors bind to ABA-responsive promoter elements (ABREs) and initiate the transcription of ABA responsive genes. It has recently been shown that increase of ABA levels in the plant cell leads to Pyrabactin resistance1/pyrabactin resistance1-like, respectively (PYR/PYL) receptor-mediated inhibition of PP2C activity, which results in the activation of the three SnRK2s and ultimately of the ABA signalling pathway (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009; Vlad et al., 2009; Gonzalez- Guzman et al., 2012).
However, disruption of the PP2A regulatory subunit ROOTS CURL IN NAPHTHYLPHTHALAMIC ACID1 (RCN1) confers ABA insensitivity in Arabidopsis, which suggests that RCN1 functions as a positive transducer of ABA signalling (Kwak et al., 2002). Pernas et al. (2007) has also reported that a catalytic subunit of PP2A functions as a negative regulator of ABA signalling.
In the last decade number of studies has focused on ABA perception by plant cells. Several receptors have been proposed; the RNA binding protein FCA, which was subsequently retracted

(Razem *et al*., 2006, Razem *et al*., 2008); a G protein- coupled receptor (GPCR), GCR2 (Liu *et al*., 2007), which has attracted various criticisms (Gao *et al*., 2007, Johnston *et al*., 2007, Illingworth *et al*., 2008, Risk *et al*., 2008) and *ABAR/ChlH/GUN5*, a Mg- chelatase (Shen *et al*., 2006), which appears not to function in barley (Muller and Hansson, 2009). Cutler *et al*. (2009) proposed a model which incorporates several of the other putative ABA receptors: *GTG*s as membrane bound ABA receptors, the *PYR*/*PYL*s as cytoplasmic receptors and *ABAR/ChlH/GUN5* as a receptor located in the chloroplast. In addition, two further GPCRs, GTG1 and GTG2, were also reported as ABA receptors (Pandey *et al*., 2009).

Recently, a number of groups using different approaches independently identified PYR/PYL/RCAR (Pyrabactin resistance1/pyrabactin1-like/ regulatory component of ABA receptor, respectively) proteins as an ABA receptors (Ma et al (2009), Nishimura et al (2010), Park et al (2009) and Santigo et al (2009). Zhao et al. (2007) previously showed that Pyrabactin is a selective agonist in seed germination of PYR1 and also induces stomatal closure in the abaxial epidermis of, *Pisum sativum*, pea plants (Puli and Raghavendra, 2012). It was also shown that pyrabactin is an ABA-agonist for PYR1 and PYL1, but it was, unexpectedly,antagonistic for PYL2 (Melcher et al., 2010). Comparison of the crystal structures of PYL1-pyrabactin-ABI1 and PYL1/PYL2-pyrabactin complexes has provided the mechanism to discriminate between productive and non-productive pyrabactin binding. In the case of PYR1 and PYL1, pyrabactin binds inside the receptor cavity and establishes interactions that stabilise the closed conformation of the gating loops, as ABA does. In the case of PYL2 however, pyrabactin also binds inside the receptor cavity but adopts a different conformation which does not promote closure of the gating loops (Melcher et al., 2010, Yuan et al., 2010, Hao et al., 2010)

Fujii et al (2009) has showed that PYR/PYL-ABA receptors are constituted from 14-member family, and all of them (except PYL13) are able to activate ABA-responsive gene expression using protoplast transfection assays. In addition, and from biochemical point, Dupeux et al (2011) characterized two families of PYR/PYL by different oligomeric states, some being dimeric (PYR1, PYL1 and PYL2) whereas others are monomeric (PYL5, PYL6 and PYL8). Thus, the dimeric receptors showed high dissociation constant for ABA (greater than 50 µM; lower affinity) than the monomeric ones (approximately 1 µM).

Park *et al*. (2009) and Ma *et al*. (2009) showed that PYR1/PYL/RCAR proteins bind to PP2Cs in the presence of ABA and prevent their repression of SnRK2 kinases. Other groups have

independently isolated the PYR/PYLs (Santiago *et al*., 2009a, Nishimura *et al*., 2009) that belong to Bet v I-fold super family (the START domain family) (Radauer *et al*., 2008). It has also shown that PYR/PYLs directly interact with signalling molecules such as SnRK2 (Cutler *et al*., 2010). Furthermore, several research groups have published the structures of PYR/PYLs with ABA-bound (Santiago *et al*., 2009b, Nishimura *et al*., 2009). In addition, Santiago et al (2009b) determined precise details of ABA bound PYR1 structure showing specific interactions with ABA. Major determinants of stereo selectivity are spatial constraints around positions 2 and 6 in the ring of the ABA molecule, rather than interactions involving polar groups which may explain the striking biological activity of the unnatural (-)-ABA stereoisomer.

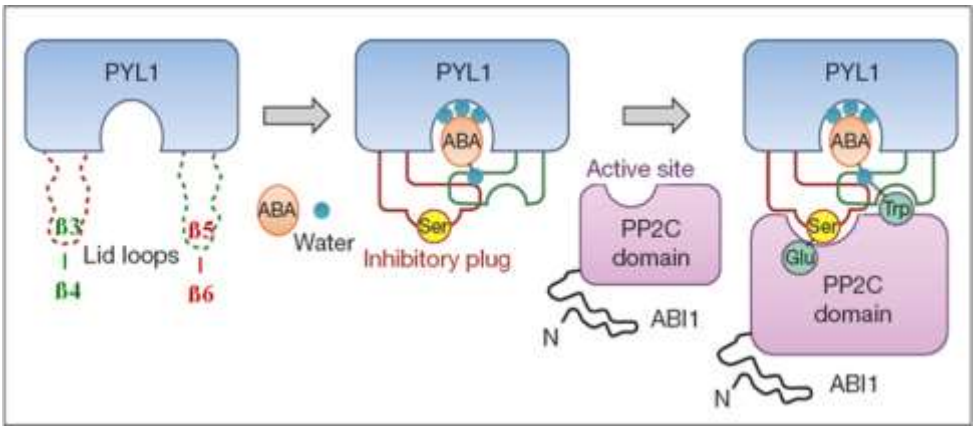


Figure 5.1 Scheme of structural mechanism for the (+)-ABA-dependent inhibition of a negative regulator ABI1 in the ABA pathway by a (+)-ABA receptor PYL1. Image adapted from (Miyazono et al., 2009)

Miyazono *et al* showed that PYL1 contacts ABI1 using the loops covering (+) –ABA (hydrophobic interface) while ABI1 interacts with PYL1 using the region near its active site that is covered by the ß3-ß4 loop of PYL1 and Trp^{300} in the ABI1 protruding domain which interacts with the hydrophobic pocket of (+)-ABA-bound PYL1. In addition, the ß3-ß4 loop of PYL1 covers the active site of ABI1, inhibiting its PP2C activity. The ß5-ß6 hydrophobic pocket is the key architecture for the PYL1-ABI1 interaction and the docking of ABI1 Trp^{300} into the PYL1 hydrophobic pocket is an essential step for the inhibition of ABI1 by PYL1 (Fig. 5.1) (Miyazono et al., 2009).

Melcher has also reported a gate-latch-lock mechanism (Melcher et al., 2009) under which ABA-binding to the open ligand-binding pocket of a PYR/PYL induces the closure of a gating loop and produces a surface by which the complex can bind and inhibit PP2Cs (Fig.5.2) (Fujii et al., 2009).

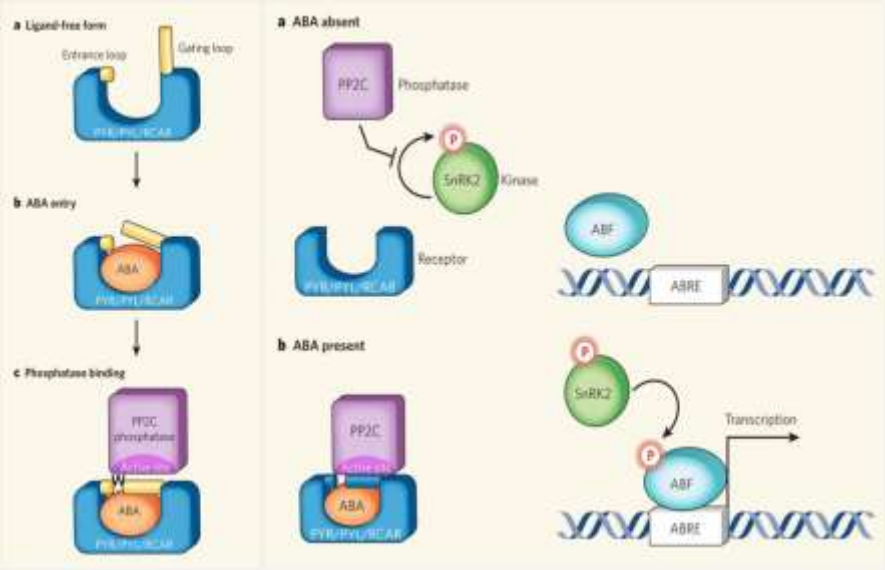


Figure 5.2 Scheme of ABA mechanisms in Arabidopsis plants shows ABA induced closure of *PYR*/*PYL* gate and binding to PP2Cs. Image adapted from (Sheard and Zheng, 2009)

ABA binding induces the closure of the ligand entry gate, which allows the receptor to bind and competitively inhibit PP2Cs. The interaction between PP2Cs and receptors further induce conformational changes that lock the receptor in the closed conformation (Melcher et al., 2010). A range of subtypes ABA receptors (PYL5-PYL13) shows an ABA-independent interaction with PP2Cs (Ma et al., 2009, Park et al., 2009). Melcher (2010) showed that pyrabactin promoted the interaction of PYR1, PYL1, PYL3, PYL5, PYL6 and PYL4 with PP2Cs (HAB1, ABI1 and ABI2) but not PYL2 due to selective antagonist (Melcher et al., 2010)

Hao et al (2011) has recently shown that in the presence of ABA most of the PYL proteins inhibited the phosphatase activity whereas PYL6 inhibited about 80% of the phosphatase activity of PP2CA. In addition, PYLs 5–9 (excluding PYL7 which has not been tested) inhibited the four PP2Cs (ABI1, HAB1, HAB2 and PP2CA) to various degrees in the absence of ligand. Furthermore, Antoni et al (2013) showed that PYL8 has a non redundant role in regulation of root sensitivity to ABA, and the root expression pattern of *PYL8* shows some similarity to that of other PYR/PYL receptors. Additionally, PYL8 is "biochemically" a monomeric receptor, with higher affinity for ABA than dimeric receptors, and showed greater capacity to inhibit *in vitro* specific PP2Cs such as ABI1, PP2CA, and HAI1 than other PYR/PYLs (Santiago et al., 2009;

Antoni et al., 2013). Thus the lack of PYL8 function leads to globally enhanced activity of PP2Cs or diminished capacity to inhibit those PP2Cs (Antoni et al., 2013). Interestingly, PYL4 showed clear inhibition of HAB2 but not the other three PP2Cs (Hao et al., 2011). Nishimura et al. (2010) showed that multiple interactions among PP2Cs and PYR/PYLs occur *in vivo*, generating a regulatory network that offers a wide range of sensitivity and combinatorial possibilities to modulate ABA signalling.

Our aim was to investigate the three PYR-PYL-RCAR protein family PYL4, 5 & 6 which were identified by transcript profiling as DC3000 responsive. The seeds corresponding to T-DNA insertion lines, *PYL4*, *PYL5* and *PYL6*, were ordered from Nottingham Arabidopsis Stock Centre "NASC" (Appendix A1.Table1. No.1-3). We also generated a *pyl5/6* double mutant and were gifted a *pyl5* over- expression line and *pyl4/5* double mutant from the Pedro Rodriguez Laboratory-Spain (Santiago et al., 2009b). For comparsion, we included a protein phosphatases triple mutant (*hab1-1/abi2-1/abi1-2*) (Rubio et al., 2009) and wild- type Col-0 or Col-5. The main aim in this work was to investigate whether *PYL4*, *5* or *6* were involved in plant pathogen interactions. We determined *pyl5* over- expression line susceptibility under a combination of abiotic and biotic conditions (as described in Chapter 4). Then we sought to generate luciferase *PYL4*, *5* and *6* transgenic lines for gene expression level and localisation and to express *PYL4*, *5* and *6* under a CaMV (35S) promoter with HA and MYC epitope tags for overexpressed and cellular localization studies using immuno-fluorescence or western blotting**.** Finally, we also sought to express GFP or YFP tagged *PYLs* and use confocal microscopy to examine histochemical localisation of the *PYL4*, *5* and *6* following pathogen challenge and other stresses.

5.2 Material and methods

General material and methods used in this Chapter are described in Chapter 2, in which, and for instance, some PCRs were carried out by using the appropriate primers that show in Appendix A 1 (Table1). Material and methods specific to this chapter are as follows:

5.2.1 Protein extraction

For total protein extraction, one frozen leaf (30-50mg) was ground in mortar before the fine powder was transferred to a 1.5 ml eppendorf tube and 100-200 µl of extraction buffer added (100 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X100, 2.5 mM DTT, 1 mM PMSF). The mixture was centrifuged at 10000 g for 5 min at 4ºC before the supernatant (total protein extract) was passed to a clean 1.5 ml microfuge tube. Total protein was quantified by the Bradford method (Bio-Rad).

5.2.2 Separation and visualisation of protein

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAG) was used for protein separation. PAG consisted of a 4% stacking gel (upper) and a 12% resolving gel (lower). The resolving and stacking gels were made up as described in Appendix A15A. Ten percent (10%) APS (Bio-Rad) and TEMED were used to polymerize the resolving and stacking gels. Gels were run in 1 X electrode (running) buffer (as described in Appendix A15D) at a constant voltage (120V) for 1-2h, stained for 2-3h in staining solution [10% Methanol (Fisher, UK), 5% acetic acid (Fisher, UK) and 0.1% Coomassie Brilliant Blue R-250 (Sigma, UK)] and distained overnight (10% Methanol and 5% acetic acid).

5.2.3 Protein "Bradford" assay

Protein standards were prepared from lysozyme (Thermo-UK) at concentrations of 1, 2, 5, 10, 15, 20 and 25µg/ml before 10 µl of each lysozyme standard was added to the sample buffer [200 µl of dye Reagent Concentrate (Bio-Rad) and 790 µl dH_2O] along with 200 µl of dye and 800 µl dH_2O as a blank sample. OD_{595} was measured against the blank. A standard curve, OD_{595} versus Lys concentration, was plotted and used to determine the protein.

5.2.4 Protein western blots

Western blotting of SDS-PAGE gels was carried out using a Bio-Rad Mini-Protean II Western Blot system following the manufacturer's instructions. The protein concentrations were determined by Bradford assay (Section 2.2.9), and 50 µg of protein sample was denaturation at 95°C for 5 min, followed by brief centrifugation then loaded alongside prestained protein marker (Bio-Labs). Following electrophoresis, proteins were blotted to Hybond-P PVDF membrane (Amersham) using 1X transfer buffer. The efficiency of protein transfer was monitored by immersing the blots into staining solution (For 1L; 100 ml Methanol, 50 ml acetic acid and 1 g Coomassie Brilliant Blue).

5.2.4.1 Western blot of CaMV: HA: PYLs

Following protein blotting (Section 2.2.10), the HA membrane was washed briefly with water before adding distain solution (10%methanol and 5% glacial acetic acid). After 10-15 min of gentle agitation the distained solution was removed and the membrane washed briefly with dH_2O before being photographed. For blocking, the membrane was pre-wetted in 100% (v/v) methanol and washed 3 times for 5 min in Tris-buffered saline (TBS), pH 7.6, and incubated in 50 ml blocking solution comprising TBS; (8g of sodium chloride, 20 ml of 1M Tris-HCl, pH7.6, /L), 5 % (w/v) non-fat dried milk and 0.1% (v/v) Tween 20, with orbital shaking at 9 rpm, for 60 min. The membrane was washed twice with TBS for 15 min, transferred to 3 ml blocking solution containing 15 µl αRAT HA monoclonal antibody 1:2000 dilutions (stock 100 µg IgG/ml, Roche, UK) and incubated for 60 min at RT with orbital shaking at 9 rpm before washed 3 x 15 min in TBS at RT. Then the membrane was incubated with the second antibody, 1 µl of αRAT.HRP in 5 ml (1:5000) TBS/3% non-fat dried milk and incubated for 60 min at RT with orbital shaking at 9 rpm before washing 3 x 15 min in TBS at RT. The western blot was developed in detection reagent A and B (Amersham- ECL Plus, Western Blotting Detection Reagents) in a ratio of 40:1 following the manufacturer's instructions.

5.2.4.2 Western blot of CaMV:MYC:PYLs

MYC-tagged protein detection followed the same procedure described in Section 2.2.10.1 except the blocking solution was phosphate-buffered saline [PBS; (80 mM of Di-sodium hydrogen orthophosphate anhydrous (Na2HPO4), 20 mM of sodium di-hydrogen orthophosphate and 100

mM sodium chloride (NaCl) pH 7.5, 5 % (w/v) non-fat dried milk and 0.1% (v/v) Tween 20] and the first antibody was Anti-myc (Invitrogen; 1:1000) and the second was anti-α Mouse-HRP, (Roche; 1:10000).

5.2.4.3 Western blot of eGFP: PYL5

Western blot of B8eGFP:PYL5 transgenic plant protein extract was carried out as described in Section 2.2.10.1, where the membrane was blocked with PBS, 5 % non-fat dried milk and 0.1% Tween with orbital shaking at 9 rpm for 60 min before being washed with PBS 3 x 15 min. The primary antibody; 3 µl of anti-GFP (Invitrogen, UK; 1:1000), was mixed with 3ml of PBS/5% non-fat dried milk and 0.1% Tween 20 and shaken at 9 rpm for 60 min before being washed 3 times in PBS for 15 min each. Then secondary antibody, 1 µl of anti-Mouse HRP (Invitrogen, UK) was added to 10 ml of PBS/5% non-fat dried milk and 0.1% Tween 20 with orbital shaking at 9 rpm for 60 min before being washed with PBS for 3 x 15 min.

5.2.4.4 Blots chemiluminescent detection

Western blotting was carried out as described above using Amersham ECL plus western blotting detection reagents using a 40:1 ratio of detection solutions A and B at 0.1ml/cm^2. The detection solution was pipetted on to the membrane and incubated for 5 min at RT before excess detection reagents were drained off. The blots wee wrapped in Saran Wrap avoiding air bubbles. The wrapped blots protein was exposed to autoradiography film in an X-ray film cassette. Film was initially exposed for 15 seconds and subsequent exposures adjusted based upon signal intensity.

5.2.5 Preparation of constructs

5.2.5.1 Luciferase construct

PCR was carried out for *PYL4*, *PYL5* and *PYL6* promoters with forward primer 5` Kpn1 and reverse primer 3` Nco1 (Appendix A1, Table1. Primers No. 18-23) at the initiating methionine encoding ATG and the pC1LUCP vector (Genebank accession AF234298). pCAMBIA C1302 was digested with Kpn1 and Nco1 (Fig .5.3 and Appendix A2.Table.2. No.1-2). The Agarose gel bands of PCR products for *PYLs* + promoters were purified using a Qiagen gel extraction kit following the manufacturer's instructions. Ligation of the *PYL* promoter fragments were carried

out with a 1:3 ratio vector to insert. *Escherichia coli* competent cells (DH5) were transformed and recombinants selected over night at 37°C using the appropriate antibiotic. Bacterial colonies were screened by PCR using Luc-up and M13 primers (Appendix A1. Table1. Primers No. 24 and 59). One verified single colony from each *PYL*/vector ligation was grown in LB liquid media O/N and the next day the recombinant plasmids were purified using a Qiagen miniprep kit, digested with Nco1 and Knp1 and visualised on ethidium stained agarose gels. One *Agrobacterium tumefaciens* transformation of each plasmid was carried out using calcium chloride heat shock competent, *A. tumefaciens* (GV3101) with rifampicin 50 mg ml^{-1} (Sigma-UK), kanamycin 25 mg ml^{-1}- (Sigma-UK) and Gentamycin 25 mg ml^{-1} (Medlford-UK) selection at 42ºC for 30 sec. Plates were incubated at 28ºC for 2 days then 8-12 colonies from each construct were checked by PCR using Luc-up and M13 primers. Positive colonies were grown O/N at 28°C shaker in 500 ml LB with antibiotics. *Agrobacterium* cultures were harvested and resuspended in 500 ml of 5% sucrose and to OD_{600} 1-2. 100 µl of Silwet L77 was added and floral inflorescences of *Arabidopsis thaliana* ecotype Col-0 or Col-5 were dipped in this Agrobacterium solution (for each *PYL* individually) for 5-10 seconds with gentle agitation. The dipped plants were laid on their side on a tray inside plastic bag for 24 h to maintain high humidity. The following day the plants were stood upright without watering for another 24h. The plants were then watered and grew as usual till seeds became mature. The seeds were harvested and transformants were selected using appropriate antibiotics as described in Chapter2.1.1.

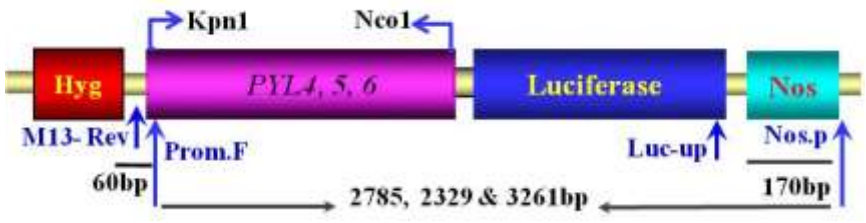


Figure 5.3 Schematic structure of pC1LUCP vector (AF234298) CAMBIA C11302. The scheme shows the structure of the Agrobacterium-mediated stable transformation vectors generated by Kpn1 and Nco1 digestion alongside the expected sizes of the PCR implications generated from M13 reverse, promoter forward, Luc-up and Nos primers for *PYL4*, *PYL5* and *PYL6* (PCR product size; 2785, 2329 & 3261bp respectively). Red colour indicates Hygromycin resistance gene, Pink colour indicates *PYL* promoters + genes, Blue indicates Luciferase, and Turquoise colour indicates *Nos* (nopaline synthase) 3 UTR (poly A signal.

5.2.5.2 Epitope tagged CaMV::HA / CAMV::MYC construct

For epitope-tagging two vectors, pCXSN-HA 1254 and pCXSN-MYC 1256, were used. These vectors contain a 35S promoter (Fig. 5.4) allowing direct cloning of genes of interest (*PYL4*, *PYL5* and *PYL6*) into vectors directing high level expression of the target protein with HA and MYC epitope-tags in Arabidopsis (Chen *et al*., 2009). PCR for *PYL4*, *PYL5* and *PYL6* was carried out using C-START and C-STOP primers [Appendix A1. Table.1 Primers No. 10-15].

The PYL PCR products were run on a 1.2% agarose gel and the bands were purified with Qiaquick PCR purification kit following the manufacturer's instructions. Plasmid vectors [pCXSN-HA (1254) and pCXSN-MYC (1256)] were digested with Xcm1 (New England-BioLabs). Ligation of the plasmids and genes of interest (1: 3) were undertaken at 37°C O/N and positive colonies were checked by PCR using C-START with Nos, CaMV with Nos or CaMV with C-STOP for each *PYL* (Fig. 5.4). A positive colony from each PYL ligation was transformed into *E. coli* competent cells (DH5) and after O/N incubation at 37°C the colonies were checked by PCR using the primer combinations shown in Fig. 5.4. Validated single colonies from each transformation were grown O/N in LB liquid with the appropriate antibiotics. Plasmids were purified using Qiaprep spin Miniprep kit following the manufacturer's instructions for Agrobacterium (GV3101) transformation. The protocol for Agrobacterium transformations and plant dipping was followed as described (5.2.5.1) and seeds from CaMV:HA/CaMV:MYC:*PYL4, PYL5* and *PYL6* were harvested.

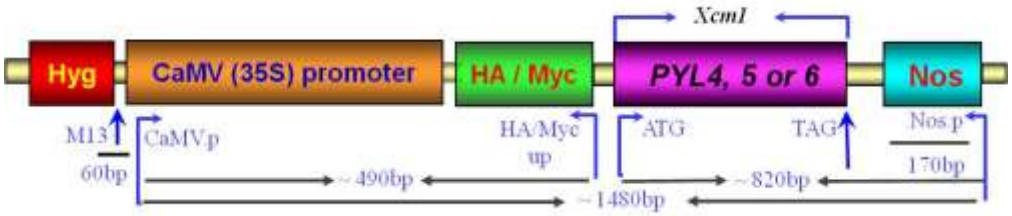


Figure 5.4 Scheme for epitope tagging to generate pCXSN-HA/Myc: *PYL4*, *5* or *6* plasmids. PCRs were carried out with different primers (M13-Reverse, CaMV-For, HA/Myc-up, C.START, C.STOP and Nos). Red colour indicates Hygromycin resistance gene, Orange colour indicates Cauliflower mosaic virus (35S) promoter, Green colour indicates HA or myc epitope tag proteins, Pink colour indicates *PYL* genes and Turquoise colour indicates *Nos*; (nopaline synthase) 3 UTR (poly A signal. The scheme also shows restriction sites of XcmI.

5.2.5.3 GFP / YFP: *PYL4*, *PYL5* and *PYL6* constructs

The objective of this section was to generate constructs containing eGFP and YFP C- terminal fusions to the *PYL4*, *PYL5* and *PYL6* promoters and transform these into *pyl4*, *pyl5* and *pyl6* mutant backgrounds. Col-0 genomic DNA was used as a template for PCR of the *PYL4*, *PYL5* and *PYL6* promoters using gene specific promoter primers forward and reverse (Appendix1. Table1. Primers No. 50-60 and Appendix10-12). PCR products were run on a 1.2% agarose gel and bands were purified using the Qiagen gel extraction. PCR purified gel bands for the three PYLs promoters and C1eGFP and YFP (pCAMBIA1302; pUC18) promoters were transformed into *E. coli* competent (DH5) cells with the appropriate antibiotic and grown over night at 37°C shaker. PCR implications for each *PYL* promoter and the vectors were digested with Sac1/Nco1 (Progema) for *PYL4* or EcoR1/Nco1 (Progema) for *PYL5* and *6* (Appendix4) before ligation of *PYL* promoters into the vectors using a ratio of 1:3 (vector to insert). One positive colony was grown in LB liquid media O/N and the following day the plasmids were purified using the Qiagen miniprep kit. This plasmid was used to transform *A. tumefaciens* (GV3101) and validated transformants were used to transform *A. thaliana* Col-0 using the floral dipping method described 5.2.5.1. Four to five weeks later seeds were harvested and transformants were selected in Hygromycin plates as described in Chapter2.1.1.

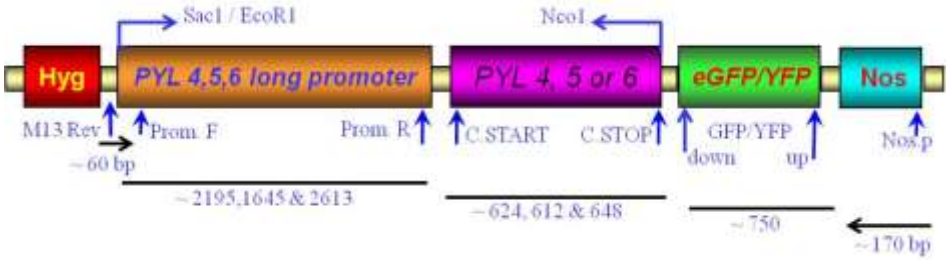


Figure 5.5 PCR and Digestion scheme of C1eGFP/YFP: *PYL4*, *5* and *6* plasmids. PCRs carried out with different primers combinations (M13.Rev, Promoter Forward, Promoter Reverse, C.START, C.STOP, GFP/YFP-up or down and Nos). Red colour indicates Hygromycin resistance gene, Orange colour indicates PYLs long promoter, Pink colour indicates *PYL* genes, Green colour indicates GFP or YFP and Turquoise colour indicates *Nos* (nopaline synthase) 3 UTR (poly A signal). The scheme also shows restriction sites of Sac1/Nco1 for *PYL4* and EcoR1/Nco1 for *PYL5* and *PYL6*.

5.2.6 Luciferin treatment

For bacterial challenge, four to five week-old plants from*PYL5-LUC* and *PYL6-LUC* were sprayed with luciferin (25ng/µl) (Promega- USA) before being kept in the dark for 30 min. Then three leaves from each plant line were inoculated with virulent DC3000, DC3000*hrpA* or mock ($MgCl_2$, 10 µM) and luciferase bioluminescence captured in 5 min intervals over 24 h using a Hamamatsu Orca II CCD camera.

For ABA treatments, 2.6 mg of ABA (Sigma, Dorset, UK) was solubilised in 200 µl ethanol diluted in 10 ml dH_2O giving a final concentration of 1mM ABA. Plants were sprayed with luciferin (25ng/µl), kept on dark for 30 min before leaves were infiltrated with serial dilutions of ABA (100 µM, 10 µM, 1 µM and 100 nM) and mock (0.2% ethanol). Assays were performed for transgenic plants under the CCD camera.

5.3 Results

The phytohormone ABA plays an important role in many plant pathogen interactions where it acts as a negative regulator of disease resistance. In some cases, ABA can also promote plant defense. For example, ABA plays a positive role in resistance against the necrotrophic pathogens *Pythium irregulare* (oomycete) and *Alternaria brassicicola* (fungus) since the ABA deficient mutants *aba2-12*, *aao3-2* and ABA signalling defective *abi4-1* were more susceptible to these pathogens (Adie et al., 2007). Thus, role of ABA in disease resistance depends on the type of pathogen, its specific way of entering the host and is therefore involved in a complicated network of synergistic and antagonistic interactions.

5.3.1 Bioinformatic studies of the *PYR/PYL/RCAR* gene family

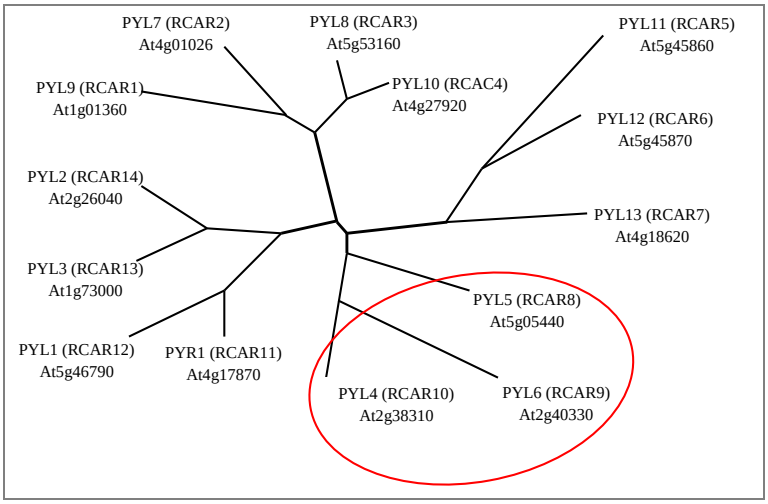


Figure 5.6 Phylogenetic tree of *PYR/PYL/RCAR* gene family in *Arabidopsis thaliana* shows the 14 members with distinct clade for *PYL 4, 5* and *6* (circled above) analysed in this study. Adapted from (Ma *et al*., 2009, Park *et al*., 2009)

The Bio-Array Resource for Arabidopsis Functional Genomics (BAR) showed that expression of *PYR1*, *PYL1*, *PYL4*, *PYL5*, *PYL6* and *PYL8* were down-regulated whilst Clade A *PP2C*s were up-regulated in Arabidopsis seedling tissues, 7 days-old, by 10 µM ABA treatment after 0.5, 1 and 3h (Fig. 5.7) (Santiago et al., 2009a, Kilian et al., 2007).

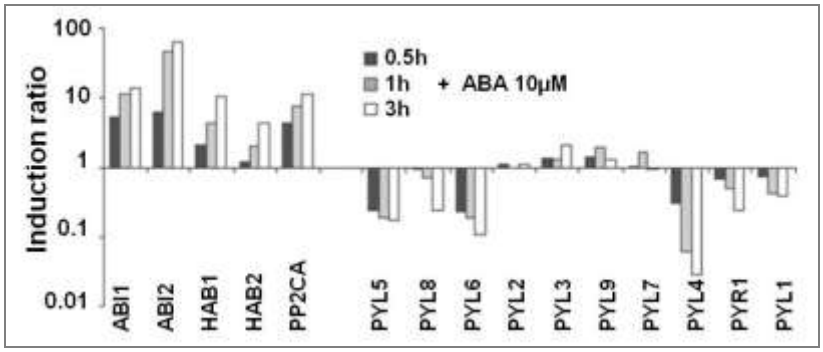


Figure 5.7 Scheme shows the regulation of clade A *PP2C*s and some *PYR/PYL* genes. Adapted from (Kilian et al., 2007, Santiago et al., 2009a)

5.3.2 Expression levels of *PYL4, PYL5* and *PYL6*

To investigate the regulation of *PYL4, PYL5* and *PYL6* genes in response to DC3000 infection, we used quantitative RT-PCR (Appendix A1. Table1. Primers No. 31-42). Col-0 plants were challenged with the virulent DC3000 wild-type strain or the type III secretion-deficient *hrpA* mutant strain and sampled before, non-inoculation (NI), and 6, 12 and 18hpi (Fig. 5.8A - 8C).

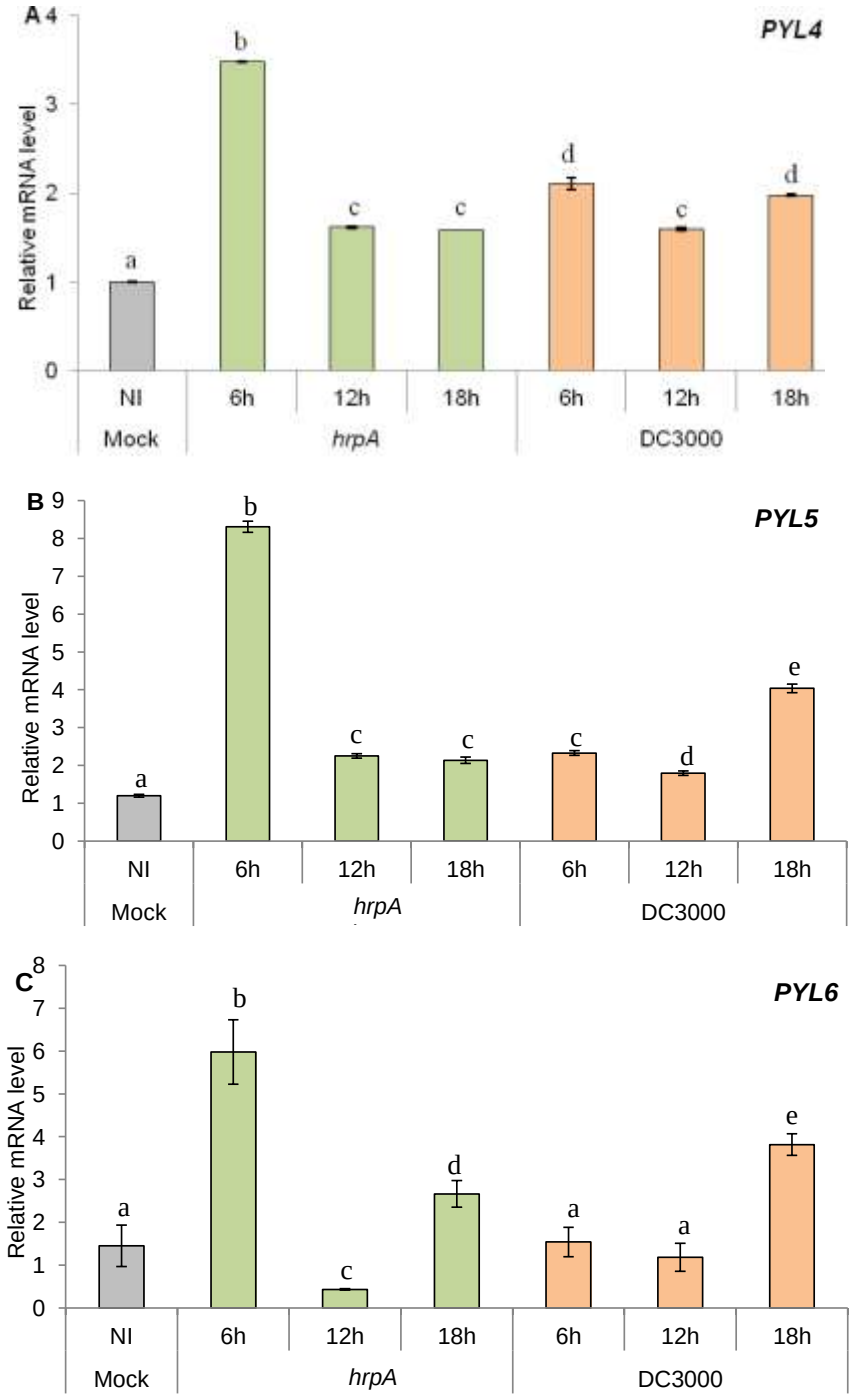


Figure 5.8 Relative level of expression of *PYL4, PYL5* and *PYL6* genes during disease development. Col-0 plants were infiltrated with (OD_{600}: 0.15; $0.75x10^8$ cfu ml^{-1}) of the virulent DC3000 wild-type or type III secretion-deficient *hrpA* mutant strain and mock (Non-Infected "NI" tissues). Steady-state levels of relative mRNA were determined at the indicated times by reverse transcript RT-PCR. Bars represent the mean of three technical replicates, three leaves per replicate. Labels "a, b, etc" above the columns represent significant differences between samples at $p < 0.05$ (Student's t-test for pair wise comparison of non-treated and treated tissues with DC3000 and *hrpA vs* mock).

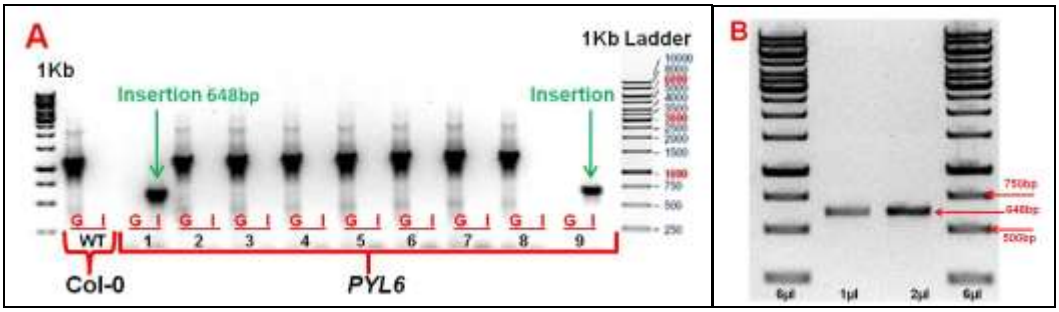
Figure 5.8A- C shows that at 6 hpi with *hrpA* the level of expression in the three *PYL* genes were significantly higher (*t-test*; 0.001, 1.42E-07 and 0.005 respectively) than non-inoculated (NI) tissues but the expression level of *PYL4* later decreased at 12hpi in *hrpA* challenged leaves. In the case of *PYL6* expression levels were lowest at 12h. Notably, in the three *PYLs* genes, expression levels at 6h after DC3000 challenge were considerably lower than 6h after *hrpA* challenge (approximately 50%, 25% and 33% for *PYL4*, *PYL5* and *PYL6*, respectively (Fig.5.8A-8C).

By contrast, the expression level of *PYL5* at 18h was considerably higher was considerably higher in DC3000 compared to *hrpA* challenge (approximately 50%) (Fig. 5.8B). The experiment was repeated twice.

5.3.3 *PYL 4, PYL5* and *PYL6* polymerase chain reaction amplification

To characterise knockout *PYL4*, *PYL5* and *PYL6*, seeds were germinated for two weeks before DNA was isolated as described in Chapter 2.1 and 2.2.1. A PCR strategy, as described in Chapter 2.2.2, was used to confirm homozygosity for *pyl4*, *pyl5* and *pyl6.* Primer sequences used to genotype *pyl4*, *pyl5* and *pyl6* knock-out mutants are showed in Appendix A1. Table 1. Primers No. 4-17)

After we confirmed the homozygocity of *pyl4* and *pyl5* we selected a *pyl6* homozygous plant (Fig. 5.9A). The PCR product for *pyl6* homozygous plant was purified (Fig. 5.9B) and sequenced. (Appendix3)



Fig**ure 5.9** Genotyping of *PYL6* plants using forward and LB3 (SAIL) primers. (A) Agarose gel of PCR for nine genomic DNA extracted from PYL6 segregating lines to screen for a homozygous *pyl6* plant in which G, for gene and I, for insertion. Expected band 648 bp. (B) 6 µl of 1Kb Ladder marker (left and right), 1 and 2 µl of purified DNA from *pyl6* homozygous plants in 1.2% of agarose gel.

The knockout (KO) lines were validated by PCR and homozygous *pyl* plants were grown for seed production. These lines were then characterised for their response to virulent DC3000 by analysing leaf phenotype and growth curves.

5.3.4 *Pseudomonas syringae* phenotype and populations in *pyl4, pyl5* and *pyl6* single mutants

We characterised the knockout *pyl* lines in response to DC3000 by analysing infection phenotypes and enumerating bacterial growth. The *pyl4, pyl5* and *pyl6* plants were grown as described in Chapter 2.1 and bacterial assays for phenotype and population were carried out under standard conditions for the three *pyls* single mutants with Col-0 as the comparator. The result showed no differences in phenotypes between the mutants or comparison with Col-0 (Fig. 5.10A-D).

Bacterial multiplication for *pyl4, pyl5* and *pyl6* plants was determined 4dpi with DC3000 low inoculum (OD_{600}: 0.0002). The result showed no statistical significant differences reported among these lines and Col-0 (t-test; 0.239, 0.742 and 0.21 respectively) (Fig. 5.11A and 11B). These experiments have been repeated at least three times under the same conditions.

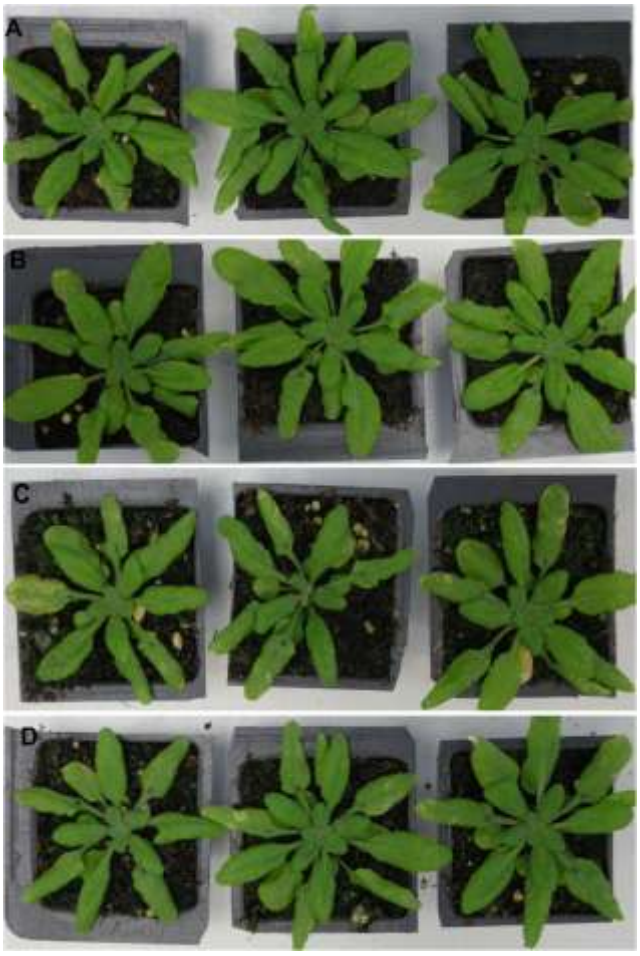
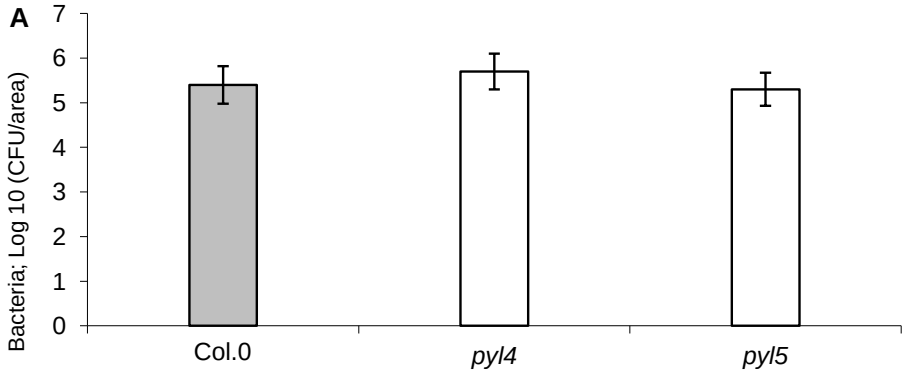


Figure 5.10 Phenotype of the ABA receptor (PYLs) single mutants 4dpi following challenge with DC3000 (O.D. 0.002; $1X10^6$ CFU/ml) compared with wild-type Col.0; (A) *pyl4*, (B) *pyl5* and (C) *pyl6* and (D) Col.0. Three plants per genotype and three leaves per plant were inoculated. Experiment was repeated three times



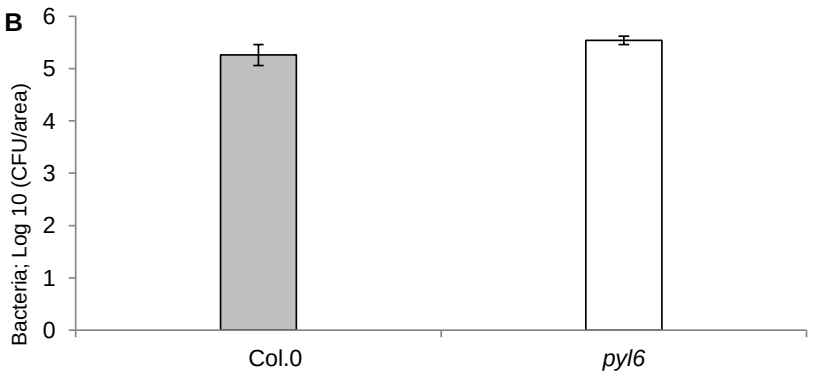


Figure 5.11 Population of DC3000 on; (A) *pyl4*, *pyl5* and (B) *pyl6* plants infected with a low inoculum of DC3000 (OD_{600}: 0.0002; $1X10^5$ CFU/ml). Y axis indicates the growth of DC3000 log 10 (CFU; colonies forming unit/area). Bacterial multiplication was determined at 4dpi compared with the control. Bars represent the mean of six biological replicates, three leaves per replicate. Errors bars represent one standard deviation. Experiments were repeated three times.

Characterization of single PYR/PYL mutants did not reveal any ABA phenotypes (Park et al., 2009). This functional redundancy likely explains why the gene family evaded detection by earlier genetic screens (Finkelstein et al., 2002b). The selectivity of pyrabactin for the receptor PYR1 enabled the genetic redundancy observed for ABA to be avoided, which illustrates the power of synthetic ligands for dissecting plant signalling networks (Park et al., 2009, Cutler and McCourt, 2005). Additionally, recent studies analysing loss-of-function *PYL* mutants revealed that the combination of several *pyr/pyl* loci was required to impair ABA signalling (Gonzalez-Guzman et al., 2012, Park et al., 2009). Subsequently, generation of *pyr1/pyl1/pyl4* triple or *pyr1/pyl1/pyl2/pyl4* quadruple mutants (Park et al., 2009) or different combinations of *pyr1/pyl4/pyl5*, *pyl4/pyl5/pyl8*, and *pyr1/pyl4/pyl8* triple mutants and *pyr1/pyl4/pyl5/pyl8* quadruple mutants (Gonzalez-Guzman et al., 2012) were required to obtain a robust ABA-insensitive phenotype suggests certain functional redundancy among *PYR/PYL* genes (Gonzalez-Guzman et al., 2012). However, Park et al., (2009) reported only *pyr1* mutant displayed a phenotype by inactivation of a single *PYR/PYL* gene.

5.3.5 *Pseudomonas syringea* phenotype and population in *pyl4/pyl5* and *pyl5/pyl6* double mutants

Plant phenotype and bacteria population assays were carried out on two double *pyl* mutants *pyl4/5,* derived from Pedro Rodriguez's laboratory-Spain and a *pyl5/6* generated in Exeter. The result showed no differences in phenotype among both double mutants and Col-0 (Fig. 5.12A - 12C)

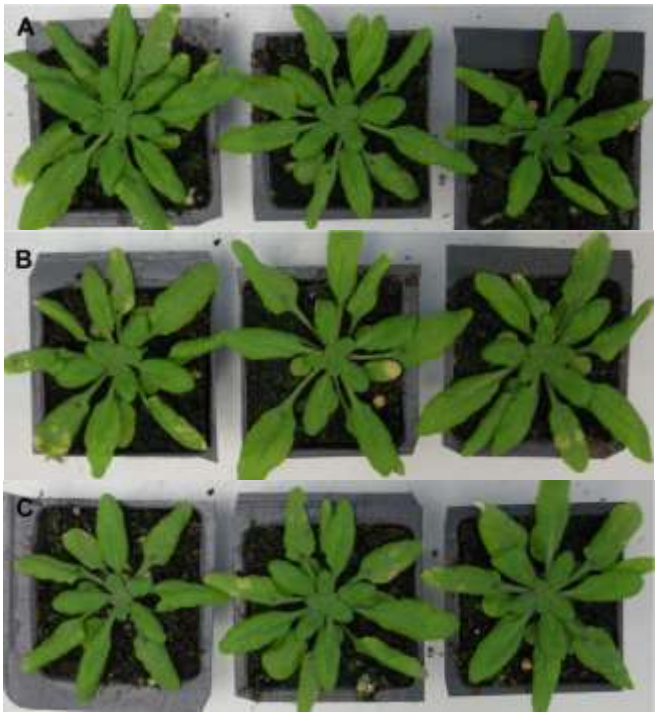


Figure 5.12 Phenotype ABA receptor double mutants 4dpi following challenge with DC3000 (O.D. 0.002; $1X10^6$ CFU/ml) compared with wild-type Col.0; (A) *pyl4/5* (B) *pyl5/6* double mutant and (C) Col-0. Three plants per genotype and three leaves per plant were inoculated. Experiment was repeated twice.

Additionally, measurements of DC3000 growth revealed that no statistical significant differences between the double mutants and Col-0 student t.test: 0.906 and 0.13 respectively (Fig. 5.13A and 13B).

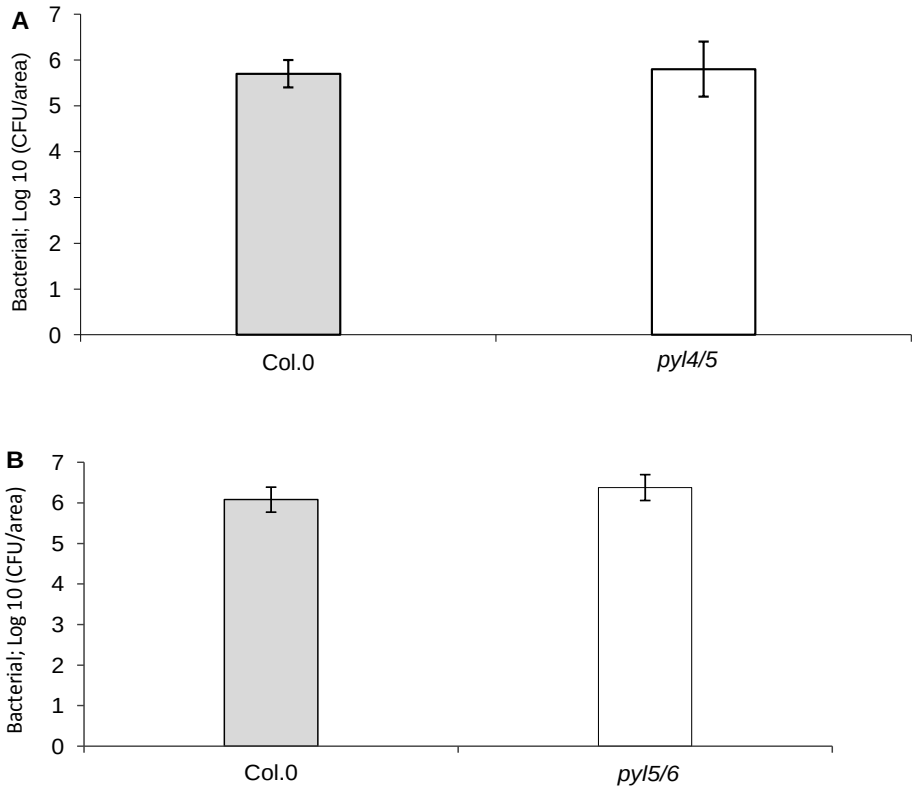


Figure 5.13 Growth of DC3000 on ABA receptor double mutants following 4dpi with low inoculum of DC3000 (OD_{600} 0.0002; $1X10^6$ CFU/ml). (A) *pyl4/pyl5* and (B) *pyl5/pyl6*. Y axis indicates the growth of DC3000 log 10 (CFU; colonies forming unit/area). Bars represent the mean of six biological replicates, three leaves per replicate. Errors bars represent one standard deviation. Experiments were repeated at least three times.

5.3.6 *Arabidopsis thaliana pyl5-* overexpression (*pyl5.OE201*) and *hab1-1/abi1-2/abi2-1*triple mutants are more susceptible to DC3000 infection than Col- 0

Characterisation of *A. thaliana* over-expressing *pyl5- OE 201* line (Santiago *et al.*, 2009b) was carried out in comparison with *hab1-1/abi2-1/abi1-2* (Rubio et al., 2009). Santiago showed that *pyl5-OE* enhanced the response to exogenous ABA, in contrast to an opposite phenotype for *HAB1*-over-expression, whereas, Rubio and co-workers reported that *hab1-1/abi2-1/abi1-2* treated with 100, 200 – 500 nM of ABA displayed an extreme response to ABA, impaired seed germination, and a partial constitutive response to endogenous ABA.

As shown in Figure 5.14A and B, *pyl5-OE201* plants displayed strong chlorosis associated with significant differences of DC3000 growth 4dpi compared with Col-0 (*t.test*: 1.20004E-05) (Fig. 5.14B). The determination of DC3000 growth at day zero shows that the same number of virulent bacteria was inoculated into the leaves (Fig. 5.14B).

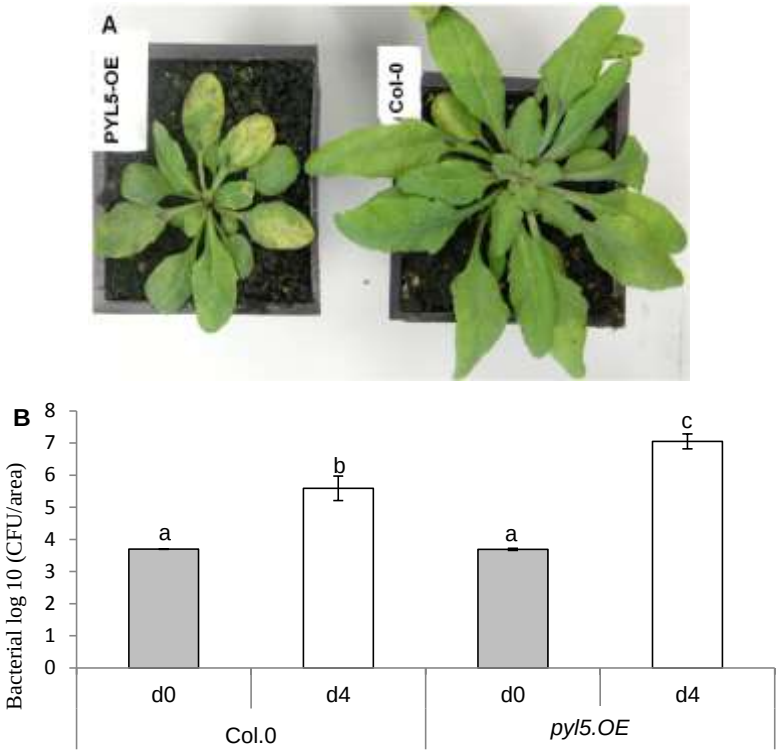


Figure 5.14 ABA receptor *pyl5-OE* plants challenged with DC3000, (A) Phenotype of DC3000 in *pyl5-OE201* and Col-0 3dpi with low inoculum (OD_{600}: 0.002). (B) Bacterial multiplication in wild type Col.0 and *pyl5-OE*. Y axis indicates the growth of DC3000 log 10 (CFU; colonies forming unit/area) and the labels "a, b, etc" above the columns represent significant differences between treatments at $p < 0.05$ (Student's t-test for pair wise comparison of Col.0). Bars represent the mean of six biological replicates, three leaves per replicate. Errors bars represent one standard deviation. Experiments were repeated twice.

Further investigation on the phenotypes of *pyl5-OE201* and *hab1-1/abi1-2/abi2-1* mutants in response to DC3000 was taken. Both mutants show drought-avoidant (Rubio et al., 2009, Santiago et al., 2009a). Our data showed a considerable chlorosis in inoculated leaves of *pyl5-OE201* and *hab1-1/abi1-2/abi2-1* 4dpi of DC3000 compared with Col-0 (Fig. 5.15A- 15C).

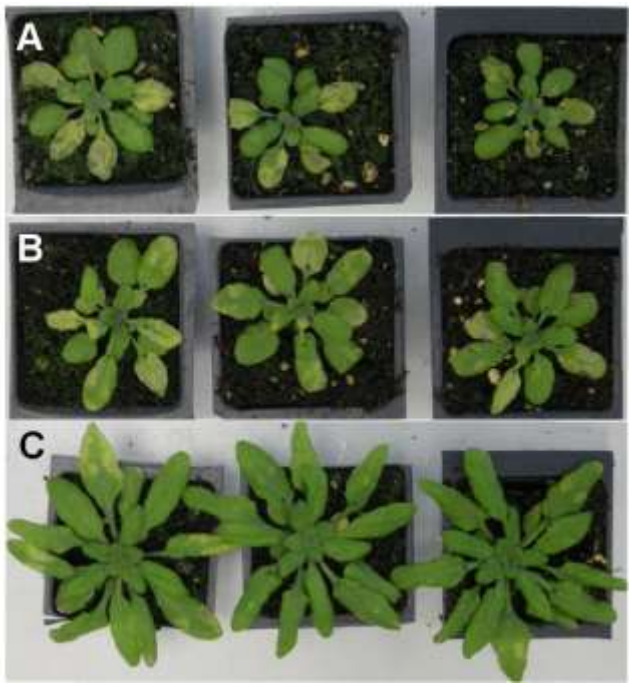


Figure 5.15 ABA-hypersensitive plants display enhanced chlorosis and necrosis 4 dpi following challenge with DC3000 (OD_{600} 0.002; $1X10^5$ CFU/ml). (A) phenotype of *pyl5-overexpression*, (B) *hab1-1/abi1-2/abi2-1* and (C) Col.0. Three of five weeks-old plants per genotype were inoculated he experiment was repeated twice.

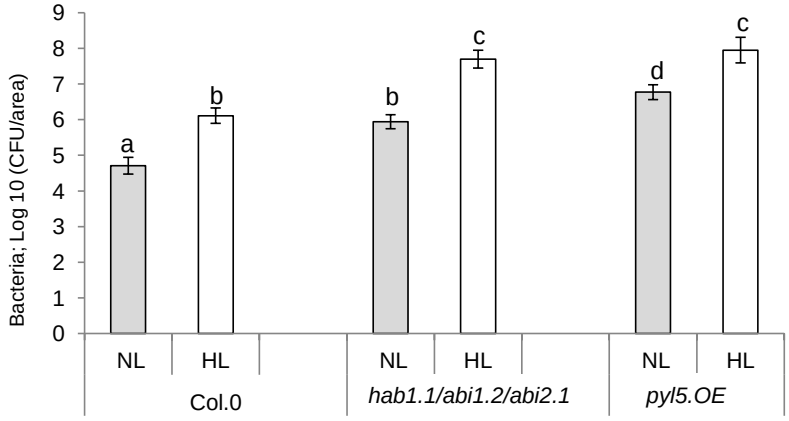


Figure 5.16 ABA-hypersensitive plants result in enhanced susceptibility. DC3000 population 4dpi in *hab1-1/abi2-1/abi1-2*, *pyl5.OE*, and Col.0 inoculated with low inoculum of DC3000 (OD_{600} 0.0002; $1X10^6$ CFU/ml). Bars represent the mean of six biological replicates, three leaves per replicate. Y axis indicates the growth of DC3000 log 10 (CFU; colonies forming unit/area) and the labels "a, b, etc" above the columns represent significant differences between treatments at $p < 0.05$ (Student's t-test for pair wise comparison of Col.0). Experiments were repeated at least three times.

In addition, DC3000 growth in *pyl5-OE* and *hab1-1/abi1-2/abi2-1* showed differences in phenotype and statistically significant differences in bacterial growth 4dpi than Col- 0 (*t.test*: 3.1E-06 and 3.9E-05 respectively) (Fig. 5.16).

As mentioned earlier, the homologous gene of *PYR/PYLs* family usually display functional redundancy to varying degrees. Santiago et al,. (2009a) used a gain-of-function approach to provide genetic evidence of the role of *PYL5* in ABA signalling. Thus, *pyl5*-over-expressing (OE) lines were generated. Expression level of these lines was determined by RT-qPCR. The expression of *PYL5* was between 15- and 20-fold higher than in wild-type. In addition, root-growth assays *pyl5.OE* lines led to hypersensitivity to ABA-mediated inhibition on growth as in double *hab1-1/abi1-2* mutant. Furthermore, although *HAB1-OE* lines were insensitive to ABA-mediated inhibition of root growth but transgenic plants over-expressing both *HAB1* and *PYL5* showed enhanced root sensitivity to ABA, similar to the phenotype found in the *pyl5.OE* lines. This suggesting that *pyl5.OE* overrides *HAB1.OE* or uses multiple Clade A PP2Cs.

Previous studies also showed that single reduction/loss-of-function alleles from *ABI1, ABI2,* and *HAB1* produced phenotypic effects on ABA signalling to a different extent and it was apparent from double mutant analyses that some functional redundancy occurs among them (Merlot et al., 2001, Saez et al., 2006). For instance, inactivation of both HAB1 and ABI1 resulted in a stronger response to ABA than that found in *hab1-1* or *abi1-2* monogenic mutants (Merlot et al., 2001). In addition, the two triple mutants, *hab1-1/abi1-2/abi2-2* and *hab1-1/abi1-2/pp2ca-1* that generated by Rubio et al,. (2009) showed an extreme response to exogenous ABA and a partial constitutive response to endogenous ABA. Furthermore, Rubio showed that the phenotype reported in these mutants suggest that PP2Cs provide a threshold of negative regulation required for a normal response to ABA. If ABI1/HAB1 and PP2CA branches regulate independently ABA signaling, the loss of one branch would be enough to overcome this threshold and, therefore, lead to ABA hypersensitivity (Rubio et al., 2009)

5.3.7 Characterization of transgenic lines expressing *PYL4, PYL5* and *PYL6* cytosolic ABA receptors:

To characterise *PYL4, PYL5* and *PYL6* cytosolic ABA receptors we generated luciferase transgenic lines for *PYL4, 5* and *6* and use the time lapse imaging to determine the expression level and localisation. In addition we aimed to express *PYL4, 5* and *6* under a CaMV (35S)

promoter with HA and MYC tags for over-expressed protein from wild type protein expressed by the host organism and for cellular localization studies by immuno-fluorescence or detection by western blot. Finally, we also sought to express GFP or YFP tagged *PYLs* and use confocal microscopy to examine histochemical localisation of the *PYL4, 5* and *6* following pathogen challenge.

5.3.7.1 Characterisation of Luciferase *PYL4, PYL5* and *PYL6* transgenic lines (*PYLs-LUC*):

Firstly we investigated the activity of the *PYL* promoters by visualising the regulation of the expression of these genes using pC1LUCP vector (backbone pCAMBIA C11302) with *PYL4, PYL5* and *PYL6* promoter as described in Section 5.2.5.1. Arabidopsis plants were transformed with a construct consisting of the firefly luciferase coding sequence under the control of the *PYL4, PYL5* and *PYL6* native promoters (Section5.2.5.1) and validated by PCR (Fig. 5.3).

Then we characterised *PYL5-LUC* and *PYL6-LUC* Luciferase reporters, but not *PYL4* which did not successfully transform. The transgenic plants of *PYL5-LUC* and *PYL6-LUC* were challenged with DC3000, *hrpA* and 10 mM $MgCl_2$ (mock) (Fig. 5.17A & 17B). These challenges showed an early and strong response to DC3000, notably the *PYL5-LUC* line displayed a high level of LUC activity at 2 hpi compared with the response to *hrpA* challenge (Fig. 5.17A), but at 6 hpi the expression level of DC3000-*PYL5-LUC* plants reached the lower point when the signal had completely disappeared at this time point of infection (Fig. 5.17A-6 hpi).

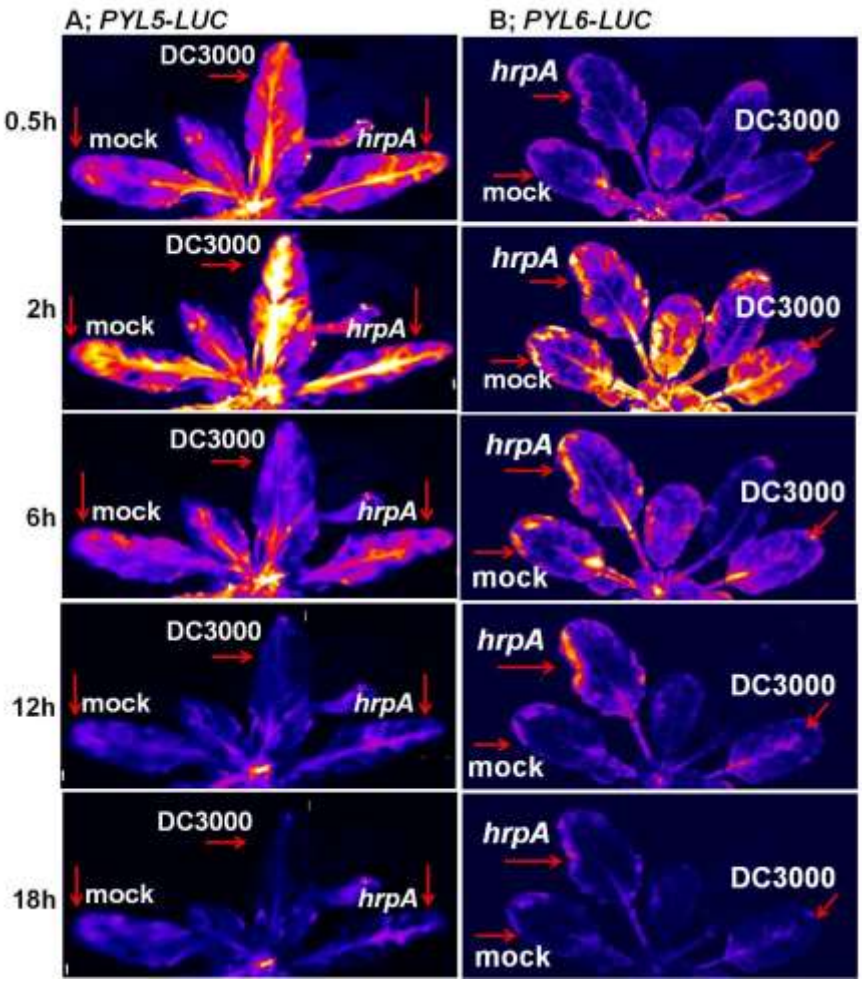


Figure 5.17 Image represents the Luciferase activity on Arabidopsis ABA receptor *PYL5* and *PYL6* transgenic plants, (A) on left represented *PYL5-LUC* and (B) on right represented *PYL6-LUC* transgenic lines. The plants were challenged with DC3000, *hrpA* (OD_{600}: 0.15; $.075X10^8$ CFU/ml) in comparison to mock (10µM $MgCl_2$) at serial time point (0.5, 2, 6, 12 and 18 hpi). After 20-30 minutes post infection the plants were imaged and luciferase bioluminescence captured in 5 min intervals over 24 h.

In Figure 5.17B, *PYL6-LUC* DC3000-challenged leaf displayed less response to DC3000 at 2hpi compared with *PYL5-LUC/*DC3000 but the signal in both lines disappeared at the same time point. By contrast, in both lines it very obvious that challenged with *hrpA* showed a low level of reporter induction compared to DC3000.

However, this result does not match with the data reported from RT-PCR assay in which the expression level of the *PYLs* at 6hpi of *hrpA* was significantly higher in *PYL5* and *PYL6* genes than DC3000 inoculated tissues (Fig.5.8.B & C). And more interestingly, *PYL6* expression was stronger in *hrpA* at 6hpi in comparison to DC3000 (Fig. 5.17A and 17B). Moreover, the expression level reached the peak at 18 hpi in *PYL5*-, *PYL6*-DC3000 (Fig. 5.8B & 8C). Overall these *PYL* genes were up-regulated at the late stages of DC3000 infection (18 hpi) whilst they were up-regulated at early stages of *hrpA*-challenged (6 hpi) (Fig. 5.8B & 8C).

The ABA receptor proteins inhibited PP2C proteins upon binding to ABA (Melcher et al., 2009, Miyazono et al., 2009, Nishimura et al., 2009, Santiago et al., 2009a, Yin et al., 2009). Therefore we treated the *PYL5-LUC* and *PYL6-LUC* transgenic plants (Section5.2.6) with different concentrations of ABA (100nM, 1µM, 10µM and 100µM) and mock (10µM MgCl) challenges to investigate the effect of ABA in the PYLs promoter activity.

In addition, the obtained result from the serial application of ABA on *PYL5-LUC* and *PYL6-LUC* showed a positive correlation in which increase of ABA concentration resulted in up regulation at 2 h post treatment in both lines *PYL5-LUC* and *PYL6-LUC* plants (Fig. 5.18A & 18B.) despite stronger luminescent in *PYL5-LUC* plants. Whilst, Hao et al., (2011) reported that, in the presence of 10 µM ABA, most of the PYL proteins completely inhibited the phosphatase activity except in PYL6, which inhibited about 80% of the phosphatase activity of PP2CA.

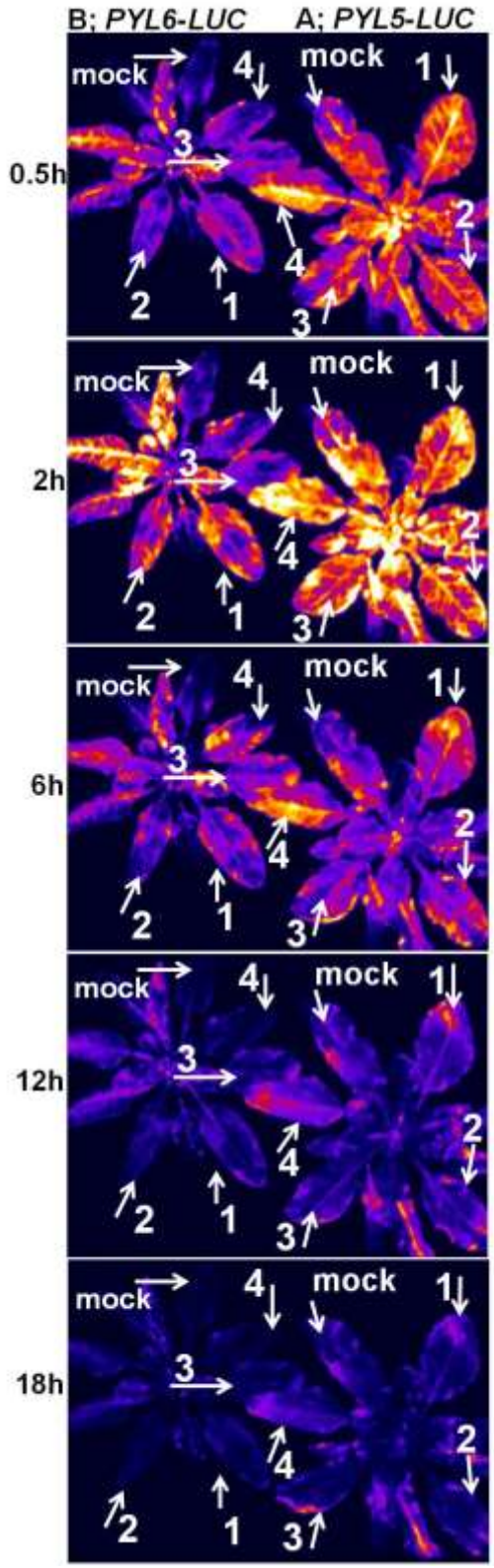


Figure 5.18 Effect of ABA treatment on Luciferase activity of *PYLs* transgenic plants. A (right) represented *PYL5-LUC* and B (left) represented *PYL6-LUC* plants. Leaves labelled 1-4 indicate treatment of different concentrations of ABA (100µM, 10µM, 1µM and 100nM respectively) in comparison with mock (10mM $MgCl_2$).

5.3.7.2 Epitope tagged CaMV::HA / CAMV::MYC PYL4, 5 and 6 lines

Here we sought to generate epitope tagged versions of the three *PYLs* so that the abundance of the protein could be monitored during infection. This would also allow *in vivo* purification of the protein products of cloned *PYL4, PYL5* and *PYL6* genes. Thus, to facilitate the three ABA receptor genes for epitope-tagging in Arabidopsis plants, two of epitope-tagging vectors (pCXSN-HA 1254; 10832bp and pCXSN-MYC 1256; 10835bp) (Chen et al., 2009a) with combination of the three *PYL* genes were carried out.(Fig. 5.4 and Appendix A6. A-F) (Section5.2.5.2).

The transgenic plants designed with a CaMV (35S) promoter followed by a triple HA or MYC tag and *PYLs* ORF, were inoculated with DC3000 (OD_{600} 0.002; $1X10^6$ CFU/ml) for phenotype in comparison with *pyl5.OE201* (as a positive control) and Col-5.

The resultant transgenic lines of CaMV:HA:*PYL4, PYL*5 and *PYL*6 showed stunted phenotype with less chlorosis compared with the Santiago *pyl5*-overexpression line (Fig. 5.19A-19F). These CaMV::HA: *PYL4,* CaMV::HA:*PYL5* and CaMV::HA:*PYL6* transgenic lines showed a high level of expression in a range of individual transformants (T1) as evidenced by the strong signal detected on western blots (Fig. 5.20A & 20B). These same lines were challenged with a low inoculum of DC3000 ($OD_{600:}$ 0.0002) and sampled at 4dpi to determine the bacterial growth. Although the western blot assays showed a strong signal in a range of plants, but differences in bacterial growth were recorded (Fig. 5.21A and 21B) and unlike *pyl5-OE201* none showed a strong phenotype and statistical differences in DC3000 growth (*t.test*; 2.4E-06) (Fig 5.21B).

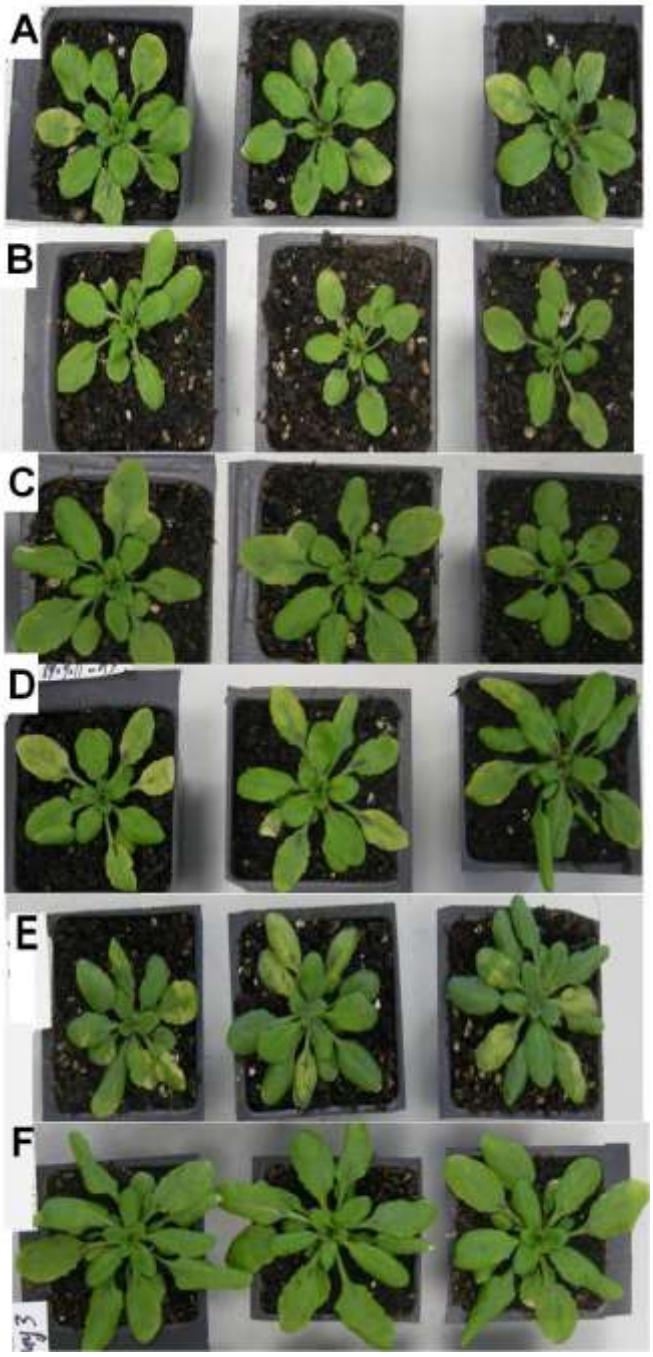


Figure 5.19 Phenotype of Arabidopsis CaMV::HA transgenic plants following 3 dpi following challenge with DC3000 (OD_{600} 0.002; $1X10^5$ CFU/ml^{-1})**,** (A) represented CaMV:*HA*:*PYL4*, (B and C) represented two lines of *HA:PYL5* and (D) represented *HA:PYL6*, (E) represented *pyl5-OE201*and (F) represented Col-5 .

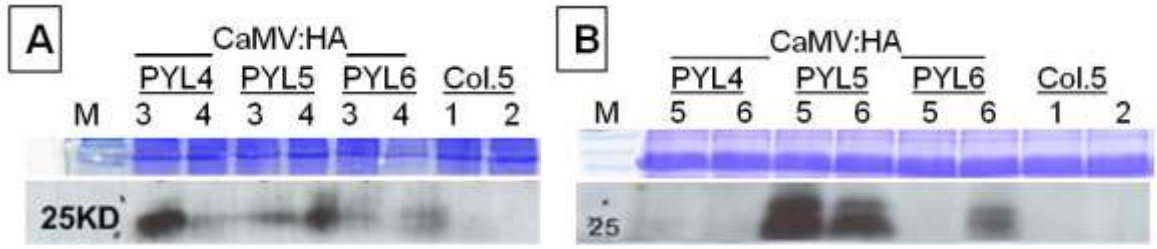


Figure 5.20 Immunoblot analysis of CaMV:HA-tagged *PYLs*. (A and B) represented the expression of HA-tagged *PYL4, PYL*5 and *PYL6* individual lines. M: indicates Protein Marker "BioLabs" 6.5-175Kd. Expected band of HA: PYLs: HA=1.1Kd, PYL4=22.44Kd, PYL5=22.67Kd and PYL6=23.85Kd (the range of HA:PYLs protein 23.5 – 25Kd)

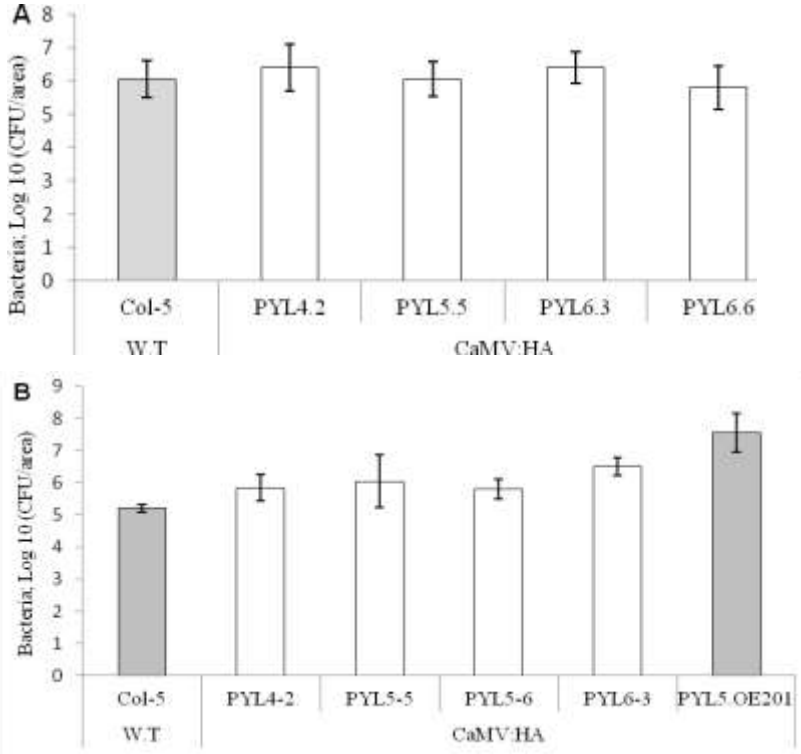


Figure 5.21 (A and B) a repeat of the DC3000 growth curve in CaMV: HA: *PYL4*, *PYL5* and *PYL6.* Plants were challenged with DC3000 (OD_{600}: 0.0002) 4dpi in comparison with Col-0 and *pyl5-OE201*. Y axis indicates the growth of DC3000 log 10 (CFU; colonies forming unit/area). Plants labelled PYL4.2 in figures 22. A and B are corresponding to *PYL4.3* in figure 5,21A, plants labelled *PYL5.5* in figure 22. A and B correspond to *PYL5.5* in figure 21.B and plants labelled PYL6.3 and PYL6.6 in figure 22. A and B correspond to *PYL6.3* and *PYL6.6* in figure 21.A and B respectively. Bars represent the mean of six biological replicates, three leaves per replicate. Errors bars represent one standard deviation. Experiments were repeated three times.

The investigation of Myc epitope-tag lines *CaMV:MYC:PYL4*, *CaMV:MYC:PYL5* and *CaMV:MYC:PYL6* resulted neither in a visible phenotype being observed (Appendix A16) nor the detection of positive lines via the western blot (Appendix A17). Despite the fact that a range of *CaMV:MYC:PYL4* line plants were stunted no phenotype was observed on DC3000 challenge in comparison to Col.5 (Appendix A16. A and B). However, the sequences of these *PYL* constructs showed mutations in the coding region Appendix A8, 9 & 10).

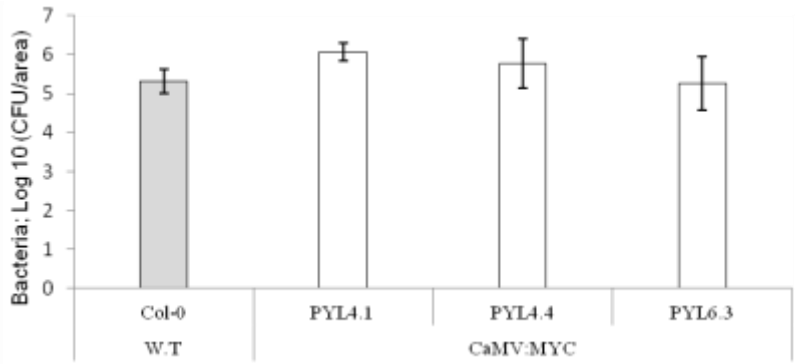


Figure 5.22 DC3000 population 4dpi in two lines of CaMV:MYC:*PYL4*, CaMV:MYC:*PYL6* and Col.5 inoculated with low inoculum of DC3000 (OD_{600} 0.0002; $1X10^6$ CFU/ml^{-1}). Bars represent the mean of six biological replicates, three leaves per replicate. Y axis indicates the growth of DC3000 log 10 (CFU; colonies forming unit/area). Experiments were repeated two times.

The investigation of CaMV:MYC plants challenged with DC3000 led to one statistical significant case of *CaMV:MYC:PYL4.1 (*student t.test; 0.0004) but no significant differences reported from CaMV:MYC:PYL4.4 and CaMV:MYC:PYL6.3 lines (t.test; 0.142 and 0.556 respectively)

Santiago et al,. (2009a) has examined the subcellular localization of PYL5 by standard biochemical techniques in protein extracts from transiently transformed Arabidopsis cells that stably express PYL5:HA line. HA–PYL5 protein was detected in both the cytosolic and nuclear fractions of Arabidopsis transgenic cells, which corroborated the detection of GFP–PYL5 in both the cytosolic and nuclear fractions by immunoblot analysis.

5.3.7.3 GFP and YFP fusions to *PYL*4, *PYL*5 and *PYL6* genes with natural promoters

We also sought to generate GFP and YFP tagged- *PYL4*, *PYL5* and *PYL6* under their native promoters in Arabidopsis. Three previous research groups have independently used fluorescent tagging to shed the light on the localisation of over- expressed *PYR*/*PYL*s. Santiago (2009a) used transient transformation of tobacco leaves inoculated with *Agrobacterium* to show that *PYL5*-GFP seemed to localise to the cytoplasm, while, Saavedra (2010) found that *PYL8*- GFP localised to both the nucleus and the cytoplasm in tobacco and onion cells, and Ma *et al.* (2009) reported that *PYL9*- (*RCAR1*) GFP localised in cytosol and in the nucleus.

Amplifications of *PYL4, 5* and *6* promoters and the genes of interest, using appropriate primers (Appendix1.Table1) and the process of transformation were carried out as described in Section 5.2.5.3. The PCR products were prepared spanning the *PYL* ORF/eGFP fusion regions and the M13/*PYL* promoter regions to verify the composition of the constructs.

In these constructs we used native *PYL* promoters which, according to the RT-PCR (Fig. 5.8), would induce a high level of expression at 6 hpi with *hrp*A. Therefore, we examined the B8-*eGFP/YFP:PYL5* homozygous plants, 5 weeks old, at 6 hpi of *hrp*A under confocal microscopy but we couldn't detect any GFP or YFP signals. We also checked seedlings from these lines 7-10 aged where they may display an early expression during the seedlings stage but again no signal has been detected.

In C1eGFP/C1YFP:*PYL4*, *PYL5* and *PYL6* sequence data we have found PCR induced mutations in both *PYL4* and *PYL5* gene coding sequences, although the mutations are in the coding region sequence, but we could not select any positive transgenic plants in hygromycin. Whilst no mutation has been found in the sequence of C1eGFP*:PYL6* construct but we couldn't select any transgenic plants from this line.

Validating constructs. Digestions of C1eGFP: *PYLs* constructs in which C1eGFP:PYL4 were digested with Nco1+ Sal1 and Nco1+ EcoR1 and both C1eGFP:PYL5 and PYL6 were digested with Pst1+Sal1 Nco1+EcoR1 (Appendix2. Table2). These results showed the expected product from each construct digestion (Fig. 5.26) and more information about the digestions with restriction sites are shown in Appendix A13

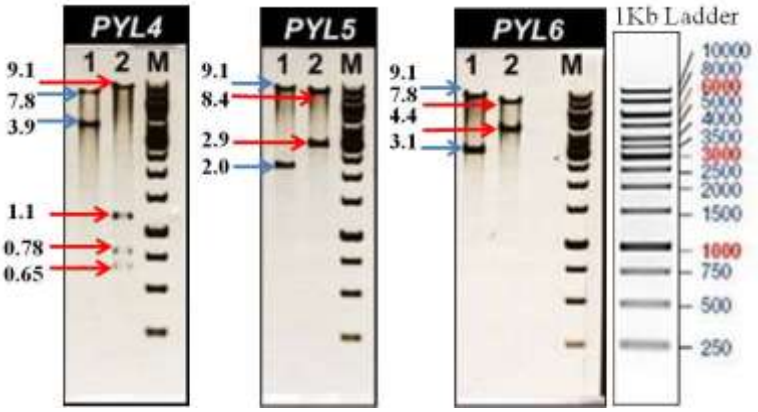


Figure 5.23 Validating GFP and YFP constructs by restriction digestion. Digestion of *PYLs* constructs (left) *PYL4*+promoter cut with (1) Nco1+Sal1 (7881+3911bp) and (2) Nco1+ EcoR1 (9186+1172+0.78+0.65bp), (middle) *PYL5*+promoter cut with (1) Nco1+EcoR1 (9186+2021bp) and (2) cut with Pst1+Sal (8430+2957bp) and (right) *PYL6*+ promoter cut with (1) Nco1+EcoR1 (9186+3143bp) and (2) *PYL6*+ promoter cut with Nco1+Sal1 (7881+4448bp).

Sequence analysis of *PYL* constructs were carried out at The Genome Analysis Centre (TGAC), Norwich, UK. The C1eGFP:*PYL4* construct showed mutations in the coding region. The first mutation changed Valine (gtc) to Isoleucine (atc) and the other changed Isoleucine (ata) to Threonine (aca) (Appendix14.A)

In addition, in the C1eGFP:*PYL5* construct sequence three mutations were found. The first two mutations did not affect the amino acid (ttg to ctg and cta to ctg both gives Leucine) but lost the BglII site. The third mutation affected the amino acid Histidine (cac) changed to Arginine (cgc) resulting in the loss of a MluI site and acquisition of a BssHII (Appendix14.B)

On the other hand, although the transformant of C1eGFP:*PYL6* was repeated twice and the construct sequence did not show any mutations but we could not select any transgenic lines, possibly due to a mutation in Hygromycin sequence.

Santiago et al,. (2009a) generated a GFP–PYL5 fusion and delivered this into leaf cells of tobacco by *A. tumefaciens* infiltration. They showed that GFP–PYL5 fusion localizes to both nucleus and cytosol, similar to the subcellular localization described for HAB1. In contrast, SWI3B, another HAB1-interacting partner, was only found in the nucleus (Saez et al., 2008, Santiago et al., 2009a)

5.4 Discussion

PYR/ PYL/ RCARs are soluble ABA receptors that function in the perception and transduction of ABA signalling. They bind to PP2Cs in the presence of ABA and prevent their repression of SnRK2 kinases (Ma et al., 2009, Park et al., 2009).

5.4.1 Regulation of *PYL4, PYL5* and *PYL6* genes in response to bacterial infection

Here we reported that the expression levels of *PYL4*, *PYL5* and *PYL6* using *A. thaliana* leaves non-inoculated (NI) or inoculated with virulent DC3000 or the *hrpA* mutant strain at 6, 12 and 18hpi showed different levels of expression (Fig. 5.8A-8C). *PYL4*, *PYL5* and *PYL6* were up-regulated at an early stage of *hrpA*-infection but later the expression level of the genes decreased (approximately 3-4 fold). At 12 hpi the three genes were down-regulated in DC3000 (Fig. 5.8B and 8C). *PYL5 & 6* transcripts accumulate at later stages of infection (18 hpi) whereas *PYL4* expression fluctuated between 6, 12 and 18 hpi (Fig. 5.8A). It is currently unclear the biologicl significance of the expression of these *PYLs*.

Santiago (2009a) showed that expression of *PYL5*, *PYL8* and *PYL6*, as well as, *PYL4*, *PYR1* and *PYL1*, were strongly down-regulated in whole-seedling tissue by ABA treatment (Santiago et al., 2009a). Chan (2011), showed that Arabidopsis plants transformed with mannose-6-phosphate reductase (*M6PR*), under salt stress, resulted in *M6PR* transgene activating the downstream abscisic acid pathway by up-regulating of *PYL4*, *PYL5* and *PYL6* genes and down-regulating protein phosphatases 2C *ABI1* and *ABI2* (Chan et al., 2011).

5.4.2 Characterisation of single and double of *pyl* lines in response to DC3000

Our investigation of *PYL4*, *PYL5* and *PYL6* knock- out lines didn't show any differences in phenotype (Fig. 5.10A-10D) or in DC3000 growth (Fig. 5.11A & 11B) probably due to the functional redundancy. In addition, the double *pyl4/pyl5* and *pyl5/pyl6* plants also behave the same as the single mutants where they didn't display any differences neither in phenotype (Fig. 5.12A-12C) nor bacterial growth as well (Fig. 5.13A & 13B). This is likely due to inherent redundancy on *pyl4/5* and *pyl5/6*. By contrast, Park (2009) generated a triple *pyr1/pyl1/pyl4* and quadruple *pyr1/pyl1/pyl2/pyl4* mutants displayed a strong ABA insensitivity which can be reversed by introducing *PYR1*- or *PYL4*-expressing transgenes.

It has also been shown that ABA does not appear to be required for the interaction of *PYL5*, *PYL6* and *PYL8* with *HAB1*, suggesting that ABA may affect the interaction between these genes and the PP2C, because they do not promote inhibition of *HAB1*activity in the absence of ABA. De Torres et al (2007) showed that foliar ABA levels increased significantly within 12 hpi in a TTSS-dependent manner and thus DC3000 multiplied more rapidly and to greater titre in ABA hypersensitive plants compared with wild type. While restriction of virulent DC3000 growth was detected in the ABA-insensitive mutants' *abi1-1* and *abi2-1*, in lines constitutively overexpressing *HAB1* and in the ABA biosynthetic mutant *aao3*. The multiplication of virulent DC3000 in an *AAO3* T-DNA knockout line (SALK_072361) was significantly restricted compared with wild type suggested that both *de novo* ABA biosynthesis induced by TTE delivery and PP2C activities collaborate to regulate pathogenicity (de Torres-Zabala et al., 2007). In contrast, Santiago showed that upon ABA binding, PYL5 underwent a conformational change that enhances the affinity for and binding of HAB1 and leads to an inhibitory ABA–PYL5–HAB1 complex (Santiago et al., 2009a). The reported bacterial growth data from *PYL5* over-expression lines supports the hypothesis that the *PYR/PYLs* are redundant ABA receptors (Klingler et al., 2010). Supporting this idea, both *pyl5-OE201* and *hab1-1/abi1-2/abi2-1* lines challenged with DC3000 displayed a considerable chlorosis and supported a high level of DC3000 growth (Fig. 5.14A & 14B, Fig. 5.15A-15C and Fig. 5.16) suggesting that both *pyl5-OE201* and *hab1-1/abi1-2/abi2-1* are opposingly involved in DC3000 virulence strategies, consistent with there interaction mechanism.

The study of PYLs is still in its infancy. A recent biochemical analysis revealed that alteration of single amino acid between valine and isoleucine that determines the distinct pyrabactin selectivity by PYL1 and PYL2 resulted in a complex regulation mechanism of ABA signalling by PYLs (Yuan et al., 2010). Subsequently it was shown that PYL4–PYL10, but not PYL7, behave as monomers in both the presence and absence of ABA (Hao et al., 2011). Thus the unbound monomeric receptors are constitutively active in PP2C inhibition in the same manner as ABA-bound receptors in vitro and in vivo (Ma et al., 2009, Park et al., 2009, Fujii et al., 2009, Hao et al., 2011, Sun et al., 2012)

5.4.3 Luciferase activity in *PYL4, PYL5* and *PYL6* transgenic lines (*PYLs-LUC*)

We investigated the activity of the *PYL* promoters by visualising the regulation of the expression of the *PYL4*, *PYL5* and *PYL6* genes by characterising *PYL promoter* Luciferase reporter lines. In the luciferase assays for transgenic lines of *PYL5* and *PYL6* we reported an earlier response to DC3000 in *PYL5-LUC* lines led to strong LUC activity at 2hpi compared with the response to *hrpA*. Furthermore, in DC3000-challenged leaves, the signal disappears faster, at about 6 hpi (Fig. 5.18A), suggesting effectors may specifically target expression of *PYL5* and further investigation is required to resolve this interesting observation.

PYL5 and PYL6 expression increases with ABA concentrations and reaches a peak at 2h in both *PYL5-LUC* and *PYL6-LUC* treated leaves (Fig. 5.19A) but by 12-18 hpi both lines showed low levels of expression in whole plants. Hao (2011) has recently shown that in the presence of ABA (10μM), most of the PYL proteins inhibited the phosphatase activity, whilst PYL6 inhibited about 80% of the phosphatase activity of PP2CA. However, it has been recently shown that *Arabidopsis thaliana PYR/PYL* sextuple mutant, *pyr1/pyl1/pyl2- /pyl4/pyl5/pyl8*, was able to germinate and grow even on 100 mM ABA and the whole rosette stomatal conductance (Gst) measurements revealed that leaf transpiration in the sextuple *pyr/pyl* mutant was higher than in the ABA-deficient *aba3-1* or ABA-insensitive *snrk2.6* mutants. Furthermore, the gradually increasing Gst values of plants lacking three, four, five, and six PYR/PYLs indicate quantitative regulation of stomatal aperture by this family of receptors (Gonzalez-Guzman et al., 2012). Thus the sextuple mutant lacked ABA-mediated activation of SnRK2s and ABA-responsive gene expression was dramatically impaired as was reported in *snrk2.2/2.3/2.6* (Fujii et al., 2009, Fujita et al., 2009). These results show that ABA perception by PYR/PYLs plays a major role in regulation of seed germination and establishment, basal ABA signalling required for vegetative and reproductive growth, stomatal aperture, and transcriptional response to the hormone (Gonzalez-Guzman et al., 2012).

5.4.4 Epitope tagged *CaMV::HA / CAMV::MYC PYL4, PYL5* and *PYL6* lines

The result of generating epitope tagged *CaMV::HA / CAMV::MYC PYL4, PYL5* and *PYL6* lines was that HA-tagged *PYL4, PYL5* and *PYL6* could be strongly detected in some lines and this was in some cases associated with stunted phenotype (Fig. 5.19 and 5.20A and 20B) however, none of the plants displayed any differences in susceptibility to DC3000 (Fig. 5.22A & 22B). Unfortunately the MYC-tagged *PYL4, PYL5* and *PYL6* lines showed mutations had occurred in the sequences during construct generation and it is likely these contributed to the absence of the expected phenotype (Appendix A16) or the detection of positive lines via the western blot (Appendix A17). Lie with the HA construct, , a range of *CaMV:MYC:PYL4* transformants were stunted but when challenged with DC3000 no significant differences in phenotype was evident (Appendix A16A and 16B). Consequently, these lines are of little sue for further work. One possibility could be that the amino terminal HA/MYC epitope tags may have affected the binding mechanism or an interference may have occurred between HA-/MYC-tagged protein and PYLs proteins or may due to the alteration of amino acids (Yuan et al., 2010)

However, Santiago et al. (2009a) successfully generated and characterised stable and transient HA epitope-tagged version of PYL5. HA–PYL5 protein was detected in both the cytosolic and nuclear fractions of Arabidopsis transgenic cells. In summary, the characterization of epitope tagged *CaMV:HA* and *CAMV:MYC:PYL4, PYL5* and *PYL6* proteins was inconclusive and needs further investigation.

5.4.5 Characterization of the PYL cytosolic ABA receptors PYL4, PYL5 and PYL6

The GFP and YFP fusions to *PYL4*, *PYL*5 and *PYL6* genes with natural promoters, showed PCR induced mutations in *PYL4* and *PYL5* gene coding sequences. However, although the C1eGFP/YFP:*PYL6* did not show any mutations but we were unable to select any transformants in hygromycin suggesting that the transformation was not successful or that there may be a mutation in the Hygromycin sequence. Whist, the transgenic seeds of B8eGFP/YFP: *PYL5* showed BASTA resistance but we were not able to detect any GFP or YFP signal neither by western nor under confocal microscopy. In addition we also examined this line at early seedling stages where a high expression was expected, but no signal was seen for both fusions, suggesting

that the GFP/YFP cassette seems to be absent from the genome or the plants have very low level of expression.

Several research groups have shown that under non-ABA inducing conditions; active PP2C phosphatases inactivate SnRK2 kinases thereby suppressing ABA signalling. Under ABA inducing conditions, PYR/PYL/RCAR receptor proteins bind to and inactivate the PP2Cs; an interaction mediated by ABA. Active SnRK2 kinases phosphorylate ABF transcription factors which then induce ABA responsive genes (Fujii et al., 2009, Ma et al., 2009, Park et al., 2009, Cutler et al., 2010). Fujii et al 2009 showed that, *in vivo*, PYR/PYLs, PP2Cs, SnRK2s and ABF transcription factors are necessary and sufficient for ABA perception, signalling and activation of ABA responsive gene expression (Fujii et al., 2009).

In contrast to our efforts, three previous studies have used fluorescent tagging to view the localisation of over-expressed *PYR*/*PYL*s; Santiago *et al*. (2009a) used *Agrobacterium* inoculation in tobacco leaves to observe the location of *PYL5*- GFP, finding that PYL5 localised to both nucleus and cytosol, similar to the subcellular localization described for HAB1. In contrast, SWI3B, another HAB1-interacting partner, was only found in the nucleus (Saez et al., 2008, Santiago et al., 2009a). Saavedra *et al*. (2010) found that *PYL8*- GFP localised to both the nucleus and the cytoplasm in tobacco and onion cells, while Ma *et al.* (2009) reported the same for *PYL9* in protoplasts. Very recently, and again in contrast to our findings, Antoni et al (2013) and Gonzalez-Guzman et al (2012) generated PYR1, PYL1, PYL2, PYL4, PYL5, PYL6, PYL7, PYL8 and PYL9 promoters by using β-glucuronidase (GUS). Hence GUS expression driven by these gene promoters was detected in guard cells. Antoni also showed that the root expression of GUS driven by PYL6 promoter was almost undetectable while expression driven by PYL7 promoter was weak and only detected after 6 h of incubation with the GUS substrate. Whereas ProPYL9:GUS lines showed GUS staining after 3 h. Based on these collective studies we would have expected to have generated viable plants reporting GFP expression and most likely the transformation was an issue but time constraints precluded following this up.

Chapter 6: General discussion

The interaction between biotic and abiotic stress in plants has been widely studied. These studies have shown that biotic and abiotic stress responses often converge on mitogen-activated protein kinase (MAPK) signalling pathways in Arabidopsis. Arabidopsis MPK3 and MPK4 function in abiotic stress and basal defense responses (Nuhse et al., 2000, Asai et al., 2002, Jonak et al., 2002, Veronese et al., 2006). Arabidopsis Oxidative Signal-Inducible1 (OXI1) regulates the activation of MPK3 and MPK6 by ROIs (Reactive oxygen intermediate) and is also required for pathogen resistance (Rentel et al., 2004). Thus, pathogen and stress response signalling share significant regulatory mechanisms with complex interactions between responses to plant hormones, pathogens, abiotic stresses and ROIs (AbuQamar et al., 2009). Recently Pham et al., (2012) showed that the Arabidopsis authentic histidine kinase 5 (AHK5) has an ability to function in response to both abiotic and biotic stimuli to affect the growth and survival of Arabidopsis.

Based on the importance of understanding the crosstalk between biotic and abiotic stress in plant responses, this study has focused on the following objectives:

6.1 Studies to identify the role of the '400' compound in Arabidopsis *Pseudomonas* interactions

First to validate the hypothetical biosynthesis pathway of the unique pathogenesis related, plant derived, 3´-*O*-β D- ribofuranosyl (400 compound). The putative biosynthetic pathway was determined by Nicholas Smirnoff and tested by analysing knockout mutants of enzymes that are predicted by biochemistry and transcriptomic profiling, to be involved in 400 compound syntheses (Chapter3, Table 3.1). Previous studies conducted in Murray Grant's laboratory showed early induction of 3´-*O*-β D- ribofuranosyl adenosine in plant tissues challenged with DC3000.

This study examined single mutants *nud6-1*, *nud8-1*, *sro-14*, *sro-2*, *prs3-a*, *glycosil-t*, *ugt1*, *ugt85a1-a*, *udp-gt.a*, *udp-gt.d* and *gt1* (Chapter3.Table3.1) and the double mutants *nud6.1/nud8.1* and *sr0.14/sro.2*. However, we did not identify any important role for these targets in the *Arabidopsis*/DC3000 infection and defence responses leading to the biosynthesis of the 400 compound (as showed in Chapter3). However, Adams-Phillips's (2010) hypothesized a role for

poly(ADP-ribosyl)ation in plant defense responses in which Adams-Phillips and colleagues have detected defense-associated expression of the poly(ADP-ribosyl)ation-related genes PARG2 and NUDT7. As they also observed PARG2 and NUDT7 altered callose deposition in the presence of a chemical PARP inhibitor. In addition, they showed that poly (ADP-Rib) glycohydrolase (PARG2) and Nudix hydrolase active on ADP-Ribose and NADH.

Previous studies have showed that *nudt7* plants displayed resistance to virulent and avirulentDC3000 (Bartsch et al., 2006; Jambunathan and Mahalingam, 2006; Ge et al., 2007; Adams-Phillips et al., 2008). Moreover, recombinant AtNUDT2, -6, -7, and -10 proteins showed both ADP-ribose and NADH pyrophosphatase activities with significantly high affinities compared with those of animal and yeast enzymes (Ogawa et al., 2005).

Straus et al, (2010) has reported a relationship between EDS1, EDS1- regulated SA and ROS by examining gene expression profiles, photo-oxidative stress and resistance phenotypes of *nudt7* mutants in combination with *eds1* and the SA-biosynthetic mutant, *sid2*. Straus was also established that EDS1 controlled steps downstream of chloroplast-derived $O2^{\bullet-}$ that led to SA-assisted H_2O_2 accumulation as part of a mechanism limiting cell death. In addition, under normal growth conditions in soil, *nudt7-1* plants displayed EDS1-dependent enhanced basal resistance and growth retardation, as well as accumulation of H_2O_2 accompanying sporadic cell death in leaves (Bartsch et al., 2006, Straus et al., 2010). The finding of this relationship between EDS1, EDS1- regulated SA and ROS that resulted in resistance phenotypes of *nudt7* may support the possibility of an alternative pathway of the 400 compound maybe through the SA-biosynthesis.

6.2 The role of high light stress in plant pathogen interactions

The second objective of this study was to investigate the interaction between abiotic and biotic stresses. Therefore, the correlation between high light stress (HL) and disease caused by the virulent *P. syringae* DC3000 strain was investigated.

6.2.1 The effect of HL/ DC3000 infection on Col-5 and phytohormones mutants

In bacterial virulence activities, PAMPs negatively regulate pathogenesis by activating innate immune signalling pathways (Block et al., 2008). Previously, Kang et al., (2003) showed that flagellin sensing (FLS2) and flagellin receptor kinase (FRK1) act as key players in PAMP perception and signalling, and the encoding Non-Host 1 (NHO1) is a glycerol kinase required for

resistance and actively suppressed by DC3000. De Torres (2009) showed that SA accumulation is elicited initially by PAMPs and later in response to TTEs, but is antagonized by coronatine. ABA levels increase in response to TTE delivery and are accelerated by coronatine and consequently a late increase of JA. Thus, TTEs and coronatine are infection-specific determinants that contribute toward suppression of these activated immune defences and pathogen nutrition (Block et al., 2008; Schwessinger and Zipfel, 2008).

In this context, DC3000 growth was primarily determined on Col-5 plants exposed to HL. The primarily investigation showed an unexpected result; HL caused enhanced susceptibility to DC3000 as evidenced by phenotypes and bacterial growth on Col-5 plants. These results led to further investigation of the crosstalk between HL and virulent DC3000. It was demonstrated that inoculation with DC3000 (OD_{600}: 0.002 inoculum) 4dpi-HL (intensity of 600µmol/m^2s^{-1}) caused a strong chlorosis phenotype in Col-5 plants exhibiting preliminary anthocyanin synthesis (Fig 4.3 and 4.22). During bacterial growth assays continues HL stress elicited a strong phenotype with leaves becoming dark reddish purple, wrinkled, brittle, inward rolling and leathery in texture with an increase in susceptibility (5d HL pre-treatment followed by 4dpi / HL post-treatment) (Fig 4.6A-B). Changes in phenotype were reported to be due to simultaneous bacterial infiltration and HL. The plants under NL/HL and HL/HL showed similar phenotype and susceptibility to DC3000 (Fig 4.6A-B), whereas HL/NL plants (5dHL pre-treatment followed by 4dNL/DC post-treatment) displayed the same response to light/DC3000 as in the control plants (NL/NL). The plants under HL/NL displayed recovery from anthocyanin accumulation and exhibited the same level of bacterial growth as in the plants under NL/NL (Fig 4.6A-B).

Interestingly, in *aao3*, HL altered the mutant response to DC3000 as the plants becoming more susceptible in comparison to *aao3* plants under NL/NL. Our findings of the *aao3*-HL/DC3000 interaction contrast with de Torres (2009) whom found that *aao3* mutant is more resistant to DC3000 under normal conditions, as stated in Chapter 4, Fig 4.8A-B. de Torres (2009) showed that ABA deficiency cannot overcome enhanced susceptibility, in *aao3*, due to loss of SA-mediated resistance, but our observation that HL promote bacterial growth in *aao3* is totally independent of ABA or as de Torres (2009) showed, ABA has feedback-regulation in its own synthesis. HL reduces SA level in Arabidopsis Col-0 plants (~2.5 fold) less than *aao3* under NL condition (Chapter 4 Fig 4.10B). However, the basal SA levels (uninfected tissues) in *aao3* were significantly higher than in Col-0 under both NL and HL conditions and even after DC3000

infection of *aao3* compared to Col-0. This result agrees with de Torres (2009) leading to the suggestion that ABA has a negative role in SA regulation under both conditions (NL and HL) alongside a role for SA in positively regulating ABA accumulation at 9dpi in DC3000.

The synthesis of SA induced by TTEs during DC3000 infection is evidenced by the additional increase of SA in plant tissues 9dpi under both condition compared with 9d-NL and –HL uninfected tissues and indicates a possible failed host response to TTEs.

Interestingly, Wang et al., (2011) showed that the well characterised circadian clock regulator, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1), is involved in R-gene-mediated resistance against downy mildew in Arabidopsis. Recently, Zhang et al (2013) showed that the defense role of CCA1 and ELONGATED HYPOCOTYL (LHY) against *P. syringae* is at least partially through circadian control of stomatal aperture but is independent of defense mediated by SA. Moreover, Zhang also found that defense activation by *P. syringae* infection can feedback regulate clock activity. As our investigation on the response of Arabidopsis plants to HL over 18 h light exposure per day for 5 days it seem that HL may impact the mechanism of stomata aperture and the circadian clock regulation. This agrees with previous studies demonstrating that the circadian clock regulation of PTI facilitates a stronger response during the period of light exposure and correlates with the time of the circadian cycle when pathogens are most abundant and the plant becomes more prone to infection due to open stomata which facilitates bacterial entry (Melotto et al., 2006, Hotta et al., 2007). In addition, our finding can be reconciled with that of Bhardwaj et al., (2011) who indicated that constitutive expression of CCA1 resulted in decreased resistance to DC3000 during the day. In summary, there appears a direct role of the circadian clock in defense control revealed in crosstalk between the circadian clock and plant innate immunity (Zhang et al., 2013) and thus more investigation requires on the circadian clock and photoreceptor/hormonal regulation on Arabidopsis responses to HL/DC3000.

A number of research groups have established the importance of JA and SA as primary signals in the regulation of the plant's immune response (Loake and Grant, 2007, Robert-Seilaniantz et al., 2011, Pieterse et al., 2012, Solano and Gimenez-Ibanez, 2013). Although our observation agreed with other studies where we reported reduction in foliar JA levels in 9d-NL and 9d-HL non-infected tissues (Fig 4.10C) compared with the foliar level of SA (Fig 4.10B), but we disagreed with the fact that JA and SA have antagonistic functions in the *Pseudomonas Arabidopsis* interaction (de Torres et al., 2009, Robert-Seilaniantz et al., 2011, Pieterse et al., 2012, Solano

and Gimenez-Ibanez, 2013) where we reported that the foliar JA levels, as for SA, reduced in Arabidopsis Col-0 and *aao3* in HL/DC3000 challenged tissues compared with those under NL/DC3000. So in fact, both hormones seem to follow the same pattern in infected tissues under HL conditions. In addition, it has been shown that JA and SA defense pathways generally antagonize each other and the elevation of resistance against necrotrophs is often correlated with increased susceptibility to biotrophs, and *vice versa* (Robert-Seilaniantz et al., 2011). However, JA accumulated to high levels in *aao3* plants, under both conditions, compared to wild-type suggests that ABA also seem to antagonize JA biosynthesis and agreed with previous studies by (Anderson et al., 2004, Adie et al., 2007, Kazan and Manners, 2008).

6.2.2 The effect of HL on flavonoid accumulation

In addition to bacterial growth determination on Arabidopsis plant tissues exposed to HL/DC3000, samples were also harvested for flavonoids measurements. LC–MS/MS analysis enabled the identification and profiling of five flavonols (kaempferol glycosides) and nine cyanidin-based anthocyanins. The five kaempferol glycosides showed an identical pattern of accumulation in *aoo3* mutant under HL and HL/DC3000 (Fig 4.13A-E). Each compound accumulated in Col-0 and *sid2-1* and all were significantly lower in the *aao3* mutants after 9dpi of DC3000 ($p < 0.05$). Whilst, two Cyanidins have reported to follow the same pattern in Col-0 and *aao3* HL-challenged tissues where; Cyanidin 3-*O*-[2″-*O*-(xylosyl) 6″-*O*-(*p*-*O*-(glucosyl) *p*-coumaroyl) glucoside] 5-*O*-[6_-*O*-(malonyl) glucoside] and Cyanidin 3-*O*-[2″-*O*-(6_-*O*-(sinapoyl) xylosyl) 6″-*O*-(*p*-*O*-(glucosyl)-*p*-coumaroyl) glucoside] 5-*O*-(6‴-*O*-malonyl) glucoside) (Fig 4.14F and I), although these Cyanidins were significantly higher in Col-0 than *aao3* tissues. Overall, all anthocyanins were enhanced by HL/DC3000 challenge except in one case of each of Col-0 and *sid2-1* (Fig 4.14A and 14E respectively). By contrast, anthocyanins were not affected by normal light, mainly in *aao3*, although accumulation of anthocyanin was observed in some cases for *sid2.1* under NL/DC3000 challenged (Fig 4.14A, C, D, G and E) and Col-0 plant tissues (Fig 4.14A, C and D) and Table6.1.

Table 6.1 Shows Flavonoid regulation in phytohormone mutants, *aao3* and *sid2.1*, in comparison to Col.0 with/without HL/DC3000 over the period of day zero (d0), 5 day , 9day, 9day/DC3000 under both conditions (NL and HL). Green shading indicates up regulation and yellow shading indicates down regulation while white shading indicates that Flavonoids are unaffected either by NL or HL with/without DC3000 infection. Flavonol members A-E and Anthocyanin members A-I are illustrated in Chapter4. Table 4.1

	Genotype	Col.0							*aao3*							*sid2.1*						
	Light condition	NL				HL			NL				HL			NL				HL		
Flavonoids group	Treatment / Member	d0	5d	9d	9dDC	5d	9d	9dDC	d0	5d	9d	9dDC	5d	9d	9dDC	d0	5d	9d	9dDC	5d	9d	9dDC
Flavonol	A																					
	B																					
	C																					
	D																					
	E																					
Anthocyanin	A																					
	B																					
	C																					
	D																					
	E																					
	F																					
	G																					
	H																					
	I																					

Our finding that anthocyanin levels remained substantially lower in plant tissues under NL conditions compared to the plant tissues under HL conditions, suggests that anthocyanin, appear to have an improtant role in responses to HL and in susceptibility to *P. syringae*. In other words our interpretation is that HL contributes directly in plant susceptibility to *P. syringae* and anthocyanin accumulation. This, to an extent, agrees with Solfanelli *et al* (2006) who reported that HL stimulated the anthocyanin biosynthesis leading to augmentation of soluble sugars in Arabidopsis plants which provides more nutrient resource to promote the bacterial growth under HL conditions. However, it has been shown that anthocyanins also contribute in plants defence against other organism. These include both direct roles as chemical repellents and more indirect roles as visual signals (Winefield et al., 2009b). Furthermore, as also showed in common with other flavonoids, certain anthocyanins have demonstrable antiviral, antibacterial, and fungicidal activities (Konczak and Zhang, 2004, Wrolstad, 2004, Winefield et al., 2009b). They have the potential, therefore, to protect plants from infections by pathogenic microorganisms (Winefield et al., 2009b).

Overall, our results from HL/DC3000-challenged in Col-5 and phytohormone mutants supports the argument that plant abiotic stress responses take precedence over biotic stress responses and that abiotic stress is detrimental to plant immunity (Anderson et al., 2004, Mauch-Mani and Mauch, 2005, Robert-Seilaniantz et al., 2007, Yasuda et al., 2008). The increase of the susceptibility under HL is expectedly due to; (a) the reduction of SA level (Chapter4. Fig 4.10B and Fig 4.11B) under HL stress, as the SA is required to suppress growth of DC3000 virulent strain (de Torres Zabala et al., 2009) and (b) is likely due to the antagonism between ABA and SA where ABA is required for full DC3000 virulence and ABA deficient *aao3* show restricted SA accumulation compared to wild type (de Torres Zabala et al., 2009). This compatible with our data in which the response of *Arabidopsis thaliana* plants to HL resulted in promotion of bacterial growth, causes low level of *ICS1*, which encodes a key regulatory enzyme of SA biosynthesis, alongside with high accumulation levels of kaempferol glycosides and Cyanidins. Thus, both HL-reduced SA and HL-increased anthocyanin and consequently potentially increased availability of carbohydrate (Matile, 2000) resulting in higher susceptibility of DC3000 in Arabidopsis-HL-challenged tissues compared with plants under NL. Furthermore,

chloroplasts are a plant organelle in which the precrsors fro a range of plant defence hormones such as SA, JA and ABA are produced. These hormones contribute to sugar status which influences flux into the shikimate pathway that leads to SA and tryptophan pathways and interacts with signalling through other phytohormones, such as ABA, that are involved in biotic challenge (Finkelstein and Gibson, 2002a, Roitsch et al., 2003, Asselbergh et al., 2008).

In the *sid2-1*-HL/DC assay, using 10 and 100 times less than the usual DC3000 inoculum suspension (OD_{600}: 0.00002 and 0.000002 respectively) showed a statistical significant increase (*t.test*: 0.00111 and 0.00119 respectively) compared with the mutant under NL which demonstrated that, SA deficient plants are also hypersusceptible to HL supporting the concept elaborated above, that limitation of nutrient resources in plant tissue may be an essential factor to determine plant susceptibility in interaction with abiotic stress (Fig 4.9A and B)

6.2.3 Effect of HL on ABA hypersensitive mutants

The ABA perception mutants, *hab1-1/abi1-2/abi2-1,* PP2C triple knockout mutant, and PYL5 ABA receptor overexpressor, *pyl5-OE,* were examined for their HL response to DC3000 challenge. The investigation revealed that *pyl5-OE* displayed more chlorosis than *hab1.1/abi1.2/abi2.1* even though both lines showed same level of bacterial growth (~2 fold) in comparison to Col-0 (Fig 4.15A-C and Fig 4.16). Our observation that HL promotes bacterial growth is supported by the increase of ABA levels alongside the reduction of SA level under HL/DC3000; 2 fold less than NL/DC3000 (Fig 4.10B). This is in agreement with the positive effects of ABA reported in promotion of callose formation together with the attenuation of SA-inducible defense responses (De Torres Zabala *et al*., 2009). In addition, Rubio et al (2009) found that the multiplicities of PP2Cs that regulate ABA signalling have diverse mechanism to effectively control ABA response both in the absence or presence of stress. Furthermore, the transcript levels of different *RCARs/PYLs* and *PP2Cs* were different during plant development and in response to environmental challenge (Ma et al., 2009, Park et al., 2009, Santiago et al., 2009a, Szostkiewicz et al., 2010). Different expression patterns of individual *PYLs* and *PP2Cs* are expected to reduce/or fine tune the numbers of combinatorial interactions in plant cells. Thus, transcript levels of *PYLs* are down-regulated under stress conditions, whereas the abundance of

PP2C transcripts are increased (Szostkiewicz et al., 2010). A concomitant change in PYLs and PP2C protein levels resulted in an ABA desensitisation of the plants under abiotic stress, hence providing a mechanism for adjustment of ABA signalling to strongly increased ABA levels (Szostkiewicz et al., 2010).

6.2.4 Effect of HL on EDS1 and its interacting partner, PAD4

The plant disease resistance signalling proteins, EDS1 and PAD4, function within the same defence pathway and are required for an identical spectrum of R genes that recognize avirulent *P. syringae* (Feys et al., 2001, Straus et al., 2010, Rietz et al., 2011). Furthermore, EDS1 controls basal resistance to virulent (host-adapted) pathogens and is indispensable for ETI mediated by TIR-NBLRR receptors to avirulent pathogens (Feys et al., 2001; Wiermer et al., 2005). Accumulation of the defense hormone SA is important for promoting basal and systemic resistance responses as part of an EDS1-regulated pathway. Evidence for a function of EDS1 in photo-oxidative stress signalling (Mateo et al., 2004; Muhlenbock et al., 2008) indicated a link between EDS1 and ROS (Straus et al., 2010) caused by oxidative stress. Therefore, we investigated the impact of DC3000 challenge on *eds1* and *pad4 Arabidopsis* mutants (OD_{600}: 0.0002) under HL. The results revealed that DC3000 challenged *pad4* mutants under HL displayed significant increased bacterial growth (*t test: 0.0001*) compared to NL/DC3000 *pad4* plants (Fig 4.18D and Fig 4.19). By contrast, *eds1* plants under both conditions showed no significant differences in bacterial growth (*t test: 0.18*), similar to our observations on *sid2-1*-HL/DC3000 challenged plants. This, once more, is possibly due to insufficient nutrient resources that would be required to support the additional bacterial growth and thus no differences have been reported between *eds1*- and *pad4*-HL/DC3000 as was shown under NL/DC3000 (Fig 4.19). Our observation agreed with that EDS1 regulates plant resistance to host-adapted biotrophic and hemi-biotrophic pathogens (Wiermer et al., 2005; Birker et al., 2009). It also controls the production of SA needed for basal defense and systemic resistance against virulent pathogens (Vlot et al., 2008; Attaran et al., 2009). Furthermore EDS1 is essential for ETI triggered by TIR domain- NB-LRR receptors (Feys et al., 2001, 2005; Wirthmueller et al., 2007). Other studies have also revealed a requirement for EDS1 in promoting leaf cell death (Rusterucci et al., 2001) and limiting the growth of DC3000 (Ochsenbein et al., 2006) in response to photo-oxidative

stress. EDS1 also responds to the status of $O2^{\bullet-}$ or $O2^{\bullet-}$–generated molecules to coordinate cell death and defense outputs and thus, enabling the plant to respond to different biotic and abiotic stresses in the environment (Straus et al., 2010).

6.2.5 Effect of HL/DC3000 on phytochrome interacting factor

HL/DC3000 challenge on phytochrome interacting factor mutants showed intrrestign and contrasting results. HL treatment of *phyA/phyB* resulted in de-elongation of leaf petioles compared with *phyA/phyB* plants under NL. Moreover, HL-challenged plants were more susceptible with a possibility of reduction in flavonoid (Fig 4.20B, 20B` and 20D). The *cry1/cry2* plants by contrast were more resistance to DC3000 under NL compared with wild type Col.0, but became more susceptible under HL/DC3000 with comparative less accumulation of flavonoids than *phyA/phyB* (Fig 4.20B` and 20C`). These observations for *phyA/phyB* suggest that auxin, gibberellin (GA) and cytokinin, hormones that are involved in stimulating cell division, cell elongation and wall-loosening factors, may be impaired in response to Arabidopsis/HL-DC3000 challenged and thus the abundance of these hormones should be measured and investigated in response to HL/DC3000 infection. Recent studies have showed that phytochrome-interacting factors 4 (PIF4) is required for hypocotyl elongation in response to high temperature and also regulates the level of auxin and gene expression related to auxin biosynthesis (Franklin et al., 2011, Gray et al., 1998, Hornitschek et al., 2012). In addition, Hao et al., (2012) showed that light signal stabilizes PAR1 protein and PAR1 interacts with PIF4 and inhibits PIF4-mediated gene activation. DNA pull-down and chromatin immunoprecipitation (ChIP) assays showed that PAR1 inhibits PIF4 DNA binding *in vitro* and *in vivo*. Transgenic plants overexpressing PAR1 (PAR1OX) are insensitive to GA or high temperature in hypocotyl elongation, similarly to the quadruple mutant *pif1/pif3/pif4/pif5*. In addition to PIF4, PAR1 also interacts with PRE1, a HLH transcription factor activated by brassinosteroid (BR) and GA(Hao et al., 2012).

6.3 Characterization of transgenic lines expressing the PYL cytosolic ABA receptors

In the final part of this project we aimed to generate new biological tools to study the characterization of *PYL4*, *PYL5* and *PYL6* genes identified by transcript profiling as DC3000 responsive.

6.3.1 Regulation of *PYL4, PYL5* and *PYL6* genes in response to DC3000 infection

Our investigation on the expression level of *PYL4*, *PYL5* and *PYL6* on *A. thaliana* leaves non-inoculated (NI), mock; 10 mM $MgCl_2$, or inoculated with virulent DC3000 the *hrpA* mutant strain (OD_{600}: 0.15) over the time course of 6, 12 and 18hpi reveals different levels of expression *vs.* mock (Fig 5.8A-C). This result suggests that *PYL4*, *PYL5* and *PYL6* are up-regulated at an early stage of *hrpA*-infection but later the expression level of the genes decreased (approximately 3-4 fold). Whilst the three genes are down-regulated up to 12 hpi of DC3000 (Fig 5.8B and 20C) *PYL5* and *PYL6* transcripts accumulated at later stages of infection (18 hpi) whereas *PYL4* expression fluctuated between 6, 12 and 18 hpi (Fig 5.8A)

6.3.2 Characterisation of single and double of *pyl* lines in response to DC3000

Based upon the expression patterns *ply4*, *pyl5* and *pyl6* knockout lines were generated. The investigation of phenotype and growth curves of DC3000 challenged single mutant *ply4*, *pyl5* and *pyl6* infected (OD_{600}: 0.002 and 0.0002 respectively) 4dpi revealed no differences between the single mutants and Col-0 control. Further investigations were carried out by generating *pyl5/6* double mutant alongside with *pyl4/5*. Both double mutants were challenged with DC3000 for phenotypes and bacterial population growth (OD_{600}: 0.002 and 0.0002 respectively). No pathogen response differences between these double mutants and Col-0 were detected, again this possibly due to inherited redundancy. Accumulating evidence suggests that PYR/ PYL/RCAR family members exhibit functional redundancy in ABA perception, and variations in ABA regulation of their binding to PP2C family members (Ma et al., 2009, Park et al., 2009). Other studies suggest that ABA either binds to PYR/PYL/RCAR proteins directly or forms molecular "glue" between PYL proteins and PP2Cs (Ma et al., 2009, Park et al., 2009, Santiago et al.,

2009b). However, we sought to examine the expression level and histochemical localisation of the *PYL4, 5* and *6* following pathogen challenge and other stresses.

6.3.3 Regulation and response of *PYL4, PYL5* and *PYL6* genes and luciferase PYL5 and PYL6 transgenic lines to bacterial infection

The investigation of *PYL4*, *PYL5* and *PYL6* genes in response to DC3000 infection revealed that the expression level of the three *PYL* genes 6 hpi by DC3000 were considerably lower than 6h after *hrpA* challenge; approximately 50%, 25% and 33% for *PYL4*, *PYL5* and *PYL6*, respectively (Fig 6.8A-C), suggesting that *PYL4* was up-regulated 6 hpi with *hrpA*, in 5 weeks-old leaf tissues, followed by *PYL6* (Fig 6.8A-C), whereas *PYL5* and *PYL6* were up-regulated 18hpi with DC3000 (Fig 6.8B and C)

PYL5-LUC plants, luciferase transgenic lines challenged with DC3000 showed a luciferase activity, high level of expression, at 2hpi (an earlier response to DC3000) with the signal disappearing at 6 hpi (Chapter 5. Fig. 5.18). This observation is contradictory to the RT-PCR result in which the highest expression level of *PYL5* in DC3000 challenged leaves was reported at 18hpi (Fig 5.8B). By contrast, *PYL6-LUC* transgenic lines showed low level of expression 2hpi (Fig 5.18B) compared with *PYL5-LUC* (Fig. 5.18A), although both lines, *PYL5-LUC* and *PYL6-LUC* displayed similar expression levels at 6 hpi of *hrpA* or 18hpi of DC3000 by RT-PCR assay (Fig 5.8B and 8C).

The fourteen member family of PYR/PYL ABA receptors, except PYL13, have been shown to be able to activate ABA-responsive gene expression using protoplast transfection assays but there sre specific signatures to this activation. Similarly, gene expression patterns obtained from public databases and GUS reporter gene analyses have revealed substantial differences among *PYL* expression (Gonzalez- Guzman et al., 2012).

In this study, application of different concentrations of ABA (100µM, 10 µM, 1 µM, 100 nM) to *PYL6-LUC* plants showed a linear increase in response to ABA and causes up regulation at 2 h post treatment (Fig 5.19B. leaf 3). As well as, treatment of *PYL5-LUC* with these concentrations of ABA also showed a gradient of expression initially from the application of 100 to 1µM and100nM ABA led to up regulation 2 h post-treatment. The luciferase activity is reported to be

high in both *PYL5-LUC* and *PYL6-LUC* 2 h per-treatment of ABA, with *PYL5-LUC* reportedly the highest. This finding is consistent microarray data analysis at the Genevestigator database (http://www.genevestigator.ethz.ch) (Zimmermann et al., 2004) revealed a general expression pattern for PYL5 in callus, cell suspension, seeds, seedlings, inflorescences, rosette leaves and roots (Santiago et al., 2009a).

6.3.4 Characterization of epitope tagged CaMV:HA and CAMV:MYC:PYL4, 5 and 6

Characterization of CaMV::HA and CAMV::MYC for *PYL*4, *PYL*5 and *PYL*6 proteins was inconclusive and needs further investigation. Although, the western blot assay with CaMV:HA:*PYL4*, 5 & *6* displayed an HA-tagged signal and resulted in plants with a stunted phenotype (Fig 5.20A-F and Fig 5.21.A and 21B), none of the plants displayed any differences in susceptibility to DC3000 (Fig 6.22A and 22B). In contrast to our efforts in the characterization of epitope tagged-PYLs, Santiago et al., (2009a) generated *PYL5*-over-expressing lines, in which Real-time quantitative PCR analysis showed that *PYL5* expression was between 15- and 20-fold higher than in wild-type and HA-tagged PYL5 protein was detected at similar levels in these lines. In addition, *pyl5-OE* lines showed higher sensitivity to ABA-mediated inhibition of seed germination than wild-type (Saez et al., 2004, Santiago et al., 2009a).

In the case of CaMV:MYC-tagged-*PYL4, 5 and 6*, mutations were identified that may have impacted the transformants. In summary, no signal was detected via western blots and no phenotype was evident in the supposedly transgenic plants (Fig 6.24 and Fig 6.23A-D), yet all plants were hygromycin resistant. However, they seem to display very low level of expression which could not be detected by western. This high level expression may have some detriemental silencing effects. It is proposed that CaMV:*MYC:PYLs* are re-transformed as N-terminus fusions to avoid/reduce potential interference by the tag protein fusion (Chen et al., 2009a). It is a powerful tag for monitoring expression of recombinant proteins in bacteria (Dreher et al. 1991; Vaughan et al. 1996), yeast (Sequi-Real et al. 1995; Weiss et al. 1998). It has also been used for the successful co-immuno-purification of interacting proteins expressed in Agrobacterium-transformed Arabidopsis cells (Ferrando et al. 2001). The c-myc-tag can be placed at the N- or C-terminus (Manstein et al., 1995, Terpe, 2003, Chen et al., 2009a).

6.3.5 Characterization of the PYL cytosolic ABA receptors PYL4, PYL5 and PYL6

Finally we attempted to express GFP or YFP tagged the *PYLs* for histochemical localisation by generating two constructs B8eGFP/YFP for *PYL5* and C1eGFP /YFP::*PYL4, 5* & *6*. The results showed that PCR induced mutations in the C1eGFP/YFP::*PYL4* and *PYL5* gene coding sequences appeared to affect the transformants (Appendix A.14B). Although the sequence of C1eGFP/YFP:*PYL6* constructs did not show any mutations, but no transformed seeds were selected on hygromycin, despite a range of PCRs and digestions (Fig 5.26 and Appendix A.13) undertaken to check the cloning efficiency of C1eGFP/YFP:*PYL4, 5* and *6* transformants. While a range of B8eGFP/YFP: *PYL5* seeds showed BASTA resistance, we could not detect any GFP or YFP signal, either by western blots or under confocal microscopy.

These lines can be further investigated, e.g. examining seedling stages in which we may detect a high level of expression would be useful. Possibly a different promoter can be used. It is unclear why transformants were not generated as Santiago et al (2009a) showed that a GFP–PYL5 fusion localizes in both cytoplasm and the nucleus and PYL5 protein acts as an intracellular hormone receptor involved in the activation of the ABA signalling pathway through inhibition of certain clade A PP2Cs (Santiago et al., 2009a). It is intyersting that one study has reported that ABI1–GFP over-expressing lines do not show any ABA response phenotypes compared with vector control lines (Moes et al., 2008), whereas, Nishimura et al., (2010) reported, YFP–ABI1 fusion expression plants showed ABA insensitive phenotypes during seed germination, root growth and in stomatal responses compared with the control YFP expression plants confirming ABI1 function. Thus functional interference by the fluorescent protein is a possibility.

6.4 Future work and additional investigation:

1- For further investigation and by taking in account the primarily findings by Murray Grant's group (unpublished data) whom had identified the 3´-*O*-β-D- ribofuranosyl adenosine (400) compound as a major discriminatory molecule induced very early in challenge-*Arabidopsis* with virulent DC3000, it is worth investigating the role of AtNUDT7, despite it not being transcriptionally regulated. AtNUDT7 accumulates as a homodimer and correct dimer formation is important for its function (Olejnik et al., 2009, Adams-Phillips et al., 2010, Olejnik et al., 2011). We investigated a range of enzymes, for instance; Nudix hydrolases 6 and 8, ribosyltransferase, glucosyl transferase and ribose-phosphate pyrophosphokinase3, that were predicted to involve in the biosynthetic of 400 compound. It may be possible to look at their expression in mutants hyperaccumulating 400 to get further evidence for a potential transcriptional role in 400 synthesis.

2- In the high light project, one of the most distinct observations was that HL causes leaf curling, dark reddish with chlorotic phenotype and become brittle with de-elongation in leaf petiole (as seen in chapter4. Fig 4.6A, Fig 4.7A-D, Fig 4.15A-C, 4.18A`-18E`, Fig 4.20A`-C` and Fig 4.23A-D). Thus, and as it has been shown, anthocyanins are functioning to attract animals for pollination and seed dispersal, and they are protect plant cells from UV radiation. Furthermore, related flavonoids serve as antibiotics against microbial pathogens and as insect repellents and are involved in signalling in plant-microbe and pollen-pistil interactions (Hahlbrock and Scheel, 1989, Mo et al., 1992, Deikman and Hammer, 1995, Winefield et al., 2009a). Accumulation of flavonoids, including anthocyanins, is stimulated by various environmental stresses including UV light, high intensity light, wounding, pathogen attack, drought, and nutrient deficiency (Swain et al., 1979, Winkel-Shirley, 2002, Winefield et al., 2009b). Therefore, we suggest more investigation on Arabidopsis in response to light intensity and pathogens attack is required. In addition, the similarity in phenotype among *pyl5.OE, hab1-1/abi1-2/abi2-1* and *phy1/phy2* plants after 5 days pre-treatment in HL (Fig 4.23B-D) needs further investigation, as does the exciting discovery of the phenotypes emerging from the photoreceptor PIF.I and PIF.H mutants. In particular

trying to understand the interaction between the clock/photoreceptors and hormonal balance under high light and pathogen stresses. The study by (Zhang et al., 2013) provides a good foundation for future work.

In addition, transformants of epitope tagged CaMV:MYC:PYLs (*4, 5* and *6)* and constructs containing eGFP and YFP fusions to the *PYL4*, *PYL5* and *PYL6* promoters require re-transformation as N-terminus to avoid/reduce the possibility that an interference between the myc-tag, GFP and YFP proteins fusion may occur in these transformants.

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Appendix:

A1. Table.1; ABA receptors nomenclatures and primers used in this Chapter of the study

PYLsnomenclature, gene number and origin				
No.	**PYRbactin resistance1/PYR 1 like**	**Regulatory component of ABA receptor**	**Gene**	**Origin**
1	PYL4	RCAR10	At2g38310	SAIL_517_C08
2	PYL5	RCAR8	At5g05440	sm_3_3493
3	PYL6	RCAR9	At2g40330	SAIL_1179_D01

Primers to check *PYL*-KO's and to make epitope tagged constructs			
No.	**Primer**	**Sequence**	**Tm**
4	*PYL4*- FP	5` TTCCAATCGTTCCAAATATCG-3`	58
5	*PYL4*- RP	5` TAAGACTCGACAACGACGGTC-3`	64
6	*PYL5*- FP	5` GGTCACCGGTGCAACTCCAACACG-3`	74
7	*PYL5*- RP	5` CAGTCTCTGGCTCGAAGTACCAACC-3`	70
8	*PYL6*- FP	5` GCCTCGAGACAGTAGAAGATTG-3`	63
9	*PYL6*- RP	5` CGTATGACTCAACGACACGTG-3`	64
10	*PYL4*-C.START	5` CATGCTTGCCGTTCACCGTCCTTC-3′	65
11	*PYL4*-STOP	5` TCACAGAGACATCTTCTTCTTGCTCAG-3′	59
12	*PYL5*-C.START	5` CATGAGGTCACCGGTGCAACTCCAAC -3′	66
13	*PYL5*-STOP	5` TTATTGCCGGTTGGTACTTCGAGCCAG- 3′	64
14	*PYL6*-C.START	5` CATGCCAACGTCGATACAGTTTCAG -3′	59
15	*PYL6*-STOP	5` TTACGAGAATTTAGAAGTGTTCTCG -3′	53
16	LB3-SAIL	5` TAGCATCTGAATTTCATAACCAATCTCGATACAC-3′	68
17	spm31 PYL5-LB	5` GCTTGTTGAACCGACACTTTTAACATAAG-3`	66

Primer for *PYL* promoter luciferase constructs (+ restriction sites Kpn1 at 5′ and Nco1 at 3′)			
No.	**Primer**	**Sequence**	**Tm**
18	*PYL4*-PRO.F	5` GTGGTACCTGACAAGATTCTTGTACAC-3`	58
19	*PYL4*-PRO.Rev	5` CAACCATGGTGATCTGAGTAATGGTG-3`	59
20	*PYL5*-PRO.F:	5` GTGGGTACCCCATTAGGAGGCTCTC-3`	64
21	*PYL5*-PRO.Rev	5` GACCCCATGGTCTCTCCTCTATCTC-3`	61
22	*PYL6*-PRO.F	5` CAGGTACCCTTTGACAATGTGTCCTC-3`	61
23	*PYL6*-PRO.Rev	5` TTGCCATGGTTAAAGCAAATCTTTTCTC-3`	57
24	Luc-up	5` TCTCGCTGCACACCACGATC-3`	68

Primers for RT-PCR standards			
No.	**Primer**	**Sequence**	**Tm**
25	*PYL4*-Standard-F	5` GTTATTCAAGAGATCTCCGCTCC-3`	56
26	*PYL4*-Standard-R	5` GCTCTCAGCCGCAGTATTCTC-3`	59
27	*PYL5*-Standard-F	5` GGTGGTGCAGATGATCCACG-3`	60
28	*PYL5*-Standard-R	5` TGCCGGTTGGTACTTCGAGC-3`	61
29	*PYL6*-Standard-F	5` GTCGTGGTACAAGACGTGGAG-3`	59
30	*PYL6*-Standard-R	5` TTAGAAGTGTTCTCGGCGAGTTT AG-3`	57
Primers for *PLY*s (*4*, *5*, *6*) RT-PCR			
No.	**Primer**	**Sequence**	**Tm**
31	*PYL4*-RT- F	5` ATAACGTTGGTAGCCTCCGTCAAG-3`	60
32	*PYL4*-RT- R	5` CCCGGAGATCGGAGAAGGGTGAAGG-3`	67
33	*PYL5*-RT- F	5` GGCGATGGACTACACGTCGGCGATC-3`	68
34	*PYL5*-RT- R	5` CACCACCACGGTACCTTCGTCGTCC-3`	67
35	*PYL6*-RT- F	5` GGAGACGGTCGAGAGGTTGGGTCG-3`	67
36	*PYL6*-RT- R	5` CTCAACGACACGTGTCCTCTTCTTG-3`	61
37	*ICS1*-F	5` CAGGCGATTAATTGAAGAAAGA-3`	61
38	*ICS1*-R	5` GGCCTGCCCTAGTTACAACC-3`	58
39	*NCED3*-F	5` AGCTCCTTACCTAGTGCCAGTC-3`	65
40	*NCED3*-R	5` CGCTCTCTGGAACAAATTCATC-3`	66
41	*Actin*-F	5` ATGGGTCGTACAACCGGTATTGT-3`	66
42	*Actin*-R	5` GTAGGCATGAGGAAGAGAGAAAC-3`	63
Primers for epitope tagged constructs			
No.	**Primer**	**Sequence**	**Tm**
43	PYL4-Myc-UP	5` CGAGCGGCCGTGGAGTCGCGTGAGAG-3`	74
44	PYL4-Myc-Down	5` CCCTTCTCCGATCTCCGGGACCGTC-3`	69
45	PYL5-Myc-UP	5` GCATACCGCGCGTGAGACACACTCTC-3`	67
46	PYL5-Myc-Down	5` CGCGTCGGACGACGAAGGTACCGTGG-3`	71
47	PYL6-Myc-UP	5` GCTCCACGTGCTCCGGCACATCAGCC-3`	73
48	PYL6-Myc-Down	5` CGGTGACGACGGTGCATGAGTCGGA-3`	69
49	CaMV	5` CACCTGTTCAAAGCAAGTGG-3`	62

Primers for eGFP and YFP fusions (+ restriction sites EcoR1 or Sac1 at 5′ and Nco1 at 3′)			
No.	**Primer**	**Sequence**	**Tm**
50	PYL4F4	5` CTAAGAGCTCATCATAAGAGTTAGGTGTGACC-3`	61
51	PYL4R1	5` AAGACCATGGACAGAGACATCTTCTTCTTG-3`	61
52	PYL5-F1	5` GGTGAATTCCCATTAGGAGGCTCTCACCATTG-3`	65
53	PYL5-R1	5` GATCCATGGATTGCCGGTTGGTACTTCG-3`	65
54	PYL6-F1	5` TAAGAATTCAGATGGAATAATGCGATGTTGCGT-3`	61
55	PYL6-R1	5` AATCCATGGACGAGAAATTTAGAAGTGTTC-3`	58
56	EGFP-up	5′ CGTCCAGCTCGACCAGGATGG-3′	64
57	EGFP-down	5′ CAACCACTACCTGAGCACCCAG- 3′	61
58	YFP-up	5′ CACTGCAGGCCGTAGCCGAAG -3′	65
59	M13-Rev	5` GGAAACAGCTATGACCATG-3`	51
60	NOS	5` CCCATCTCATAAATAACGTCATGC-3`	54

A2. Table.2 List of enzymes with appropriate buffer used in the project

No.	**Enzyme**	**Sequence**	**Cut site**	**Buffer**
1.	Kpn1	GGTACC	GGTAC/C C/CATGG	NEB1+ BSA
2.	Nco1	CCATGG	C/CATGG GGTAC/C	NEB3
3.	Xcm1	CCANNNNNNNNNTGG	CCANNNNN/NNNNTGG GGTNNNN/NNNNNACC	NEB2
4.	EcoR1	GAATTC	G/AATTC CTTAA/G	NEB4
5.	Sac1	GAGCTC	GAGCT/C C/TCGAG	NEB1+ BSA
6.	Sal1	GTCGAC	G/TCGAC CAGCT/G	NEB3+ BSA
7.	Pst1	CTGCAG	CTGCA/G G/ACGTC	NEB3+ BSA

A3. Sequence analysis of *pyl6* PCR product. The sequence was carried out at The Genome Analysis Centre (TGAC).

PYL6 Sequences producing significant alignments: Score (bits) Value E

AT2G40330.1, Symbols: *PYL6, RCAR9, PYR1-like 6* 989 0.0

```
Sequence of pyl6 KO PCR with PYL6; Forward/LB3 Query:
       263 cgtatgactcaacgacacgtgtcctcttcttgccgtcggagtcctcctccgactcatgca 322
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 639 cgtatgactcaacgacacgtgtcctcttcttgccgtcggagtcctcctccgactcatgca 580

Query: 323 ccgtcgtcaccgacttgtagttcatgagtctgtggtccccaccaacgacgctgaaactga 382
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 579 ccgtcgtcaccgacttgtagttcatgagtctgtggtccccaccaacgacgctgaaactga 520

Query: 383 tgacgtggcgatcatcgtccatgatctcaagccgctctaagctaaacgccgcggggagac 442
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 519 tgacgtggcgatcatcgtccatgatctcaagccgctctaagctaaacgccgcggggagac 460

Query: 443 cagagacgactctgacctctctcaccgacccaacctctcgaccgtctccgataaccacgt 502
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 459 cagagacgactctgacctctctcaccgacccaacctctcgaccgtctccgataaccacgt 400

Query: 503 ggcagcttttcacgaagtgtttgtacgcttgagggtgttcgaagcggcttaggatcgacc 562
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 399 ggcagcttttcacgaagtgtttgtacgcttgagggtgttcgaagcggcttaggatcgacc 340

Query: 563 agactgtggaaaccggagcctccacgtcttgtaccacgacggagaagcactgagaaggac 622
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 339 agactgtggaaaccggagcctccacgtcttgtaccacgacggagaagcactgagaaggac 280

Query: 623 caaccacgtgcgtgtgggaaagctccacgtgctccggcacatcagccatcccgcgcgtga 682
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 279 caaccacgtgcgtgtgggaaagctccacgtgctccggcacatcagccatcccgcgcgtga 220

Query: 683 ggctcacttttgaacctgtttctgatggtggtggggatagtttcttacggtggcgttgg 742
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 219 ggctcacttttgaacctgtttctgatggtggtggggatagtttcttacggtggcgttgg 160

Query: 743 ctgcttctgcggcggtgga 761
           ||||||||||||||||||
Sbjct: 159 ctgcttctgcggcggtgga 141
```

A4. Sequences and translation of *PYL4*, *PYL5* and *PYL6* genes:

a) *PYL4*: At2g38310

```
ATGCTTGCCG TTCACCGTCC TTCTTCCGCC GTATCAGACG GAGATTCCGT TCAGATTCCG ATGATGATCG
CGTCGTTTCA AAAACGTTTT CCTTCTCTCT CACGCGACTC CACGGCCGCT CGTTTTCACA CACACGAGGT
TGGTCCTAAT CAGTGTTGCT CCGCCGTTAT TCAAGAGATC TCCGCTCCAA TCTCCACCGT TTGGTCCGTC
GTACGCCGCT TTGATAACCC ACAAGCTTAC AAACACTTTC TCAAAAGCTG TAGCGTCATC GGCGGAGACG
GCGATAACGT TGGTAGCCTC CGTCAAGTCC ACGTCGTCTC TGGTCTCCCC GCCGCTAGCT CCACCGAGAG
ACTCGATATC CTCGACGACG ACGCCACGTC ATCAGCTTCA GCGTTGTTGG TGGTGACCAC CGGCTCTCTA
ACTACCGATC CGTAACGACC CTTCACCCTT CTCCGATCTC CGGGACCGTC GTTGTCGAGT CTTACGTCGT
TGATGTTCCT CCAGGCAACA CAAAGGAAGA GACTTGTGAC TTCGTTGACG TTATCGTACG ATGCAATCTT
CAATCTCTTG CGAAAATAGC  CGAGAATACT GCGGCTGAGA  GCAAGAAGAA GATGTCTCTG TGA
```

```
Met L A V H R P S S A V S D G D S V Q I P Met Met I A S F Q K R F P S L S R D
S T A A R F H T H E V G P N Q C C S A V I Q E I S A P I S T V W S V V R R F D
N P Q A Y K H F L K S C S V I G G D G D N V G S L R Q V H V V S G L P A A S S
T E R L D I L D D E R H V I S F S V V G G D H R L S N Y R S V T T L H P S P I
S G T V V V E S Y V V D V P P G N T K E E T C D F V D V I V R C N L Q S L A K
I A E N T A A E S K K K Met S L Stop
```

b) *PYL5*: At5g05440

```
ATGAGGTCAC CGGTGCAACT CCAACACGGC TCAGACGCCA CTAACGGTTT CCACACGCTG CAGCCTCACG
ATCAGACCGA TGGTCCGATC AAGAGAGTGT GTCTCACGCG CGGTATGCAT GTCCCTGAAC ACGTTGCGAT
GCACCACACA CACGACGTTG GTCCGGACCA GTGTTGCTCC TCGGTGGTGC AGATGATCCA CGCGCCGCCT
GAGTCCGTGT GGGCTCTTGT GCGGCGTTTC GATAATCCGA AGGTTTACAA GAACTTCATC AGACAGTGCC
GTATCGTCCA AGGCGATGGA CTACACGTCG GCGATCTCCG GGAGGTCATG GTGGTCTCTG GACTCCCGGC
GGTCTCGAGC ACCGAGAGGC TCGAGATCTT GGACGAGGAG CGTCACGTGA TAAGCTTTAG TGTCGTTGGT
GGGGACCACA GGCTCAAGAA CTACCGATCG GTGACGACAC TACACGCGTC GGACGACGAA GGTACCGTGG
TGGTGGAGTC TTACATCGTT GATGTGCCGC CGGGAAACAC GGAGGAGGAA ACTCTAAGCT TCGTTGATAC
TATCGTCCGG TGCAACCTTC  AGTCTCTGGC TCGAAGTACC  AACCGGCAAT AA
```

```
Met R S P V Q L Q H G S D A T N G F H T L Q P H D Q T D G P I K R V C L T R
G Met H V P E H V A Met H H T H D V G P D Q C C S S V V Q Met I H A P P E S V
W A L V R R F D N P K V Y K N F I R Q C R I V Q G D G L H V G D L R E V Met V
V S G L P A V S S T E R L E I L D E E R H V I S F S V V G G D H R L K N Y R S
V T T L H A S D D E G T V V V E S Y I V D V P P G N T E E E T L S F V D T I V
R C N L Q S L A R S T N R Q Stop
```

c) *PYL6*: At2g40330

```
ATGCCAACGT CGATACAGTT TCAGAGATCC TCCACCGCCG CAGAAGCAGC CAACGCCACC GTAAGAAACT
ATCCCCACCA CCATCAGAAA CAGGTTCAAA AAGTGAGCCT CACGCGCGGG ATGGCTGATG TGCCGGAGCA
CGTGGAGCTT TCCCACACGC ACGTGGTTGG TCCTTCTCAG TGCTTCTCCG TCGTGGTACA AGACGTGGAG
GCTCCGGTTT CCACAGTCTG GTCGATCCTA AGCCGCTTCG AACACCCTCA AGCGTACAAA CACTTCGTGA
AAAGCTGCCA CGTGGTTATC GGAGACGGTC GAGAGGTTGG GTCGGTGAGA GAGGTCAGAG TCGTCTCTGG
TCTCCCCGCG GCGTTTAGCT TAGAGCGGCT TGAGATCATG GACGATGATC GCCACGTCAT CAGTTTCAGC
GTCGTTGGTG GGGACCACAG ACTCATGAAC TACAAGTCGG TGACGACGGT GCATGAGTCG GAGGAGGACT
CCGACGGCAA GAAGAGGACA CGTGTCGTTG AGTCATACGT CGTTGACGTA CCGGCGGGTA ACGATAAGGA
AGAGACTTGT AGCTTTGCTG ATACTATAGT ACGGTGCAAC TTGCAATCGC TGGCTAAACT CGCCGAGAAC
ACTTCTAAAT TCTCGTAA
```

```
Met P T S I Q F Q R S S T A A E A A N A T V R N Y P H H H Q K Q V Q K V S L T
R G Met A D V P E H V E L S H T H V V G P S Q C F S V V V Q D V E A P V S T V
W S I L S R F E H P Q A Y K H F V K S C H V V I G D G R E V G S V R E V R V V
S G L P A A F S L E R L E I Met D D D R H V I S F S V V G G D H R L Met N Y K
S V T T V H E S E E D S D G K K R T R V V E S Y V V D V P A G N D K E E T C S
F A D T I V R C N L Q S L A K L A E N T S K F S Stop
```

A5. Theoretical primers design for *PYL4*, *PYL5* and *PYL6*:

Primers Key for *PYLs*: ..... Promoter Forward primer ..... Reverse primer

a) *PYL4 + promoter*

PYL4 (650bp) + promoter (2135bp) and 3' untranslated; cut with **Sac1**at 5' and **Nco1** at 3'

PYL4-long-For: CTAA**GAGCTC**ATCATAAGAGTTAGGTGTGACC

PYL4-long-Rev: AAGA**CCATGG**ACAGAGACATCTTCTTCTTG

gcaattttgcaccgagaatcctaatttgtgtccttcattttaaaggtttaaaaagcatgtttataaaaac
atggaactggatggtttgtgagatatgccatcaaattagacaaaagggtccattaagcctgtgaaaaaaa
aagaaaaaaaaaa**ctaagagctcataagagttaggtgtgacc**tgacaagattcttgtacacccgaacccg
acttgtgaactgtgaagtcaacctctaattaacagttatttttattcctacaaatacggatccaacatt
attctcaattagttgaagtaatgtctgtctatttgcacattttcaaacccataatcaaacaaaagttaca
tatcctgacccgctgaccatattatacactcatctgtgtatatctcaaatctcacttgcaggtaattctc
tctagtacttttatgtagatatatagatattagtagaatgttagcaagaaaagatgtaaaacatatatat
caactatattagaacccgtcctttgtgcgagtttttgatttttatgataatttaagttaacaaaaactg
aattaattttgctttatatattaagatgtgtgtgaagctcaatacacaatattaatctattcttatcaaa
aaaatttatattcaaaatttacagcaattataaagaactaatagcattgtattatgacttatcattaaga
tggggtttgtgtccatgtccggagttgttaaaggtttgaaaacccagagttatgaatgtaaagaaacaag
acagaatgcttgaaaacagagcatcaagtaaatgttttgtgtttatattattatgcagctaaaatggaac
cggtgacggcattccaacttggccaatggatgtctagactcgctatattcttcaaggcagacgacacatt
cttccccaacctcgaaattgaattcctcgacggcaacaaaaatatatcttcgaacgttttgttgtgtctg
atcagccaactccacatctttaggtaaactctaatatcctagagaataatagaaatctataatactagtt
acaatgtagaagaagagacaacctataacgaag**ctagatactatagccaacgtaacc**aaaccaaacaaca
aaagtatgttgtgatagaccagtttttctttccaaaatacggcccaattaaaagtttggttctaaaaaca
atttgggccttagtttgtatagtttaaaatgtgtttctt**agtgaggattggtttttcatgcc**cctttgtt
tgtgtgctccttttttttgtatattgaagatcatatcaaagcaaaagcaatacaatacttttcattcaaaa
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tttttttgcaattgaaaactcatatatatagtatggctagatatatgcaattgaaaactcatatatcta
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tttgatcttacattccaatcgttccaaatatcgaaactcttaaagcgtattcaatcaaacgaatctcgtc
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atattataattctagtgttgatatacgagaattatatattggtcacttgcccaattaaaaatatcgtgta
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ttttttcgttatcaccactcacatgaactctgcattaaaaactctatctctctcaaatcgaaaggcacag
cccaacttttcgcaagtcgctgtaaagtttgatttgcttctttttatatacacacatacttctcctccat
acactttcctcttcaatcctcagttttttttctaagccctaataccatctcaaagaagagatcaagattt
gaaatcaagaaga**caccattactcagatcaacATG**CTTGCCGTTCACCGTCCTTCTTCCGCCGTATCAGA
CGGAGATTCCGTTCAGATTCCGATGATGATCGCGTCGTTTCAAAAACGTTTTCCTTCTCTCTCACGCGAC
TCCACGGCCGCTCGTTTTCACACACACGAGGTTGGTCCTAATCAGTGTTGCTCCGCCGTTATTCAAGAGA
TCTCCGCTCCAATCTCCACCGTTTGGTCCGTCGTACGCCGCTTTGATAACCCACAAGCTTACAAACACTT
TCTCAAAAGCTGTAGCGTCATCGGCGGAGACGGCGATAACGTTGGTAGCCTCCGTCAAGTCCACGTCGTC
TCTGGTCTCCCCGCCGCTAGCTCCACCGAGAGACTCGATATCCTCGACGACGAACGCCACGTCATCAGCT
TCAGCGTTGTTGGTGGTGACCACCGGCTCTCTAACTACCGATCCGTAACGACCCTTCACCCTTCTCCGAT
CTCCGGGACCGTCGTTGTCGAGTCTTACGTCGTTGATGTTCCTCCAGGCAACACAAAGGAAGAGACTTGT
GACTTCGTTGACGTTATCGTACGATGCAATCTTCAATCTCTTGCGAAAATAGCCGAGAATACTGCGGCTG
AGAGCAAGAAGAAGATGTCTCTGTGAtgagtctttgtcgttgtcgggtagtttcgttagatccgacgtcg
ttttctagatttttagccgtcgtgtgatctatgtttttcggcttatgtgtgaaaaaaaagttacattag
tgaattaatctctcatgcatatcataatccttcttttaatttttgtattttacatatcccataaagaacc
gatttggatagccctattccggctttcaccacccaaagataataatattcaaactgaaagaatgtggttg
tgttgtccgctaattaaaagtgtgattttcaagtttaatt

b) *PYL5 + promoter*

PYL5 (612bp) + promoter (1717) +3' untranslated; PYL5 cut with **EcoR1 at 5`** and Nco1 at 3`

PYL5-long-For1: GGT**GAATTC**CCATTAGGAGGCTCTCACCATTG

PYL5-long-Rev1: GAT**CCATGG**ATTGCCGGTTGGTACTTCG

ttgtgaataatttttttccagacaactcttttaaaagagacttaccattggactatgaaaaatctttcaaaaaaat
cagaagctctcatcttacaaattcaaaagatttatacttaaaaaatggtgagatc**ccattaggaggctctcaccatt**
ggagatggtcttatgagttccgtgaagctctctcgttattcttacagggcatgatttcgtataatatttttaaaaaa
acaaacgattgggccttgaatttttaattagaaagttaaacaaaatctaaaaacccacatgtggtctcttgttttttt
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aatgggcaacaagaacaaaagaaaatagagaaaaaaaggaaaggataatcttccacctaaacaagtccaaagaagat
tgcaagttgcaacatccacttgcttcgtccggcgt**aagctctttgataaggtctctca**ccgtcctccgactttctct
ctccatacatacaccacatttacatacgccatttacacatatatgacaaacccatcaacatctgtgcatgcacgtgt
gctcatgcatgtatgtagtttttacttcgccatgatgaccatgtctatcttaaaatttaccacaatcaatcaatcat
gcgcgttacagtcgtgtgatattcaaaaattgtacatgttatttctcataactatcactttagtagattaagatcag
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agataaaactaattctgaaatttcagtttttttctatgaacaatttattaacagtattcatatgtagataaaccttgt
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agctctcttctttttctcatcaaacttatttctctctcgatcgcaatatatacgattccataaattctcccaaaaaca
aattaa**gagatagaggagaga**tcATGAGGTCACCGGTGCAACTCCAACACGGCTCAGACGCCACTAACGGTTTCCAC
ACGCTGCAGCCTCACGATCAGACCGATGGTCCGATCAAGAGAGTGTGTCTCACGCGCGGTATGCATGTCCCTGAACA
CGTTGCGATGCACCACACACACGACGTTGGTCCGGACCAGTGTTGCTCCTCGGTGGTGCAGATGATCCACGCGCCGC
CTGAGTCCGTGTGGGCTCTTGTGCGGCGTTTCGATAATCCGAAGGTTTACAAGAACTTCATCAGACAGTGCCGTATC
GTCCAAGGCGATGGACTACACGTCGGCGATCTCCGGGAGGTCATGGTGGTCTCTGGACTCCCGGCGGTCTCGAGCAC
CGAGAGGCTCGAGATCTTGGACGAGGAGCGTCACGTGATAAGCTTTAGTGTCGTTGGTGGGGACCACAGGCTCAAGA
ACTACCGATCGGTGACGACACTACACGCGTCGGACGACGAAGGTACCGTGGTGGTGGAGTCTTACATCGTTGATGTG
CCGCCGGGAAACACGGAGGAGGAAACTCTAAGCTTCGTTGATACTATCGTCCGGTGCAACCTTCAGTCTCTGGCTCG
AAGTACCAACCGGCAATAAtctcatctttcttatataaattgcaattatgtatctaatttttttgttgttctattt
cttttagatgttcgatcttctttacaaggaagaaaatttcgagtacctttctttctttttaaatagatatatcggc
ttagaaagaattgtaatttaatggggatttctttgggagatttatgttggaaatttcgaagtactgttgggggattc
acaaaactttggatttggagggtgttagtactggtacataaaacattttaaggtgaatctgttaaatgaattaatcc
atttgttgttttgtacatggtatcattctttgtgacattgtttaatttcttgtactcttttaaatgttactcttaac
cgtttttttctttttgtggtttgtaaatgaatatttgatgcatcggtattgttaatgatagacttattaatttatttc
ttcatagtatgtaacatt

c) *PYL6 + promoter*

PYL6 (648bp)+promoter (2613bp)3261)+3' untranslated; PYL5 cut with
**EcoR1 at 5`** and Nco1 at 3`
PYL6F1: TAA**GAATTC**AGATGGAATAATGCGATGTTGCGT
PYL6R1: AAT**CCATGG**ACGAGAATTTAGAAGTGTTC

ttagaaaaagtttataacattcggtacatatcaaagttttgaacttcggatgcatataagctataaagttttgatct
ttgaactttggatactctcacatctaattttcatccttaagaatgg**agatggaataatgcgatgttgcgt**agtggtt
ttattgacttggcaagttttgattattcaagaaacttggcttagttt**aatggaaatatccagtagtgg**agtttttg
gtctgcagactttcctcaggttgtctgagattacataagcaatgaggtatgaataataaagttatcattagagagtt
atctatctgtattttctacaaaaggcaaattgggtatttgaaagtgcaaatgggacatagttgtatattatttaaaa
ggctttaactattccattctggtctccaaaatcgtttttcttgctcgaatcttgaagatcttctaagcctttagaaa
aatgaagaaatcaagttggcaaactgttaattaattaaatccaatgaattggtgggtattgcacctccacaagttcc
ctttgacaatgtgtcctcttctccgcaagaatgagatgcaactattttggacaatttctccttttaattacttttg
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tttttcgatacttatttttgaaatttaagtgcattttgtttataatcatagttggggatttaataatttaggtgg
aataatattttatgcatattggagatttatttctcgagaaatattcacaaacaaacaaacaaccggcttttgcacaa
aaataataatgacatggagtcgagttgtttatactaatgtttgtcactatctcaccattggaatttaaatcttggat
atataatacatgttttgacaaaaaaaaaagttggcacaaattatgttgttgtaacaaaatacgtctgatgcattgat
ttgagattgactctttgaacccaaaaatgtatcaccgtaatattaatacattttccacatcaacttgtaatctattt
gtgttcagtaatatattggtgagtgattatgtcacctaacccgaaagcttataagatgagcaatctttatatcgagg
atggtgcaaaaaatagcaaattgtaacttttgtccttcttattctat**aaaatgtcagatataattagtactgca**aa
ttgctcgcttattcgtattaatcttaacaactagggctacttgggaatatgtatagttgttggaaaaactaatgcat
tagataacccttattatttttcccaattgtgttttgttattatgcccaccactcaaatccaagagtccaatatatt
catatgatttgaatatacgtttgtggaagaaaacacacccaagaaatattgtccaattcgtaaatttccctgacttt
acgttggttacttttagcaaactgcttagtttgattttcttctaattcgtcttattaatattctgttcttttctttg
ttgaaaaggaatttgattaatacttttttttagtatcgtcgaaaggctcaagttgattacaagatgaggaaagattat
tatgatggacccatgacatgatatcttcaccaacttggaacattgaaagtcctatggagttatttttgaaatttta
ccttagtcagaaggttccaacaaaattaaaaatccaccaaaaacgcatccaagaatagtaccaactgaaaatagatc
ggcaatcacaaagacaaaggtcggtcctcccaaccaatttgtttcaagaatttttcaaacctatgcataaattaag
tatagctcttaaccaatatatatagcttaaccaactcgacacatagtgatcatgttcttataatctttaataacgtt
ttagagttagctatatatatagcctcgagacagtagaagattgaaatatctatgcaaaacctcaaagataatgttca
atgtttttatattctgaatattgatataatcggtgagatagagatgagttcagaatttttttctcggtatcttaaat
gatattgatttcattttctcaactttcattcaagagtgtttttggttaaaacataaaaacgtccattctaattatag
taatgcttgtgtggttacgacaacatgctcattcattgtaataaactggtgaaatatatagcaccattgacaaatag
acagcctcagtaacaaagcaaggaaaaacaaaattaaaatttaataaccggttatattaaatatcgagaaccagtcc
gtagaatttcatgggaaaccgggccggtccttgatataaagaaagagagagacgtttcttaactgaagcagtacaca
aactccaaacaaatcccaagtggacaaccgaagaacccacacaaactaactctctttcctaatccacatacttgcat
ttttatatataaacactctgtccttatatagttctgtatattacatgtaaatatctctcattaatacaacctcacga
agaaaaccatttgttttcttagagagagccaagaatattaaaagagatata**gagaaaagatttgctttaat**aATGCC
AACGTCGATACAGTTTCAGAGATCCTCCACCGCCGCAGAAGCAGCCAACGCCACCGTAAGAAACTATCCCCACCACC
ATCAGAAACAGGTTCAAAAAGTGAGCCTCACGCGCGGGATGGCTGATGTGCCGGAGCACGTGGAGCTTTCCCACACG
CACGTGGTTGGTCCTTCTCAGTGCTTCTCCGTCGTGGTACAAGACGTGGAGGCTCCGGTTTCCACAGTCTGGTCGAT
CCTAAGCCGCTTCGAACACCCTCAAGCGTACAAACACTTCGTGAAAAGCTGCCACGTGGTTATCGGAGACGGTCGAG
AGGTTGGGTCGGTGAGAGAGGTCAGAGTCGTCTCTGGTCTCCCCGCGGCGTTTAGCTTAGAGCGGCTTGAGATCATG
GACGATGATCGCCACGTCATCAGTTTCAGCGTCGTTGGTGGGGACCACAGACTCATGAACTACAAGTCGGTGACGAC
GGTGCATGAGTCGGAGGAGGACTCCGACGG**C**AAGAAGAGGA**CACGTGTCGTTGAGTCATACG**TCGTTGACGTACCGG
CGGGTAACGATAAGGAAGAGACTTGTAGCTTTGCTGATACTATAGTACGGTGCAACTTGCAATCGCTGGCTAAACTC
GCCGAGAACACTTCTAAATTCTCGTAAttacatttttttcaatctttttatttttatttttatttttctatatttctctc
tttcaaaatttatctttttatttttgggattctcgaggtggttttggattttaagatttaagtatttaactatcgtcg
gggatttttcgaaactaaaacaaaaaacaagaattatatcaaacaagatggttttggttttttcgaagttagggtttt
tagggtctgttaaatgtatgtctcaacgatactttggttttaccccttaaaaccatttcttcttgtacagctctcgta
gctttattatataaacattgattgtttagtta

A5. Theoretical design of binary vector pCXSN-*HA/MYC:PYLs*

Key; ..... CaMV promoter ..... HA or Myc epitope tag; Text: *PYL* genes

a) pCXSN-*HA:PYL4*

pCXSN-*HA:PYL4*

```
acaaagggtaatatccggaaacctcctcggattccattgcccagctatctgtcactttattgtgaagata
gtggaaaaggaaggtggctcctacaaatgccatcattgcgataaaggaaaggccatcgttgaagatgcct
ctgccgacagtggtcccaaagatggacccccacccacgaggagcatcgtggaaaaagaagacgttccaac
cacgtcttcaaagcaagtggattgatgtgataacatggtggagcacgacacacttgtctactccaaaaat
atcaaagatacagtctcagaagaccaaagggcaattgagacttttcaacaaagggtaatatccggaaacc
tcctcggattccattgcccagctatctgtcactttattgtgaagatagtggaaaaggaaggtggctccta
caaatgccatcattgcgataaaggaaaggccatcgttgaagatgcctctgccgacagtggtcccaaagat
                                                          CaMV
Ggacccccacccacgaggagcatcgtggaaaaagaagacgttccaaccacgtcttcaaagcaagtggatt
gatgtgatatctccactgacgtaagggatgacgcacaatcccactatccttcgcaagacccttcctctat
ataaggaagttcatttcatttggagaggacctcgacctcaacacaacatatacaaaacaaacgaatctca
agcaatcaagcattctacttctattgcagcaatttaaatcatttcttttaaagcaaaagcaattttctga
                                                HA-tag
aaattttcaccatttacgaacgatactcgaggggatccatgtacccatacgatgttccagattacgct
         PYL4.C.START
ccaataccATGCTTGCCGTTCACCGTCCTTCTTCCGCCGTATCAGACGGAGATTCCGTTCAGATTCCGAT
                                            PYL4-tag-Up
GATGATCGCGTCGTTTCAAAAACGTTTTCCTTCTCTCTCACGCGACTCCACGGCCGCTCGTTTTCACACA
CACGAGGTTGGTCCTAATCAGTGTTGCTCCGCCGTTATTCAAGAGATCTCCGCTCCAATCTCCACCGTTT
GGTCCGTCGTACGCCGCTTTGATAACCCACAAGCTTACAAACACTTTCTCAAAAGCTGTAGCGTCATCGG
CGGAGACGGCGATAACGTTGGTAGCCTCCGTCAAGTCCACGTCGTCTCTGGTCTCCCCGCCGCTAGCTCC
ACCGAGAGACTCGATATCCTCGACGACGAACGCCACGTCATCAGCTTCAGCGTTGTTGGTGGTGAC
                                                  PYL4-tag-Down
CACCGGCTCTCTAACTACCGATCCGTAACGACCCTTCACCCTTCTCCGATCTCCGGGACCGTCGTTGTCG
AGTCTTACGTCGTTGATGTTCCTCCAGGCAACACAAAGGAAGAGACTTGTGACTTCGTTGACGTTATCGT
ACGATGCAATCTTCAATCTCTTGCGAAAATAGCCGAGAATACTGCGG
              PYL4.C.STOP
CTGAGAGCAAGAAGAAGATGTCTCTGTGAagtattggggatccactagcgaatttccccgatcgttcaaa
catttggcaataaagtttcttaagattgaatcctgttgccggtcttgcgatgattatcatataatttctg
                                                  NOS.Primer
ttgaattacgttaagcatgtaataattaacatgtaatgcatgacgttatttatgagatgggttttatga
ttagagtcccgcaattatacatttaatacgcgatagaaaacaaaatatagcgcgcaaactaggataaatt
atcgcgcgcggtgtcatctatgttactagatcggaattc
```

b) pCXSN-*HA:PYL5*

pCXSN-*HA:PYL5*

gtaacgccagggttttcccagtcacgacgttgtaaaacgacggccagtgccaagcttgcatgcctgcaggtcaacatggtggagcacgacacacttgtct
actccaaaaatatcaaagatacagtctcagaagaccaaagggcaattgagacttttcaacaaagggtaatatccggaaacctcctcggattccattgccca
gctatctgtcactttattgtgaagatagtggaaaaggaaggtggctcctacaaatgccatcattgcgataaaggaaaggccatcgttgaagatgcctctgcc
gacagtggtcccaaagatggacccccacccacgaggagcatcgtggaaaaagaagacgttccaaccacgtcttcaaagcaagtggattgatgtgataa
catggtggagcacgacacacttgtctactccaaaaatatcaaagatacagtctcagaagaccaaagggcaattgagacttttcaacaaagggtaatatccg
gaaacctcctcggattccattgcccagctatctgtcactttattgtgaagatagtggaaaaggaaggtggctcctacaaatgccatcattgcgataaaggaa
aggccatcgttgaagatgcctctgccgacagtggtcccaaagatggacccccacccacgaggagcatcgtggaaaaagaa

CaMV

Gacgttccaaccacgtcttcaaagcaagtggattgatgtgatatctccactgacgtaagggatgacgcacaatcccactatccttcgcaagacccttcctct
atataaggaagttcatttcatttggagaggacctcgacctcaacacaacatatacaaaacaaacgaatctcaagcaatcaagcattctacttctattgcagca

HA-tag

Atttaaatcatttcttttaaagcaaaagcaattttctgaaaattttcaccatttacgaacgatactcgagggggatccatgtacccatacgatgttccagattac

PYL5.C.START

gctccaatacATGAGGTCACCGGTGCAACTCCAACACGGCTCAGACGCCACTAACGGTTTCCACAC

PYL5-tag-Up

GCTGCAGCCTCACGATCAGACCGATGGTCCGATCAAGAGAGTGTGTCTCACGCGCGGTATGC
ATGTCCCTGAACACGTTGCGATGCACCACACACACGACGTTGGTCCGGACCAGTGTTGCTCC
TCGGTGGTGCAGATGATCCACGCGCCGCCTGAGTCCGTGTGGGCTCTTGTGCGGCGTTTCGA
TAATCCGAAGGTTTACAAGAACTTCATCAGACAGTGCCGTATCGTCCAAGGCGATGGACTAC
ACGTCGGCGATCTCCGGGAGGTCATGGTGGTCTCTGGACTCCCGGCGGTCTCGAGCACCGAG
AGGCTCGAGATCTTGGACGAGGAGCGTCACGTGATAAGCTTTAGTGTCGTTGGTGGGGACCA

PYL5-tag-Down

CAGGCTCAAGAACTACCGATCGGTGACGACACTACACGCGTCGGACGACGAAGGTACCGTG
GTGGGAGTCTTACATCGTTGATGTGCCGCCGGGAAACACGGAGGAGGAAACTCTAAGCTTC

PYL5.C.STOP

GTTGATACTATCGTCCGGTGCAACCTTCAGTCTCTGGCTCGAAGTACCAACCGGCAATAAagta
ttggggatccactagcgaatttccccgatcgttcaaacatttggcaataaagtttcttaagattgaatcctgttgccggtcttgcgatgattatcatataatttctg

NOS. primer

ttgaattacgttaagcatgtaataattaacatgtaatgcatgacgttatttatgagatgggttttatgattagagtcccgcaattatacatttaatacgcgataga
aaacaaaatatagcgcgcaaactaggataaattatcgcgcgcggtgtcatctatgttactagatcggaattc

c) pCXSN-*HA:PYL6*

pCXSN-*HA:PYL6*

acaaagggtaatatccggaaacctcctcggattccattgcccagctatctgtcactttattgtgaagatagtggaaaaggaaggtggctcctacaaatgcca
tcattgcgataaaggaaaggccatcgttgaagatgcctctgccgacagtggtcccaaagatggacccccacccacgaggagcatcgtggaaaaagaag
acgttccaaccacgtct**t**caaagcaagtggattgatgtgataacatggtggagcacgacacacttgtctactccaaaaatatcaaagatacagtctcagaa
gaccaaagggcaattgagacttttcaacaaagggtaatatccggaaacctcctcggattccattgcccagctatctgtcactttattgtgaagatagtggaaa
aggaaggtggctcctacaaatgccatcattgcgataaaggaaaggccatcgttgaagatgcctctgccgacagtggtcccaaagat

CaMV

Ggacccccacccacgaggagcatcgtggaaaaagaagacgttccaaccacgtcttcaaagcaagtggattgatgtgatatctccactgacgtaaggga
tgacgcacaatcccactatccttcgcaagacccttcctctatataaggaagttcatttcatttggagaggacctcgacctcaacacaacatatacaaaacaaa
cgaatctcaagcaatcaagcattctacttctattgcagcaatttaaatcatttcttttaaagcaaaagcaattttctgaaaattttcaccatttacgaacgatac

HA-tag PYL6.C.START

tcgaggggggatccatgtacccatacgatgttccagattacgctccaatacATGCCAACGTCGATACAGTTTCAGAGCCT
CCACCGCCGCAGAAGCAGCCAACGCCACCGTAAGAAACTATCCCCACCACCATCAGAAACA

PYL6-tag-UP

GGTTCAAAAAGTGAGCCTCACGCGCGGGATGGCTGATGTGCCGGAGCACGTGGAGCTTTCC
CACACGCACGTGGTTGGTCCTTCTCAGTGCTTCTCCGTCGTGGTACAAGACGTGGAGGCTCC
GGTTTCCACAGTCTGGTCGATCCTAAGCCGCTTCGAACACCCTCAAGCGTACAAACACTTCG
TGAAAAGCTGCCACGTGGTTATCGGAGACGGTCGAGAGGTTGGGTCGGTGAGAGAGGTCAG
AGTCGTCTCTGGTCTCCCCGCGGCGTTTAGCTTAGAGCGGCTTGAGATCATGGACGATGATC

PYL6-tag-Down

GCCACGTCATCAGTTTCAGCGTCGTTGGTGGGGACCACAGACTCATGAACTACAAGTCGGTG
ACGACGGTGCATGAGTCGGAGGAGGACTCCGACGGCAAGAAGAGGACACGTGTCGTTGAGT
CATACGTCGTTGACGTACCGGCGGGTAACGATAAGGAAGAGACTTGTAGCTTTGCTGATACT

PYL6.C.STOP

ATAGTACGGTGCAACTTGCAATCGCTGGCTAAACTCGCCGAGAACACTTCTAAATTCTCGTA
Aagtattggggatccactagcgaatttccccgatcgttcaaacatttggcaataaagtttcttaagattgaatcctgttgccggtcttgcgatgattatcatata

NOS.primer

atttctgttgaattacgttaagcatgtaataattaacatgtaatgcatgacgttatttatgagatgggttttatgattagagtcccgcaattatacatttaatacgc
gatagaaaacaaaatatagcgcgcaaactaggataaattatcgcgcgcggtgtcatctatgttactagatcggaattc

d) pCXSN-*Myc-PYL4*

tcaaagatacagtctcagaagaccaaagggcaattgagacttttcaacaaagggtaatatccggaaacctcctcgga
ttccattgcccagctatctgtcactttattgtgaagatagtggaaaaggaaggtggctcctacaaatgccatcattg
cgataaaggaaaggccatcgttgaagatgcctctgccgacagtggtcccaaagatggacccccacccacgaggagca
tcgtggaaaaagaagacgttccaaccacgtc**ttcaaagcaa**gtggattgatgtgataacatggtggagcacgacaca
cttgtctactccaaaaatatcaaagatacagtctcagaagaccaaagggcaattgagacttttcaacaaagggtaat
atccggaaacctcctcggattccattgcccagctatctgtcactttattgtgaagatagtggaaaaggaaggtggct
cctacaaatgccatcattgcgataaaggaaaggccatcgttgaagatgcctctgccgacagtggtcccaaagatgga

CaMV

Cccccacccacgaggagcatcgtggaaaaagaagacgttccaaccacgtc**ttcaaagcaagtgg**attgatgtgatat
ctccactgacgtaagggatgacgcacaatcccactatccttcgcaagacccttcctctatataaggaagttcatttc
atttggagaggacctcgacctcaacacaacatatacaaaacaaacgaatctcaagcaatcaagcattctacttctat
tgcagcaatttaaatcatttcttttaaagcaaaagcaattttctgaaaattttcaccatttacgaacgatactcgag

Myc-tag **PYL4.C-START**

ggggatcc**atggaacaaaagttgatttctgaagaagatct**tccaatatGCTTGCCGTTCACCGTCCTTCTTCCGCC

PYL4-Myc-Down

GTATCAGACGGAGATTCCGTTCAGATTCCGATGATGATCGCGTCGTTTCAAAAACGTTTTCCTTCTCTCTCACGCGA
CTCCACGGCCGCTCGTTTTCACACACACGAGGTTGGTCCTAATCAGTGTTGCTCCGCCGTTATTCAAGAGATCTCCG
CTCCAATCTCCACCGTTTGGTCCGTCGTACGCCGCTTTGATAACCCACAAGCTTACAAACACTTTCTCAAAAGCTGT
AGCGTCATCGGCGGAGACGGCGATAACGTTGGTAGCCTCCGTCAAGTCCACGTCGTCTCTGGTCTCCCCGCCGCTAG
CTCCACCGAGAGACTCGATATCCTCGACGACGAACGCCACGTCATCAGCTTCAGCGTTGTTGGTGGTGACCACCGGC

PYL4-Myc-UP

TCTCTAACTACCGATCCGTAACGACCCTTCACCCTTCTCCGATCTCCGGGACCGTCGTTGTCGAGTCTTACGTCGTT
GATGTTCCTCCAGGCAACACAAAGGAAGAGACTTGTGACTTCGTTGACGTTATCGTACGATGCAATCTTCAATCTCT

PYL4.C-STOP

TGCGAAAATAGCCGAGAATACTGCGGCTGAGAGCAAGAAGAAGATGTCTCTGTGAgtattggggatccactagcgaa
Tttccccgatcgttcaaacatttggcaataaagtttcttaagattgaatcctgttgccggtcttgcgatgattatca

NOS. primer

tataatttctgttgaattacgttaagcatgtaataattaacatgtaat**gcatgacgttatttatgagatggg**ttttt
atgattagagtcccgcaattatacatttaatacgcgatagaaaacaaaatatagcgcgcaaactaggataaattatc
gcgccggtgtcatctatgttactagatcggaattc

e) pCXSN-*Myc:PYL5*

gaaggtggctcctacaaatgccatcattgcgataaaggaaaggccatcgttgaagatgcctctgccgacagtggtcc
caaagatggacccccacccacgaggagcatcgtggaaaaagaagacgttccaaccacgtcttcaaagcaagtggatt
gatgtgataacatggtggagcacgacacacttgtctactccaaaaatatcaaagatacagtctcagaagaccaaagg
gcaattgagacttttcaacaaagggtaatatccggaaacctcctcggattccattgcccagctatctgtcactttat
tgtgaagatagtggaaaaggaaggtggctcctacaaatgccatcattgcgataaaggaaaggccatcgttgaagatg
cctctgccgacagtggtcccaaagatggacccccacccacgaggagcatcgtggaaaaagaagacgttccaaccacg

CaMV

Tcttcaaagcaagtggattgatgtgatatctccactgacgtaagggatgacgcacaatcccactatccttcgcaaga
cccttcctctatataaggaagttcatttcatttggagaggacctcgacctcaacacaacatatacaaaacaaacgaa
tctcaagcaatcaagcattctacttctattgcagcaatttaaatcatttcttttaaagcaaaagcaattttctgaaa

MYC-tag **PYL5-C.START**

attttcaccatttacgaagaggggatcc**atggaacaaaagttgatttctgaagaagatct**tccaatactATGAGGT
CACCGGTGCAACTCCAACACGGCTCAGACGCCACTAACGGTTTCCACACGCTGCAGCCTCACGATCAGAC

PYL5-tag-Up

CGATGGTCCGATCAAGAGAGTGTGTCTCACGCGCGGTATGCATGTCCCTGAACACGTTGCGATGCACCACACACACG
ACGTTGGTCCGGACCAGTGTTGCTCCTCGGTGGTGCAGATGATCCACGCGCCGCCTGAGTCCGTGTGGGCTCTTGTG
CGGCGTTTCGATAATCCGAAGGTTTACAAGAACTTCATCAGACAGTGCCGTATCGTCCAAGGCGATGGACTACACGT
CGGCGATCTCCGGGAGGTCATGGTGGTCTCTGGACTCCCGGCGGTCTCGAGCACCGAGAGGCTCGAGATCTTGGACG
AGGAGCGTCACGTGATAAGCTTTAGTGTCGTTGGTGGGGACCACAGGCTCAAGAACTACCGATCGGTGACGACACTA

PYL5-tag-Down

CACGCGTCGGACGACGAAGGTACCGTGGTGGGAGTCTTACATCGTTGATGTGCCGCCGGGAAACACGGAGGAGGAAA

PYL5-C.STOP

CTCTAAGCTTCGTTGATACTATCGTCCGGTGCAACCTTCAGTCTCTGGCTCGAAGTACCAACCGGCAATAAgtattg
gggatccactagcgaatttccccgatcgttcaaacatttggcaataaagtttcttaagattgaatcctgttgccggt

NOS.primer

cttgcgatgattatcatataatttctgttgaattacgttaagcatgtaataattaacatgtaatgcatgacgttatt
tatgagatgggttttttatgattagagtcccgcaattatacatttaatacgcgatagaaaacaaaatatagcgcgcaa
actaggataaattatcgcgcgcggtgtcatctatgttactagatcggaattc

f) pCXSN-*Myc:PYL6*

gaaggtggctcctacaaatgccatcattgcgataaaggaaaggccatcgttgaagatgcctctgccgacagtggtcc
caaagatggacccccacccacgaggagcatcgtggaaaaagaagacgttccaaccacgtcttcaaagcaagtggatt
gatgtgataacatggtggagcacgacacacttgtctactccaaaaatatcaaagatacagtctcagaagaccaaagg
gcaattgagacttttcaacaaagggtaatatccggaaacctcctcggattccattgcccagctatctgtcactttat
tgtgaagatagtggaaaaggaaggtggctcctacaaatgccatcattgcgataaaggaaaggccatcgttgaagatg
cctctgccgacagtggtcccaaagatggacccccacccacgaggagcatcgtggaaaaagaagacgttcca

CaMV

Accacgtc**ttcaaagcaagtgg**attgatgtgatatctccactgacgtaagggatgacgcacaatcccactatccttc
gcaagacccttcctctatataaggaagttcatttcatttggagaggacctcgacctcaacacaacatatacaaaaca
aacgaatctcaagcaatcaagcattctacttctattgcagcaatttaaatcatttcttttaaagcaaaagcaat

MYC-tag

Tttctgaaaattttcaccatttacgaacgatactcgaggggatcc**atggaacaaaagttgatttctgaagaagat**

PYL6-C.START

Cttccaatact**ATGCCAACGTCGATACAGTTTCAG**AGCCTCCACCGCCGCAGAAGCAGCCAACGCCACCGTAAGAA

PYL6-tag-Up

ACTATCCCCACCACCATCAGAAACAGGTTCAAAAAGTGAGCCTCACGCGCGGGATGGCTGATGTGCCGGAGCACGTG
GAGCTTTCCCACACGCACGTGGTTGGTCCTTCTCAGTGCTTCTCCGTCGTGGTACAAGACGTGGAGGCTCCGGTTTC
CACAGTCTGGTCGATCCTAAGCCGCTTCGAACACCCTCAAGCGTACAAACACTTCGTGAAAAGCTGCCACGTGGTTA
TCGGAGACGGTCGAGAGGTTGGGTCGGTGAGAGAGGTCAGAGTCGTCTCTGGTCTCCCCGCGGCGTTTAGCTTAGAG
CGGCTTGAGATCATGGACGATGATCGCCACGTCATCAGTTTCAGCGTCGTTGGTGGGGACCACAGACTCATG

PYL6-tag-Down

AACTACAAGTCGGTGACGACGGTGCATGAGTCGGAGGAGGACTCCGACGGCAAGAAGAGGACACGTGTCGTTGAGTC
ATACGTCGTTGACGTACCGGCGGGTAACGATAAGGAAGAGACTTGTAGCTTTGCTGATACTATAGTACGGTGCAA

PYL6-C.STOP

CTTGCAATCGCTGGCTAAACTCGCCGAGAACACTTCTAAATTCTCGTAAgtattggggatccactagcgaatttccc
cgatcgttcaaacatttggcaataaagtttcttaagattgaatcctgttgccggtcttgcgatgattatcatata

NOS.primer

atttctgttgaattacgttaagcatgtaataattaacatgtaatgcatgacgttatttatgagatgggtttttatgattagagtcccgcaattatacatttaatacgc
gatagaaaacaaaatatagcgcgcaaactaggataaattatcgcgcgcggtgtcatctatgttactagatcggaattc

A6. 35S.*MYC:PYL4* construct sequence:

a) Sequence of *MYC.PYL4*.C-START

CATAGACGGAGAT**CGTTCAGATTCCGATGATGATCGCGTCGTTTCAAAAACGTTTTCCTTCTCTCTCACGCGACTCC**
ACGGCCGCTCGTTTTCACACACACGAGGTTGGTCCTAATCAGTGTTGCTCCGCCGTTATTCAAGAGATCTCCGCTCC
AATCTCCACCGTTTGGTCCGTCGTACGCCGCTTTGATAACCCACAAGCTTACAAACACTTTCTCAAAAGCTGTAGCG
TCATCGGCGGAGACGGCGATAACGTTGGTAGCCTCCGTCAAGTCCACGTCGTCTCTGGTCTCCCCGCCGCTAGCTCC
ACCGAGAGACTCGATATCCTCGACGACGAACGCCACGTCATCAGCTTCAGCGTTGTTGGTGGTGACCACCGGCTCTC
TAACTACCGATCCGTAACGACCCTTCACCCTTCTCCGATCTCCGGGACCGTCGTTGTCGAGTCTTACGTCGTTGATG
TTCCTCCAGGCAACACAAAGGAAGAGACTTGTGACTTCGTTGACGTTATCGTACGATGCAATCTTCAATCTCTTGCG
AAAATAGCCGAGAATACTGCGGCTGAGCAAGAAGAAGATGTCTCTGTGAAGTATTGGGGATCCACTACGAATTTCCC
CGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAAT
TTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGA

>At2g38310 PYL4

ATGCTTGCCG TTCACCGTCC TTCTTCCGCC GTATCAGACG GAGATTC**CGT TCAGATTCCG ATGATGATCG**
CGTCGTTTCA AAAACGTTTT CCTTCTCTCT CACGCGACTC CACGGCCGCT CGTTTTCACA CACACGAGGT
TGGTCCTAAT CAGTGTTGCT CCGCCGTTAT TCAAGAGATC TCCGCTCCAA TCTCCACCGT TTGGTCCGTC
GTACGCCGCT TTGATAACCC ACAAGCTTAC AAACACTTTC TCAAAAGCTG TAGCGTCATC GGCGGAGACG
GCGATAACGT TGGTAGCCTC CGTCAAGTCC ACGTCGTCTC TGGTCTCCCC GCCGCTAGCT CCACCGAGAG
ACTCGATATC CTCGACGACG AACGCCACGT CATCAGCTTC AGCGTTGTTG GTGGTGACCA CCGGCTCTCT
AACTACCGAT CCGTAACGAC CCTTCACCCT TCTCCGATCT CCGGGACCGT CGTTGTCGAG TCTTACGTCG
TTGATGTTCC TCCAGGCAAC ACAAAGGAAG AGACTTGTGA CTTCGTTGAC GTTATCGTAC GATGCAATCT
TCAATCTCTT GCGAAAATAG CCGAGAATAC TGCGGCTGAG AGCAAGAAGA AGATGTCTCT GTGA

Score = 1062 bits (575), Expect = 0.0
Identities = 585/589 (99%), Gaps = 4/589 (1%)
Strand=Plus/Plus

```
Query  4   AGACGGAGA-T-CGTTCAGATTCCGATGATGATCGCGTCGTTTCAAAAACGTTTTCCTTC  61
           ||||||||| | ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36  AGACGGAGATTCCGTTCAGATTCCGATGATGATCGCGTCGTTTCAAAAACGTTTTCCTTC  95

Query  62  TCTCTCACGCGACTCCACGGCCGCTCGTTTTCACACACACGAGGTTGGTCCTAATCAGTG  121
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  96  TCTCTCACGCGACTCCACGGCCGCTCGTTTTCACACACACGAGGTTGGTCCTAATCAGTG  155

Query  122 TTGCTCCGCCGTTATTCAAGAGATCTCCGCTCCAATCTCCACCGTTTGGTCCGTCGTACG  181
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  156 TTGCTCCGCCGTTATTCAAGAGATCTCCGCTCCAATCTCCACCGTTTGGTCCGTCGTACG  215

Query  182 CCGCTTTGATAACCCACAAGCTTACAAACACTTTCTCAAAAGCTGTAGCGTCATCGGCGG  241
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  216 CCGCTTTGATAACCCACAAGCTTACAAACACTTTCTCAAAAGCTGTAGCGTCATCGGCGG  275

Query  242 AGACGGCGATAACGTTGGTAGCCTCCGTCAAGTCCACGTCGTCTCTGGTCTCCCCGCCGC  301
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276 AGACGGCGATAACGTTGGTAGCCTCCGTCAAGTCCACGTCGTCTCTGGTCTCCCCGCCGC  335

Query  302 TAGCTCCACCGAGAGACTCGATATCCTCGACGACGAACGCCACGTCATCAGCTTCAGCGT  361
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336 TAGCTCCACCGAGAGACTCGATATCCTCGACGACGAACGCCACGTCATCAGCTTCAGCGT  395

Query  362 TGTTGGTGGTGACCACCGGCTCTCTAACTACCGATCCGTAACGACCCTTCACCCTTCTCC  421
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  396 TGTTGGTGGTGACCACCGGCTCTCTAACTACCGATCCGTAACGACCCTTCACCCTTCTCC  455
```

```
Query 422 GATCTCCGGGACCGTCGTTGTCGAGTCTTACGTCGTTGATGTTCCTCCAGGCAACACAAA  481
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 456 GATCTCCGGGACCGTCGTTGTCGAGTCTTACGTCGTTGATGTTCCTCCAGGCAACACAAA  515

Query 482 GGAAGAGACTTGTGACTTCGTTGACGTTATCGTACGATGCAATCTTCAATCTCTTGCGAA  541
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 516 GGAAGAGACTTGTGACTTCGTTGACGTTATCGTACGATGCAATCTTCAATCTCTTGCGAA  575

Query 542 AATAGCCGAGAATACTGCGGCTGAG--CAAGAAGAAGATGTCTCTGTGA  588
          |||||||||||||||||||||||||  ||||||||||||||||||||||
Sbjct 576 AATAGCCGAGAATACTGCGGCTGAGAGCAAGAAGAAGATGTCTCTGTGA  624
```

Reverse Complement; *MYC.PYL4*.C-STOP

TCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCG
CAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACCTCGACCTCAACACAACATATACAAAACAA
ACGAATCTCAAGCAATCAAGCATTCTACTTCTATTGCAGCAATTTAAATCATTTCTTTTAAAGCAAAAGCAATTTTC
TGAAAATTTTCACCATTTACGAACGATACTCGAGGGGGATCCATGGAACAAAAGTTGATTTCTGAAGAAGATCTTCC
AATACTC**ATGCTTGCCGTTCACCGTCCTTCTTCCGCCGTATCAGACGGAGATTCCGTTCAGATTCCGATGATGATCG**
CGTCGTTTCAAAAACGTTTTCCTTCTCTCTCACGCGACTCCACGGCCGCTCGTTTTCACACACACGAGGTTGGTCCT
AATCAGTGTTGCTCCGCCGTTATTCAAGAGATCTCCGCTCCAATCTCCACCGTTTGGTCCGTCGTACGCCGCTTTGA
TAACCCACAAGCTTACAAACACTTTCTCAAAAGCTGTAGCGTCATCGGCGGAGACGGCGATAACGTTGGTAGCCTCC
GTCAAGTCCACGTCGTCTCTGGTCTCCCCGCCGCTAGCTCCACCGAGAGACTCGATATCCTCGACGACGAACGCCAC
GTCATCAGCTTCAGCGTTGTTGGTGGTGACCACCGGCTCTCTAACTACCGATCCGTAACGACCCTTCACCCTTCTCC
GATCTCCGGGACCGTCGTTGTCGAGTCTTACGTCGTTGATGTTCCTCCAGGCAACACAAAGGAAGAGACTTGTGACT
TCGTTGACGTTATCGTACGATGCAATCTTCAATCTCTGCGAAAAAGCCGGAGAATTCTG

>At2g38310 PYL4

ATGCTTGCCG TTCACCGTCC TTCTTCCGCC GTATCAGACG GAGATTCCGT TCAGATTCCG ATGATGATCG
CGTCGTTTCA AAAACGTTTT CCTTCTCTCT CACGCGACTC CACGGCCGCT CGTTTTCACA CACACGAGGT
TGGTCCTAAT CAGTGTTGCT CCGCCGTTAT TCAAGAGATC TCCGCTCCAA TCTCCACCGT TTGGTCCGTC
GTACGCCGCT TTGATAACCC ACAAGCTTAC AAACACTTTC TCAAAAGCTG TAGCGTCATC GGCGGAGACG
GCGATAACGT TGGTAGCCTC CGTCAAGTCC ACGTCGTCTC TGGTCTCCCC GCCGCTAGCT CCACCGAGAG
ACTCGATATC CTCGACGACG AACGCCACGT CATCAGCTTC AGCGTTGTTG GTGGTGACCA CCGGCTCTCT
AACTACCGAT CCGTAACGAC CCTTCACCCT TCTCCGATCT CCGGGACCGT CGTTGTCGAG TCTTACGTCG
TTGATGTTCC TCCAGGCAAC ACAAAGGAAG AGACTTGTGA CTTCGTTGAC GTTATCGTAC GATGCAATCT
TCAATCTCTT GCGAAAATAG CCGAGAATAC TGCGGCTGAG AGCAAGAAGA AGATGTCTCT GTGA

Score = 1070 bits (579), Expect = 0.0

```
Identities = 589/593 (99%), Gaps = 3/593 (1%)
Strand=Plus/Plus

Query  316  ATGCTTGCCGTTCACCGTCCTTCTTCCGCCGTATCAGACGGAGATTCCGTTCAGATTCCG  375
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGCTTGCCGTTCACCGTCCTTCTTCCGCCGTATCAGACGGAGATTCCGTTCAGATTCCG  60

Query  376  ATGATGATCGCGTCGTTTCAAAAACGTTTTCCTTCTCTCTCACGCGACTCCACGGCCGCT  435
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   ATGATGATCGCGTCGTTTCAAAAACGTTTTCCTTCTCTCTCACGCGACTCCACGGCCGCT  120

Query  436  CGTTTTCACACACACGAGGTTGGTCCTAATCAGTGTTGCTCCGCCGTTATTCAAGAGATC  495
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CGTTTTCACACACACGAGGTTGGTCCTAATCAGTGTTGCTCCGCCGTTATTCAAGAGATC  180

Query  496  TCCGCTCCAATCTCCACCGTTTGGTCCGTCGTACGCCGCTTTGATAACCCACAAGCTTAC  555
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TCCGCTCCAATCTCCACCGTTTGGTCCGTCGTACGCCGCTTTGATAACCCACAAGCTTAC  240
```

```
Query  556  AAACACTTTCTCAAAAGCTGTAGCGTCATCGGCGGAGACGGCGATAACGTTGGTAGCCTC  615
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AAACACTTTCTCAAAAGCTGTAGCGTCATCGGCGGAGACGGCGATAACGTTGGTAGCCTC  300

Query  616  CGTCAAGTCCACGTCGTCTCTGGTCTCCCCGCCGCTAGCTCCACCGAGAGACTCGATATC  675
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CGTCAAGTCCACGTCGTCTCTGGTCTCCCCGCCGCTAGCTCCACCGAGAGACTCGATATC  360

Query  676  CTCGACGACGAACGCCACGTCATCAGCTTCAGCGTTGTTGGTGGTGACCACCGGCTCTCT  735
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CTCGACGACGAACGCCACGTCATCAGCTTCAGCGTTGTTGGTGGTGACCACCGGCTCTCT  420

Query  736  AACTACCGATCCGTAACGACCCTTCACCCTTCTCCGATCTCCGGGACCGTCGTTGTCGAG  795
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AACTACCGATCCGTAACGACCCTTCACCCTTCTCCGATCTCCGGGACCGTCGTTGTCGAG  480

Query  796  TCTTACGTCGTTGATGTTCCTCCAGGCAACACAAAGGAAGAGACTTGTGACTTCGTTGAC  855
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  TCTTACGTCGTTGATGTTCCTCCAGGCAACACAAAGGAAGAGACTTGTGACTTCGTTGAC  540

Query  856  GTTATCGTACGATGCAATCTTCAATCTCT-GCGAAAA-AGCCGGAGAATTCTG  906
            |||||||||||||||||||||||||||| ||||||| ||||| ||||| |||
Sbjct  541  GTTATCGTACGATGCAATCTTCAATCTCTTGCGAAAATAGCCG-AGAATACTG  592
```

b) *35S.MYC.PYL4*-tag-Down

ATTTACGTCGTTGTGTTCTCCAGGCACACAAAGGAAGAGACTTGTGACTTCGTTGACGTTATCGTACGATGCAATCT
TCAATCTCTTGCGAAAATAGCCGAGAATACTGCGGCTGAGCAAGAAGAAGATGTCTCTGTGAAGTATTGGGGATCCA
CTACGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATG
ATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATG
GGA

```
Score =  515 bits (570),  Expect = 2e-150 Identities = 307/314 (98%), Gaps = 7/314
(2%)
Strand=Plus/Plus

Query  541  TTACGTCGTTGATGTTCCTCCAGGCAACACAAAGGAAGAGACTTGTGACTTCGTTGACGT  600
            |||||||||||  |||||  ||||||||  ||||||||||||||||||||||||||||||
Sbjct  3    TTACGTCGTTG-TGTTC-TCCAGGCA-CACAAAGGAAGAGACTTGTGACTTCGTTGACGT  59

Query  601  TATCGTACGATGCAATCTTCAATCTCTTGCGAAAATAGCCGAGAATACTGCGGCTGAGAG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  60   TATCGTACGATGCAATCTTCAATCTCTTGCGAAAATAGCCGAGAATACTGCGGCTGAG--  117

Query  661  CAAGAAGAAGATGTCTCTGTGA-GTATTGGGGATCCACTAGCGAATTTCCCCGATCGTTC  719
            |||||||||||||||||||||| ||||||||||||||| ||||||||||||||||||||
Sbjct  118  CAAGAAGAAGATGTCTCTGTGAAGTATTGGGGATCCACTA-CGAATTTCCCCGATCGTTC  176

Query  720  AAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTAT  779
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  177  AAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTAT  236

Query  780  CATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTT  839
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  237  CATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTT  296

Query  840  ATTTATGAGATGGG  853
            ||||||||||||||
Sbjct  297  ATTTATGAGATGGG  310
```

c) Reverse complement of *35S.MYC.PYL4*-tag-Up

TCACGTCTTCAAAGCAAGT**GGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCT**TCG
CAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACCTCGACCTCAACACAACATATACAAAACAA
ACGAATCTCAAGCAATCAAGCATTCTACTTCTATTGCAGCAATTTAAATCATTTCTTTTAAAGCAAAAGCAATTTTC

MYC-tag

TGAAAATTTTCACCATTTACGAACGATACTCGAGGGGGATCCATGGAACAAAAGTTGATTTCTGAAGAAGATCT

PYL4

TCCAATACTCATGCTTGCCGTTCACCGTCCTTCTTCCGCCGT**ATCAGACGGAGATTCCGTTCAGATTCCGATGATGA**
TCGCGTCGTTCAAAAACGTTC

```
Score = 246 bits (272), Expect = 2e-69
Identities = 145/149 (98%), Gaps = 4/149 (2%)
Strand=Plus/Plus
Query  1    CGATACTCGAGGGGGATCCATGGAACAAAAGTTGATTTCTGAAGAAGATCTTCCAATACT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  255  CGATACTCGAGGGGGATCCATGGAACAAAAGTTGATTTCTGAAGAAGATCTTCCAATACT  314

Query  61   ---GCTTGCCGTTCACCGTCCTTCTTCCGCCGTATCAGACGGAGATTCCGTTCAGATTCC  117
               |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315  CATGCTTGCCGTTCACCGTCCTTCTTCCGCCGTATCAGACGGAGATTCCGTTCAGATTCC  374

Query  118  GATGATGATCGCGTCGTTTCAAAAACGTT  146
            |||||||||||||||| ||||||||||||
Sbjct  375  GATGATGATCGCGTCG-TTCAAAAACGTT  402
```

A7. *35S.MYC.PYL5* construct sequence:

a) *MYC.PYL5*.C-START

ACCCCAAGCCATAACGGTTTCACACGCTGCAGCCTCACGATCAGACCGATGGTCCGATCAAGAGAGTGTGTCTCACG
CGCGGTATGCAT**GTCCCTGAACACGTTGCGATGCACCACACACACGACGTTGGTCCGGACCAGTGTTGCTCCTCGGT**
GGTGCAGATGATCCACGCGCCGCCTGAGTCCGTGTGGGCTCTTGTGCGGCGTTTCGATAATCCGAAGGTTTACAAGA
ACTTCATCAGACAGTGCCGTATCGTCCAAGGCGATGGACTACACGTCGGCGATCTCCGGGAGGTCATGGTGGTCTCT
GGACTCCCGGCGGTCTCGAGCACCGAGAGGCTCGAGATCTTGGACGAGGAGCGTCACGTGATAAGCTTTAGTGTCGT
TGGTGGGGACCACAGGCTCAAGAACTACCGATCGGTGACGACACTACACGCGTCGGACGACGAAGGTACCGTGGTGG
TGGAGTCTTACATCGTTGATGTGCCGCCGGGAAACACGGAGGAGGAAACTCTAAGCTTCGTTGATACTATCGTCCGG
TGCAACCTTCAGTCTCTGGCTCGAAGTACCAACCGGCAATAAAGTATTGGGGATCCACTACGAATTTCCCCGATCGT
TCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTT
GAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGA

>At5g05440 *PYL5*

ATGAGGTCAC CGGTGCAACT CCAACACGGC TCAGACGCCA CTAACGGTTT CCACACGCTG CAGCCTCACG ATCAGACCGA
TGGTCCGATC AAGAGAGTGT GTCTCACGCG CGGTATGCAT **GTCCCTGAAC ACGTTGCGAT GCACCACACA CACGACGTTG**
GTCCGGACCA GTGTTGCTCC TCGGTGGTGC AGATGATCCA CGCGCCGCCT GAGTCCGTGT GGGCTCTTGT GCGGCGTTTC
GATAATCCGA AGGTTTACAA GAACTTCATC AGACAGTGCC GTATCGTCCA AGGCGATGGA CTACACGTCG GCGATCTCCG
GGAGGTCATG GTGGTCTCTG GACTCCCGGC GGTCTCGAGC ACCGAGAGGC TCGAGATCTT GGACGAGGAG CGTCACGTGA
TAAGCTTTAG TGTCGTTGGT GGGGACCACA GGCTCAAGAA CTACCGATCG GTGACGACAC TACACGCGTC GGACGACGAA
GGTACCGTGG TGGTGGAGTC TTACATCGTT GATGTGCCGC CGGGAAACAC GGAGGAGGAA ACTCTAAGCT TCGTTGATAC
TATCGTCCGG TGCAACCTTC AGTCTCTGGC TCGAAGTACC AACCGGCAAT AA

```
Score = 1051 bits (569),  Expect = 0.0
Identities = 574/576 (99%), Gaps = 2/576 (0%)
Strand=Plus/Plus

Query  8    GCCA-TAACGGTTT-CACACGCTGCAGCCTCACGATCAGACCGATGGTCCGATCAAGAGA  65
            ||||  |||||||||  ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  37   GCCACTAACGGTTTCCACACGCTGCAGCCTCACGATCAGACCGATGGTCCGATCAAGAGA  96

Query  66   GTGTGTCTCACGCGCGGTATGCATGTCCCTGAACACGTTGCGATGCACCACACACACGAC  125
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97   GTGTGTCTCACGCGCGGTATGCATGTCCCTGAACACGTTGCGATGCACCACACACACGAC  156

Query  126  GTTGGTCCGGACCAGTGTTGCTCCTCGGTGGTGCAGATGATCCACGCGCCGCCTGAGTCC  185
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  157  GTTGGTCCGGACCAGTGTTGCTCCTCGGTGGTGCAGATGATCCACGCGCCGCCTGAGTCC  216

Query  186  GTGTGGGCTCTTGTGCGGCGTTTCGATAATCCGAAGGTTTACAAGAACTTCATCAGACAG  245
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  217  GTGTGGGCTCTTGTGCGGCGTTTCGATAATCCGAAGGTTTACAAGAACTTCATCAGACAG  276

Query  246  TGCCGTATCGTCCAAGGCGATGGACTACACGTCGGCGATCTCCGGGAGGTCATGGTGGTC  305
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  277  TGCCGTATCGTCCAAGGCGATGGACTACACGTCGGCGATCTCCGGGAGGTCATGGTGGTC  336

Query  306  TCTGGACTCCCGGCGGTCTCGAGCACCGAGAGGCTCGAGATCTTGGACGAGGAGCGTCAC  365
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  337  TCTGGACTCCCGGCGGTCTCGAGCACCGAGAGGCTCGAGATCTTGGACGAGGAGCGTCAC  396

Query  366  GTGATAAGCTTTAGTGTCGTTGGTGGGGACCACAGGCTCAAGAACTACCGATCGGTGACG  425
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  397  GTGATAAGCTTTAGTGTCGTTGGTGGGGACCACAGGCTCAAGAACTACCGATCGGTGACG  456

Query  426  ACACTACACGCGTCGGACGACGAAGGTACCGTGGTGGTGGAGTCTTACATCGTTGATGTG  485
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
Sbjct  457  ACACTACACGCGTCGGACGACGAAGGTACCGTGGTGGTGGAGTCTTACATCGTTGATGTG  516

Query  486  CCGCCGGGAAACACGGAGGAGGAAACTCTAAGCTTCGTTGATACTATCGTCCGGTGCAAC  545
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  517  CCGCCGGGAAACACGGAGGAGGAAACTCTAAGCTTCGTTGATACTATCGTCCGGTGCAAC  576

Query  546  CTTCAGTCTCTGGCTCGAAGTACCAACCGGCAATAA  581
            |||||||||||||||||||||||||||||||||||
Sbjct  577  CTTCAGTCTCTGGCTCGAAGTACCAACCGGCAATAA  612
```

b) Reverse Complement of *MYC.PYL5*.C-STOP

TCACCGTCTTTCAAAGCAAGTGGATTGATGTGATATCTCCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGAC
CCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACCTCGACCTCAACACAACATATACAAAACAAACGAATCTCAAGCAA
TCAAGCATTCTACTTCTATTGCAGCAATTTAAATCATTTCTTTTAAAGCAAAAGCAATTTTCTGAAAATTTTCACCATTTACGAAC
GATACTCGAGGGGGATCC**ATGGAACAAAAGTTGATTTCTGAAGAAGATCT**TCCAATACTC**ATGAGGTCACCGGTGCAACTCCAACA**
CGGCTCAGACGCCACTAACGGTTTCCACACGCTGCAGCCTCACGATCAGACCGATGGTCCGATCAAGAGAGTGTGTCTCACGCGCG
GTATGCATGTCCCTGAACACGTTGCGATGCACCACACACACGACGTTGGTCCGGACCAGTGTTGCTCCTCGGTGGTGCAGATGATC
CACGCGCCGCCTGAGTCCGTGTGGGCTCTTGTGCGGCGTTTCGATAATCCGAAGGTTTACAAGAACTTCATCAGACAGTGCCGTAT
CGTCCAAGGCGATGGACTACACGTCGGCGATCTCCGGGAGGTCATGGTGGTCTCTGGACTCCCGGCGGTCTCGAGCACCGAGAGGC
TCGAGATCTTGGACGAGGAGCGTCACGTGATAAGCTTTAGTGTCGTTGGTGGGGACCACAGGCTCAAGAACTACCGATCGGTGACG
ACACTACACGCGTCGGACGACGAAGGTACCGTGGTGGTGGAGTCTTACATCGTTGATGTGCCGCCGGGAAACACGGAGGAGGAAAC
TCTAAGCTTCGTGATACTATCGTCCGGCACCTTT

>At5g05440 *PYL5*

ATGAGGTCAC CGGTGCAACT CCAACACGGC TCAGACGCCA CTAACGGTTT CCACACGCTG CAGCCTCACG
ATCAGACCGA TGGTCCGATC AAGAGAGTGT GTCTCACGCG CGGTATGCAT GTCCCTGAAC ACGTTGCGAT
GCACCACACA CACGACGTTG GTCCGGACCA GTGTTGCTCC TCGGTGGTGC AGATGATCCA CGCGCCGCCT
GAGTCCGTGT GGGCTCTTGT GCGGCGTTTC GATAATCCGA AGGTTTACAA GAACTTCATC AGACAGTGCC
GTATCGTCCA AGGCGATGGA CTACACGTCG GCGATCTCCG GGAGGTCATG GTGGTCTCTG GACTCCCGGC
GGTCTCGAGC ACCGAGAGGC TCGAGATCTT GGACGAGGAG CGTCACGTGA TAAGCTTTAG TGTCGTTGGT
GGGGACCACA GGCTCAAGAA CTACCGATCG GTGACGACAC TACACGCGTC GGACGACGAA GGTACCGTGG
TGGTGGAGTC TTACATCGTT GATGTGCCGC CGGGAAACAC GGAGGAGGAA ACTCTAAGCT TCGTTGATAC
TATCGTCCGG TGCAACCTTC AGTCTCTGGC TCGAAGTACC AACCGGCAAT AA

```
 Score = 1046 bits (566),  Expect = 0.0
 Identities = 569/570 (99%), Gaps = 1/570 (0%)
 Strand=Plus/Plus

Query  319  ATGAGGTCACCGGTGCAACTCCAACACGGCTCAGACGCCACTAACGGTTTCCACACGCTG  378
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGAGGTCACCGGTGCAACTCCAACACGGCTCAGACGCCACTAACGGTTTCCACACGCTG  60

Query  379  CAGCCTCACGATCAGACCGATGGTCCGATCAAGAGAGTGTGTCTCACGCGCGGTATGCAT  438
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CAGCCTCACGATCAGACCGATGGTCCGATCAAGAGAGTGTGTCTCACGCGCGGTATGCAT  120

Query  439  GTCCCTGAACACGTTGCGATGCACCACACACACGACGTTGGTCCGGACCAGTGTTGCTCC  498
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GTCCCTGAACACGTTGCGATGCACCACACACACGACGTTGGTCCGGACCAGTGTTGCTCC  180

Query  499  TCGGTGGTGCAGATGATCCACGCGCCGCCTGAGTCCGTGTGGGCTCTTGTGCGGCGTTTC  558
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TCGGTGGTGCAGATGATCCACGCGCCGCCTGAGTCCGTGTGGGCTCTTGTGCGGCGTTTC  240
```

```
Query  559  GATAATCCGAAGGTTTACAAGAACTTCATCAGACAGTGCCGTATCGTCCAAGGCGATGGA  618
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  GATAATCCGAAGGTTTACAAGAACTTCATCAGACAGTGCCGTATCGTCCAAGGCGATGGA  300

Query  619  CTACACGTCGGCGATCTCCGGGAGGTCATGGTGGTCTCTGGACTCCCGGCGGTCTCGAGC  678
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CTACACGTCGGCGATCTCCGGGAGGTCATGGTGGTCTCTGGACTCCCGGCGGTCTCGAGC  360

Query  679  ACCGAGAGGCTCGAGATCTTGGACGAGGAGCGTCACGTGATAAGCTTTAGTGTCGTTGGT  738
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  ACCGAGAGGCTCGAGATCTTGGACGAGGAGCGTCACGTGATAAGCTTTAGTGTCGTTGGT  420

Query  739  GGGGACCACAGGCTCAAGAACTACCGATCGGTGACGACACTACACGCGTCGGACGACGAA  798
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GGGGACCACAGGCTCAAGAACTACCGATCGGTGACGACACTACACGCGTCGGACGACGAA  480

Query  799  GGTACCGTGGTGGTGGAGTCTTACATCGTTGATGTGCCGCCGGGAAACACGGAGGAGGAA  858
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GGTACCGTGGTGGTGGAGTCTTACATCGTTGATGTGCCGCCGGGAAACACGGAGGAGGAA  540

Query  859  ACTCTAAGCTTCGT-GATACTATCGTCCGG  887
            |||||||||||||| ||||||||||||||
Sbjct  541  ACTCTAAGCTTCGTTGATACTATCGTCCGG  570
```

c) *35S.MYC.PYL5*-tag-Down

TGGGGTTTCTCGTTGTGGCCGCCGGGAACACGGAGGAGGAACTCTAAGCTTCGTTGATACTATCGTCCGGTGCAACC
TTCAGTCTCTGGCTCGAAGTACCAACCGGCAATAAAGTATTGGGGATCCACTACGAATTTCCCCGATCGTTCAAACA
TTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTAC
GTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGG

```
Score =  360 bits (398),  Expect = 8e-104
 Identities = 220/226 (98%), Gaps = 6/226 (2%)
 Strand=Plus/Plus

Query  556  TCGTTGATGTGCCGCCGGGAAACACGGAGGAGGAAACTCTAAGCTTCGTTGATACTATCG 615
            |||||| || |||||||||| |||||||||||| ||||||||||||||||||||||||
Sbjct  10   TCGTTG-TG-GCCGCCGGGAA-CACGGAGGAGGAA-CTCTAAGCTTCGTTGATACTATCG  65

Query  616  TCCGGTGCAACCTTCAGTCTCTGGCTCGAAGTACCAACCGGCAATAA-GTATTGGGGATC 674
            |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  66   TCCGGTGCAACCTTCAGTCTCTGGCTCGAAGTACCAACCGGCAATAAAGTATTGGGGATC 125

Query  675  CACTAGCGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATC 734
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  126  CACTA-CGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATC 184

Query  735  CTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTA  780
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  185  CTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTA  230
```

d) Rev. complement. *35S.MYC.PYL5*-tag-Up

TCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCG
CAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACCTCGACCTCAACACAACATATACAAAACAA
ACGAATCTCAAGCAATCAAGCATTCTACTTCTATTGCAGCAATTTAAATCATTTCTTTTAAAGCAAAAGCAATTTTC
TGAAAATTTTCACCATTTACGAACGATACTCGAGGGGGATCCATGGAACAAAAGTTGATTTCTGAAGAAGATCTTCC

AATACTCATGAGGTCACCGGTGCAACTCCAACACGGCTCAGACGCCACTAACGGTTCCACACGCTGCAGCCTCACGA
TCAGACCGATCCA

Score = 226 bits (250), Expect = 2e-63
Identities = 132/134 (99%), Gaps = 2/134 (1%)
Strand=Plus/Plus

```
                                MYC-tag
Query  1    GAGGGGGATCCATGGAACAAAAGTTGATTTCTGAAGAAGATCTTCCAATACT-ATGAGGT  59
            ||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  263  GAGGGGGATCCATGGAACAAAAGTTGATTTCTGAAGAAGATCTTCCAATACTCATGAGGT 322

Query  60   CACCGGTGCAACTCCAACACGGCTCAGACGCCACTAACGGTTTCCACACGCTGCAGCCTC 119
            |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  323  CACCGGTGCAACTCCAACACGGCTCAGACGCCACTAACGG-TTCCACACGCTGCAGCCTC 381

Query  120  ACGATCAGACCGAT  133
            ||||||||||||||
Sbjct  382  ACGATCAGACCGAT  395
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A8. *35S.MYC.PYL6* construct sequence:

MYC.PYL6.C-START
CCTCCCGGCCGCAGAGCAGCCACGCCACCGTAAG**AACTATCCCCACCACCATCAGAAACAGGTTCAAA**
AAGTGAGCCTCACGCGCGGGATGGCTGATGTGCCGGAGCACGTGGAGCTTTCCCACACGCACGTGGTT
GGTCCTTCTCAGTGCTTCTCCGTCGTGGTACAAGATGTGGAGGCTCCGGTTTCCACAGTCTGGTCGAT
CCTAAGCCGCTTCGAACACCCTCAAGCGTACAAACACTTCGTGAAAAGCTGCCACGTGGTTATCGGAG
ACGGTCGAGAGGTTGGGTCGGTGAGAGAGGTCAGAGTCGTCTCTGGTCTCCCCGCGGCGTTTAGCTTA
GAGCGGCTTGAGATCATGGACGATGATCGCCACGTCATCAGTTTCAGCGTCGTTGGTGGGGACCACAG
ACTCATGAACTACAAGTCGGTGACGACGGTGCATGAGTCGGAGGAGGACTCCGACGGCAAGAAGAGGA
CACGTGTCGTTGAGTCATACGTCGTTGACGTACCGGCGGGTAACGATAAGGAAGAGACTTGTAGCTTT
GCTGATACTATAGTACGGTGCAACTTGCAATCGCTGGCTAAAACTCGCCGAGAACACTTCTAAATTCT
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AA

>At2g40330 *PYL6*

ATGCCAACGT CGATACAGTT TCAGAGATCC TCCACCGCCG CAGAAGCAGC CAACGCCACC GTAAGA**AACT**
ATCCCCACCA CCATCAGAAA CAGGTTCAAA AAGTGAGCCT CACGCGCGGG ATGGCTGATG TGCCGGAGCA
CGTGGAGCTT TCCCACACGC ACGTGGTTGG TCCTTCTCAG TGCTTCTCCG TCGTGGTACA AGACGTGGAG
GCTCCGGTTT CCACAGTCTG GTCGATCCTA AGCCGCTTCG AACACCCTCA AGCGTACAAA CACTTCGTGA
AAAGCTGCCA CGTGGTTATC GGAGACGGTC GAGAGGTTGG GTCGGTGAGA GAGGTCAGAG TCGTCTCTGG
TCTCCCCGCG GCGTTTAGCT TAGAGCGGCT TGAGATCATG GACGATGATC GCCACGTCAT CAGTTTCAGC
GTCGTTGGTG GGGACCACAG ACTCATGAAC TACAAGTCGG TGACGACGGT GCATGAGTCG GAGGAGGACT
CCGACGGCAA GAAGAGGACA CGTGTCGTTG AGTCATACGT CGTTGACGTA CCGGCGGGTA ACGATAAGGA
AGAGACTTGT AGCTTTGCTG ATACTATAGT ACGGTGCAAC TTGCAATCGC TGGCTAAACT CGCCGAGAAC
ACTTCTAAAT TCTCGTAA

Score = 1103 bits (597), Expect = 0.0
Identities = 614/621 (99%), Gaps = 5/621 (1%)
Strand=Plus/Plus

```
Query  1    CCTCC-CGGCCGCAG-AGCAGCC-ACGCCACCGTAAG-AACTATCCCCACCACCATCAGA  56
            ||||| | ||||||| ||||||| |||||||||||||| ||||||||||||||||||||||
Sbjct  29   CCTCCACCGCCGCAGAAGCAGCCAACGCCACCGTAAGAAACTATCCCCACCACCATCAGA  88
```

```
Query  57   AACAGGTTCAAAAAGTGAGCCTCACGCGCGGGATGGCTGATGTGCCGGAGCACGTGGAGC 116
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  89   AACAGGTTCAAAAAGTGAGCCTCACGCGCGGGATGGCTGATGTGCCGGAGCACGTGGAGC 148

Query  117  TTTCCCACACGCACGTGGTTGGTCCTTCTCAGTGCTTCTCCGTCGTGGTACAAGATGTGG 176
            |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  149  TTTCCCACACGCACGTGGTTGGTCCTTCTCAGTGCTTCTCCGTCGTGGTACAAGACGTGG 208

Query  177  AGGCTCCGGTTTCCACAGTCTGGTCGATCCTAAGCCGCTTCGAACACCCTCAAGCGTACA 236
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  209  AGGCTCCGGTTTCCACAGTCTGGTCGATCCTAAGCCGCTTCGAACACCCTCAAGCGTACA 268

Query  237  AACACTTCGTGAAAAGCTGCCACGTGGTTATCGGAGACGGTCGAGAGGTTGGGTCGGTGA 296
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  269  AACACTTCGTGAAAAGCTGCCACGTGGTTATCGGAGACGGTCGAGAGGTTGGGTCGGTGA 328

Query  297  GAGAGGTCAGAGTCGTCTCTGGTCTCCCCGCGGCGTTTAGCTTAGAGCGGCTTGAGATCA 356
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  329  GAGAGGTCAGAGTCGTCTCTGGTCTCCCCGCGGCGTTTAGCTTAGAGCGGCTTGAGATCA 388

Query  357  TGGACGATGATCGCCACGTCATCAGTTTCAGCGTCGTTGGTGGGGACCACAGACTCATGA 416
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  389  TGGACGATGATCGCCACGTCATCAGTTTCAGCGTCGTTGGTGGGGACCACAGACTCATGA 448

Query  417  ACTACAAGTCGGTGACGACGGTGCATGAGTCGGAGGAGGACTCCGACGGCAAGAAGAGGA 476
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  449  ACTACAAGTCGGTGACGACGGTGCATGAGTCGGAGGAGGACTCCGACGGCAAGAAGAGGA 508

Query  477  CACGTGTCGTTGAGTCATACGTCGTTGACGTACCGGCGGGTAACGATAAGGAAGAGACTT 536
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  509  CACGTGTCGTTGAGTCATACGTCGTTGACGTACCGGCGGGTAACGATAAGGAAGAGACTT 568

Query  537  GTAGCTTTGCTGATACTATAGTACGGTGCAACTTGCAATCGCTGGCTAAAACTCGCCGAG 596
            |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  569  GTAGCTTTGCTGATACTATAGTACGGTGCAACTTGCAATCGCTGGCTAAA-CTCGCCGAG 627

Query  597  AACACTTCTAAATTCTCGTAA  617
            ||||||||||||||||||||
Sbjct  628  AACACTTCTAAATTCTCGTAA  648
```

a) Reverse Complement; *MYC.PYL6*.C-STOP

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CAATACTC**ATGCCAACGTCGATACAGTTTCAGAGATCCTCCACCGCCGCAGAAGCAGCCAACGCCACC**
GTAAGAAACTATCCCCACCACCATCAGAAACAGGTTCAAAAAGTGAGCCTCACGCGCGGGATGGCTGA
TGTGCCGGAGCACGTGGAGCTTTCCCACACGCACGTGGTTGGTCCTTCTCAGTGCTTCTCCGTCGTGG
TACAAGATGTGGAGGCTCCGGTTTCCACAGTCTGGTCGATCCTAAGCCGCTTCGAACACCCTCAAGCG
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GTGCATGAGTCGGAGGAGGACTCCGACGGCAAGAAGAGGACACGTGTCGTTGAGTCATACGTCGTTGA

CGTACCGGCGGGTAACGATAAGGAAGAGACTTGTAGCTTTGCTGATACTATAGTACGGTGCAACTGCA
TCGCCGGCAAGTT

>At2g40330 *PYL6*

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GTAAGAAACT ATCCCCACCA CCATCAGAAA CAGGTTCAAA AAGTGAGCCT CACGCGCGGG
ATGGCTGATG TGCCGGAGCA CGTGGAGCTT TCCCACACGC ACGTGGTTGG TCCTTCTCAG
TGCTTCTCCG TCGTGGTACA AGACGTGGAG GCTCCGGTTT CCACAGTCTG GTCGATCCTA
AGCCGCTTCG AACACCCTCA AGCGTACAAA CACTTCGTGA AAAGCTGCCA CGTGGTTATC
GGAGACGGTC GAGAGGTTGG GTCGGTGAGA GAGGTCAGAG TCGTCTCTGG TCTCCCCGCG
GCGTTTAGCT TAGAGCGGCT TGAGATCATG GACGATGATC GCCACGTCAT CAGTTTCAGC
GTCGTTGGTG GGGACCACAG ACTCATGAAC TACAAGTCGG TGACGACGGT GCATGAGTCG
GAGGAGGACT CCGACGGCAA GAAGAGGACA CGTGTCGTTG AGTCATACGT CGTTGACGTA
CCGGCGGGTA ACGATAAGGA AGAGACTTGT AGCTTTGCTG ATACTATAGT ACGGTGCAAC
TTGCAATCGC TGGCTAAACT CGCCGAGAAC ACTTCTAAAT TCTCGTAA

Score = 1110 bits (601), Expect = 0.0
Identities = 610/614 (99%), Gaps = 2/614 (0%)
Strand=Plus/Plus

```
Query  145  ATGCCAACGTCGATACAGTTTCAGAGATCCTCCACCGCCGCAGAAGCAGCCAACGCCACC 204
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGCCAACGTCGATACAGTTTCAGAGATCCTCCACCGCCGCAGAAGCAGCCAACGCCACC  60

Query  205  GTAAGAAACTATCCCCACCACCATCAGAAACAGGTTCAAAAAGTGAGCCTCACGCGCGGG 264
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GTAAGAAACTATCCCCACCACCATCAGAAACAGGTTCAAAAAGTGAGCCTCACGCGCGGG 120

Query  265  ATGGCTGATGTGCCGGAGCACGTGGAGCTTTCCCACACGCACGTGGTTGGTCCTTCTCAG 324
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  ATGGCTGATGTGCCGGAGCACGTGGAGCTTTCCCACACGCACGTGGTTGGTCCTTCTCAG  180

Query  325  TGCTTCTCCGTCGTGGTACAAGATGTGGAGGCTCCGGTTTCCACAGTCTGGTCGATCCTA  384
            |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  181  TGCTTCTCCGTCGTGGTACAAGACGTGGAGGCTCCGGTTTCCACAGTCTGGTCGATCCTA  240

Query  385  AGCCGCTTCGAACACCCTCAAGCGTACAAACACTTCGTGAAAAGCTGCCACGTGGTTATC  444
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AGCCGCTTCGAACACCCTCAAGCGTACAAACACTTCGTGAAAAGCTGCCACGTGGTTATC  300

Query  445  GGAGACGGTCGAGAGGTTGGGTCGGTGAGAGAGGTCAGAGTCGTCTCTGGTCTCCCCGCG  504
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GGAGACGGTCGAGAGGTTGGGTCGGTGAGAGAGGTCAGAGTCGTCTCTGGTCTCCCCGCG  360

Query  505  GCGTTTAGCTTAGAGCGGCTTGAGATCATGGACGATGATCGCCACGTCATCAGTTTCAGC  564
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GCGTTTAGCTTAGAGCGGCTTGAGATCATGGACGATGATCGCCACGTCATCAGTTTCAGC  420

Query  565  GTCGTTGGTGGGGACCACAGACTCATGAACTACAAGTCGGTGACGACGGTGCATGAGTCG  624
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GTCGTTGGTGGGGACCACAGACTCATGAACTACAAGTCGGTGACGACGGTGCATGAGTCG  480
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Query  625  GAGGAGGACTCCGACGGCAAGAAGAGGACACGTGTCGTTGAGTCATACGTCGTTGACGTA  684
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GAGGAGGACTCCGACGGCAAGAAGAGGACACGTGTCGTTGAGTCATACGTCGTTGACGTA  540

Query  685  CCGGCGGGTAACGATAAGGAAGAGACTTGTAGCTTTGCTGATACTATAGTACGGTGCAAC  744
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  CCGGCGGGTAACGATAAGGAAGAGACTTGTAGCTTTGCTGATACTATAGTACGGTGCAAC  600

Query  745  T-GCA-TCGCCGGC  756
            | ||| |||| |||
Sbjct  601  TTGCAATCGCTGGC  614
```

b) *35S.MYC.PYL6*-tag-Down

GGCCTCGACGCAGAGAGAACGTGTCGTTGAGTCATACGTCGTTGACGTACCGGCGGGTAACGATAAGGAAGAGACTT
GTAGCTTTGCTGATACTATAGTACGGTGCAACTTGCAATCGCTGGCTAAACTCGCCGAGAACACTTCTAAATTCTCG
TAAAGTATTGGGGATCCACTACGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCT
GTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCAT
GACGTTATTTATGAGATGGG

```
Score =  545 bits (604),  Expect = 1e-159
 Identities = 309/311 (99%), Gaps = 2/311 (0%)
 Strand=Plus/Plus

Query  568  ACGTGTCGTTGAGTCATACGTCGTTGACGTACCGGCGGGTAACGATAAGGAAGAGACTTG  627
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19   ACGTGTCGTTGAGTCATACGTCGTTGACGTACCGGCGGGTAACGATAAGGAAGAGACTTG  78

Query  628  TAGCTTTGCTGATACTATAGTACGGTGCAACTTGCAATCGCTGGCTAAACTCGCCGAGAA  687
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79   TAGCTTTGCTGATACTATAGTACGGTGCAACTTGCAATCGCTGGCTAAACTCGCCGAGAA  138

Query  688  CACTTCTAAATTCTCGTAA-GTATTGGGGATCCACTAGCGAATTTCCCCGATCGTTCAAA  746
            |||||||||||||||||| |||||||||||||||| ||||||||||||||||||||||||
Sbjct  139  CACTTCTAAATTCTCGTAAAGTATTGGGGATCCACTA-CGAATTTCCCCGATCGTTCAAA  197

Query  747  CATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCAT  806
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  198  CATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCAT  257

Query  807  ATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATT  866
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  258  ATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATT  317

Query  867  TATGAGATGGG  877
            ||||||||||
Sbjct  318  TATGAGATGGG  328
```

c) Reverse complement of *35S.MYC.PYL6*-tag-Up

TCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCG
CAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACCTCGACCTCAACACAACATATACAAAACAA
ACGAATCTCAAGCAATCAAGCATTCTACTTCTATTGCAGCAATTTAAATCATTTCTTTTAAAGCAAAAGCAATTTTC
TGAAAATTTTCACCATTTACGAACGATACTCGAGGGGGATCCATGGAACAAAAGTTGATTTCTGAAGAAGATCTTCC

AATACTCATGCCAACGTCGATACAGTTTCAGAGATCCTCCACCGCCGCAGAAGCAGCCAACGCCACCGTAAGAAACT
ATCCCCACCACCATCAGAAACAGGTCAAAAAGTAGCTCCCGCA

Score = 269 bits (298), Expect = 2e-76
Identities = 161/165 (98%), Gaps = 4/165 (2%)
Strand=Plus/Plus

```
Query  1    CGATACTCGAGGGGGATCCATGGAACAAAAGTTGATTTCTGAAGAAGATCTTCCAATACT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  255  CGATACTCGAGGGGGATCCATGGAACAAAAGTTGATTTCTGAAGAAGATCTTCCAATACT  314

Query  61   -ATGCCAACGTCGATACAGTTTCAGAG--CCTCCACCGCCGCAGAAGCAGCCAACGCCAC  117
             |||||||||||||||||||||||||  ||||||||||||||||||||||||||||||
Sbjct  315  CATGCCAACGTCGATACAGTTTCAGAGATCCTCCACCGCCGCAGAAGCAGCCAACGCCAC  374

Query  118  CGTAAGAAACTATCCCCACCACCATCAGAAACAGGTTCAAAAAGT  162
             |||||||||||||||||||||||||||||||||| |||||||||
Sbjct  375  CGTAAGAAACTATCCCCACCACCATCAGAAACAGG-TCAAAAAGT  418
```

A9. *PYL4*: At2g38310

PYR1-LIKE4 (RCAR10); ATGC = Exon, atgc = Intron

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tac

PYL4-C.START: 5′ CATGCTTGCCGTTCACCGTCCTTC 3′
PYL4-STOP: TCACAGAGACATCTTCTTCTTGCTCAG
PYL4.KO.FP: TTCCAATCGTTCCAAATATCG
PYL4.KO.RP: TAAGACTCGACAACGACGGTC
PYL4-Standard-F: 5′ GTTATTCAAGAGATCTCCGCTCC
PYL4-Standard-R: 5′ GCTCTCAGCCGCAGTATTCTC

A10. *PYL5:* At5g05440

PYR1-LIKE5 (RCAR8); ATGC = Exon, atgc = UTR, atgc = Intron

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PYL5.KO.FP: 5` AAACACAAAGCCTTCACATCC-3`
PYL5.KO.RP: 5` AAGTTTTGTGAATCCCCCAAC-3`
PYL5-RT- F: 5` GGCGATGGACTACACGTCGGCGATC-3`
PYL5-RT- R: 5` CACCACCACGGTACCTTCGTCGTCC-3`
PYL5-Standard-F: 5′ GGTGGTGCAGATGATCCACG-3`
PYL5-Standard-R: 5′ TGCCGGTTGGTACTTCGAGC-3`

A11. *PYL6*: At2g40330

PYR1-LIKE6 (RCAR9); ATGC = Exon, atgc = Intron

cgtatttatgtaaagtttgttgatgaaatgacaaataatttgaaaattttacgtttccaaattagggaattaaagatataatgctgtattacctgacaccatttatttt
ggcctagcattatatatgccataaagaaaattcttaagattatatcacataactcaatacatgcattacgccttggttcaagtttaattatcactaattgatattatt
ccatctttgcttttaggattgaacgctttgattatatagctggtgagcaaattcatttaaaacaccaagtagtattctactgtttatacatttcaataaaattcaaatg
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atagtagatatgccaatgattatattaatcaaattgtttgtgtaatacattattatgtaggttatcaaaattaatgaaattagacataataatatcggaaatatcgta
acaatataccccatgcattatagctgaatcactatatagtctatattgtttcatgattaactaaattaaaagacaaataataaatatgtaaaatacggcgcaaga
agaagattatgaaaataactaaacaatcaatgtttatataataaagctacgagagctgtacaagaagaaatggttttaagggtaaaaccaaagtatcgttgag
acatacatttaacagaccctaaaaaccctaacttcgaaaaaccaaaaccatcttgtttgatataattcttgtttttgttttagtttcgaaaaatccccgacgatag
ttaaatacttaaatcttaaaatccaaaaccacctcgagaatcccaaaaataaaagataaattttgaaagagagaaatatagaaaataaaataaaaataaaaa
gattgaaaaaatgtaaCGAGAATTTAGAAGTGTTCTCGGCGAGTTTAGCCAGCGATTGCAAGTTGCAC
CGTACTATAGTATCAGCAAAGCTACAAGTCTCTTCCTTATCGTTACCCGCCGGTACGTCAAC
GACGTATGACTCAACGACACGTGTCCTCTTCTTGCCGTCGGAGTCCTCCTCCGACTCATGCAC
CGTCGTCACCGACTTGTAGTTCATGAGTCTGTGGTCCCCACCAACGACGCTGAAACTGATGA
CGTGGCGATCATCGTCCATGATCTCAAGCCGCTCTAAGCTAAACGCCGCGGGGAGACCAGA
GACGACTCTGACCTCTCTCACCGACCCAACCTCTCGACCGTCTCCGATAACCACGTGGCAGC
TTTTCACGAAGTGTTTGTACGCTTGAGGGTGTTCGAAGCGGCTTAGGATCGACCAGACTGTG
GAAACCGGAGCCTCCACGTCTTGTACCACGACGGAGAAGCACTGAGAAGGACCAACCACGT
GCGTGTGGGAAAGCTCCACGTGCTCCGGCACATCAGCCATCCCGCGCGTGAGGCTCACTTTT
TGAACCTGTTTCTGATGGTGGTGGGGATAGTTTCTTACGGTGGCGTTGGCTGCTTCTGCGGCG
GTGGAGGATCTCTGAAACTGTATCGACGTTGGtattaaagcaaatcttttctctatatctcttttaatattcttggctctctctaag
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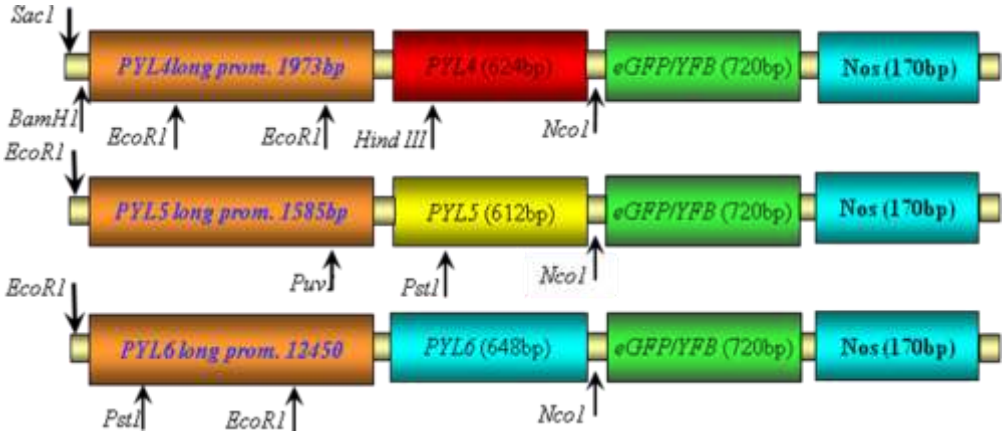
attataagaacatgatcactatgtgtcgagttggttaagctatatatattggttaagagctatacttaatttatgcataggtttgaaaaaattcttgaaacaaattg
gttgggaggaccgacctttgtctttgtgattgccgatctattttcagttggtactattcttggatgcg

PYL6.KO.FP: 5` GCCTCGAGACAGTAGAAGATTG- 3′
PYL6.KO.RP: 5` CGTATGACTCAACGACACGTG- 3′
PYL6-C.START: 5′ CATGCCAACGTCGATACAGTTTCAG- 3′
PYL6-STOP: 5′ TTACGAGAATTTAGAAGTGTTCTCG- 3′

A12. Digestion scheme shows restriction sites on C1*eGFP/YFP:PYL4, 5* & *6* plasmids; the scheme shows the digestion of C1eGFP or C1YFP/PYL4, 5 & 6 cut with either Sac1 and Nco1 for PYL4 or cut with EcoR1 and Nco1 for PYL5, PYL6; Orange colour indicates PYL promoters, Red colour indicates *PYL* genes, Green colour indicates GFP/YFP proteins and Turquoise colour indicates Nos; colour indicates Nos (nopaline synthase) 3 UTR (poly A signal).



A13. Sequence of C1*GFP:PYLs*;

a) C1eGFP*:PYL4*

C1eGFP:PYL4, RCAR10 | PYR1-like 4

```
                Mutation 1
Query: 301 cgtcaagtccacatcgtctctggtctccccgccgctagctccaccgagagactcgatatc 360
           |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 489 cgtcaagtccacgtcgtctctggtctccccgccgctagctccaccgagagactcgatatc 548

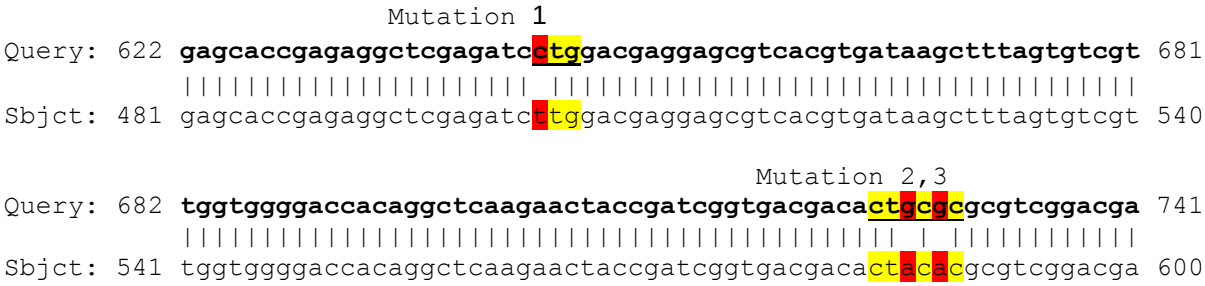
Query: 361 ctcgacgacgaacgccacgtcatcagcttcagcgttgttggtggtgaccaccggctctct 420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 549 ctcgacgacgaacgccacgtcatcagcttcagcgttgttggtggtgaccaccggctctct 608

Query: 421 aactaccgatccgtaacgacccttcacccttctccgatctccgggaccgtcgttgtcgag 480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 609 aactaccgatccgtaacgacccttcacccttctccgatctccgggaccgtcgttgtcgag 668

Query: 481 tcttacgtcgttgatgttcctccaggcaacacaaaggaagagacttgtgacttcgttgac 540
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 669 tcttacgtcgttgatgttcctccaggcaacacaaaggaagagacttgtgacttcgttgac 728
                                       Mutation 2
Query: 541 gttatcgtacgatgcaatcttcaatctcttgcgaaaacagccgagaatactgcggctgag 600
           |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct: 729 gttatcgtacgatgcaatcttcaatctcttgcgaaaatagccgagaatactgcggctgag 788
```

b) *C1eGFP:PYL5*

C1eGFP:PYL5, RCAR8 | Polyketide cyclase/dehydrase and lipid transport superfamily protein



A14. Reagent Preparation and Stock Solutions for protein separation

a) Resolving and Stacking Gels:

	Reagent / Gel / %		30% Acrylamide	Tris-HCl*	10% SDS*	10% APS*	TEMED*	dH_2O*
1	Resolving	12%	4 ml	2.5 ml (1.5 M, pH 8.8)	100 µl	50 µl	5 µl	3.4 ml
2	Stacking	4%	1.5 ml	1.25 ml (0.5 M, pH 6.8)	100 µl	50 µl	10 µl	7.25 ml

* see abbreviation for full names.

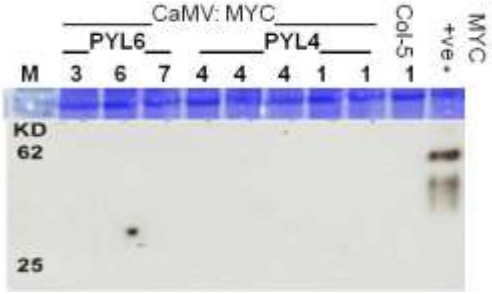
b) For 1.5Molar (M) of Tris-HCL, pH 8.8; 27.22g of Tris base was dissolved in 100 ml of deionised water (dH_2O), pH was adjusted to 8.8 with HCl. The volume was completed to 150 ml with dH_2O. Stock solution was stored at 4 °C.

c) For 0.5M of Tris-HCL, pH 6.8; 9g of Tris base was dissolved in 100 ml of dH_2O, pH was adjusted to 6.8 with HCl. The volume was completed to 150 ml with dH_2O. Stock solution was stored at 4 °C.

d) 10x electrode (Running) buffer, pH 8.3; 30.3 g of Tris base, 144.0 g of Glycine and 10.0 g of SDS were dissolved in 800 ml dH_2O. The volume was made up to 1,000 ml dH_2O and stored at 4 °C.

A15. Arabidopsis CaMV::MYC:PYL4, PYL5 and PYL6 transformed plants challenged with DC3000 3dpi (A and B) two lines of CaMV:*MYC:PYL4.1 &.2*, (C) CaMV:*MYC*:*PYL6* and (D) Col.5

A16. Immounoblot analysis for CaMV: MYC: *PYL4* and *PYL*6 individual lines for expression level M: Prestained Protein Marker "BioLabs" 6.5-175Kd, (+ve) positive control; Invitrogen Positope Antibody control protein (~62Kd). Expected bands for *MYC: PYL4* & *PYL6* = ~23.6 and ~25Kd respectively.



A17. Websites used in the project;

http://www.invitrogen.com/site/us/en/home.html
http://arabidopsis.info/
http://biocyc.org
http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAS_PROG_DEF=megaBlast&BLAST_SPEC=blast2seq
http://orders.genome-enterprise.com/
http://signal.salk.edu/
http://signal.salk.edu/tdnaprimers.2.html
http://tools.neb.com/NEBcutter2/
http://web.expasy.org/translate/
http://www.arabidopsis.org/.
http://www.arabidopsis.org/cgi-bin/Blast/TAIRblast.pl
http://www.biochemj.org/bj/section.htm
http://www.bioinformatics.org/sms/rev_comp.html
http://www.ebi.ac.uk/Tools/msa/clustalw2/
http://www.faculty.ucr.edu/~jkzhu/
http://www.nature.com/
https://www.genevestigator.com/gv/user/gvLogin.jsp
https://www.genevestigator.com/gv/user/gvLogin.jsp
http://web.expasy.org/translate/
www.technologica.co.uk

A18. Paper Published: Manuscript stage:

"High Light stress alters the susceptibility of *Arabidopsis thaliana* abscisic acid deficient mutants to *Pseudomonas syringae".* Authors: Ibrahim A. Alzwiy[1], Marta De-Torres-Zabala[2], Venura Perera[3], Hannah Florance[3], Nick Smirnoff[3] and Murray Grant[*].