Gene expression study of candidate *Arabidopsis* defence genes in response to the bacterial wilt pathogen, *Ralstonia solanacearum*

By

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Submitted in partial fulfilment of the requirement of the degree

Magister Scientiae Biotechnology

In the Faculty of Natural Agricultural Sciences

Department of Plant Sciences

University of Pretoria

Pretoria

January 2015

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Declaration

I, Degracious Moloko Kgoale hereby declare that this dissertation herewith submitted for the degree *Magister Scientiae Biotechnology* to the University of Pretoria comprises of my own work and has not been previously submitted for any degree at any other university.

Degracious Moloko Kgoale

January 2015

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Acknowledgments

I would like to send forth my heartfelt acknowledgements and profound gratitude to the following individuals, organisations and institutes:

- First of all, thank you **God** for granting me life and the opportunity to live and learn. Thank you for your protection, guidance and courage to face yet another day.
- To my supervisor **Prof. Dave Berger**, thank you for seeing potential in me especially when I had no believe in myself for achieving this. Your support, inputs and critical analysis of this dissertation is greatly appreciated. You are the best. May God Bless you abundantly.
- To my Co-supervisor **Dr Sanushka Naidoo**, you are thus far the sweetest, supportive and most caring person I have ever met. Thank you for everything. I will forever cherish your words of encouragement throughout my journey as a student. Your comprehensive inputs and believe you had in me when I was down and ready to give up. I pray to God to grant you all your desires.
- To the **MPPI** research group, thank you all for the warmth, laughter, support and the unity as a group.
- To past and present **EPPI** and **FMG** members, thank you for accommodating me in your lab so that I could complete my lab experiments. **Ronishreee**, thank you for the help with qBase.
- **Jane Breedenkamp** and **Dr S. Phillips** thanks so much for everything.
- To my mother, **Lenah Mmakoma Kgoale**, thank you for your financial support, love and care you provided throughout the years.
- To my family and friends, thank you for the love and care especially, from you **Lebogang Maphokoane.**
- Thank you to the **National Research Foundation of South Africa** (NRF) for the Grand-holder bursary awarded to me for my project.
- To the **University of Pretoria**, **Forestry and Agricultural Biotechnology Institute** (FABI), the **Plant Sciences** and **Genetics departments** for allowing me to be student and utilizing your academic and social facilities.
- Last but not least, thank you to my grandmother, **Francinah Teti Kgoale** for being there for me.

Preface

Arabidopsis thaliana is a model plant widely used to gain insights in the so called plant-pathogen interactions. This is so because plants are sessile organisms living in an environment rich in disease causing microbes. In contrast, plants cannot relocate to avoid pathogen attacks like animals. As a result, plants rely on their immune responses to rapidly detect invading pathogens so as to fight and defend themselves against pathogens. Over the years, *Ralstonia solanacearum* has been recognised as an economically important causal agent of bacterial wilt in a vast distribution of plants ranging from important solanaceous crops, leguminous plants, a few monocotyledonous plants (Genin & Boucher, 2002) to major forest trees such as *Eucalyptus* (Coutinho et al., 2000). It has been documented that *R. solanacearum* affects over 200 plant species representing 50 botanical families (Hayward, 1991). Thus it is of utmost importance to gain as much insight into this devastating phytopathogen in order to accomplish better control methods. Chemical control is not an option for bacterial diseases, in contrast to fungal diseases. Integrated Pest Management (IPM) of bacterial wilt is considered to be a sustainable approach (Smith, 2000). Hence there is a need to generate disease resistant plants as part of IPM of bacterial wilt across the world.

The first step in understanding resistance is to study compatible host plant responses to infection with *R. solanacearum*. A fascinating discovery was that *A. thaliana* is a compatible host to bacterial wilt. This has built a strong foundation, a step forward to gaining knowledge as to what happens in a host plant upon *R. solanacearum* infection. The study of the *A. thaliana* - *R. solanacearum* pathosystem has revealed that different ecotypes of *Arabidopsis* have different responses to the bacterium. Resistant and tolerant ecotypes of *Arabidopsis* are due to the possession of the recessive gene, *AtRRS1-R* on chromosome five. The RRS1-R protein physically interacts with an effector protein from the Type III Secretion System (T3SS) of *R. solanacearum* called PopP2 to elicit Effector Triggered Immunity (ETI) (Coutinho et al., 2000; Deslandes et al., 1998; Deslandes et al., 2002; Deslandes et al., 2003). The interaction thereof is strongly suggested to localise in the nucleus of the host plant involving at least two more *A. thaliana* proteins, RPS4 (Narusaka et al., 2009) and RD9 (Rivas, 2012). This further suggests that *Arabidopsis* responses to bacterial wilt involve an extensive cascade of distinct genes.

With the latter in mind, the established *A. thaliana* - *R solanacearum* pathosytem, with the *Eucalyptus* isolate denoted, BCCF402 (isolated from *Eucalyptus* plantations in the Republic of Congo in Africa) has facilitated the basic pathogenicity modelling in *A. thaliana* plants (Van der

Linden et al., 2013). Through the generation of RT-PCR-based Suppressive Subtractive Hybridisation cDNA (SSH) libraries and hybridization of the library clones on a microarray slide, following successive microarray studies, a pool of more candidate defence response genes showing differential regulation upon BCCF402 inoculation in the tolerant ecotype, Kil-0 have been revealed. Furthermore, a whole genome microarray study also revealed several genes up-regulated upon BCCF402 in Kil-0 (Naidoo, 2008b). Also, the interaction of the susceptible ecotype, Be-0 inoculated with BCCF401 also revealed many genes differentially regulated due to the pathogen (Naidoo et al., 2011). Therefore, this somewhat confirms that defence responses in *A. thaliana* against bacterial wilt involves an extensive cascade of gene interactions. Thus far, the identified genes include Transferases, Pathogenesis related (*PR*) family genes, Resistance (*R*) genes, Kinases (including cell wall receptor kinases) and other putative and unknown genes (Beyene, 2007).

Previous studies, namely (i) SSH libraries from Kil-0 inoculated with BCCF402 and control plants, (ii) microarray hybridization experiment prepared from SSH libraries, and (iii) a whole genome microarray experiment of Kil-0 challenged with BCCF402 and control plants have been used to extract eight candidate defence response genes for further expression profiling in *A. thaliana*.

The **aims** of this MSc project were:

i) To choose *A. thaliana* candidate defence response genes based on previous microarray data from *A. thaliana* plants inoculated with *R. solanacearum* (BCCF402)
ii) To determine the differential expression trends of the candidate defence response genes in an *A. thaliana* ecotype Be-0 that is susceptible to *R. solanacearum* (BCCF402); and
iii) To determine the differential expression trends of the candidate defence response genes in an *A. thaliana* ecotype Kil-0 that is tolerant to *R. solanacearum* (BCCF402); and
iv) Establish the time points at which these genes are up-regulated in *A. thaliana* ecotype Kil-0.

Chapter 1 of this dissertation is the literature published to date focusing on the *A. thaliana – R. solanacearum* pathosytem and some of the technologies used to uncover distinct genes that play a crucial role in defence response in *A. thaliana* against pathogens.

Chapter 2 focuses on the methods and materials used in determining the results of this dissertation, which are found in Chapter 3. The methods include the construction of two independent plant pathogen challenges that were used for the expression profiling of eight

candidate defence response genes. Chapter 2 also sheds light as to how the genes were identified and how gene specific primers were developed based on the *A. thaliana* Col-0 genome for expression profiling purposes.

In **Chapter 3,** the outcomes of the experiments carried out for this MSc project are revealed. This includes the results of the viability of the development of the gene specific primers, the relative expression profiles of the chosen genes and their confirmation thereof. Also the results of what is known about each specific gene across various expression profiling experiments, available in the Genevestigator database (http://www.genevestigator.com), is shown.

Chapter 4 focuses on the discussions and the future prospects stemming from this dissertation. This chapter basically discusses the obtained results (chapter 3), and sheds some light on each gene's possible role in defence against *R. solanacearum*.

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CHAPTER 1

Literature Review:

Plant host defence responses with a focus on the *Arabidopsis thaliana* – *Ralstonia solanacearum* pathosystem

1.1 Introduction

A vast number of phytopathogens exist all around the world and affect majority of botanical species. These pathogens include viruses, bacteria, fungi, nematodes and insect pathogens. They infect plants and cause a variety of symptoms, such as tumors, rots, leaf spots, browning, wilts, galls and possible plant death (Dangl & Jones, 2001). The well-known pathogens such as *Pseudomonas syringe* (bacterial), *Botrytis cinerea* (fungal) and *Phytopthora infestans* (oomycetes) are among the former phytopathogens that have shown to cause world devastating economical loses. This also includes the soil-borne, gram-negative bacteria, *Ralstonia solanacearum* that causes bacterial wilt in many solanaceaous plants across the world (Hayward, 1991; Kunkel & Brooks, 2002).

Ralstonia solanacearum is a significant threat to most botanical species around the world. It is known as a root infecting phytopathogen that makes way into the host plant roots through root wound openings and natural pores at sites of secondary root growth. Once it gains entry into the host plant, it moves towards the vascular system where it extensively multiplies and colonises in the xylem vessels of the plant, thereby producing exopolysaccharides that block the plants water movement from the roots to the aerial parts. Eventually the plant wilts and ultimately dies (Denny, 2000).

Eucalyptus and most solanaceous crops are among the widely affected plant species that are affected by *R. solanacearum* (Genin & Boucher, 2002; Swanson et al., 2005). In order to understand the role of *R. solanacearum* and its contribution to economic loses, plant-pathogen interaction studies have been implemented and have revealed insights about the pathogen itself and its mode of infection. Virulence of the pathogen in host plants is attributed to its Type II Secretion System (T2SS) and Type III Secretion System (T3SS), which produces cell wall degrading enzymes to assist the pathogen in piercing into the host plant tissues, effector proteins that serve to suppress the plants basal immunity and macro-molecules such as exopolysaccharides that play a role in clogging the xylem vessels of the plant (Gabriel et al., 2006; Salanoubat et al., 2002).

The identification of the model plant, *A. thaliana* as a host of *R. solanacearum* has helped in understanding more about the pathogen itself and resistance of plants to the pathogen (Deslandes et al., 1998). However, a lot is still to be elucidated in terms of uncovering the core resistance of plants to *R. solanacearum*, more importantly focusing on plant immune responses to

the pathogen. In this way, the pathosystem serves to elucidate important fundamental aspects of microbial pathogenesis and associate host responses and also develop more effective disease-control methods.

Eucalyptus species contribute widely to the forestry industries across different countries in the world, including South Africa (Myburg et al., 2014). It is also a major component in the cosmetics and paper and pulp industries. The identification of *R. solanacearum* in *Eucalyptus* plantations in Africa has aided the established *A. thaliana – R. solanacearum* pathosystem with the *Eucalyptus* isolates and thus serves as a foundation to develop novel molecular tools required to uncover long-term solutions against the pathogen and possibly other *Eucalyptus* pathogens.

Stemming from the *A. thaliana – R. solanacearum* pathosytem, different insights uncovered from the interaction will be reviewed. The focus of this review is on the investigations conducted on the virulent African isolate from *Eucalyptus* species*, R. solanacearum* (BCC402) in an interaction with *A. thaliana*.

1.2 An overview of plant-pathogen interactions

1.2.1 Plant defences

Plants are sessile organisms that on a daily bases, face various biotic stresses and have to utilize different mechanisms to combat such invasive pathogen attack. These mechanisms comprise of a network of single and synergistic defensive strategies (Rafiqi et al., 2009). There are at least three forms of defence strategies deployed by plants, i.e. preformed, induced or a combination of both defence responses collectively referred to as Innate immunity (Rafiqi et al., 2009). Preformed responses comprise of cell wall structural barriers such as the epidermal cuticle, thorns, hairs and trichomes against insects, and antimicrobial compounds and toxins against pathogens. Induced responses are as a result of the recognition of the Pathogen Associated Molecular Patterns (PAMPs) by Pattern Recognition Receptors (PRRs) on the cell membrane of the plant cells that in turn signal a cascade of intercellular molecular responses (Dangl & McDowell, 2006). These responses are also known as plant basal responses.

1.2.2 Plant preformed responses

Preformed responses are those involved in the first lines of defence responses in plants. These include structural barriers and the production of antimicrobial compounds. Plants upper epidermal layer have structural barriers which serve to protect plants against intruders. Physical structures like waxy cuticle, trichomes, thorns, spikes, root hairs or bark, act as natural defence weapons for the internal environment against pathogens (Naidoo, 2008b). Cell wall components such as cellulose, pectins and lignin also serve as natural barriers against pathogen invasions.

Antimicrobial compounds take account of naturally synthesized substances by the host to deter a pathogen attack. They consist of antimicrobial peptides, proteins and non-protenaceous secondary metabolites such as flavonoids, terpenoids, phenolic acids etc. (Cowan, 1999). Cyanogenic glycosides, glucosinolates and phytoalexins are activated upon tissue damage and pathogen attack (Osbourn, 1996). Chitosan controls pathogenic microorganisms and activates several defence responses inducing or inhibiting different biochemical activities during the plant-pathogen interaction (Bautista-Baños et al., 2006).

1.2.3 Plant basal defences

Plants have molecular strategies to deter pathogens. When a pathogen physically associates with its host plant, PRR proteins are alerted to trigger defence response. As the pathogen persists to invade the cells of the host plant, the interaction of the PRRs with PAMPs signals the activation of Pathogen Triggered Immunity (PTI) explained in the next section. Some of the outcomes of PTI are deposition of callose for cell wall strengthening, Programmed Cell Death to restrict pathogen spread, the production of phytoalexins around the site of infection and an Oxidative Burst through the generation of Reactive Oxygen Species (ROS) (Montesano et al., 2003; Pieterse et al., 2009). Furthermore, ambient phytohormones are also produced (Pieterse et al., 2009). Salicylic acid (SA) gives rise to Systemic Acquired Resistance (SAR) accomplished through the activation of Pathogen-Related genes (*PR* genes), usually against biotrophic pathogens. Ethylene and Jasmonic acid (ET/JA) give rise to Induced Systemic Resistance (ISR) against necrotrophic pathogens. Collectively these outcomes have the potential to resist development of disease symptoms.

1.2.4 Plant induced defences

The first model describing plant-pathogen interactions was proposed to consist of a gene-for-gene resistance hypothesis, stating that for each gene that triggers a reaction in the host, there is a subsequent gene in the pathogen that conditions pathogenicity (Flor, 1971). The gene-for-gene hypothesis simply illustrates that for every Resistance gene (*R*-gene) in the host, there is a corresponding Avirulence (*Avr*) gene in the pathogen. This model profound the receptor-ligand model, where there is direct interaction also portrayed through the interaction between Flax and Flax rust (Flor, 1956). This led to the common understanding of R proteins serving as receptors that specifically bind to a matching Avr ligand to activate the defence machinery of the plant.

In an effort to explain the model, van der Biezen and Jones, 1998 explain how a signalling protein interacts with a pathogenic protein within the host cell. In tomato, a protein kinase, Pto interacts with the avirulent protein AvrPto from *Pseudomonas syringae*. However, Prf (an NB-LRR protein) is recruited to activate defence signalling upon pathogen invasion. This suggests an indirect interaction of Pto and AvrPto which was contradictory to what was initially speculated, i.e. the direct protein-ligand interaction (gene-for-gene interaction). In order to explain the indirect association of plant proteins and pathogenic proteins within the host, the guard hypothesis was thus proposed, which better puts the conundrum in order (Lahaye, 2004).

The guard hypothesis proposes that specific R proteins monitor or guard a host protein which targets the pathogen Avr protein. The *R* gene detects the modulation of the guarded protein, then activates the plant's innate immunity (Lahaye, 2004). Thus if the R protein is inadequate or absent, the host target is not guarded from the Avr protein, thus the pathogen is able to spread through the host unnoticed and enhance disease symptoms in the host.

Referring back to the example of van der Biezen and Jones, 1998, Pto serves as the guarding protein that recognises effectors from *P. syringae*. AvrPto is an effector protein injected into the plant cell by the T3SS. AvrPto binds to Pto and inactivates the effector. Prf is the guarded R protein. Prf is signalled by the active association of AvrPto-Pto unity, which activates plant defences against the invading parasite.

Furthermore, evidence of the guard model is explained in the interaction of *A. thaliana* and *P. syringae*. AvrB is the effector protein that interacts with RIN4, which guards RMP1, R protein. Also RIN4 serves a dual function as it also binds with *P. syringae* AvrPM1 effector. Remarkably RIN4

has been shown to also associate with AvrRpt2 effector when guarding the RPS2 R protein (Lahaye, 2004). While the guard model provides some understanding of the *R-Avr* gene interaction, the number of actions a single guard protein can perform, remains to be determined. In the case of RIN4, the mechanism of detection by the guard protein to activate defence responses is also under investigation.

Today, advances have been made to broaden the understanding of plant pathogen interactions. Dangl and Jones, 2006 have proposed the two-layered defence response model known as the zig-zag model. This model accounts both the gene-for-gene model and the guard hypothesis to illustrate how a pathogen is recognised by the host and how defence responses are perceived by the plant to diminish the pathogen. Figure 1 below lists the general overview of events that possibly take place within the host.

Plants detect PAMPs such as peptidoglycans, lipopolysaccharides, bacterial flagellin, fungal chitin and cold shock proteins. This is due cause of transmembrane PRR proteins on the cell membrane (CM) of the host that binds to the PAMPs. Effective response in plants results in PTI. Ambient production of callose and lignin, programme cell death, production of phytoalexins and Oxidative Burst is able to restrict invasion of the pathogen in the host (Lewis et al., 2009). However, in other cases, pathogens have a way of overriding this immune response by the activation of the T3SS which the pathogen uses to inject effector proteins into the host. Injected effectors act to suppress PTI resulting in Effector Triggered Susceptibility (ETS). In this way, the pathogen is able to spread and colonise host cells promoting disease in the host. As a counteracting mechanism, the host plant is able to signal the activation of *R* genes in the lead of detecting effector proteins in the host cellular compartments. These *R* genes encode Resistance proteins that indirectly or directly interact with effector proteins to confer Effector Triggered Immunity (ETI). A direct and indirect association is illustrated in Rafiqi et al., 2009 schematically representing an R-protein and Avr protein interaction, stemming from the gene-for-gene and guard model interactions.

Some pathogens evolve to produce modified effectors that manipulate R proteins resulting in the second line of ETS, such as in the case of a pathogen injecting more effectors that further overrides the first hand ETI. To counteract such a response, co-evolution of plant *R* genes aids the plants to produce R proteins that activate the second, more effective ETI. In this case, it is believed to be synergistic responses of multiple activation of *R* genes and reporter genes (Fig. 1, Dangl &

McDowell, 2006). The R proteins could possibly be dismantling the structure of the effector proteins or blocking the active sites of the effectors to render them non-functional.

Overall, ETI is believed to be the more rapid and advanced defence response. However, the exact timing in plant defence responses is unknown. These responses can occur in parallel or alternatively, depending on the pathogenic events that occur between the plant and the pathogen.

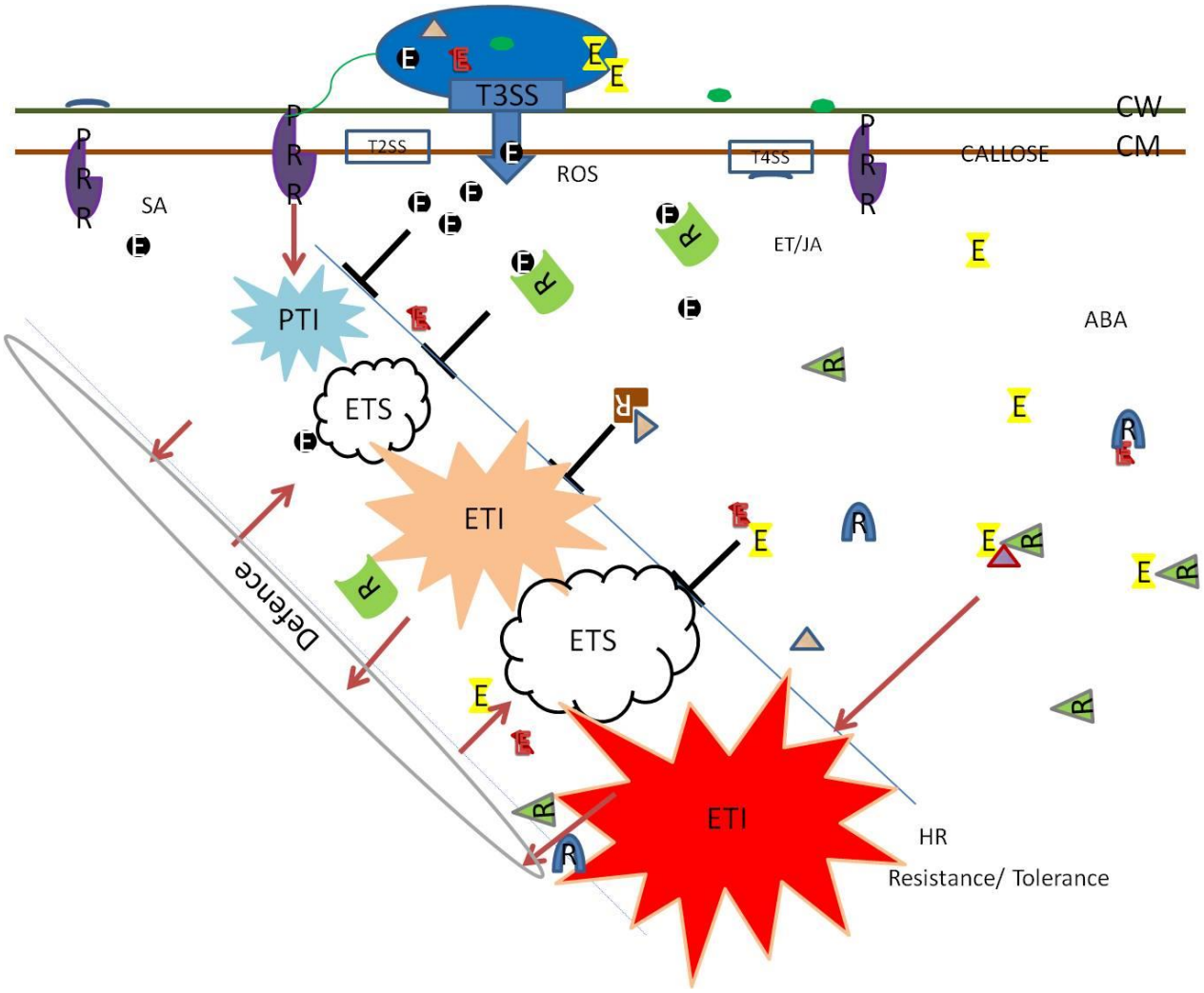

Figure 1.1: A schematic representation of the plant pathogen arms race (adapted from (Dangl & McDowell, 2006). Pathogen Associated Molecular Patterns (PAMPS) produced by bacteria are perceived by Pattern Recognition Receptors (PRRs) resulting in PAMP triggered immunity (PTI). Bacteria secrete effectors (E) into the plant cell via the T3SS. If the corresponding *R*-gene is not present in the plant, Effector Triggered Susceptibility (ETS) ensues. Plants respond by the production of *R* genes (R) which recognise specific effectors resulting in effector-triggered immunity (ETI), associated with a threshold for hypersensitive response (HR). The pathogen evolves new effectors to circumvent detection and ETS occurs. Once again, the plant evolves *R* genes, which target specific effectors, resulting in ETI associated with Resistance/tolerance and Hypersensitive Responses (HR).

1.2.5 Pathogen virulence strategies

Pathogens as well are equipped with their own mechanisms to ensure their survival and success in obstructing the plants structure in order to feed on the plant's nutrients within the tissues, and ultimately causing devastating diseases on the host plants. The defensive strategies deployed by pathogens to infect host plants involve the suppression of host defences, manipulation of host ubiquitination machinery and transcription, alteration of vesicle trafficking and modulation of hormone signalling (Hu et al., 2008). Most pathogens have evolved to deter the first line of plant responses. Pathogenic gram negative bacteria have at least six classes of secretions systems, type one to six which are used to manipulate host defence response (Baron & Coombes, 2007; Bingle et al., 2008)

Thus a continuous arms-race exists between plants and pathogens. Victory depends on which defence responses are more rapid and effective between the host and the pathogen. Therefore one can categorize plant-pathogen interactions into two classes, a compatible and an incompatible interaction. This is based on the plant's susceptibility or resistance to the host-specific pathogen, respectively (Hu et al., 2008).

A compatible interaction is one that involves a host plant showing susceptibility to a virulent invading pathogen. However, the plant's defence responses are activated upon recognition of the pathogen but to a certain extent, either delayed by the pathogen' s virulent factors or suppressed effectively. In this case the pathogen is able to cause disease to the host and possibly death. This is most likely the case for necrotrophic pathogens that infect and kill off the host, such as *R. solanacearum*.

An incompatible interaction on the other hand, is one that involves a tolerant/resistant plant and an avirulent pathogen. In a tolerant interaction, the plant is infected by the pathogen but not severely affected suggesting that the plants innate responses are activated to a level exceeding that of the pathogen, however the pathogen as well gives a good battle and as it is able to commensally benefit from certain parts of the host tissue. This is mostly associated with biotrophic pathogens that are able to live and survive in certain host tissue without causing disease to the host (Hu et al., 2008). In a resistant interaction, the host is neither infected nor affected by the host-specific pathogen, suggesting that the pathogen's attacks are weak and

ineffective to the plants innate responses, and in some cases, this interaction is termed non-host interaction.

1.3 The *A. thaliana – R. solanacearum* (solanaceous isolates) pathosystem

R. solanacearum is the causative agent of bacterial wilt in a broad range of plants. Due to its broad host range, including trees, shrubs and crops, it is considered one of the most economically important bacterial pathogens resulting in global economic losses (Hayward, 1991). Tomato being the most affected crop due to bacterial wilt globally, has been a model crop in investigations carried out to gain insights about the pathogen. This led to the sequencing of *R. solanacearum* GMI1000 isolate of tomato together with UW551 a geranium isolate (Gabriel et al., 2006; Salanoubat et al., 2002). Thus genome sequences of these strains have provided knowledge of putative pathogenicity factors required for a typical pathogen infection and host-specific pathosystem. *R. solanacearum* has a diverse number of genes involved in colonization and wilting of host plants, including lytic enzymes and exopolysaccharides, known to be secreted by the T2SS and T3SS.

The investigations in plant-pathogen interactions conducted in the model plant, *A. thaliana* have revealed that the model plant is a host of GMI1000 (Deslandes et al., 1998). This resulted in an explosion of information regarding both disease resistance and susceptibility to the pathogen. Different *A. thaliana* ecotypes behave differently to *R. solanacearum*. Ecotype Columbia (Col-5) originally from the United States of America (USA) is susceptible to GMl1000 and Nd-1 originally from The Nederlands, is resistant to GMI1000 (Deslandes et al., 1998). It was identified that resistance in Nd-1 and susceptibility in Col-5 is due to the locus, Resistance to Ralstonia solanacearum 1 (*RRS-1*) on chromosome V in the genome of *A. thaliana* (Deslandes et al., 1998). Furthermore, it is known that the *RRS-1* gene in Nd-1 consists of recessive RRS-1R alleles which confer resistance to *R. solanacearum*. However, in the susceptible ecotype Col-5, the *RRS-1* gene consists of dominant *RRS-1S* susceptibility alleles (Deslandes et al., 2002). Both encoded proteins are of similar structure but only differ in length (Deslandes et al., 2002). The proteins consist of a combination of two domains, namely the N-terminal TIR-NBS-LRR domain found in several cytoplasm *R* genes (Bernoux et al., 2008) and a C-terminal WRKY domain usually an element of transcription factors.

The RRS-1 proteins associates with the PopP2 Type lll effector proteins of *R. solanacearum* (belonging to the YopJ/AvrRxv protein family) to activate defence responses (Deslandes et al., 2003). In Nd-1, the interaction of the host proteins and the pathogen effectors results in disease resistance. In Col-5, the interactions results in the susceptibility of the host to bacterial wilt.

Literature suggests the interaction of these molecules to be a physical interaction that initiates in the cytoplasm and co-localize into the nucleus (Deslandes et al., 2003). Alternatively, the localization of PopP2 into the nucleus promotes an accumulation of RRS1 into the nucleus (Rivas, 2012). This is due to the Nuclear Localization Signal (NLS) sequence carried on the RRS1 protein (Bernoux et al., 2008). It is believed that delivery of PopP2 into the nucleus also promotes a relocation of Responsive to Dehydration 19 (RD19), a Cysteine Protease protein that is usually expressed in mobile vacuole-associated cellular compartments into the nucleus (Rivas, 2012). It is proposed that RD19 physically associates with PopP2 in the nucleus and thus is recognised by the RRS1 leading to either the modification of RRS1 transcriptional activity or transcriptional activation by additional transcriptional factors (Rivas, 2012). Therefore, the RD19-RRS1-R-PopP2 protein complex initiates effective immune defence responses and confers resistance in Nd-1.

Additionally, the resistance in *A. thaliana* against *R. solanacearum* is also linked to the *Resistance to Pseudomonas syringae 4* (*RPS4*) genes, which is also mapped on chromosome five in the *Arabidopsis* genome. RRS1-R and RPS4 knock out lines have revealed an enhanced susceptibility to *R. solanacearum* (Narusaka et al., 2009). These genes in *A. thaliana* are said to provide a dual resistance- gene system against pathogens. Williams et al., 2014 showed how RPS4 and RRS1 work in a paired manner. The authors resolved the crystal structures of the proteins and showed that the proteins have a shared surface. This allows RRS1 and RPS4 to form heterodimers and for RRS1 to repress RPS4. The WRKY domain of RRS1 acts as a bait for effectors and once the effector binds to the WRKY domain, derepression of RPS4 takes place and RPS4 is activated. A tetramer is thought to be produced that leads to the host defence signalling.

1.4 The *A. thaliana – R. solanacearum* (tree isolates) pathosystem

Bacterial wilt in *Eucalyptus* plantations was discovered in 1980 in Brazil, China, Taiwan, Thailand, Indonesia, Vietnam, Australia and Venezuela (Coutinho et al., 2000). Across the African continent, it was first reported in the new millennium in South Africa, Uganda and the Democratic Republic of Congo (DRC) (Coutinho et al., 2000, Fouché-Weich et al., 2006). Currently, high impact in

bacterial wilt is reported in China, Uganda and DRC (Xue et al., 2011). Again the African isolates have been identified to effectively cause bacterial wilt in *A. thaliana*, thereby establishing the *A. thaliana* interaction studies with Congo Kissoki (CK) strain, BCCF402 from *Eucalyptus*. The latter pathosytem is the main focus of this review.

The *A. thaliana – R. solanacearum* (CK strain, BCCF402) has confirmed that Col-0 is susceptible and Nd-1 is resistant to bacterial wilt. Additionally, Bensheim (Be-0) originally from Germany was identified as being susceptible to bacterial wilt while Killean (Kil-0) from the United Kingdom (UK) is tolerant to bacterial wilt (Van der Linden et al., 2013). Proceeding studies from the Molecular Plant Pathogen Interactions (MPPI) research group have uncovered new insights into the pathosystem itself that point to novel strategies to enhance disease resistance in plants.

R. solanacearum CK isolate (BCCF402) associates in a compatible interaction with Be-0 and Col-0. In the latter interactions, Be-0 and Col-0 show wilting symptoms as early as 3-4 days post inoculation (dpi) where at least one or two leaves wilt. Disease progression continues to 12dpi where by then the host is completely dead. Bacterial numbers within the infected plants are around 10^{12} cfu/g fresh weight (Deslandes et al., 1998). However, an incompatible interaction is illustrated in Nd-1 and Kil-0 with the CK isolate. No wilting symptoms have been detected in both ecotypes for up to 19 dpi. However, there is a difference between the bacterial numbers in Nd-1 compared to Kil-0. Bacterial numbers remain low in Nd-1, which means it is resistant, but bacterial numbers build up to a high level in Kil-0, which resulted in this response being classified as tolerance (Van der Linden et al., 2013).

Tolerance in Kil-0 has been identified to also be associated with the RRS-1 protein that associates with PopP2 effector of CK strain. A genetic hybrid cross between Be-0 and Kil-0 produced the F1 generation that were all susceptible, indicating tolerance is recessive. Further, F2 plants segregated in a 1:3 ratio of tolerance to susceptibility, which was interpreted as single gene tolerance. Cleaved Amplified Polymorphic Sequence (CAPS) marker analysis showed that this was due to the *RRS1* gene. Inoculation with a *R. solanacearum* knockout of the *PopP2* gene showed that tolerance in Kil-0 was dependent on the PopP2-RRS1 interaction (Van der Linden et al., 2013)

1.5. Gene expression studies of the *A. thaliana – R. solanacearum* pathosystem

Generally, genetic determinants of resistance to pathogens are poorly understood. It seems like a complex and most complicated network of genetic material. As discussed in the previous section,

Nd-1 resistance and Kil-0 tolerance to *R. solanacearum* is associated with the ambient interaction of the RRS1-R and PopP2 effector. However, in order to further understand this resistance, it is crucial to learn more on the transcriptional level of resistance to pathogenesis viewing the changes induced upon pathogen attack on a transcriptional level. Hu et al., 2008 elucidated the transcriptional changes that occur within the *A. thaliana – R. solanacearum* pathosystem.

Susceptible Col-5 and RRS-1-containing resistant Nd-1 were root inoculated with *R. solanacearum* GMI1000 wild-type and GMI1000ΔPopP2 which lacks the PopP2 avirulence gene. General changes in gene expression of the pathosystem were identified through a microarray analysis with ATH1 affymetrix gene chips. Disease symptoms were observed in both compatible interactions, Col-5-GMI1000 and Nd-1-GMI1000ΔPopP2 with the same disease intensity and kinetics. Disease symptoms were observed at 5 dpi. No disease symptoms were observed in the incompatible interaction, Nd-1- GMI1000 interaction.

Looking at the global gene expression patterns of the compatible and the incompatible interactions, only a few genes were differentially expressed in the early hours post inoculation (6h-24h pi). At the onset of disease symptoms, 353 genes were up regulated in the compatible interactions. These up regulated genes were activated between 1 dpi to 5 dpi and remained highly expressed throughout the study. These were the ABA-senescence, basal resistance-associated, metabolic processes, signal transduction and transcriptional regulation genes. Down regulated included developmental genes, auxin and cytokinin signalling genes. The genes were strongly down regulated throughout the study. The up regulated genes had similar expression profiles in incompatible interactions; however the down regulated genes seemed unchanged in the compatible interaction. Remarkably, ATPase plasma membrane-type, putative (At3g47950) gene was only specifically up regulated in Nd-1 plants and not in Col-5. However, there was no evidence of linkage to the presence of RRS-1 as CH1.2 transgenic Col-0 plants inoculated with GMI1000 and GMI1000ΔPopoP2 did not show expression of At3g47950. Furthermore the gene was also not linked to the presence or absence of PopP2 since CH1.2 and Col-5 inoculated GMI1000 and GMI1000ΔPopP2 did not show expression of the gene. Some genes were only observed to be up regulated in Col-5 only. This finding could be explained by the presence of widely diverging gene sequences with rapid evolution within different genotypes (Hu et al., 2008). The authors made an important observation based on their results i.e. the plant-pathogen interaction is a complex network of gene regulation effecting changes at the molecular and developmental level of the host (Hu et al., 2008).

In addition, Hu et al., 2008 knocked out 45 different *A. thaliana* genes in Col-0 and challenged the wild-type and mutant plants with the virulent and avirulent *R. solanacearum* isolate (GMI1000). They observed that knockouts of the abscisic acid signalling genes (ABI1 and ABI2) increased resistance to the virulent strain of *R. solanacearum* while *wrky53* and *putative kinase* knockout plants developed wilt symptoms slower and later than in the wild-type Col-0 in response to virulent *R. solanacearum* challenge.

Naidoo et al., 2011 also looked into expression profiling of *A. thaliana* genes inoculated with *R. solanacearum*, BCCF401, an Africa isolate from *Eucalyptus* plantations during early and late time points. The study revealed severe virulence in Col-5 inoculated with BBCF401. This indicated that the model plant, *A. thaliana* is a host for the forest pathogen (Fouché-Weich, 2004; Naidoo et al., 2011). The onset of disease appearance was observed at 10 dpi with complete plant death at 14 dpi. Microarray expression profiling was performed using 7200 *Arabidopsis* cDNA elements, containing at least 5000 unigenes from EST collections, representing 20% of the *Arabidopsis* genome (Naidoo et al., 2011). The identified differentially regulated genes highly correlated with the expression profiles of the susceptible interaction, Col-5 inoculated with GMI1000. The majority of the genes correlated with the late wilt responses in both susceptible interactions, Col-5 inoculated with BCCF401 and GMI1000. The differentially regulated genes included methyl jasmonate/ethylene defence response marker genes, *basic endochitinase* (*PR-3*) and *pre-hevein like protein* (*PR-4*) which were up regulated. Down regulated genes consisted of salicylic acid defence signalling pathway genes such as *PR-5*. Some basal defence genes, at least a subset of 38 genes, were induced. Several others were also induced pertaining to water deprivation genes from the dehydrin family protein (*response to dehydration 17* and *19* (*RD17* and *RD19*)), cold-regulated genes (*COR78 and COR4130*), *late embryogenic abundant protein 5* and *NAC transcription factor*. The expression profiles of *PR-3*, *seed imbibitions protein homologue (Sip1)*, *tyrosine amino transferase (TAT)* and *PR-4* were validated with Real Time - quantification Polymerase Chain Reaction (RT-qPCR), which showed a highly correlated expression profile to that observed in the microarray study.

The overall study revealed 141 differentially regulated genes in the susceptible interaction, Col-5 inoculated with BCCF401 with the majority of genes being differentially expressed at late wilt. Thirty three of the genes were differentially regulated during both early and late time points. Only six genes were differentially regulated only during early wilt. Approximately 102 genes were differentially regulated at late wilt. This suggest that transcriptional events of signalling networks

such as priming (Conrath et al., 2002) and activation of basal defence responses only occur during late wilt in a compatible interaction (Naidoo et al., 2011).

Following on from these studies in *Arabidopsis*, McLeod and Naidoo, 2005 (unpublished) constructed SSH libraries prepared from pooling total RNA from Kil-0 challenged with *R. solanacearum* isolate CK (BCCF402) over a time-course after inoculation (McLeod & Naidoo, 2005). Libraries consisted of pools of the total RNA from the inoculated plant material (unsubtracted tester, see Fig 3.) at (4h, 24h, 32h, 48h & 96h and 30min, 2h, 8h, 24h, & 7days). The same was done for the uninoculated (control) plant material (unsubtracted driver, see Fig 3.). The resulting cDNAs were cloned into pGEMT-easy vector and a sub-set of 222 was sequenced to identify the genes. The identity of the sequenced clones can be obtained from SSHdb (http://sww.sshdb.bi.up.ac.za). A set of 1052 clones from the SSH library was subsequently spotted onto a microarray slide and hybridisations were performed using RNA isolated from Kil-0 inoculated and Kil-0 uninoculated material at 4 dpi (Naidoo et al. 2008). Eight genes were differentially regulated, five were up regulated while three were down regulated. The 5 up regulated genes were identified as: AT4G30270 (*endoxyloglucan transferase*), AT2GG21660 (*glycine rich RNA-binding protein*), AT1G59870 (*ATP transporter*), AT1G21250 (*cell wall associated kinase*) and AT1G79245 (*unknown protein*). The three down regulated genes were identified as: AT1G78370 (*Glutathione transferase*), AT3G01500 (*carbonic anhydrase*), AT5G49740 (*ferric chelate reductase*). These results were based on the National Center for Biotechnological Information (NCBI) databases accessed in December 2008.

As described in the earlier sections, several techniques have been applied to study the defence responses in the *A. thaliana* - *R. solanacearum* pathosystem. This included microarray analysis (Hu et al., 2008; Naidoo, 2008b, Naidoo et al., 2011,) SSH (McLeod & Naidoo, 2005), RT-qPCR (Hu et al., 2008; Naidoo et al., 2011) and knock-out studies (Hu et al., 2008; Van der Linden et al., 2013). The principles of two of these techniques, SSH and RT-qPCR, which form the basis for the selection of the candidate genes identified in this study, are high-lighted below.

1.6. Suppressive Subtractive Hybridization (SSH) technology

Suppressive subtractive hybridization is a technology developed in the 20th century to detect the differential level of expression between two samples. It is used to identify genes with differential expression patterns such as those involved in the regulation of basic biological processes. The two

main SSH applications include cDNA subtraction and genomic DNA subtraction. This means that the SSH technology is one of the powerful and popular methods for generating subtracted cDNA or genomic DNA libraries (van den Berg et al., 2004). This is accomplished by its well established suppression PCR technique which combines normalisation and subtraction in a single procedure.

Suppression PCR is based on the inverted terminal repeats (Fig. 2) of genomic DNA (gDNA). The mechanism of this is as follows; in the first PCR cycle, upon the denaturation phase, the single-stranded (ss) DNA fragments flanked by inverted terminal repeats (ITR) form either self-annealing "pan-like" structures or DNA-primer hybrid structures (Fig. 2). In the case of the "pan-like" structures, the primer is unable to bind to its complementary binding sites and therefore suppresses the PCR. In the case of DNA-primer hybrids, the primer binds to the corresponding ITR binding site and the DNA Taq-polymerase proceeds with the elongation of the original structure ensuring the persistence of suppression during further PCR cycles (Fig. 2).

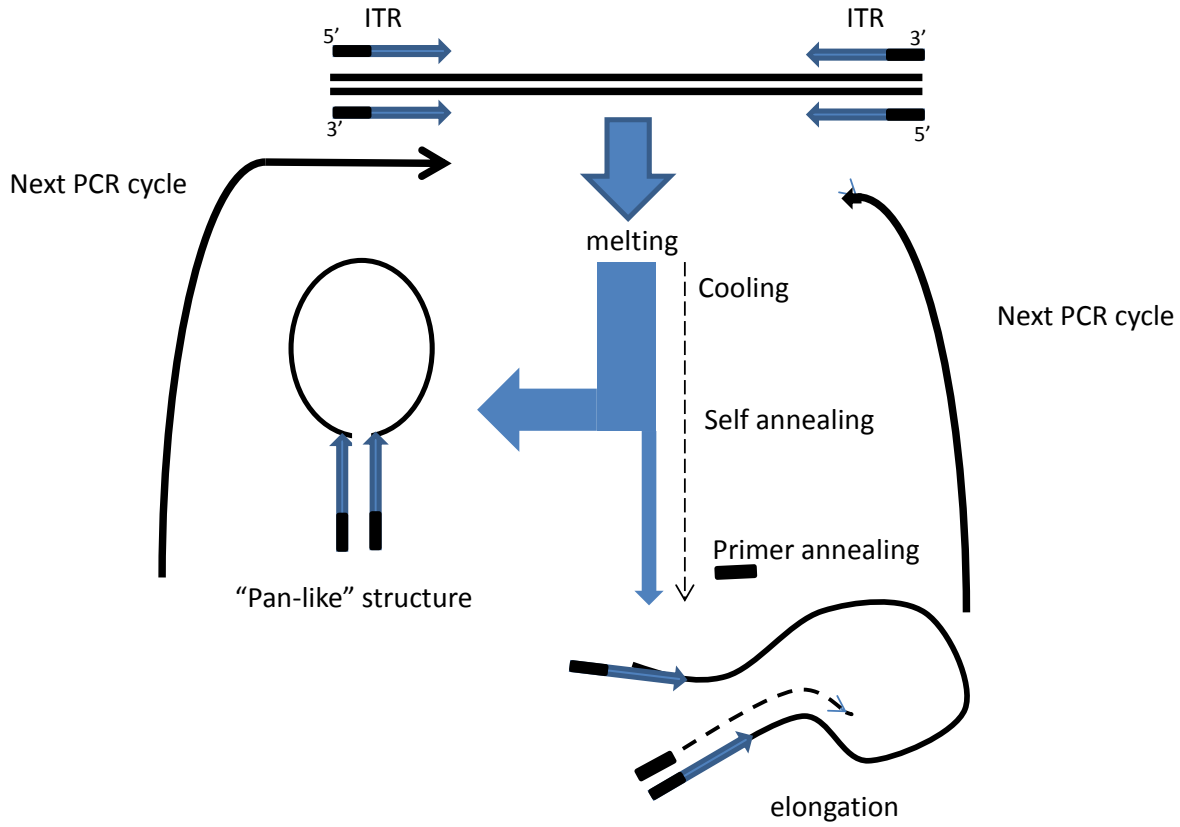

Figure 1.2: An outline of PCR suppression by inverted terminal repeats (ITR) (adapted from: http://www.evrogen.com/technologies/SSH.shtml)

This principle forms the fore step of the SSH technique. The SSH technique is applicable to many comparative and functional genetic studies for the identification of diseases, genes involved in

developmental processes, tissue specific genes or other differentially expressed genes involved in signalling pathways and as well, genomic DNA fragments can be recovered for more comparison studies (Diatchenko et al., 1996). The advantage of this method is that it does not require prior sequence information of the samples and most importantly it is able to identify less-abundant and more-abundant transcripts. However, the disadvantages include producing libraries consisting of multiples of the same gene repeatedly and redundant genes.

The procedure involves starting with RNA isolations from two sample sets, a tester sample (from the experimental group) and the driver sample (from the control group), followed by cDNA synthesis. Ideally tester samples contain transcripts that are not present in the driver samples (Diatchenko et al., 1996). The experimental group is the inoculated plant material while the driver group is the control plant material. The tester sample is digested with Rsal restriction enzyme that cleaves four bases at the 5' end to create blunt ends. The digested tester sample is then sub-divided into two equal volumes; one subdivision is ligated with adapator-1 and the other with adaptor-2 (Fig. 3). Each sample is hybridised with excess driver sample.

This step is aimed at subtracting all common cDNA molecules and obtains single stranded tester molecules. Therefore, the tester samples are mixed together to allow the second hybridization which aims to enrich the tester hybrids (Fig 1.3). Therefore, through PCR, there would be an exponential amplification of the tester hybrids.

SSH screening technique involves taking these tester hybrids and then cloning them into vectors and sequencing them to identify the genes. In the case of McCleod and Naidoo, 2005 (unpublished), the four libraries are comprised of two forward libraries where the tester transcripts were from the two independent pathogen trials of the inoculated plant material. The reverse libraries were from the same two independent studies, however, the tester samples were the transcripts from the control plant material and the driver transcripts were the inoculated transcripts. From the SSH screen, the clones were used as probes on a microarray slide for further analysis of the identified genes.

Van den Berg et al., 2004 constructed SSH libraries from *Pennisetum glaucum* (L.), Pearl Millet and *Musa acuminata*, Banana inoculated with *Bacillus spp.* and *Fusaruim oxysporum*, respectively. The aim was to determine differentially regulated transcripts during the two independent plant-pathogen interactions using the SSH technique which allows the determination of transcript regulation within hosts. The undertaken SSH technology revealed libraries containing 960 pearl

millet clones and 736 banana clones (Van den Berg et al., 2004). The independent libraries were arrayed on silanized microarray glass slides to allow identification of exclusive transcripts differentially regulated during plant defence responses. The authors were able to quantitatively determine the up regulated transcripts and identify if they were rare and abundant genes.

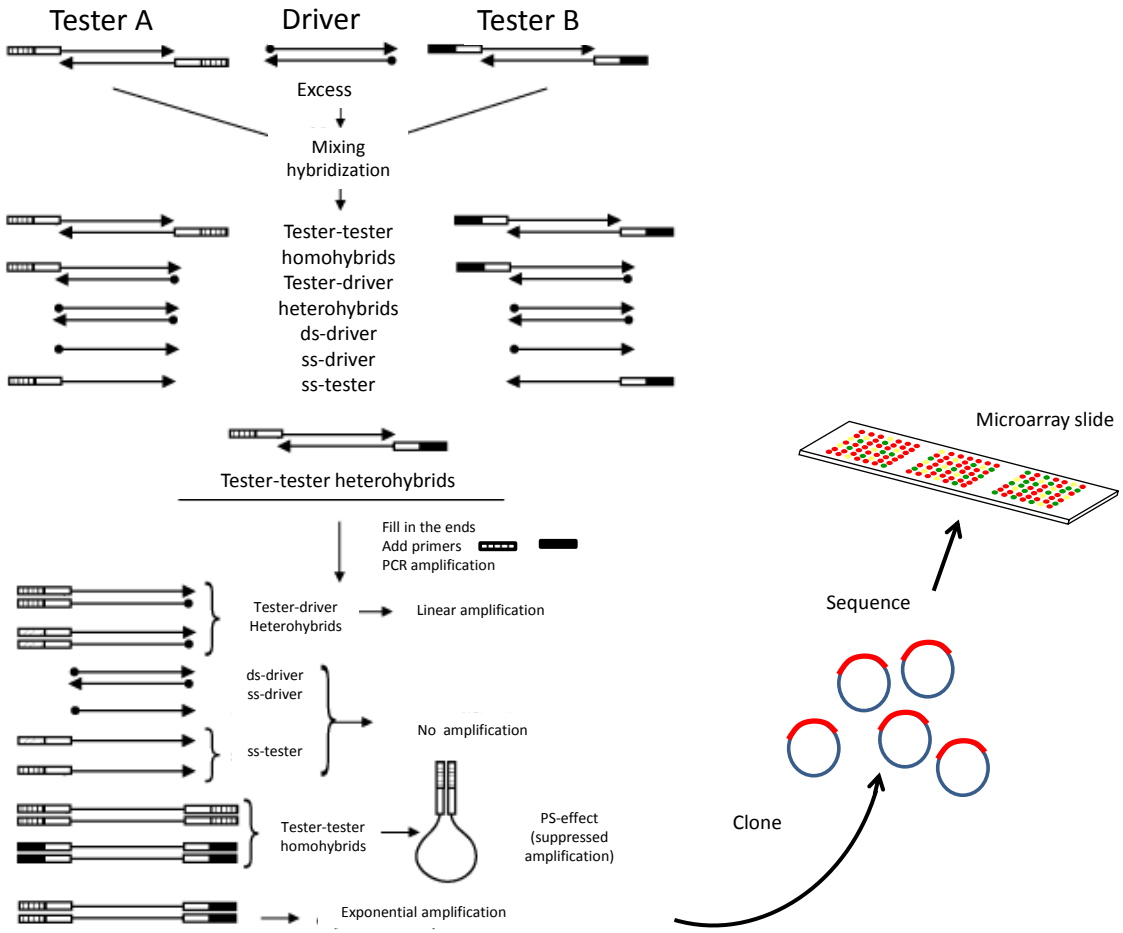

Figure 1.3: A Schematic diagram outlining Suppression Subtractive hybridization, followed by selection of clones and microarray preparation, as described by McCleod and Naidoo, 2005 (unpublished) and van den Berg et al., 2004.

1.7. Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR)

Polymerase Chain Reaction (PCR) is a powerful technique which was developed around the mid-1980s to rapidly detect and amplify very low amounts of DNA (Okubara et al., 2005). The technique is based on three temperature depended phases, which lead the denaturing of double stranded DNA into single stranded DNA. Then the annealing of specific primers to the corresponding sequences on complementary strands following the elongation into dsDNA by DNA

polymerase. Advances of this technique have led to the development of Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) which requires the synthesis of cDNA from RNA. In so doing, one is able to amplify the nucleic acids at the transcript level. The advantage thereof permits the analysis of minute quantities of starting material (Heid et al., 1996). Disadvantages of the two techniques is that there is a requirement of post-PCR sample analysis which may lead to post-PCR carry over contamination and a labour intensive quantification procedure of the PCR product, usually on agarose gel electrophoresis.

Then more techniques were developed to compensate for the extra independent quantification steps of conventional PCR. This gave rise to the development of Real-time PCR which differs from conventional PCR in that the PCR product is monitored as it is amplified either directly by binding an intercalating DNA fluorescent dye or probes. Indirectly via fluorescence that is generated using the exonuclease activity of *Taq* polymerase (Okubara et al., 2005). The advantages of real time PCR is that there is no requirement of post-PCR sample handling, it is a much faster and higher throughput assay, it has a large dynamic range of starting target template , it is extremely accurate and less labor-intensive (Derveaux et al., 2010). Today, two real-time PCR techniques exist, according to the MIQE guidelines nomenclature (Bustin et al., 2009) qPCR (to detect and measure minute quantities of nucleic acids at genomic DNA level) and RT-qPCR (to detect and measure quantities of nucleic acids at transcript level). The mechanisms of these techniques are solemnly based on the principles of conventional PCR and RT-PCR with the addition of basic fluorescence chemistry methods (Okubara et al., 2005). These include SYBR™ Green I, TaqMan™, molecular beacons and Scorpion™. They have all been developed to monitor amplicon formation during PCR cycles (Okubara et al., 2005).

qPCR is an extremely sensitive, accurate, robust and cost-effective method for quantifying gene transcripts from cells. It is considered as the golden standard for medium throughput gene expression analysis (Derveaux et al., 2010). At least two reference genes are required, according to MIQE guidelines, to determine relative expression of target genes in so doing eliminating the level of variation across biological representatives. However, if a single reference is used, there should be clear and adequate evidence that endorses its invariant expression under the set experiment (Bustin et al., 2009).

1.8. Conclusion

Based on previous studies on the *A. thaliana- R. solanacearum* interaction, various insights into possible defence mechanisms have been gained. In particular, previous studies within the MPPI group revealed a pathosytem where *A. thaliana* ecotype Kil-0 was tolerant (Van der Linden et al., 2012) and Be-0 was susceptible (Fouché-Weich, 2004). Using this pathosystem, various gene discovery experiments were undertaken to identify defence gene candidates important for tolerance to *R. solanaceram*. These studies were: (i) SSH libraries prepared from Kil-0 CK strain challenge and unchallenged material (McLeod and Naidoo, 2005) (ii) a microarray hybridisation prepared from the SSH library (Naidoo, 2008a) and (iii) a whole genome microarray experiment (Naidoo, 2008b).

All the above mentioned studies were based on the Kil-0 ecotype, which led to the selection of various candidate genes, which may play a role in defence against the pathogen. Prior to determining which candidate genes to target, using gene function studies, it is prudent to prioritise the candidate genes based on independent experiments. Thus, the aim of this study is to select and profile the expression of the candidate defence genes in independent *A. thaliana - R. solanacearum* interaction trials and to determine based on bioinformatics analyses, which candidates should be prioritised for functional genetic characterisation.

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Chapter 2

Materials and Methods

2.1 Candidate gene selection and Bioinformatics analysis

2.1.1 Selection of candidate defence genes

Six candidate defence response genes were selected from four Suppressive Subtractive Hybridization (SSH) libraries hybridised for microarray studies and compiled as Expressed Sequence Tags (ESTs) into a database (SSHdb) (http://sshdb.bi.up.ac.za) in a format illustrated in Appendix A (Coetzer, 2009). In addition, two genes were selected from a whole genome microarray experiment (Naidoo, 2008b). Thus a total of eight candidate defence response genes were selected for this MSc study (see Table 3.1). SSH library clones (cloned in pGEM®-T plasmid vector) were obtained from -80°C glycerol stocks of *Escherichia coli* cells. The *E. coli* cells were cultured on Luria-Bertani (LB) media (prepared from 1% Bacto Tryptone, 0.5% Bacto Yeast extract, 1% NaCl, 1.2% Bacto agar and 100µg/ml Ampicillin) for 48 hours at 37°C. Single colonies were selected and cultured in 5ml of liquid LB broth (LB without the bacto agar) overnight at 37°C with shaking at 110rpm. Plasmids were isolated using the Invisorb® Spin Plasmid Mini Two kit (Invitek GmbH, Germany). Purified plasmids were sequenced in both the forward and reverse orientation using T7 and SP6 primers, respectively (Promega, USA). The sequenced clones were used for genomic level comparisons in order to identify and confirm the selected candidate genes.

2.1.2 Genomic level comparisons

ESTs from the Suppressive Subtractive Hybridization database (SSHdb), Naidoo, R (Naidoo, 2008a) and probe sequences from Naidoo, S (Naidoo, 2008b) were subjected to a BLASTN and BLASTX analyses conducted in The Arabidopsis Information Resource (TAIR, v.10) database (http://www.arabidopsis.org) to identify and confirm the selected candidate genes. The sequenced SSH library clones were also analysed in TAIR. Full length genomic DNA (gDNA) and coded DNA (cDNA) sequences of each gene (representing the Col-0 genome) were downloaded from TAIR, v.10 for designing primers. Individual gene sequences of the Kil-0 genome were also obtained from the 1001 Genome Project (http://www.1001genomes.org). Altogether these sequences were imported into CLCBio Main Workbench 6.0 (Qiagen, Valencia CA, USA) for nucleotide and protein alignments for analysis of genomic level variations among the two genotypes, Col-0 and Kil-0. The SSHdb ESTs were also aligned with the sequenced clones to validate the SSHdb with frozen libraries. Unfortunately, there was no genomic data for Be-0.

2.1.3 Bioinformatics analysis

Bioinformatics analyses were performed for the eight candidate defence response genes (*AtDRG, AtGPX1*, *AtLTP3, AtPAH2, AtPNT, AtXTH22, AtXTH24* and *AtWAK1*), five well known defence response genes (*AtPR1, AtPR2, AtPR3, AtPR4, AtPDF1.2*) and *AtRRS-1* using the Genevestigator v3 tool (Zimmermann et al., 2005)(http://www.genevestigator.com). Available microarray expression profiles of the above mentioned genes were investigated in response to Pathogen Associated Molecular Patterns (PAMPs) treatments, pathogen challenges and hormone treatment. The Affymetrix data was available as log 2 signal values. These values were analysed on Microsoft Office Excel 2007 and the biological replicate expression data were averaged. Log 2 ratios of the gene under treated versus control conditions were calculated and plotted. Error bars were calculated as representatives of the standard error of the mean of the biological replicates available for each treatment.

2.2 Designing primers

Individual full length cDNAs obtained from TAIR were imported into Primer Designer 4 software (Scientific & Educational Software, Cary, North Carolina, USA). The primer type criteria selected was that of a PCR primer pair. The selected parameters included: the length of 20bp long, GC% between 40%-60%, melting temperature between 55°C to 60°C, matching pairs of the GC content to be more or less 5 and the stability of the 5' vs. 3' to be 1.5kcal. Table 3.1 in Chapter 3 illustrates the list of the designed gene specific primers. Each gene specific primer pair was aligned with Col-0 and Kil-0 cDNA sequences in search for Single Nucleotide Polymorphisms (SNPs) among the genotypes that would potentially affect the viability of the primer sets across the different genotypes.

2.3 Plant growth and maintenance

A. thaliana seeds, ecotypes Be-0 (Bensheim, Germany) and Kil-0 (Killean, United Kingdom) were surface sterilized with 70% ethanol for 5 min, 1.5% sodium hypochloride (Jik) for 30 min and then rinsed thrice with distilled water. The sterilized seeds were suspended in 1ml of 0.1% (w/v) agar bacteriological and evenly disseminated on Murashige and Skoog (MS) media plates (Murashige & Skoog, 1962) before being covered with foil and incubated at 4°C for 24 hours. The plates were then subjected to room temperature incubation for 24 hours. Then foil was removed and the plates were incubated at 20°C under long day conditions (16 hours lights/8 hours' darkness) for

two weeks. Seedlings were transferred from the medium agar with sterile forceps and planted in Jiffy® pots (Jiffy Products International AS, Norway), where they were grown in the growth room for four weeks at temperature 25.5-26.5˚C, light intensity of 300-500lum/sqf, relative humidity between 75%-100%. The conditions were monitored by the HOBO® data logger (Onset Computer Corporation, Bourne, USA). Plants were fertilized twice per week with Multifeed® Classic solution (www.biggreengarden.co.za) for two weeks and watered with distilled water every second day. To avoid determinate growth of the plants, emerging florescence were cut as the plants grew (Deslandes et al., 1998).

2.4 *R. solanacearum* BCCF402 growth conditions

Bacto-agar Glucose Triphenyltetrazolium (BGT) media plates were prepared by adding 10g peptone powder, 1g Casmino acid, 1g Yeast extract and 15g Agar bacteriological in a litre of filtered distilled water and autoclaved for 20 min at 121°C. A 1.25% Triphenyltetrazolium Chloride (TTC) solution (in absolute ethanol) and 20% Glucose were added to the autoclaved solution and poured into Petri dishes. Frozen glycerol stocks of *R. solanacearum* strain CK (BCCF402) were streaked out on the medium and incubated while inverted (to prevent condensed water droplets from washing off the colonies) at 28˚C for 48 hours. For inoculation purposes, six loops of mucoid (displaying a virulent phenotype) bacterial colonies were transferred to 200ml of sterile liquid B media (BGT media without the TTC solution and the agar) and cultured for 24 hours at 26˚C in a 200rpm shaking incubator.

2.5 Inoculation of *A. thaliana* with BCCF402 and harvesting plant material

Plants roots of Be-0 and Kil-0 were wounded by cutting the Jiffy® pots horizontally according to Fouché-Weich, 2004 (Deslandes et al., 1998). Then the plants were soaked in $1x10^8$ cfu/ml of *R. solanacearum* BCCF402 liquid culture for 30min. The concentration was determined from measuring an OD_{600} of the overnight culture using a spectrophotometer, assuming that an OD_{600} of 1 is $1x10^9$ cfu/ml. From the assumption, the obtained OD_{600} is then diluted to a final concentration of $1x10^8$ cfu/ml. The plants were transferred to wet vermiculite and grown under long day conditions as previously mentioned in section 2.3. Control plants were mock-inoculated with sterile B media corresponding with the concentration as per bacterial culture. Trial 1 material was obtained from frozen plant tissue previously harvested at 4dpi by Naidoo, R. (2008a) kept at -80°C. In the case of trial 2, inoculated plant tissue was harvested at 0, 4, 7 and 10 dpi and

immediately frozen in liquid nitrogen. Control plants were only harvested at 0 dpi assuming that mock-inoculated plants remain healthy across all time points.

2.6 Monitoring disease progression

Observations were made on the plants every second day post inoculation for a period of 19 days to monitor the progression of disease symptom development. The degree of the wilt symptoms was observed and scored on a scale of 0-5 described Table 1, adapted from the classic wilting symptoms (Deslandes et al., 1998). The Disease Index (DI) was also calculated with the formula: DI = $[\sum (n_i \times v_i)/ (V \times N)]$ where n_i = number of plants with the respective disease score, v_i = disease score from 0-5, V = highest possible disease score and N = the total number of plants used for scoring disease symptoms (Deslandes et al., 1998; Naidoo et al., 2011).

Table 2.1: Disease scoring of wilt symptom development of *A. thaliana* inoculated with *R. solanacearum*

Score	Plant symptoms
0	No symptoms
0.5	One wilting leaf
1	25% of the plant leaves wilted
2	25%-50% wilted leaves
3	55%-75% wilted leaves
4	75%-100% wilted leaves
5	Completely dead plant.

2.7 RNA isolations

Total RNA was isolated from frozen control and inoculated plant material using RNeasy Mini Kit (Qiagen Inc, Valencia, California) following the manufacturer's instructions. The extracted samples were digested with RNAse-free DNAse I enzyme (Qiagen Inc., Valencia, California) to eliminate genomic DNA contamination. The DNAse I was inactivated by a heat shock treatment at 65°C for 10 min. The RNA was purified from residual DNAse I using the RNeasy Mini Kit (Qiagen, Valencia, California) following the manufacturer's protocol. Total RNA yield and quality was determined by measuring the absorbance at 260nm using the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, USA) followed by a visual assessment on 1.2% Formaldehyde Agarose Gel Electrophoresis. Furthermore, to verify that the RNA was rid of genomic DNA, the RNA samples

were subjected to PCR using intron spanning primer pair (AtACT2_F, TGGAATCCACGAGACAACCT and AtACT2_R, TGGACCTGCCTCATCATACT) that amplifies a 372bp fragment of gDNA. The cycle conditions were: 1 cycle of denaturation at 95°C for 5min; 30 cycles of denaturation at 95°C for 30sec, primer annealing at 60°C for 30sec and elongation at 72°C for 1min; 1 cycle of final extension at 72°C for 5min and a final hold at 4°C. Amplicons were visualised on a 2% (w/v) agarose gel.

2.8 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

First strand cDNA was synthesised from 1µg of purified total RNA in a 20µl total reaction. The reaction set up was as follows: 1µl RNase inhibitor, 1µl Oligo $(dT)_{18}$ primer (0.5µg/µl), RNase-free water and incubated for 10min at 85°C and chilled on ice for 2min. This was followed by an addition of 1µl dNTPs (10mM), 4µl of 5X buffer and 1µl of Tetro Reverse Transcriptase (Bioline, MA, USA). The reaction sample was incubation at 45°C for 30min, 85°C for 5min and immediately chilled on ice. Then 2µl of the first strand cDNA was subjected to 2µl of 10X buffer, 1µl of the forward and the reverse primer (10mM), 2µl dNTPs (2mM), 0.2 µl KAPA Taq polymerase (KAPA Biosystems, Cape Town, South Africa). The PCR conditions included 95°C for 5 min, 30 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, a final extension cycle of 72°C for 5 min and a hold at 4°C. The results were analysed on 2% (w/v) agarose gel. The cDNA was stored in aliquots at -20°C.

2.9 Reverse Transcriptase – quantitative Polymerase Chain Reaction (RT-qPCR)

A two step reverse transcriptase PCR was performed using the LightCycler® 480 Real Time PCR instrument (version 1.2, Roche Diagnostics GmbH, Germany). The LightCycler® 480 Master^{PLUS} SYBR Green I system (Roche) was used for real-time PCR in a 10µl reaction. The reactions comprised of 5µl SYBR Green 1, 0.5µl of the forward primers and reverse primer, 2µl of RNAse-free water and 2µl of cDNA. The RNAse- free water was spiked with 1125ng/µl of Yeast tRNA (Sigma) to enable nucleic acids saturation across the PCR plate well surfaces, in so doing, preventing losses of target cDNA to the sides of the wells. Also to normalize reaction conditions across all reactions and protect minute quantities of the cDNA from degradation, especially in diluted cDNA samples for construction of standard curves. Standard curves for each gene were generated from a serial dilution range of 1:0, 1:5, 1:10, 1:20, 1:50, 1:100, 1:150, 1:300, and 1:750

of the synthesised cDNA. All PCR reactions were performed in triplicate including three biological replicates. Relative quantification was performed with the LightCycler® 480 software using the Second Derivative maximum software, which calculates the crossing point values during the quantification cycles. The RT-qPCR cycling conditions were as follows: 1 cycle of Initiation at 95°C for 10min; 40 cycles of quantification at 95°C for 10 sec, 60°C for 12 sec, 72°C for 12 sec with data acquisition performed at 72°C; 1 cycle of melting curve analysis at 95°C for 1 sec, 65°C for 60 sec and 95°C for 1 min with continuous data acquisition at 95°C; 1 cycle of Cooling phase at 40°C for 30 sec. The products were analysed on 2% (w/v) agarose gel to verify that the correct fragment was amplified. All relative quantification data was exported from the Lightcycler® 480, imported and analysed in Biogazelle *qBASE*plus software v1.0 (Hellemans et al., 2007). The experimental design followed the sample maximization setup, as set in the Minimum Information for Publication of Quantitative Real Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Normalization of the target genes was based on a set of reference genes that had the most stable expression across a range of samples as calculated by the software. Table 2, shows reference genes that were designed and used by de Castro (De Castro, 2010) for RT-qPCR. Significance was determined using a two-tailed Student *t*-test ($p<0.05$ and $p<0.01$) between three biological replicates analysed in Microsoft Office Excel 2007.

Table 2.2. RT-qPCR primer sequences for reference genes selected for normalisation

Gene name	AGI number	Forward primer 5'-3'	Reverse primer 5'-3'	Average Tm (°C)
AtACT2	AT3G18780	TGGAATCCACGAGACAACCT	TGGACCTGCCTCATCATACT	60
AtCBP20	AT5G44200	AGCTCGTGGATTACGGTACT	TTCTCCGGTCTCATGTCACT	60
AtELF1a	AT1G07920	ACAGGCGTTCTGGTAAGGAG	CCTTCTTGACGGCAGCCTTG	60
AtTUB4	AT5G44340	GAGCGAACAGTTCACAGCTA	GCTGCTTGCTTACACAGCTT	60
AtUBQ5	AT3G62250	GGTGGTGCTAAGAAGAGGAA	TCGATCTACCGCTACAACAG	60

2.10 Sequencing

Amplicons from RT-qPCR, visualised on the 2% (w/v) agarose gel were cut and the fragments were recovered using the Zymoclean™ gel DNA recovery kit (Zymo Reasearch, USA) following the manufacture's protocol. The quality and the concentration of the amplicons were determined by measuring the absorbance at 260nm using the Nanodrop ND-100 Spectrophotometer (Nanodrop

Technologies, USA). Approximately, 60ng of the amplicon was added to a solution of 2µl Big Dye, 0.5µl (10mM) primer (the forward or the reverse primer of each target gene, described in Table 3.1), 1µl 5X sequencing buffer and nuclease free water to make a total of 10µl reaction. The cycling conditions were: 1 cycle of initiation at 96°C for 1 min; 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min; and a final hold at 4°C. Thereafter, the sequencing products were precipitated in 30:1 (v/v) Ethanol/NaOAc solution and thoroughly washed with 70% Ethanol before subjected to Automated Sanger Sequencing Technology using the ABI 3500xl Genetic Analyzer (Applied Biosystems, Foster City, USA) which utilises capillary electrophoresis technology. The data file from the ABI 3500xl Genetic Analyzer (Applied Biosystems, Foster City, USA) were analysed on Molecular Evolutionary Genetics Analysis (MEGA) 6 (Tamura et al., 2011) to obtain the sequences which were further analysed by BLASTN in TAIR to obtain the Hit-definitions and the E-values to confirm the identities of the genes. Furthermore, the sequences were aligned with gene specific sequences using CLCBio Main Workbench 6.0 (Qiagen, Valencia CA, USA).

2.11 References

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Chapter 3

Results

3.1 Selection of candidate defence response genes

Previously, within the MPPI group pathogen challenges with *Ralsonacearum solanacearum* isolate CK (BCC402) revealed a pathosystem where *Arabidopsis* ecotype Kil-0 was tolerant and ecotype Be-0 was susceptible (Van der Linden et al., 2013). Based on the pathosystem, various gene discovery experiments were undertaken to identify defence gene candidates important for tolerance to *R. solanacearum*. These studies were: (i) SSH libraries prepared from Kil-0 CK strain challenge and unchallenged material (McLeod & Naidoo, 2005) (ii) a microarray hybridisation prepared from the SSH library (Naidoo, 2008a) and (iii) a whole genome microarray experiment (Naidoo, 2008b). A list of the candidate defence response genes, and their BLAST descriptions, based on the non-redundant database, are indicated in Table 3.1.

A further BLAST analysis was conducted for each candidate gene sequences against The Arabidopsis Information Resource (TAIR) version 10 to confirm the identity of the sequences and determine the *Arabidopsis* gene identifier (Table 3.2). The hit definition in the database matched the ones in Table 3.1 and the E-values were also significant. The clones from the SSHdb were re-sequenced to further confirm and validate that the genes selected were the correct genes. The sequences were subjected to BLASTN or BLASTX analysis in the *Arabidopsis* protein database and aligned to the representative genome sequences of *Arabidopsis* using CLCBio Main Workbench 6.0 (Qiagen, Valencia CA, USA). Figures 3.1-3.8 show the alignments of *AtDRG*, *AtGPX1*, *AtPAH2*, *AtWAK1*, *AtXTH22*, *AtXTH24*, *AtLTP3* and *AtPNT* respectively. All aligned sequeces include the Kil-0 gene sequences which were obtained from the 1001 genome project (http://signal/.salk.edu/atg1001/3.0/gegrowser.php), the Col-0 gene sequences obtained from TAIR, version 10 (http://www.arabidopsis.org), the sequences obtained from the SSHdb (http://sshdb.bi.up.ac.za) which were previously sequenced in the Kil-0 background and the newly sequenced clones from the SSH libraries (in the Kil-0 bacground) stored at -80°C . All *AtDRG* sequences aligned with each other as seen in Fig. 3.1, there was only one SNP between Kil-0 and Col-0 on the selected gene region, at nucleotide 2903 (G) in Kil-0 and 3587 in Col-0 (Fig. 3.1). However, the other nucleotide mismatches were due to sequencing error of the non-proofreading DNA polymerase enzyme used in Sanger senquencing technology on the ABI 3500xl Genetic Analyzer machine (Applied Biosystems, Foster City, USA). *AtGPX1* and *ATPAH2* gene specific sequences also align perfectly on the selected gene regions with no indication of SNPs (Fig 3.2 and Fig 3.3). In the case of *AtWAK1*, a poor EST sequence was obtained as indicated by the N's in the sequence which represent unidentified nucleotides (Fig 3.4), however, the sequences aligned with

each other with no SNPs between Kil-0 and Col-0 on the selected gene fragment. In Fig. 3.5 the alignments are shown only for Kil-0, Col-0 and SSHdb_EST for *AtXTH22*. There was one SNP between Kil-0 and Col-0 on the selected region. This is illustrated by the gap at nucleotide 323 (-) in Kil-0 and 310 (T) in Col-0 (Fig. 3.5). The sequence obtained from the clone in the SSH library stored at -80°C was an incorrect clone as indicated in Fig. 3.13, hence only the Kil-0, Col-0 and the SSHdb_XTH22_EST were aligned (Fig 3.5). In Fig. 3.6 the Kil-0 *AtXTH24* sequence was reverse completemented using CLCBio Main Workbench 6.0 (Qiagen, Valencia CA, USA) in order to obtain a feasible alignment with Col-0 *AtXTH24*, SSHdb_XTH24_EST and Clone_XTH24 sequences. This suggest that the *AtXTH24* gene in Kil-0 and Co-l-0 is differently oriented as they lie on different strands. In Kil-0 it probably lies in the negative strand while in Col-0 it lies in the positive strand. Thus only 59 nucleotides from the Kil-0 sequence was aligned. For Figure 3.7 and 3.8, only the Kil-0, Col-0 and the probes are indicated, because there were no SSHdb EST matching those identities, baring in mind that *AtLTP3* and *AtPNT* were selected from the whole genome microarray study (Naidoo,2008b).

Table 3.1 Candidate defence response genes based on *Arabidopsis* SSHdb ESTs and *Arabidopsis* whole genome microarray probes*

Clone/ Probe name	BLAST result (N/X)	BLAST Acc number	BLAST HitDef	E-value	Source
M64_AF2-F9_F	N	NM_124238	*Arabidopsis thaliana* disease resistance protein (CC-NBS-LRR class), putative (at5g48620) mRNA, complete cds	0.0e+00	(McLeod & Naidoo, 2005), unpublished
M64_AF1-B11_F	X	CAA61965	Glutatione peroxidase [*Arabidopsis thaliana*]	1.2e-48	(McLeod& Naidoo, 2005), unpublished
M64_AF1-B5_F	X	NP_199101	Lipin family protein [*Arabidopsis thaliana*]	5.6e-29	(McLeod& Naidoo, 2005), unpublished
M64_SF5-G1_F	X	NP_200564	TCH4 (TOUCH 4); Hydrolase, acting on glycosly bonds/ xyloglucan:xyloglucosyl tranferase [*Arabidopsis thaliana*]	1.4e-65	(McLeod& Naidoo, 2005), unpublished
M64_AR2-A1_R	X	AAF81356	Identical to wall-associated kinase 1 from *Arabidopsis thaliana* gb|AJ009696 and contains Eukaryotic protein kinase PF|00069 and EGF-like PF:00008 domains	2.3e-47	(Naidoo, 2008a)
M64_AR4-E9_R	X	NP_194756.1	Xyloglucan endotransglucosylase/hydrolase protein 24 [*Arabidopsis thaliana*]	0.004	(Naidoo, 2008a)
AF159800*	X	AAM06088.1	Non-specific lipid transfer protein precursor-like protein [*Arabidopsis thaliana*]	4e-06	(Naidoo, 2008b)
AC016795*	X	NP_187783.1	Polynucleotidyl transferase , ribonuclease H-like superfamily protein [*Arabidopsis thaliana*]>gb|AAF23193.1|AC016795_6 unknown protein [*Arabidopsis thaliana*]	6e-04	(Naidoo, 2008b)

Table 3.2: TAIR BLAST results of the candidate gene sequences

Gene name	BLAST (N/X)	ATG number	BLAST HitDef	Score (bits)	E-value	Clone name (SSHdb)/ Probe name
AtDRG	N	AT5G48620	Disease resistance protein (CC-NBS-LRR class) family	821	0	M64_AF2-F9-F
AtGPX1	X	AT2G25080	Glutathione peroxidase 1	191	1e-49	M64_AF1-B11_F
AtPAH2	X	AT5G42870	Phosphatidic acid phosphohydrolase 2	123	2e-29	M64_AF1-B5_F
AtWAK1	X	AT1G21250	Cell wall-associated kinase 1	251	7e-68	M64_AR2-A1_R
AtXTH22	X	AT5G57560	Xyloglucan endotransglucosylase/hydrolase 22	251	8e-68	M64_SF5-G1_F
AtXTH24	X	AT4G30270	Xyloglucan endotransglucosylase/hydrolase 24	44	3e-05	M64_AR4-E9_R
AtLTP3	X	AT5G59320	Lipid transfer protein 3	49	9e-07	AF159800
AtPNT	X	AT3G11770	Polynucleotidyl transferase, ribonuclease H-like suprefamily protein	44	3e-05	AC016795

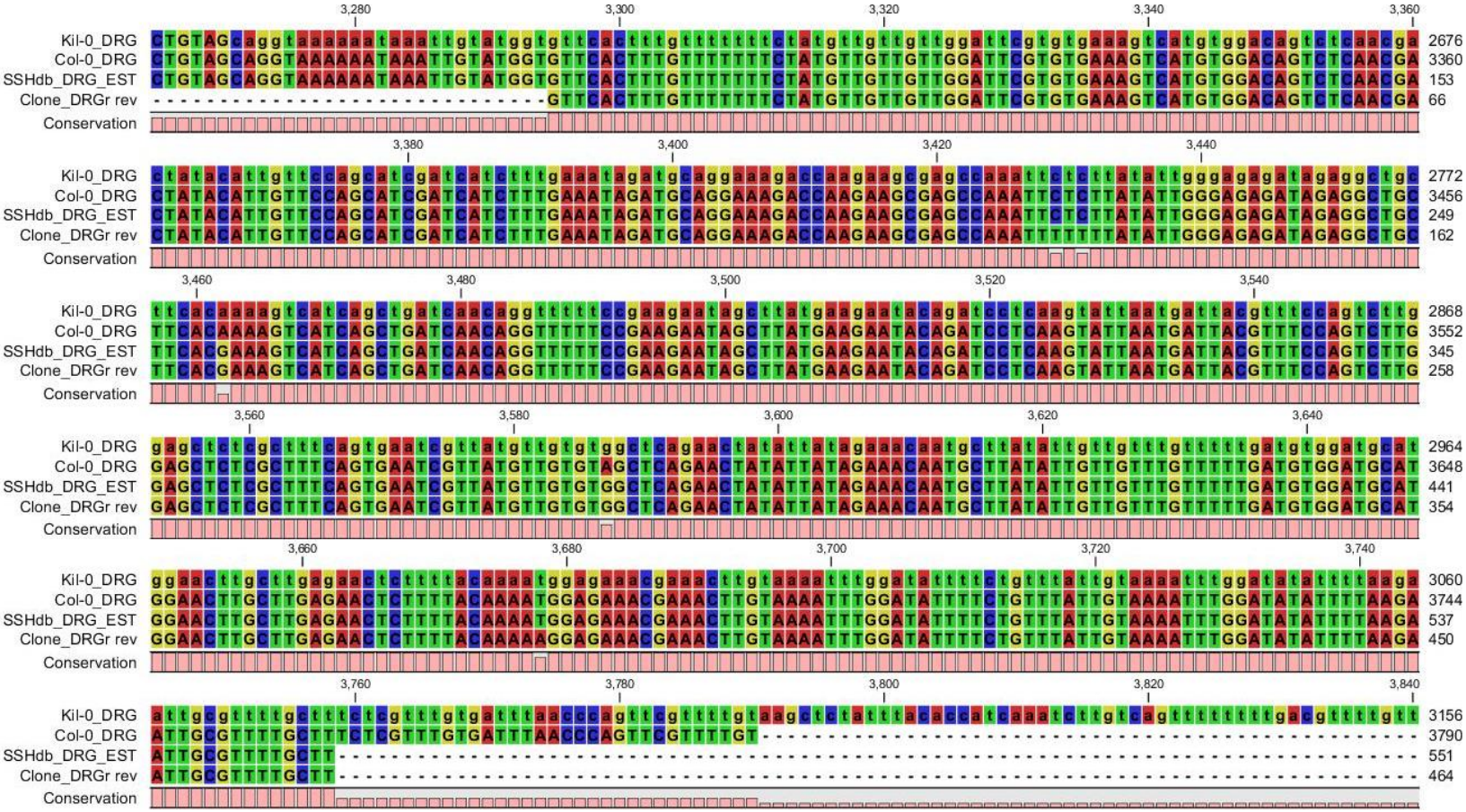


Figure 3.1 Alignment of nucleotide sequence of Kil-0 and Col-0 DRG to the SSHdb_DRG_EST (previously sequenced) and Clone_DRG (AF2-F9_F) sequence from the SSH library. Dash lines indicate a lack of nucleotide sequence, numbers on the far right indicate the nucleotide positions of each sequence according to the length of the sequence and the numbers on top of the sequence alignments indicate the positions of the nucleotides based on the length of the Col-0 gene sequence.

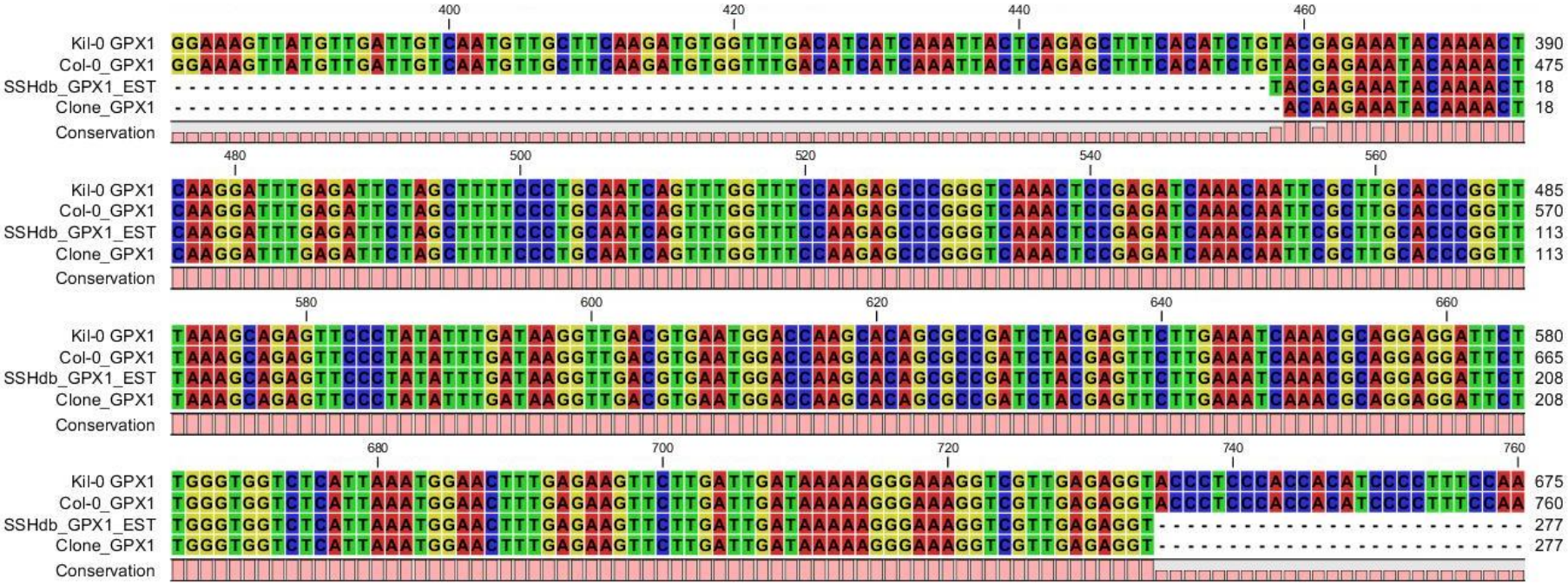


Figure 3.2 Alignment of nucleotide sequence of Kil-0 and Col-0 GPX1 to the SSHdb_GPX1_ EST (previously sequenced) and Clone_GPX1 (AF1-B11_F) sequence from the SSH library. Dash lines indicate a lack of nucleotide sequence, numbers on the far right indicate the nucleotide positions of each sequence according to the length of the sequence and the numbers on top of the sequence alignments indicate the positions of the nucleotides based on the length of the Col-0 gene sequence.

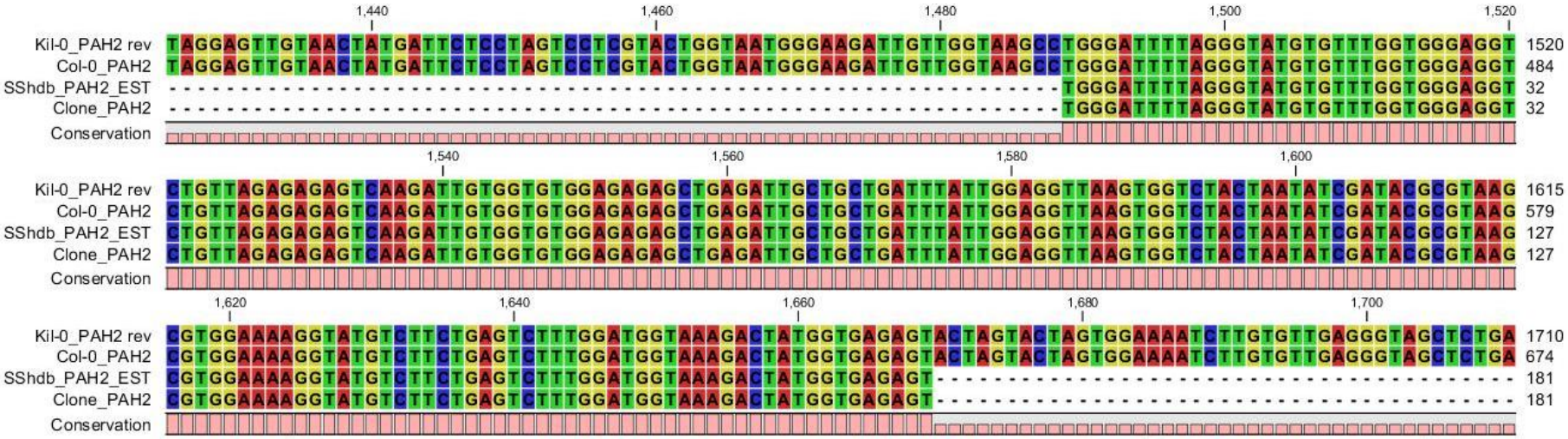


Figure 3.3 Alignment of nucleotide sequence of Kil-0 and Col-0 PAH2 to the SSHdb_PAH2_ EST (previously sequenced) and Clone_PAH2 (AF1-B5_F) sequence from the SSH library. Dash lines indicate a lack of nucleotide sequence, numbers on the far right indicate the nucleotide positions of each sequence according to the length of the sequence and the numbers on top of the sequence alignments indicate the positions of the nucleotides based on the length of the Kil-0 gene sequence.

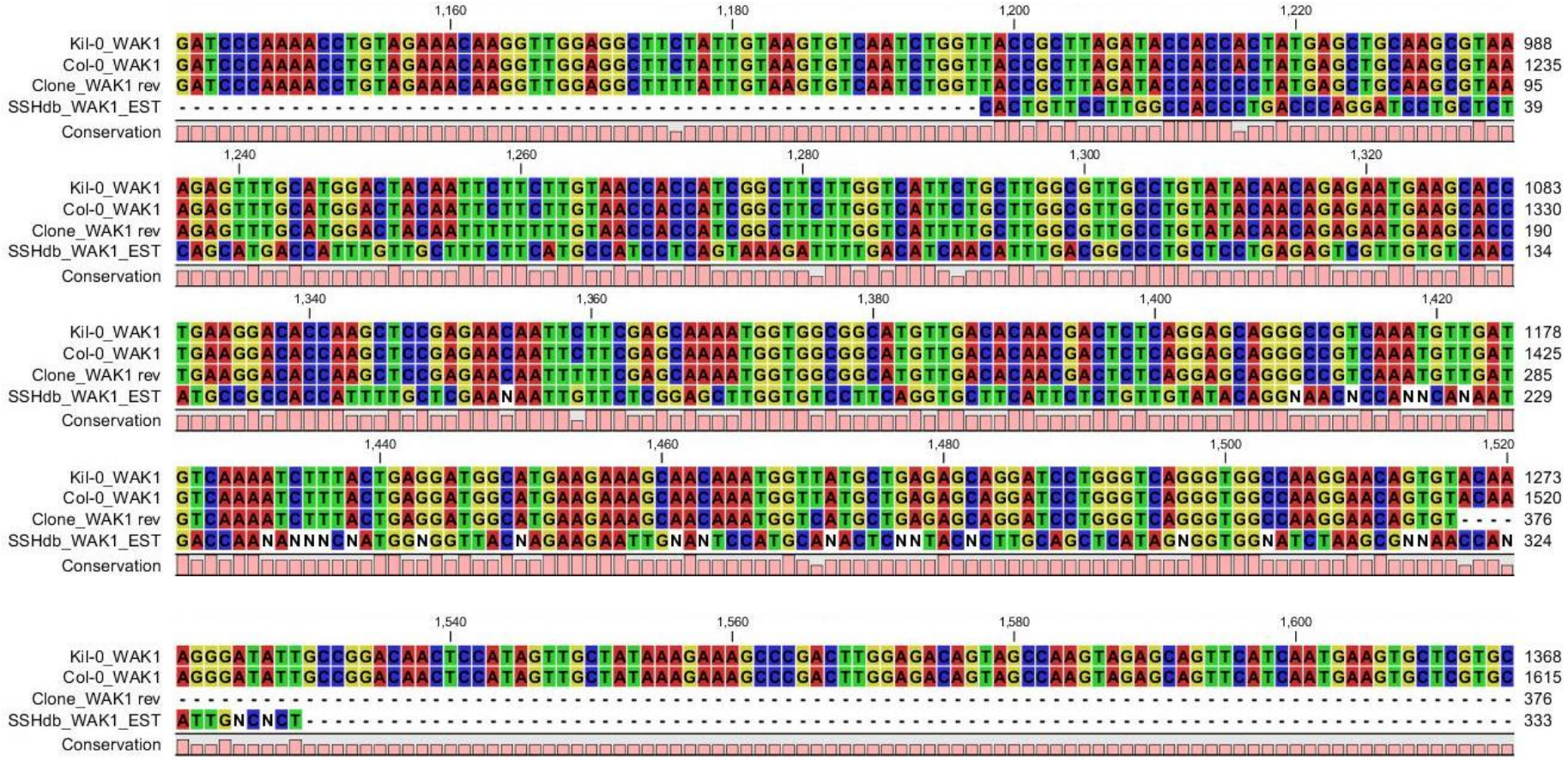


Figure 3.4 Alignment of nucleotide sequence of Kil-0 and Col-0 WAK1 to the SSHdb_WAK1_EST (previously sequenced) and Clone_WAK1 (AR2-A1_F) sequence from the SSH library. Dash lines indicate a lack of nucleotide sequence, numbers on the far right indicate the nucleotide positions of each sequence according to the length of the sequence and the numbers on top of the sequence alignments indicate the positions of the nucleotides based on the length of the Col-0 gene sequence. The N represents unknown nucleotide from Sanger sequencing.

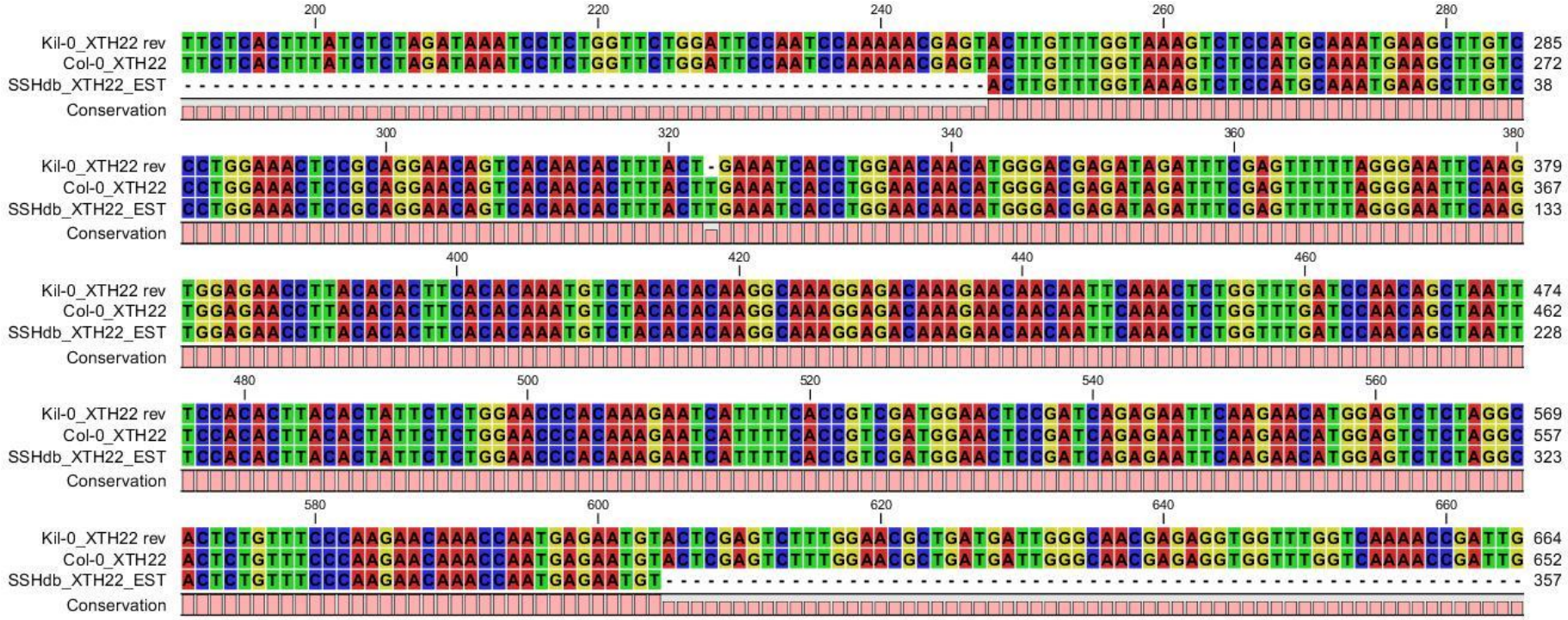


Figure 3.5 Alignment of nucleotide sequence of Kil-0 and Col-0 XTH22 to the SSHdb_XTH22_EST (previously sequenced) and Clone_XTH22 (SF5-G1_F) sequence from the SSH library. Dash lines indicate a lack of nucleotide sequence, numbers on the far right indicate the nucleotide positions of each sequence according to the length of the sequence and the numbers on top of the sequence alignments indicate the positions of the nucleotides based on the length of the Kil-0 gene sequence.

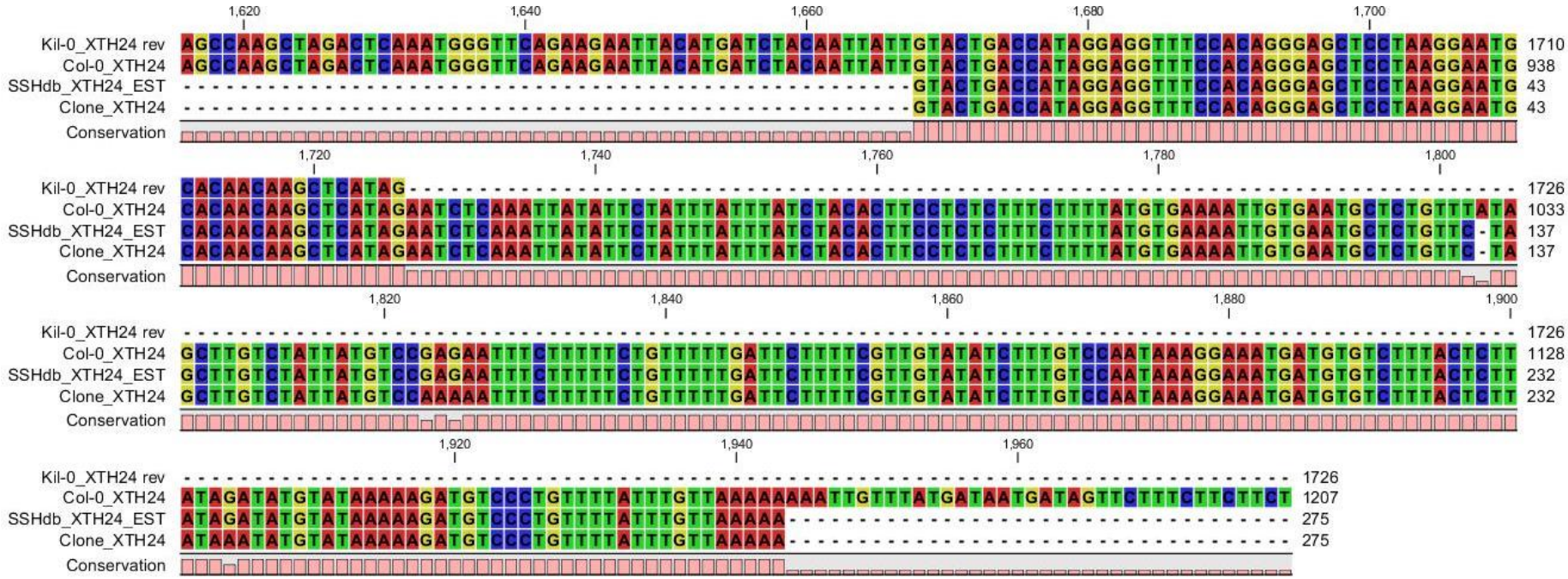


Figure 3.6 Alignment of nucleotide sequence of Kil-0 and Col-0 XTH24 to the SSHdb_XTH24_ EST (previously sequenced) and Clone_XTH24 (AR4-E9_R) sequence from the SSH library. Dash lines indicate a lack of nucleotide sequence, numbers on the far right indicate the nucleotide positions of each sequence according to the length of the sequence and the numbers on top of the sequence alignments indicate the positions of the nucleotides based on the length of the Kil-0 gene sequence.

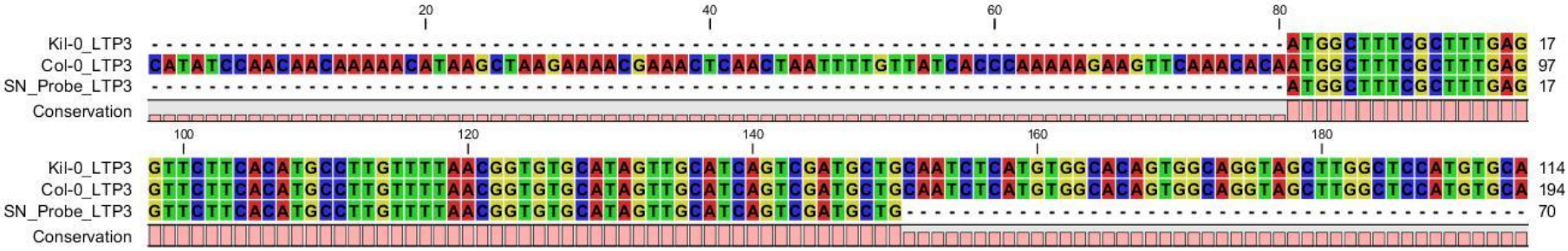


Figure 3.7 Alignment of nucleotide sequence of Kil-0 and Col-0 LTP3 to the SN_Probe_LTP3 sequence from the Arizona whole genome microarray. Dash lines indicate a lack of nucleotide sequence, numbers on the far right indicate the nucleotide positions of each sequence according to the length of the sequence and the numbers on top of the sequence alignments indicate the positions of the nucleotides based on the length of the Col-0 gene sequence.

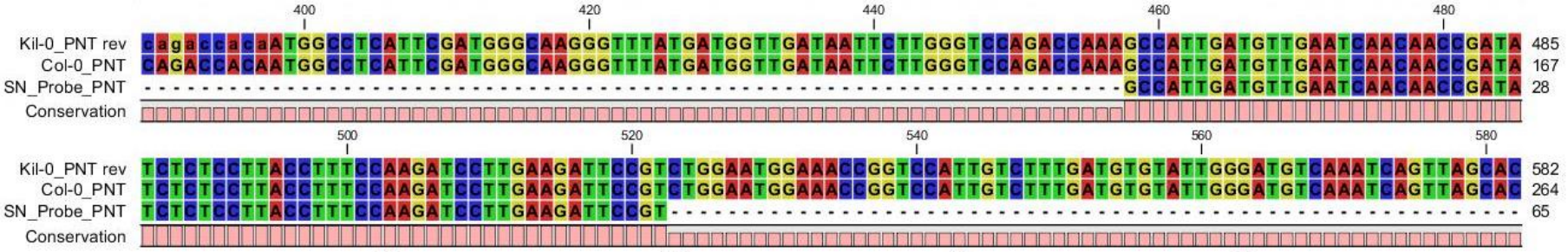


Figure 3.8 Alignment of nucleotide sequence of Kil-0 and Col-0 PNT to the SN_Probe_PNT sequence from the Arizona whole genome microarray. Dash lines indicate a lack of nucleotide sequence, numbers on the far right indicate the nucleotide positions of each sequence according to the length of the sequence and the numbers on top of the sequence alignments indicate the positions of the nucleotides based on the length of the Kil-0 gene sequence.

In addition, a BLASTN and BLASTX analysis was further conducted for the SSH library clones to further validate that the correct SSHdb_EST corresponds with the correct SSH library clone and the correct gene identity. Figures 3.9-3.16 show the BLAST analysis alignments of *AtDRG*, *AtGPX1*, *AtPAH2*, *AtWAK1*, *AtXTH22*, *AtXTH24*, *AtLTP3* and *AtPNT* respectively. In the case of *AtDRG* a nucleotide alignment is shown because Clone_DRG extends to the 3' UTR (Fig. 3.9). In the case of, *AtGPX1*, *AtPAH2*, *AtWAK1*, *AtXTH22*, *AtXTH24*, *AtLTP3* and *AtPNT*, the amino acid alignments are shown because the sequences of the SSH library clones lie within the coding regions of the genes. The BLASTX analysis for AtXTH22 indicates that the SSH library clone aligns with an unknown protein (At1G73885) with an alignment score of 129 and the E-value is 8e-33 (Fig 3.13). This indicates that the SSH library clone that was obtained from the -80°C freezer stocks was incorrect. This could be due to selecting the incorrect well of the SSH library 96 well plate. Despite this, further analysis of *AtXTH22* was continued. In the case of *AtLTP3* and *AtPNT*, the probes were aligned to the TAIR database gene specific sequences (Fig 3.15 and 3.16), as there are no SSH library clones of these genes, because they are selected from the whole genome microarray study (Naidoo, 2008b).

AtDRG

Query: 18 ctatgttgttgttggattcgtgtgaaagtcatgtggacagtctcaacgactatacattgt 77

Sbjct: 3312 ctatgttgttgttggattcgtgtgaaagtcatgtggacagtctcaacgactatacattgt 3371

Query: 78 tccagcatcgatcatctttgaaatagatgcaggaaagaccaagaagcgagccaaannnnn 137

Sbjct: 3372 tccagcatcgatcatctttgaaatagatgcaggaaagaccaagaagcgagccaaattctc 3431

Query: 138 nnatattgggagagatagaggctgcttcacgaaagtcatcagctgatcaacaggtttttc 197

Sbjct: 3432 ttatattgggagagatagaggctgcttcacaaaagtcatcagctgatcaacaggtttttc 3491

Query: 198 cgaagaatagcttatgaagaatacagatcctcaagtattaatgattacgtttccagtctt 257

Sbjct: 3492 cgaagaatagcttatgaagaatacagatcctcaagtattaatgattacgtttccagtctt 3551

Query: 258 ggagctctcgctttcagtgaatcgttatgttgtggctcagaactatattatagaaaca 317

Sbjct: 3552 ggagctctcgctttcagtgaatcgttatgttgtgtagctcagaactatattatagaaaca 3611

Query: 318 atgcttatattgttgtttgtttttgatgtggatgcatggaacttgcttgagaactctttt 377

Sbjct: 3612 atgcttatattgttgtttgtttttgatgtggatgcatggaacttgcttgagaactctttt 3671

Query: 378 acaaaaaggagaaacgaaacttgtaaaatttggatattttctgtttattgtaaaatttgg 437

||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct: 3672 acaaaatggagaaacgaaacttgtaaaatttggatattttctgtttattgtaaaatttgg 3731

Query: 438 atatattttaagaattgcgttttgctt 464

|||||||||||||||||||||||||||

Sbjct: 3732 atatattttaagaattgcgttttgctt 3758

Figure 3.9 A BLASTN alignment of the nucleotide sequence of Clone_DRG (Query) to the gene specific sequence (Sbjct) in TAIR. The numbers indicate the positions of the nucleotides of the alignments relative to complementary alignments to each other. The alignment score was 821 and the E-value was 0.00.

AtGPX1

Query: 48 YEKYKTQGFEILAFPCNQFGFQEPGSNSEIKQFACTRFKAEFPIFDKVDVNGPSTAPIYE 227

YEKYKTQGFEILAFPCNQFGFQEPGSNSEIKQFACTRFKAEFPIFDKVDVNGPSTAPIYE

Sbjct: 125 YEKYKTQGFEILAFPCNQFGFQEPGSNSEIKQFACTRFKAEFPIFDKVDVNGPSTAPIYE 184

Query: 228 FLKSNAGGFLGGLIKWNFEKFLIYKKGKVVERY 326

FLKSNAGGFLGGLIKWNFEKFLI KKGKVVERY

Sbjct: 185 FLKSNAGGFLGGLIKWNFEKFLIDKKGKVVERY 217

Figure 3.10 A BLASTX alignment of the predicted amino acid sequence of Clone_GPX1 (Query) to the gene specific sequence (Sbjct) in TAIR. The numbers indicate the positions of the nucleotides of the alignments relative to complementary alignments to each other. The alignment score was 191 and the E-value was 1e-49.

AtPAH2

Query: 51 KIVGKPGILGYVFGGRSVRESQDCGVERAEIAADLLEVKWSTNIDTRKRGKGMSSESLDG 230

KIVGKPGILGYVFGGRSVRESQDCGVERAEIAADLLEVKWSTNIDTRKRGKGMSSESLDG

Sbjct: 146 KIVGKPGILGYVFGGRSVRESQDCGVERAEIAADLLEVKWSTNIDTRKRGKGMSSESLDG 205

Query: 231 KDYGEST 251

KDYGEST

Sbjct: 206 KDYGEST 212

Figure 3.11 A BLASTX alignment of amino acid sequence of Clone_PAH2 (Query) to the gene specific sequence (Sbjct) in TAIR. The numbers indicate the positions of the nucleotides of the alignments relative to complementary alignments to each other. The alignment score was 123 and the E-value was 2e29.

AtWAK1

Query: 1 DPKTCRNKVGGFYCKCQSGYRLDTTPMSCKRKEFAWTTIFFVTTIGFLVILLGVACIQQR 180

DPKTCRNKVGGFYCKCQSGYRLDTT MSCKRKEFAWTTI VTTIGFLVILLGVACIQQR

Sbjct: 299 DPKTCRNKVGGFYCKCQSGYRLDTTTMSCKRKEFAWTTILLVTTIGFLVILLGVACIQQR 358

Query: 181 MKHLKDTKLREQFFEQNGGGMLTQRLSGAGPSNVDVKIFTEDGMKKATNGHAESRILGQG 360

MKHLKDTKLREQFFEQNGGGMLTQRLSGAGPSNVDVKIFTEDGMKKATNG+AESRILGQG

Sbjct: 359 MKHLKDTKLREQFFEQNGGGMLTQRLSGAGPSNVDVKIFTEDGMKKATNGYAESRILGQG 418

Query: 361 GQGTV 375

GQGTV

Sbjct: 419 GQGTV 423

Figure 3.12 A BLASTX alignment of amino acid sequence of Clone_WAK1 (Query) to the gene specific sequence (Sbjct) in TAIR. The numbers indicate the positions of the nucleotides of the alignments relative to complementary alignments to each other. The alignment score was 251 and the E-value was 7e-68.

AtXTH22

Query: 63 RYLWAFGILFALISGGLAAGAYNEGATDFKETPVYKEAIQSRDLLDEAKSSNSEDVFESN 242

+YLWAFGILFALISGGLAAG YNEGATDFKETPVYKEAIQSRDLLDEA+SSNSEDVFESN

Sbjct: 112 QYLWAFGILFALISGGLAAGTYNEGATDFKETPVYKEAIQSRDLLDEAESSNSEDVFESN 171

Query: 243 PTKVAPSL 266

PT+VAP++

Sbjct: 172 PTEVAPTI 179

Figure 3.13 A BLASTX alignment of amino acid sequences of Clone_XTH22 (Query) to the gene specific sequence (Sbjct) in TAIR. The numbers indicate the positions of the nucleotides of the alignments relative to complementary alignments to each other. The alignment score was 129 and

the E-value was 8e-33. Note: Clone_XTH22 was identified as the an unknown protein (At1G73885) in TAIR.

AtXTH24

Query: 3 TDHRRFPQGAPKECTTSS 56

TDHRRFPQGAPKECTTSS

Sbjct: 252 TDHRRFPQGAPKECTTSS 269

Figure 3.14 A BLASTX alignment of amino acid sequences of Clone_XTH24 (Query) to the gene specific sequence (Sbjct) in TAIR. The numbers indicate the positions of the nucleotides of the alignments relative to complementary alignments to each other. The alignment score was 44 and the E-value was 3e-05.

AtLTP3

Query: 1 MAFALRFFTCLVLTVCIVASVDA 69

MAFALRFFTCLVLTVCIVASVDA

Sbjct: 1 MAFALRFFTCLVLTVCIVASVDA 23

Figure 3.15 A BLASTX alignment of amino acid sequences of Probe_PNT (Query) to the gene specific sequence (Sbjct) in TAIR. The numbers indicate the positions of the nucleotides of the alignments relative to complementary alignments to each other. The alignment score was 49 and the E-value was 9e-07.

AtPNT

Query: 1 AIDVESTTDISPYLSKILEDS 63

AIDVESTTDISPYLSKILEDS

Sbjct: 21 AIDVESTTDISPYLSKILEDS 41

Figure 3.16 A BLASTX alignment of amino acid sequence of Probe_PNT (Query) to the gene specific sequence (Sbjct) in TAIR. The numbers indicate the positions of the nucleotides of the alignments relative to complementary alignments to each other. The alignment score was 44 and the E-value was 3e-05.

3.2 In silico expression analysis of candidate defence response genes

The genevestigator database was analysed to identify the expression of the candicate defence response genes under various biotic stress challenges. Figure 3.17-3.24 show the expression patterns of the candidate defence response genes (*AtGPX1*, *AtLTP3*, *AtPAH2*, *AtPNT*, *AtXTH22*, *AtXTH24* and *AtWAK1*) alongside the expression of known defence response genes (*AtPR1*, *AtPR2*, *AtPR3*, *AtPR4*, *AtPDF1.2* and *AtRRS1*) under hormone treatment (Fig 3.17- 3.18), Pathogen Associated Molecular Patterns (PAMPs) treatment (Fig. 3.19 and 3.20) and pathogen challenges (Fig. 3.21-3.24)

Salicylic acid (SA) is one of the key plant hormones produced in response to a variety of pathogens. SA is necessary for the establishment of both local and systemic-acquired resistance (SAR). SA application induces accumulation of pathogenesis-related (PR) proteins (Edgar et al., 2006). Figure 3.17 show that *AtPR1* is up regulated by treatment of plants with SA from 4-52 hours post treatment. However, as expected, markers of MeJA/ET defence, *AtPR4* and *AtPDF1.2*, appear to be down regulated. *AtWAK1* is down regulated at 28 and 52 hours post treatment.

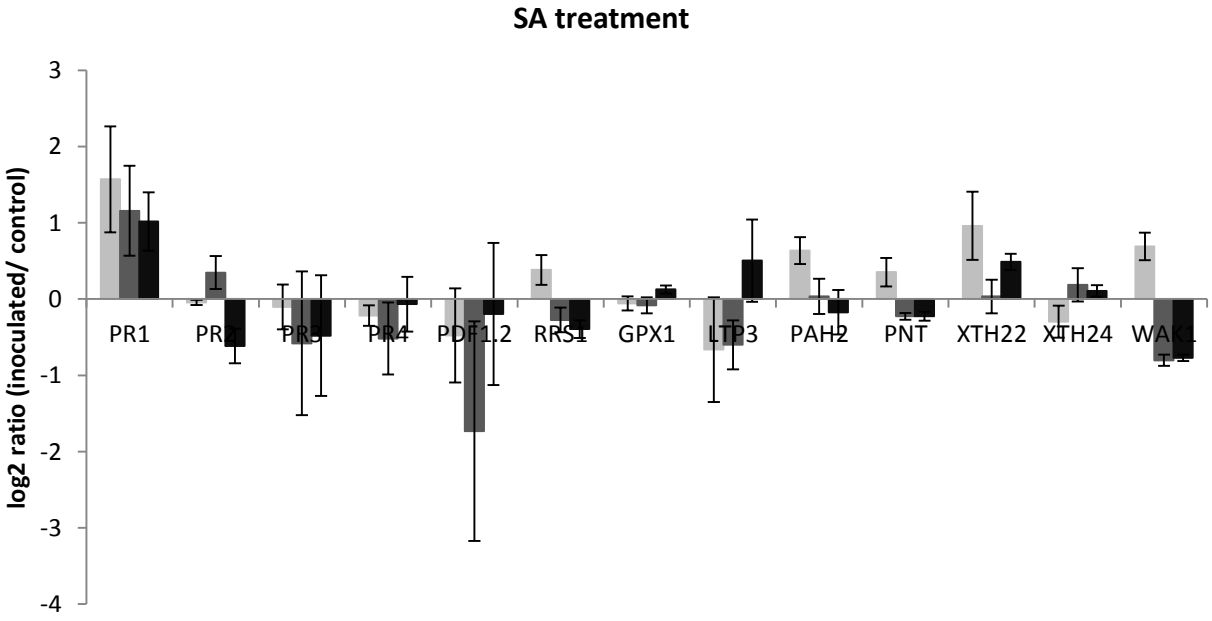


Figure 3.17: Expression of candidate defence response genes in *A. thaliana* Col-0 plants treated with salicylic acid at 4 (light shaded bars), 28 (medium shaded bars) and 52 (dark shaded bars) hours. The expression is relative to untreated plants. Error bars represent standard error of the mean of the biological replicates (n=3).

Treatment of plants with methyl jasmonate (MeJA), also a key plant hormone in response to a variety of pathogens (Dong, 1998), induces the expression of *AtLTP3* and *AtXTH24*. However, *AtPR1* and *AtPR3* as expected seem to be down regulated. Unexpectedly, *AtPR4* and *AtPDF1.2* were down regulated. However, it could be due to lack of information considering only one time point was investigated.

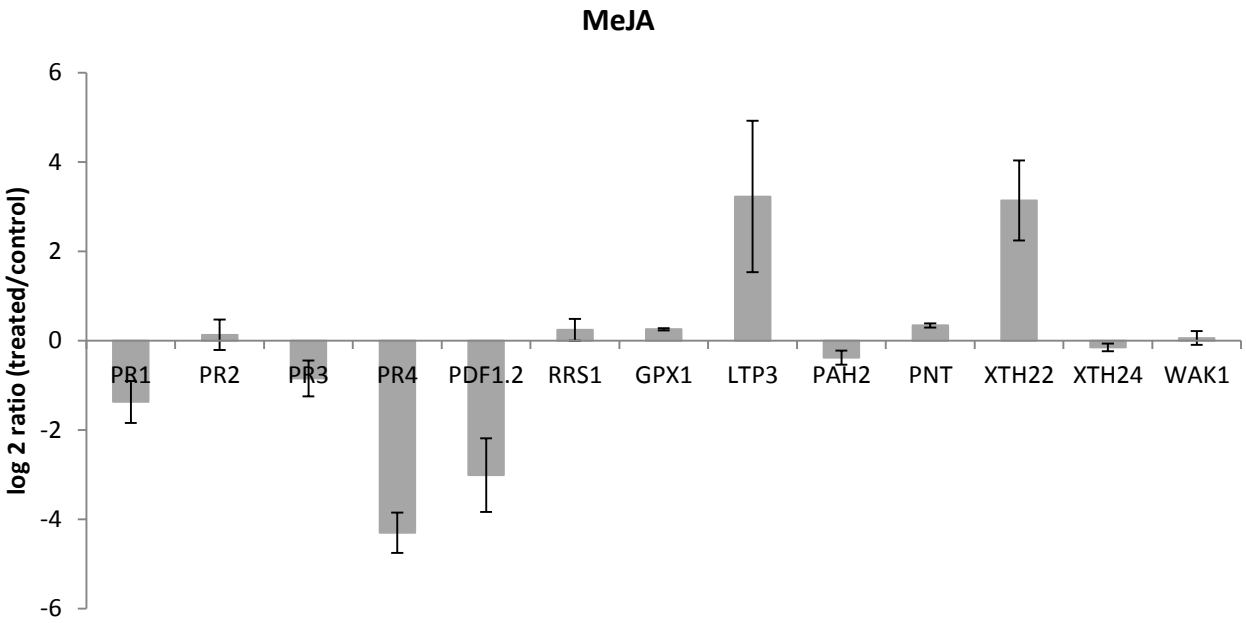


Figure 3.18: Expression of candidate defence response genes in *A. thaliana* plants treated with MeJA at 1h. The expression is relative to untreated plants. Error bars represent standard error of the mean of the biological replicates (n=3).

It appears that at 60 min after the treatment of plants with PAMP, EF-Tu Factor the expression of *AtPR1* was down regulated. The expression of *AtXTH22* appears to be up regulated (Fig.3.19). There is possibility that EF-Tu activates the expression of *AtXTH22* as early a 1h post treatment.

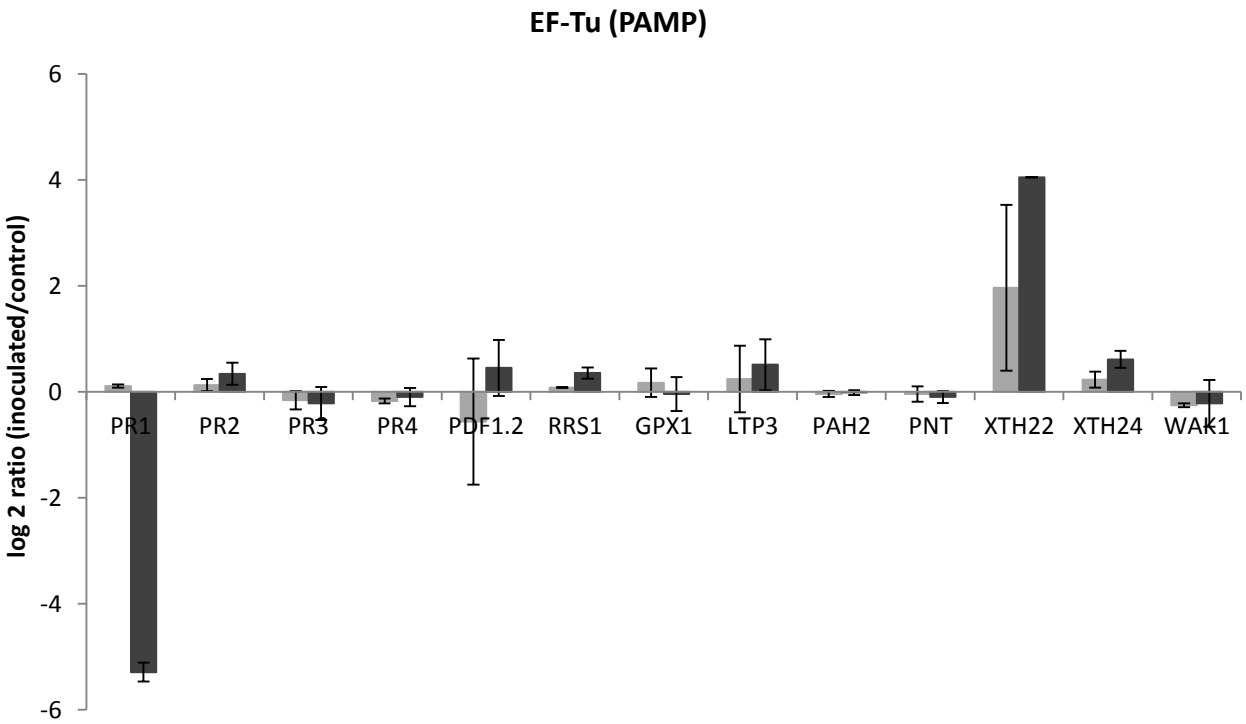


Figure 3.19 Expression of candidate defence response genes in *A. thaliana* Ler-0 plants treated with elicitor elongation factor EF-TU at 30 (light shaded bars) and 60 (dark shaded bars) minutes. Expression levels obtained from Genevestigator microarray database (www.genevestigator.com). The expression is relative to untreated plants. Error bars represent standard error of the mean of the biological replicates (n=2).

AtPR3 and *AtPR4* seem to be induced during treatment of the plants with oligogalacturonides from pathogens. *AtWAK1* appears up regulated by oligogalacturonides which is expected since it codes for oligogalacturonides receptors (Brutus et al., 2010). However, *AtXTH22* and *AtXTH24* appear down regulated at 30min and 60min.

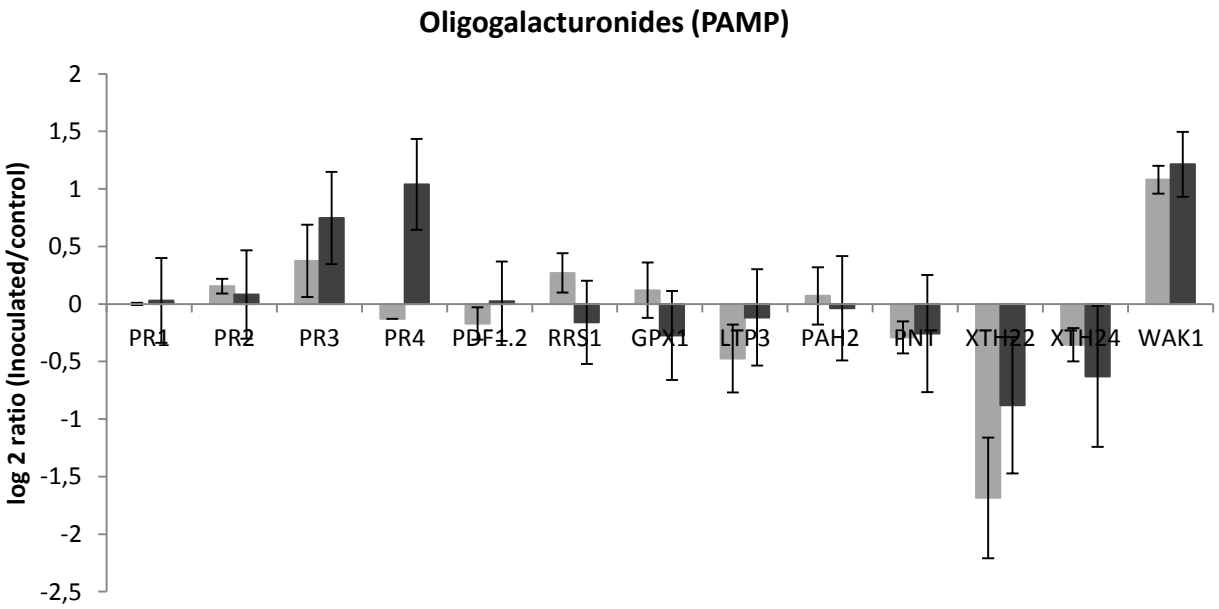


Figure 3.20 Expression of candidate defence response genes in *A. thaliana*, Col-0 plants treated with fungal oligogalacturonides as elicitors of defence responses at 1h (light shaded bars) and 3h (dark shaded bars) post inoculation. The expression is relative to untreated plants. Error bars represent standard error of the mean of the biological replicates (n=3).

Treatment of plants with fungal pathogens such as *Botrytis cinerea* induces the expression of known *PR* genes, *PR1*, *PR2*, *PR3*, *PR4* and *PDF 1.2* respectively (Fig 3.21). *AtXTH24* seem to be slightly up regulated. *AtGPX1* and *AtXTH22* appear a bit down regulated. *AtXTH24* looks up regulated at 48 hours post inoculation. *AtWAK1* also appears up regulated at 18 hours post inoculation.

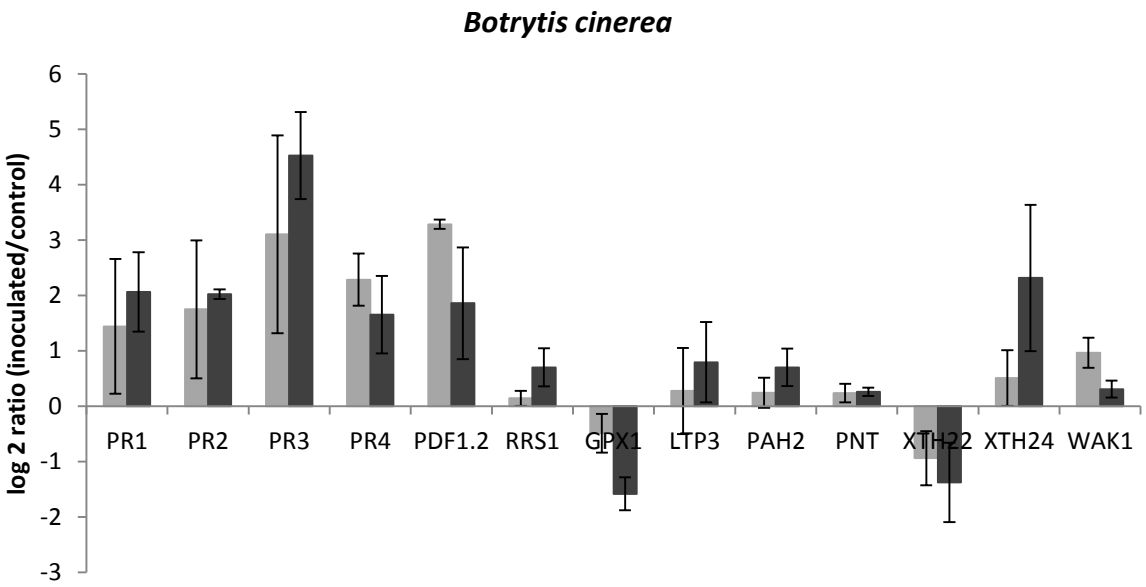


Figure 3.21 Expression of candidate defence response genes in *Arabidopsis* Col-0 plants inoculated with *B. cinerea* at 18 (light shaded bars) and 48 (dark shaded bars) days post inoculation. The expression is relative to untreated plants. Error bars represent standard error of the mean of the biological replicates (n=3).

PR genes were induced by treatment of plants with *Phytophthora infestans* (Fig 3.22). *AtPR1* is highly induced at 24 hours post inoculation whilst *AtPDF1.2* was highly induced as early as 6 hours post inoculation. *AtXTH24* appears up regulated at 12 hours post inoculation. *AtWAK1* seemed to be induced at 6 and 24 hours post inoculation. However, *AtXTH24* appears down regulated at 24 hours post inoculation (Fig 3.22).

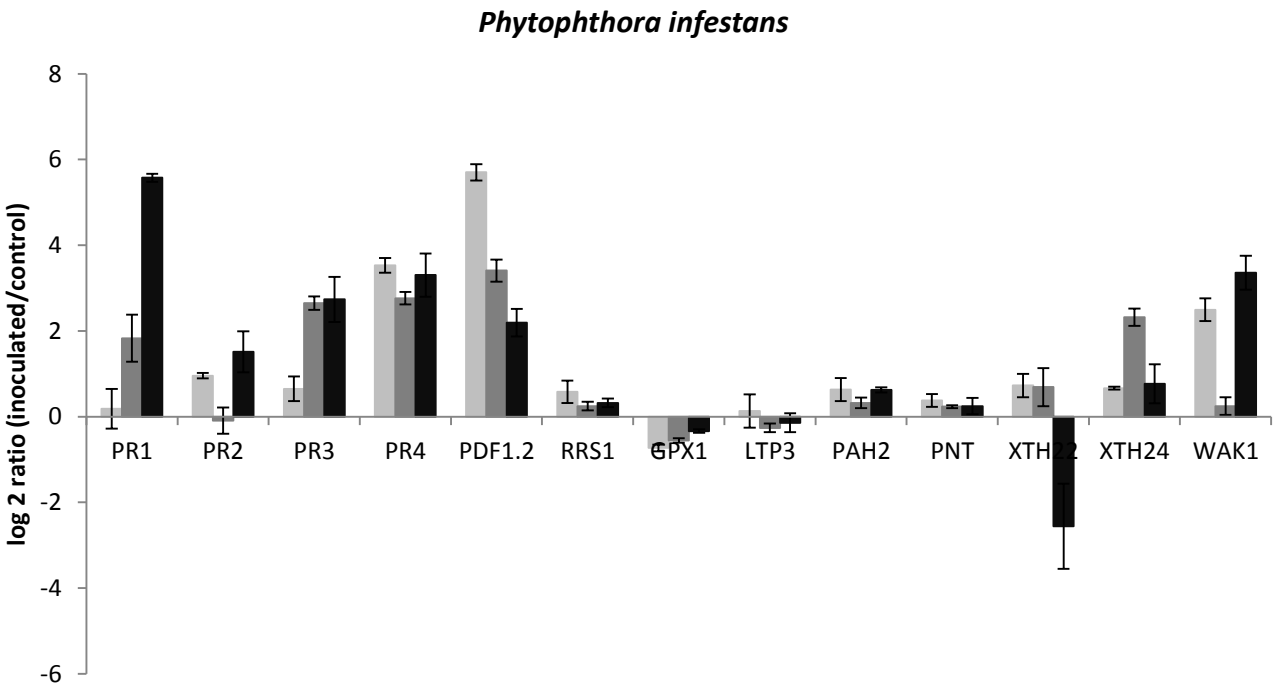


Figure 3.22: Expression of candidate defence response genes in susceptible *Arabidopsis* Col-0 plants inoculated with *P. infestans* at 6 (light shaded bars), 12 (medium shaded bars) and 24 (dark shaded bars) hours post inoculation. The expression is relative to untreated plants. Error bars represent standard error of the mean of the dividends of the inoculated and control biological replicates (n=3).

AtPR2 was up regulated by inoculation of plants with bacterial pathogen, *Pseudomonas syringae* (Fig 3.23). However, *AtPR3* and *AtPDF1.2* appear down regulated. *AtXTH22* was also down regulated by *P. syringae*. *AtWAK1* was up regulated by the bacterial plant pathogen (Fig 3.23).

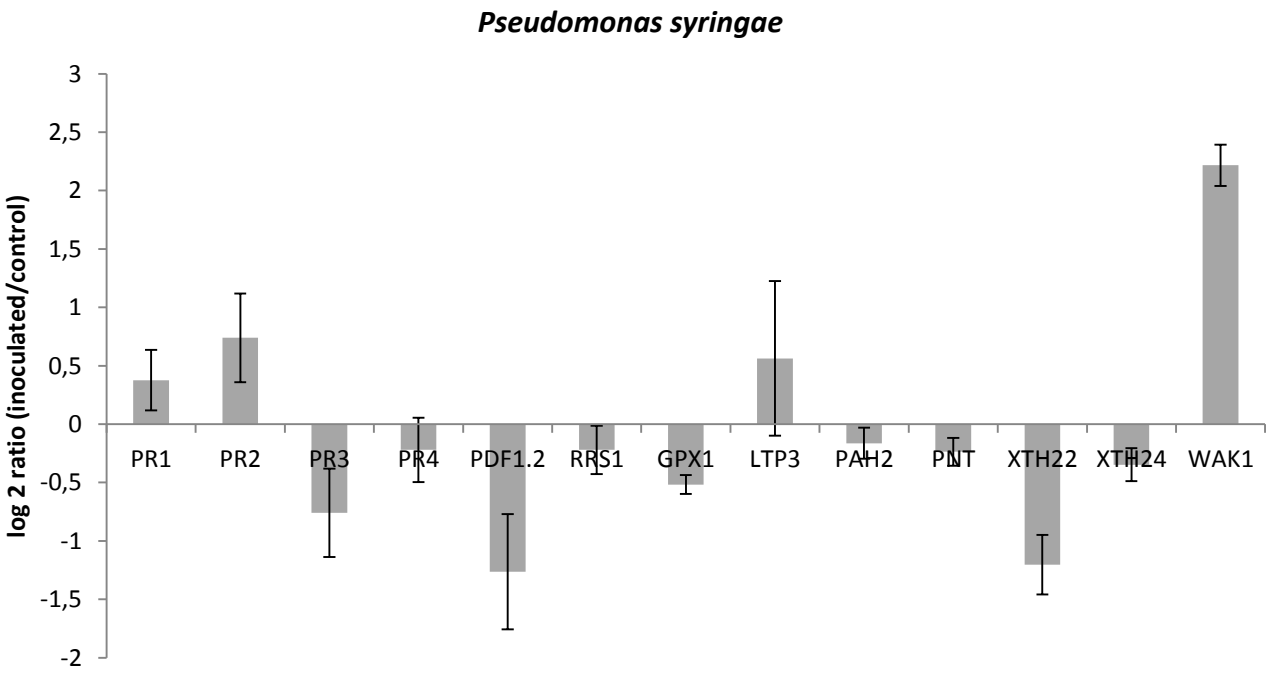


Figure 3.23: Expression of candidate defence response genes in *A. thaliana*, Col-5 plants inoculated with the bacterial plant pathogen *P. syringae* (DC3000). The expression is relative to untreated plants. Error bars represent standard error of the mean of the biological replicates (n=3).

When plants were inoculated with the bacterial wilt pathogen, *R. solanacearum AtPR3* appeared up regulated by bacterial wilt. *AtPDF1.2* was down regulated. However, *AtLTP3* was highly up regulated by *R. solanacearum*. *AtXTH24* also appears up regulated.

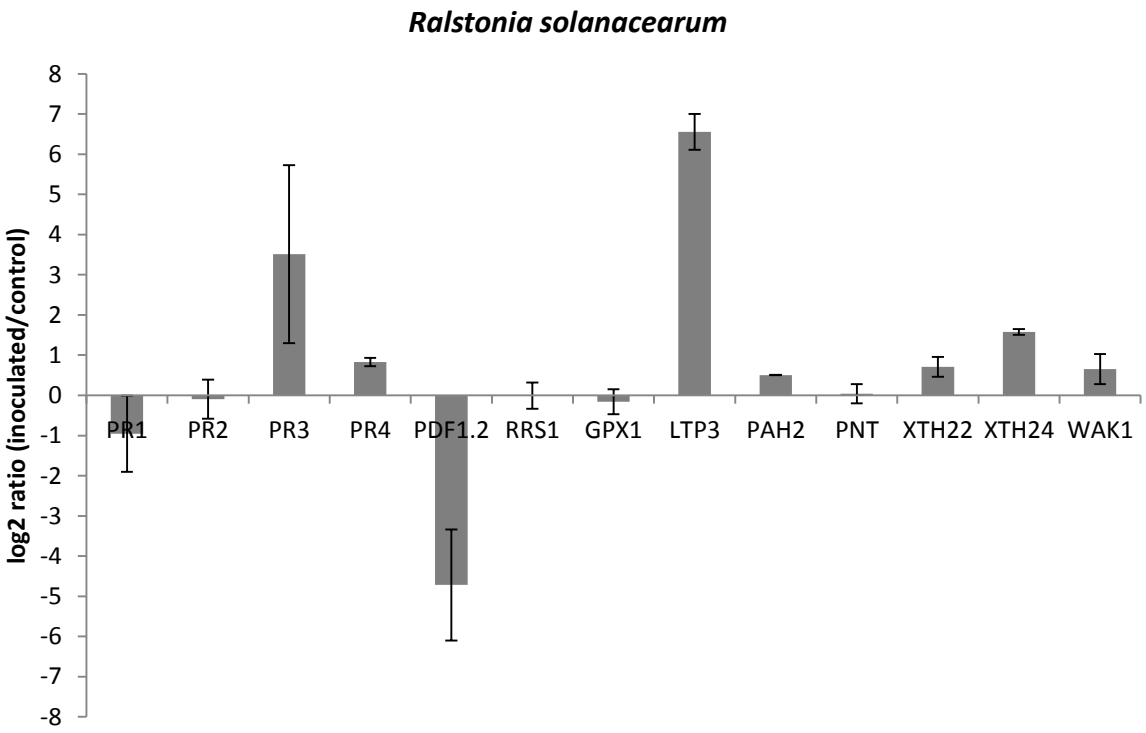


Figure 3.24: Expression of candidate defence response genes in a susceptible plant interaction where Col-0 plants are inoculated with *R. solanacearum* (GMI1000). The expression is relative to untreated plants. Error bars indicate the standard error calculated from two replicates of inoculated plants (n=2).

The overall *in silico* data shows possibility of the candidate defence genes potential role in defence responses against biotic stresses. Although the data appears non significant, it gives a fair indication of the potential defence responses of the selected genes. More time points would be able to elucidate more insights into the puzzle of defence related responses. There was no obtainable data for *AtDRG* in Genevestigator analysis, as there is no probe available for the transcript.

3.3 Expression profiling of candidate defence response genes in trial 1

3.3.1 Primer design

Primers for RT-qPCR were designed based on the full length cDNA sequence of Col-0 available from TAIR. Fig 3.25 shows a schematic representation for each gene and the primer pair positions. Some of the primer pairs span introns such as *AtGPX1*, *AtLTP3* and *AtXTH24* (Fig. 3.25 B, C and G) and others lie within the exon such as in the cases of *AtPAH2*, *AtPNT* and *AtXTH22* (Fig 3.25 D, E and F). The *AtDRG* primer pair was designed at the 3' UTR of the gene (Fig 3.25A). This was to ensure that the AtDRG was specifically targeted since the gene sequence is closely conserved with the sequence of At5G43470 and At5G35450. The *AtWAK1* primer pair was designed in a manner where the forward primer lies within the exon and the reverse primer is situated in the 3' UTR of the gene (Fig 3.25H). This was to ensure that only *AtWAK1* is targeted since it is highly conserved with its five family members, *AtWAK1-AtWAK5*. Table 3.3 show the primer pair sequences and the product sizes for each target gene. It is import for RT-qPCR amplicons not to exceed the size of 300bp in avoidance of super coiling of the amplicons or development of secondary structures that can inhibit efficiency of RT-qPCR.

Table 3.3 Designed RT-qPCR primers for candidate defence response genes

Gene name	AGI number	Forward primer 5'-3'	Reverse primer 5'-3'	Amplicon size (bp)
AtDRG	AT5G48620	TGGACAGTCTCAACGACTAT	AGATAGAGGCTGCTTCACAA	119
AtGPX1	AT2G25080	TCACCGTTAAGGACATTGAT	ATAAGGTTGACGTGAATGGA	284
AtLTP3	AT5G95320	TCCATGTGCAACCTATCTAT	CACTAACTGCAACAACATCA	239
AtPAH2	AT5G42870	CCTTGTCAGAGCCGATTAAC	CTAATCCTGAAGTGGTCGAG	287
AtPNT	AT5G11770	GAAGATTCCGTCTGGAATGG	AACTTCTGTCTCTTCCTCCG	134
AtXTH22	AT5G57560	ATGGAACTCCGATCAGAGAA	TCTTGTCCTAATGCCTCGAA	232
AtXTH24	AT4G30270	TAAGCTTGTTCCTGGTAACT	AGAATCATATTGACCGTCGA	252
AtWAK1	AT1G21250	TTGATTGGTGGTCACATCTT	ATACTAACCAGTAGCCAATC	272

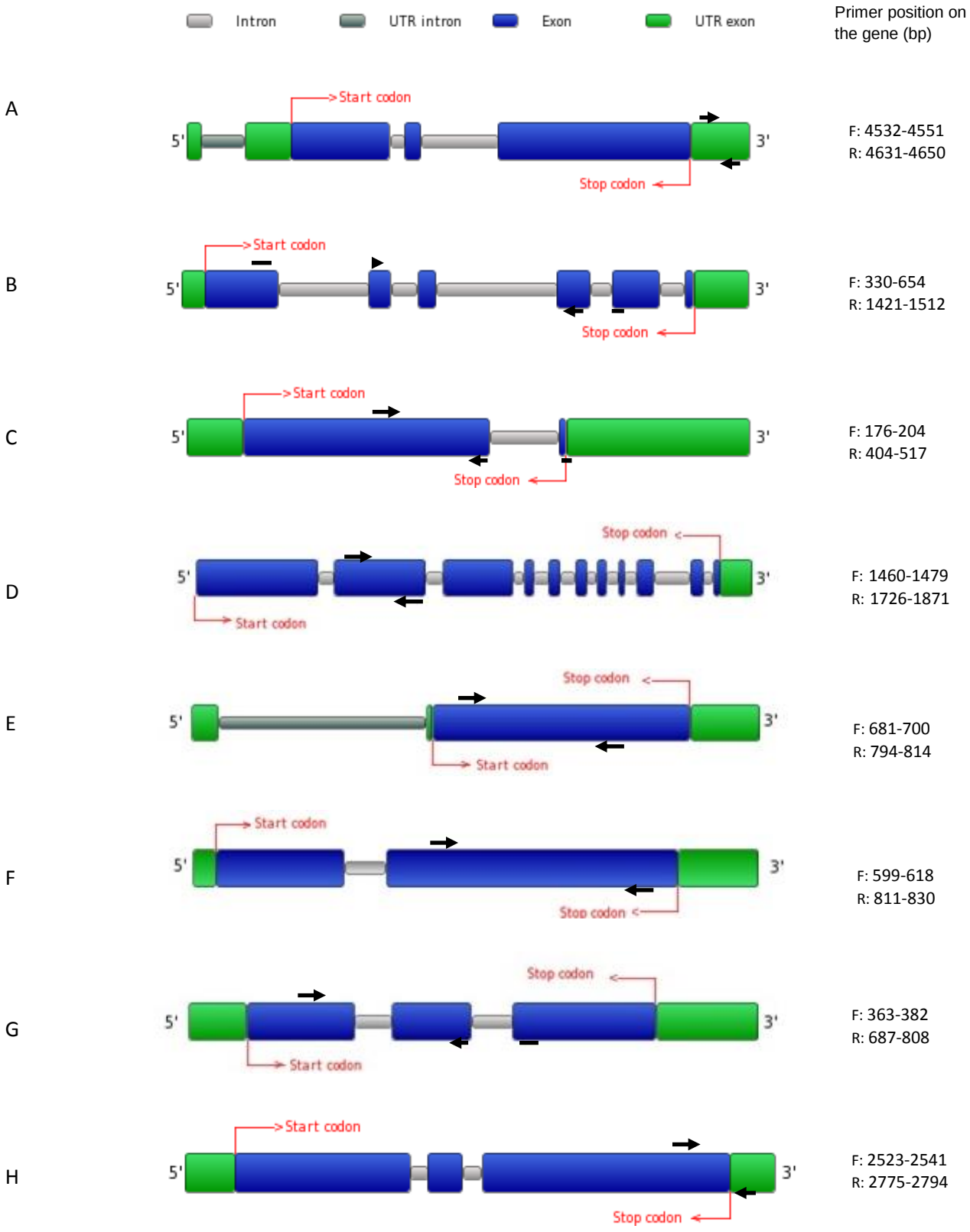


Figure 3.25 Col-0 specific gene structures of the eight candidate defence response genes obtained from Plaza (www.bioinformatics.psb.ugent.be/plaza). The genes structures show the RT-qPCR primer pair positions designed for each gene. Forward primers are indicated by top positioned arrows and reverse primers are indicated by the bottom arrows. A) *AtDRG*, B) *AtGPX1*, C) *AtLTP3*, D) *AtPAH2*, E) *AtPNT*, F) *AtXTH22*, G) *AtXTH24*, and H) *AtWAK1*.

3.4 Trial 1: RNA isolations from *A. thaliana* inoculated with *R. solanacearum* (BCC 402/CK) at 4dpi

A previous plant trial, designated Trial 1, was conducted by Naidoo, R (Naidoo, 2008a). Kil-0 and Be-0 plants were inoculated and mock inoculated with *R. solanacearum*, CK. The material was harvested at 4dpi. Total RNA was isolated from this plant material. Figure 3.26 shows the RNA extracted from these samples. The concentrations are indicated in Table 3.4- 3.6. Figure 3.26A shows an absense of RNA in lane 5 and 6. Thus re-extractions were necessary and the RNA thereof is indiated in Fig. 3.26 B and C. In order to maximize the RNA yield, a second elution of RNA were performed for all the samples. Table 3.4, 3.5 and 3.6 show the quality and concentration of the RNA from the first extractions, second extractions and the pooled first and second extractions respectively. Although all RNA subunits can be seen in Fig 3.26A, in Fig. 3.26B and C they are absent because the electrophoresis gel was run for a shorter period of time. The OD260nm/230nm ratio is an indication of the purity of the RNA and a value of 1.8-2 indicates high quality RNA free of protein contamination. The purity and concentration is high for most of the RNA samples except Be-0 3 control (Table 3.4). Kil-0 3 control yielded the least RNA concentration in the second extractions (Table 3.5). However, the overall pooled first and second extractions show purified and good concentrations of RNA (Table 3.6). Moreover, Table 3.5 showed that the second elution provided additional RNA and is thus recommended for further RNA isolations.

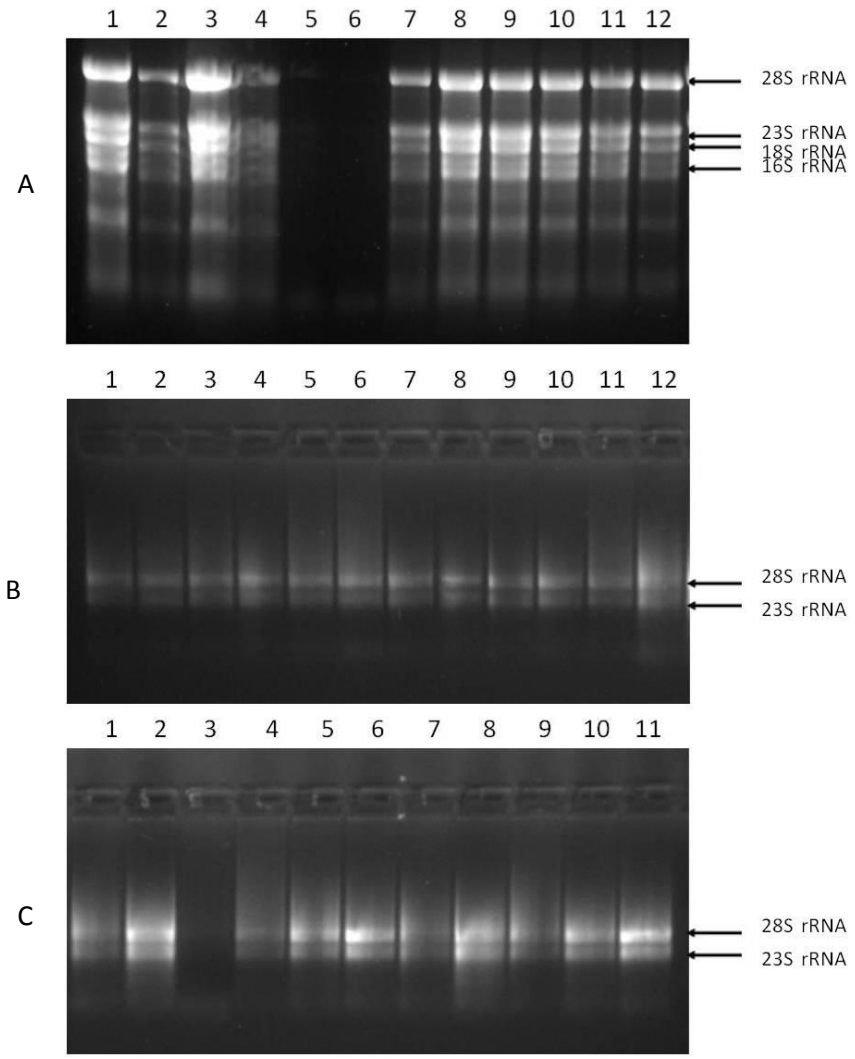


Figure 3.26: A 1.2% (w/v) agarose gel illustrating total RNA extracted at 4dpi from *A. thaliana* (Trial 1). A) Lane 1-3 represents Kil-0 inoculated biological replicates. Lane 4-6 represents Kil-0 control biological replicates. Lane 7-9 represents Be-0 inoculated biological replicates and lane 10-12 represents Be-0 control biological replicates. B) Total RNA re-extracted from stored Be-0 leaf material and column eluted twice. Lane 1-3 represents Be-0 control biological replicates from the first column elution; lane 4-6 represents Be-0 inoculated biological replicates from the first column elution; lane 7-9 represents Be-0 inoculated biological replicates from second column elution and lane 10-12 represents Be-0 control biological replicates from second column elution. C) Total RNA re-extracted from stored Kil-0 leaf material and column eluted twice. Lane 1-2 represents Kil-0 control biological replicates from the first column elution, lane 3 represents Kil-0 control biological replicates where both column elution 1 and 2 were pooled together. Lane 4-6 represents Kil-0 inoculated biological replicates from first column elution; lane 7-9 represents Kil-0 control biological replicates from the second column elution and lane 10-11 represents Kil-0 inoculated biological replicates from the second column elution.

Table 3.4 Quality and concentration of RNA samples from the first extractions of trial 1 plant tissue

Sample	OD260/230	Concentration (ng/µl)
Be-0 1 control	2.12	526.5
Be-0 2 control	2.10	384.7
Be-0 3 control	1.5	91.0
Kil-0 1 control	2.14	639.5
Kil-0 2 control	2.16	1059.1
Kil-0 3 control	2.13	2028.8
Be-0 1 inoculated	2.05	150.1
Be-0 2 inoculated	2.17	933.8
Be-0 3 inoculated	2.11	490.6
Kil-0 1 inoculated	2.15	868.4
Kil-0 2 inoculated	2.19	260.4
Kil-0 3 inoculated	2.16	1182.0

Table 3.5 Quality and concentrations of RNA samples from the second extractions from trial 1 plant tissue

Sample	OD260/230 at E1	OD260/230 at E2	Conc. E1(ng/µl)	Conc. E2(ng/µl)
Be-0 1 control	2.15	2.13	1644.6	355.9
Be-0 2 control	2.17	2.18	943.1	957.5
Be-0 3 control	2.15	2.10	1014.3	473.7
Kil-0 1 control	2.20	2.17	851.5	1086.1
Kil-0 2 control	2.19	2.14	763.6	1802.3
Kil-0 3 control	2.12	2.10	57.7	66.5
Be-0 1 inoculated	2.15	2.13	993.0	870.9
Be-0 2 inoculated	2.11	2.12	402.8	234.8
Be-0 3 inoculated	2.21	2.11	185.2	228.0
Kil-0 1 inoculated	2.13	2.22	405.8	785.5
Kil-0 2 inoculated	2.19	2.17	668.0	1462.8
Kil-0 3 inoculated	2.16	2.19	575.7	1006.6

*E1 represents Elution 1 and E2 represents Elution 2

Table 3.6 Quality and concentration of Pooled RNA samples from extraction 1 and extraction 2 of trial 1 plant tissue

Sample	OD260/230	Concentration (ng/µl)
Be-0 1 control	2.15	790.1
Be-0 2 control	2.19	700.6
Be-0 3 control	2.15	526.9
Kil-0 1 control	2.16	954.7
Kil-0 2 control	2.18	1261.1
Kil-0 3 control	2.19	370.4
Be-0 1 inoculated	2.19	753.0
Be-0 2 inoculated	2.08	263.5
Be-0 3 inoculated	2.12	190.8
Kil-0 1 inoculated	2.13	562.0
Kil-0 2 inoculated	2.14	1053.7
Kil-0 3 inoculated	2.13	686.2

3.4.1 RT-PCR of candidate defence response genes for Trial 1

Following total RNA isolations, RT-PCR was conducted with gene specific primers (Table 3.3) to confirm the integrity of the cDNA. All gene specific primers amplified correct size amplicons of all target and reference genes as indicated in Fig. 3.27. Single products were obtained in all primer sets with little to null primer dimers in both Be-0 and Kil-0 (Fig. 3.27). It can be confirmed from the figure that there was no gDNA contamination as the intron spanning primer pairs (AtGPX1, AtLTP3, AtXTH24, AtACT2 and AtCPB20) amplified the correct size products. These are further confirmed by the 372bp gDNA product (Fig.3.27F) for AtACT2 and the 242bp product from cDNA. In Be-0 control and inoculated samples, the product seem to be in low concentrations as indicated by the less intense fragment (Fig. 3.27A and B) as compared to Kil-0 control and inoculated samples (Fig. 3.27C and D), which showed more intensity fragment bands. Figure 3.27E showed the non-template RT-PCR controls for all the target and reference genes. Fragments around 50bp indicate primer dimers as seen in lane 12 for AtTUB4 primers (Fig 3.27E).

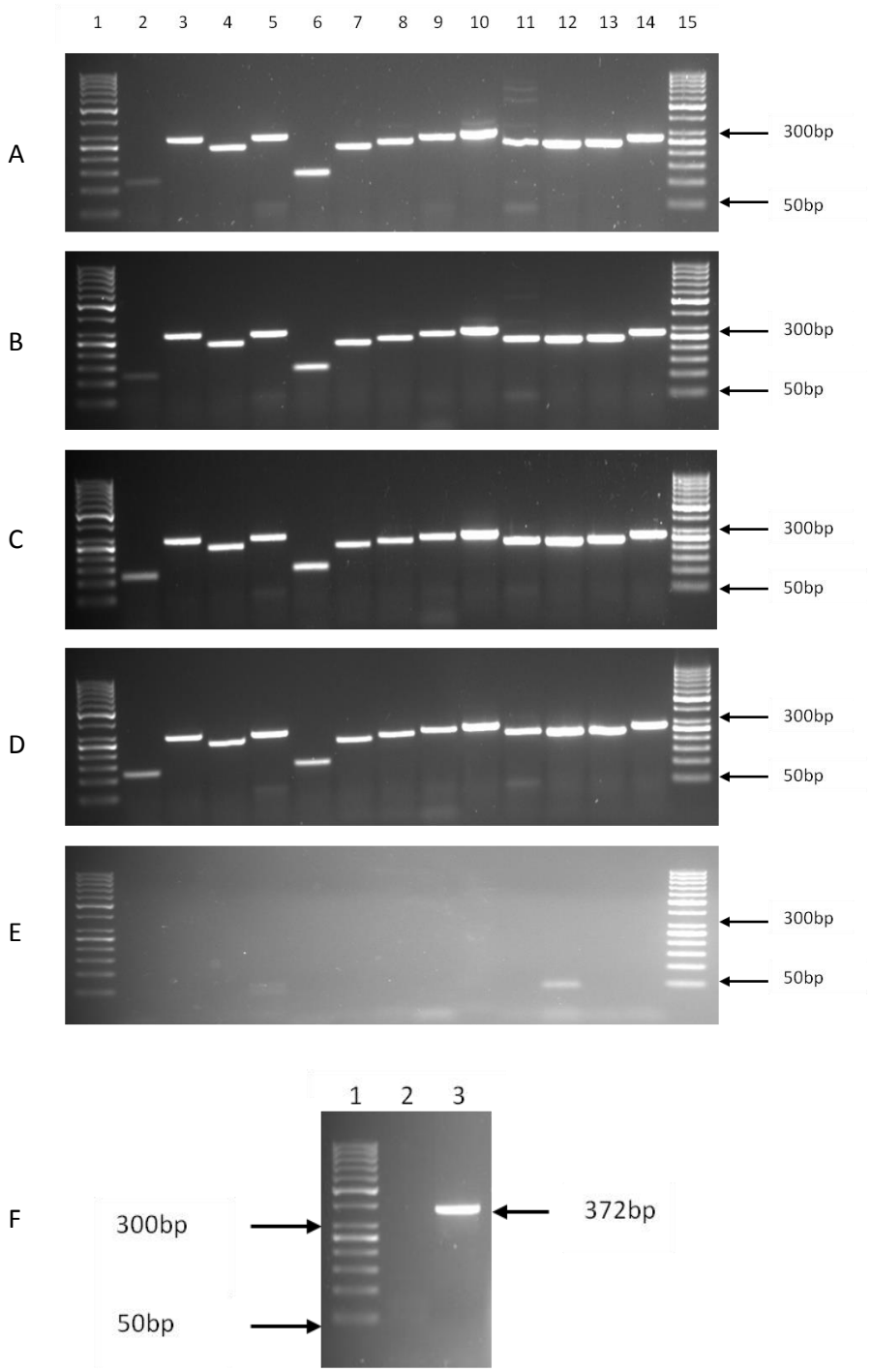


Figure 3.27 A 2 % (w/v) agarose gel illustrating RT-PCR products of Be-0 and Kil-0 inoculated and control cDNA samples. All three biological replicates of all control and inoculated samples were pooled together. Lane 1 and 15 is a 50bp ladder (Fermentas). Lanes 2-9 represent all target genes and lanes 10-14 represent reference genes. Lane 2: *AtDRG*; lane 3: *AtGPX1*; lane 4*: AtLTP3*; lane 5: *AtPAH2*; lane 6: *AtPNT*; lane 7: *AtXTH22*; lane 8: *AtXTH24*; lane 9: *AtWAK1*; lane 10: *AtACT2*; lane 11: *AtCBP20*; lane 12: *AtELF1α*; lane 13: *AtTUB4* and lane 14: *AtUBQ5*. A) Be-0 control B) Be-0 inoculated C) Kil-0 control and D) Kil-0 inoculated. E) No template controls. F) *AtACT2* amplified from Kil-0 inoculated genomic DNA in lane 3 and no template control in lane 2.

3.4.2 RT-qPCR using the LightCycler 480 and gene specific primers

Relative expression profiling of candidate defence response genes and reference genes was performed at 4dpi using the LightCycler (Roche) and qBase v1.2 software from Biogazelle. Melt curve analysis produced distinct single peaks (Fig 3.28 and Fig. 3.29) for most of the candidate defence response and reference genes. This excluded *AtWAK1* which indicated two peaks (Appendix B). It is precisely important to perform a melt curve analysis which indicates the temperature at which RT-qPCR amplicons denature and the fluorescent signal of the interchelating dye is detectable, to validate that only a single product was amplified. Reference genes were chosen based on the best M and CV value analyses in qBase v1.2 software from Biogazelle. The best combination chosen was *AtTUB4* and *AtUBQ5* with an M value of 0.325 and CV of 0.113. The technical reproducibility of the experiments was 92.5% as according to qBase v1.2 software. This is the measure of the high similarities between the technical replicates, allowing only 0.5 differences between the Cp values between technical replicates.

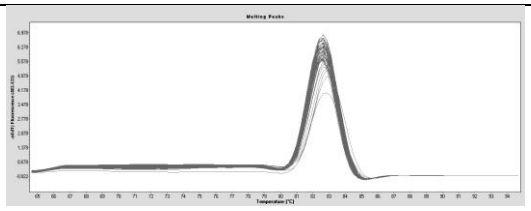
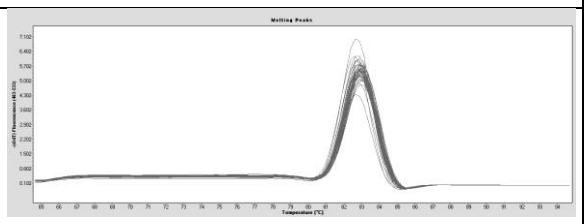
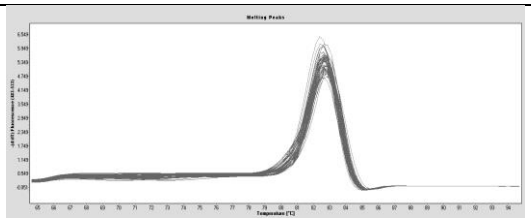
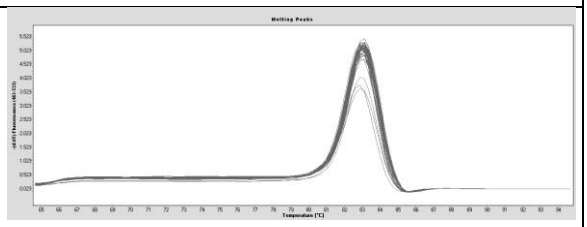
Gene	Standard plate	Quantification plate
AtTUB4		
AtUBQ5		

Figure 3.28 Melt curves of RT-qPCR products for the reference genes from the LightCycler 480 software (Roche). The X-axis indicates the temperature (°C) and the Y-axis indicates (d/dT) Fluorescence (483-533).

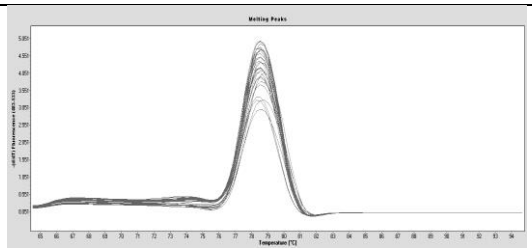
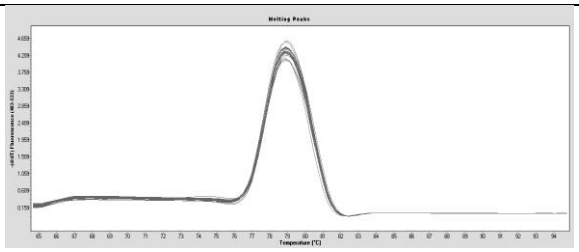
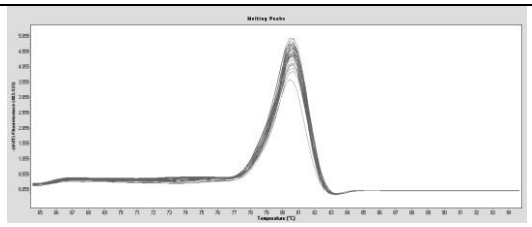
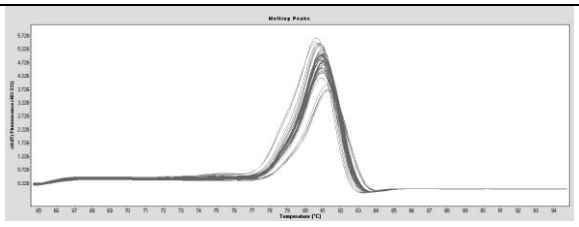
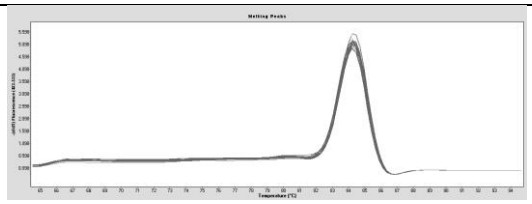
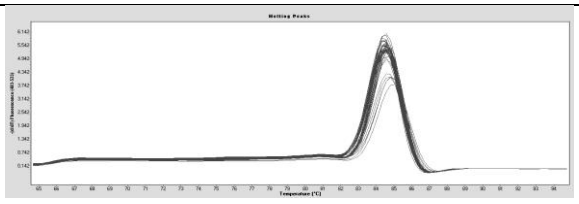
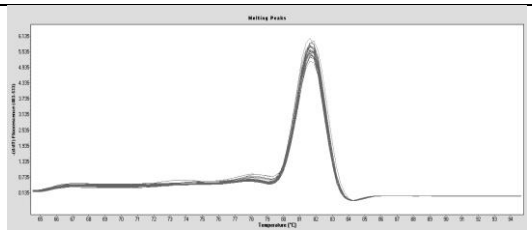
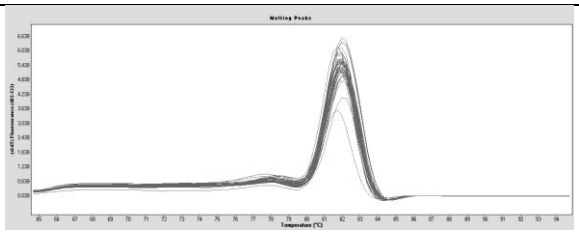
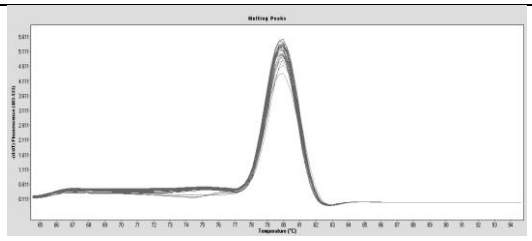
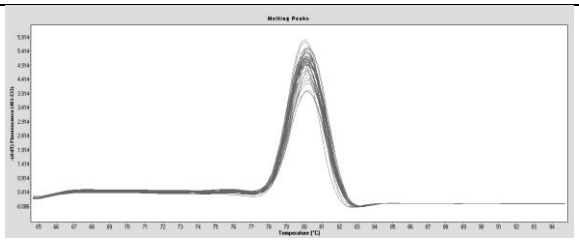
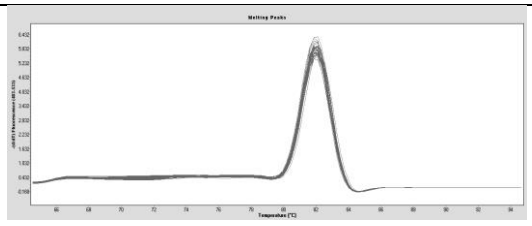
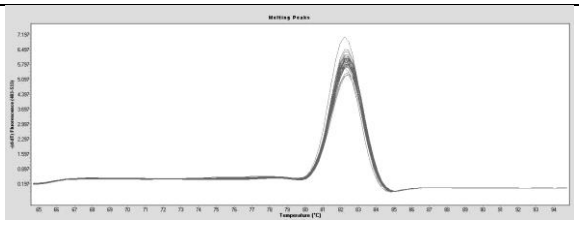
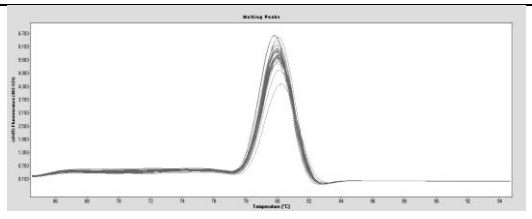
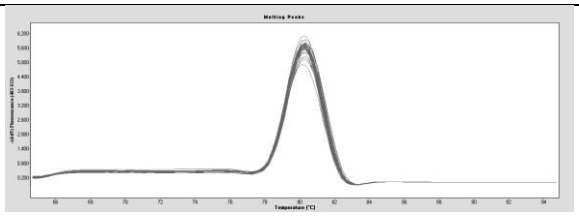
Gene	Standard plate	Quantification plate
AtDRG		
AtGPX1		
AtLTP3		
AtPAH2		
AtPNT		
AtXTH22		
AtXTH24		

Figure 3.29 Melting curves of RT-qPCR products for the candidate defence response genes from the LightCycler 480 software (Roche). The X-axis indicates the temperature (°C) and the Y-axis indicates (d/dT) Fluorescence (483-533).

Figure 3.30 shows the relative expression levels of the candidate defence response genes, *AtGPX1, AtLTP3, AtPAH2, AtPNT, AtXTH22* and *AtXTH24* respectively. The relative expression levels of these genes are normalised to reference genes, *AtTUB4* and *AtUBQ5*. *AtGPX1* is significantly expressed in Kil-0 inoculated at 4dpi as compared to Kil-0 control. There is no significant difference in Be-0 inoculated and control samples (Fig 3.30A). At 4dpi, *AtLTP3*, *AtPAH2* and *AtPNT* show no differential expression levels between inoculated samples and control samples, but it seems as though the level of expression of *AtLTP3* and *AtPAH2* was more in ecotype Be-0 than in Kil-0 (Fig 3.30B and C). *AtPNT* in Be-0 and Kil-0 was the same, there is no differential expression pattern in Be-0 and Kil-0 (Fig 3.30D). Moreover, *AtXTH22* and *AtXTH24* were significantly up regulated upon pathogen inoculation at 4dpi in Kil-0. *AtXTH22* seemed down regulated in Be-0 and again its expression levels were more in Be-0 than in Kil-0 (Fig 3.30E). *AtXTH24* was more or less similar in expression in both Kil-0 and Be-0 (Fig 3.30 F).

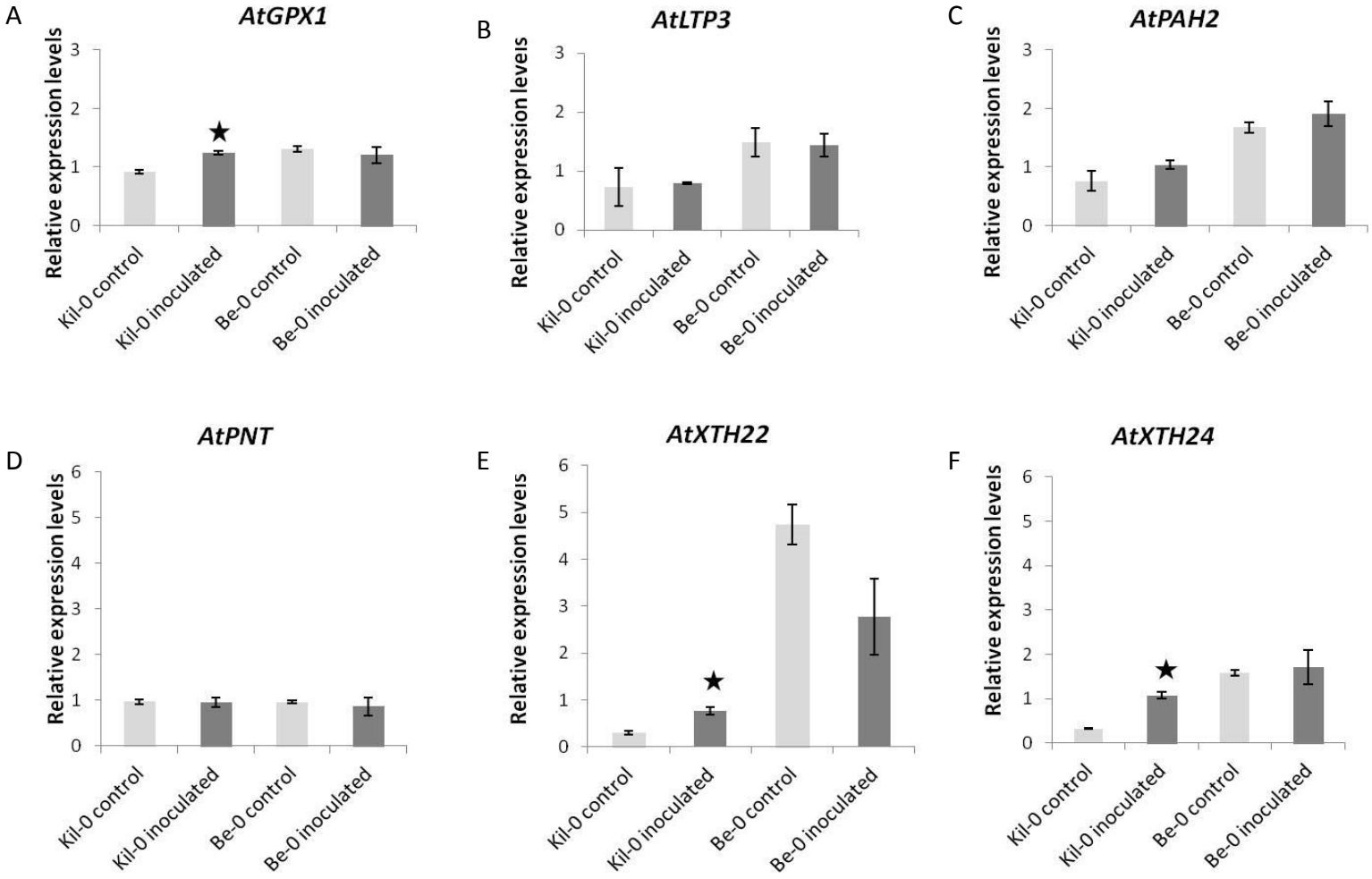


Figure 3.30. The relative expression profiles of candidate defence response genes in Kil-0 and Be-0 following inoculation with *R. solanacearum* after normalization with *AtTUB4* and *AtUBQ5*. Error bars show the standard error of the mean of the biological replicates (n=3). Significance is relative to the control in each graph and was calculated by the Student's t-test which is indicated by the black star ★ $p < 0.05$. A) *AtGPX1*, B) *AtLTP3*, C) *AtPAH2*, D) *AtPNT*, E) *AtXTH22* and F) *AtXTH24.*

Figure 3.31 shows the expression profile of *AtDRG* in Be-0 and Kil-0. *AtDRG* seems to be expressed at higher levels in Kil-0 than in Be-0, however, upon pathogen inoculation, *AtDRG* is down regulated in Kil-0. The expression of *AtDRG* is very low in Be-0. In Fig. 3.27A and B), the less intense bands on the gel supports that *AtDRG* is expressed at low levels in Be-0.

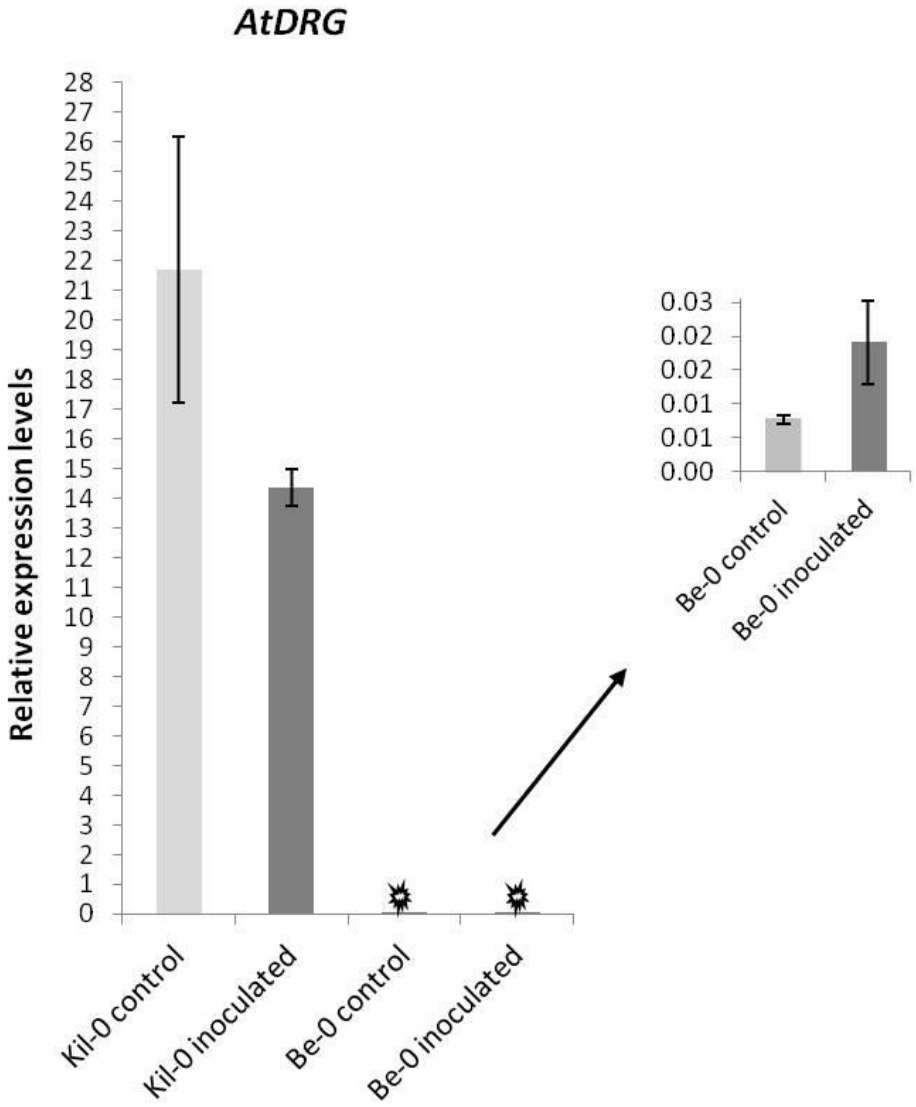


Figure 3.31 The relative expression profiles of *AtDRG* in Kil-0 and Be-0 following inoculation with *R. solanacearum* (BCC402) after normalization with *AtTUB4* and *AtUBQ5*. Error bars show the standard error of the mean of the biological replicates (n=3). Significance is relative to the control in each graph and was calculated by the Student's t-test which is indicated by the black star ★ p< 0.05✡indicate the relative expression level of less than 0.02.

Table 3.7 shows comparisons of the expression levels of the candidate defence response genes with the previous data from Naidoo (2008a) and Naidoo (2008b) at 4dpi in ecotype Be-0 and Kil-0. There is no known information for *AtGPX1*, *AtPAH2*, *AtXTH22*, and *AtDRG* in both Be-0 and Kil-0 in the previous data. However, *AtXTH24* was shown to be up regulated in Kil-0 in the previous data and there is no data for Be-0. *AtLTP3* seems to be significantly up regulated in Kil-0 as compared to Be-0 at 4dpi. *AtPNT* was down regulated in both Kil-0 and Be-0. In Trial 1, *AtGPX1* and *AtLTP3* were up regulated in Kil-0 compared to Be-0. *AtGPX1*, *AtXTH22* and *AtXTH24* are up significantly up regulated in Kil-0. *AtDRG* appeared up regulated in Be-0 and down regulated in Kil-0.

Table 3.7: Comparison of expression levels of candidate defence response genes (Fig 3.30 and 3.31) with previous data at 4dpi in Be-0 and Kil-0 indicated in log2 fold (inoculated/control)

Gene	Previous data					Current data (RT-qPCR)			
	Be-0	**P<0.05**	**Kil-0**	**P<0.05**	**Source**	**Be-0**	**P<0.05**	**Kil-0**	**P<0.05**
AtGPX1	N/D	N/D	N/D		SSHdb	-0.12	N	0.44	Y
AtLTP3	1.00	N	2.00	Y	RT-qPCR data	-0.04	N	0.11	N
AtPAH2	N/D	N/D	N/D		SSHdb	0.19	N	0.45	N
AtPNT	-0.1	N	-0.5	N	RT-qPCR data	0.14	N	0.03	N
AtXTH22	N/D	N/D	N/D		SSHdb	0.78	N	1.36	Y
AtXTH24	N/D	N/D	1.69		Naidoo (2008)	0.12	N	1.72	Y
AtDRG	N/D	N/D	N/D		SSHdb	1.17	N	-0.59	N

N/D= Not determined; N= No; Y=Yes

3.5 Expression profiling of selected candidate defence response genes in Trial 2

A. thaliana ecotypes, Kil-0 and Be-0 were inoculated with *R. solanacearum* CK strain and monitored for 15 dpi, and was designated Trial 2. Kil-0 showed delayed disease development as compared to Be-0 which developed disease symptoms as soon as 4dpi (Fig 3.32). Kil-0 showed slight disease symptom development only after 7-8 dpi. Disease symptoms remained mild throughout the course of the trial as seen on Figure 3.32, where at 15dpi, the disease index was only 0.1 in Kil-0 as compared to Be-0 which was 0.9 indicating no life in the plants.

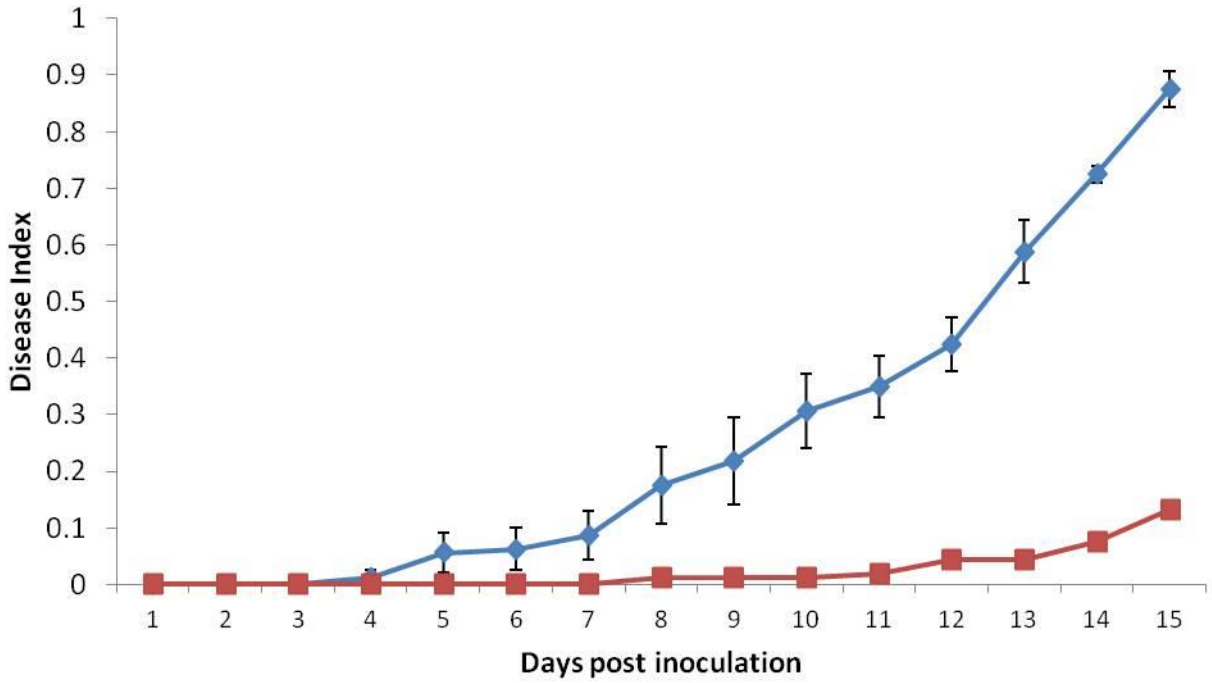


Figure 3.32: Disease Index of Be-0 (blue) and Kil-0 (red) after inoculation with *R. solanacearum* (BCC 402). Disease progression was monitored over a period of 15 dpi on four biological replicates per ecotype. Each replicate is comprised of four individual plants. Error bars indicate the standard error of the mean of four replicate disease indexes (n=4).

Total RNA was isolated from all harvested plant material at 0dpi, 4dpi, 7dpi and 10dpi. 0dpi is designated as the control samples for all the other time points. RNA quality was analysed on the denaturing formaldehyde agarose gel (Fig 3.33). All isolated Be-0 samples appeared intact as indicated by the four RNA subunits (Fig. 3.33A). Lane 4 on Fig. 3.33B indicated an absence of RNA, suggesting that Kil-0 biological replicate 1 harvested at 4dpi was unsuccessfully isolated. This necessitated for a re-isolation of the sample, which was successfully isolated (result not shown).

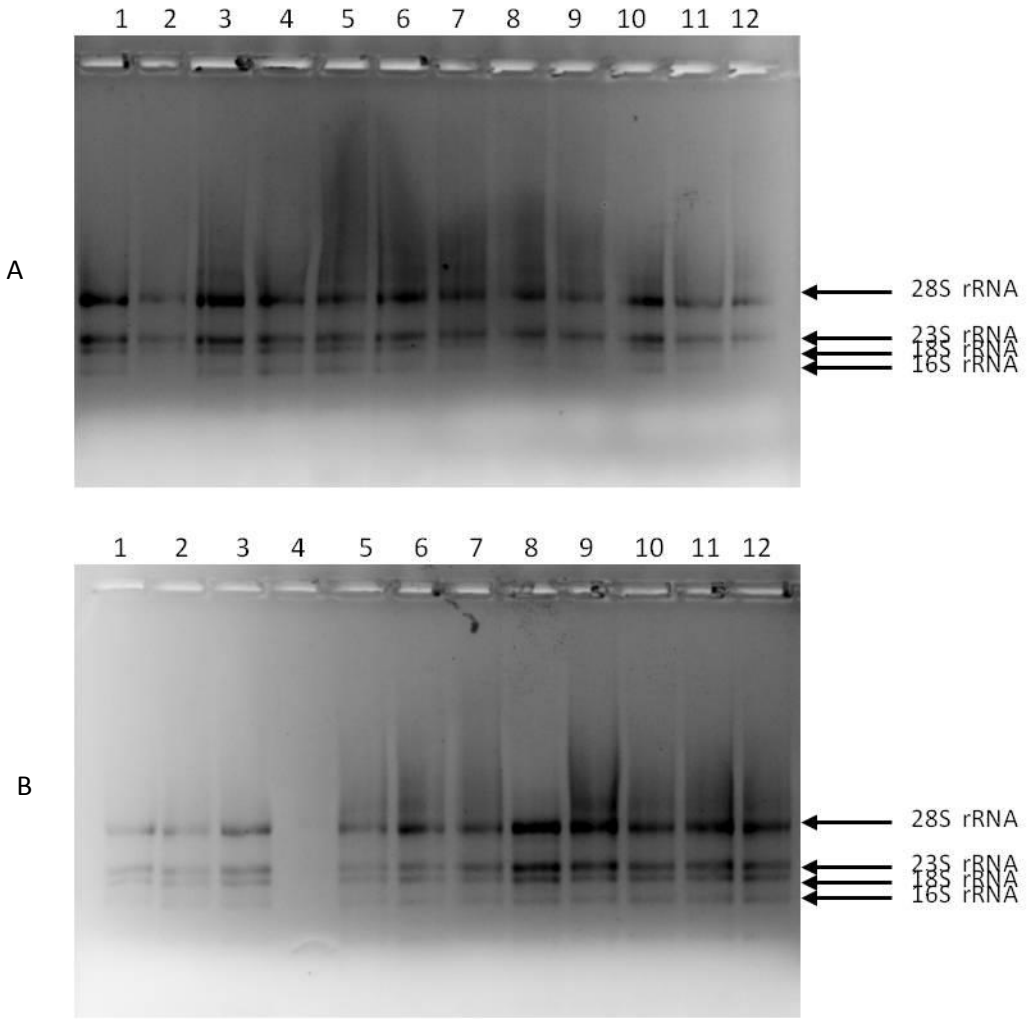


Figure 3.33: Total RNA isolated from Be-0 (A) and Kil-0 (B) at different time points post inoculation with *R. solanacearum*. All time points include three biological replicates. Every three lanes represent three biological replicates for each treatment. Lanes 1-3: Control plants at 0dpi, lanes 4-6: inoculated plants at 4dpi. Lanes 7-9: inoculated plants at 7dpi and lanes 10-12: inoculated plants at 10dpi.

Unfortunately the isolated RNA had genomic DNA contamination as indicated by the 372bp fragment on the gels (Fig 3.34). All Be-0 and Kil-0 RNA samples had gDNA contamination. The smears on the gels indicate the RNA. Therefore the RNA samples were subjected to DNAse1 treatment to shred the gDNA contamination followed by RNA clean up. Fig. 3.35 shows the purified RNA samples free of gDNA. Lane 4 shows the positive control of a 372bp fragment of *AtACT2* amplified from Kil-0 gDNA. The smears indicate the unamplified RNA.

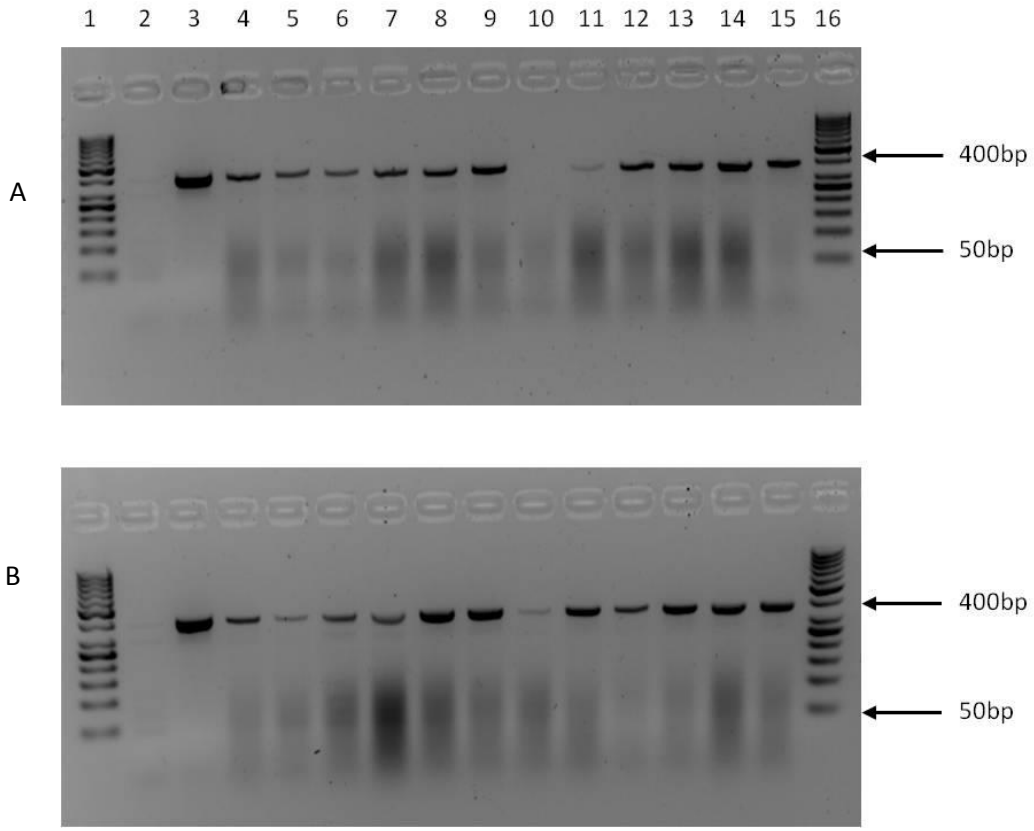


Figure 3.34: A 2% (w/v) agarose gel showing genomic DNA contamination in all isolated total RNA from Be-0 (A) and Kil-0 (B) inoculated and control samples amplified with *AtACT2* primers. Lanes 1 and 16: 50bp ladder, lanes 2: NTC, lanes 3: gDNA template, lanes 4-6: RNA at 0dpi, lanes 7-9: RNA at 4dpi, lanes 10-12: RNA at 7dpi and lanes 13-15: RNA at 10dpi.

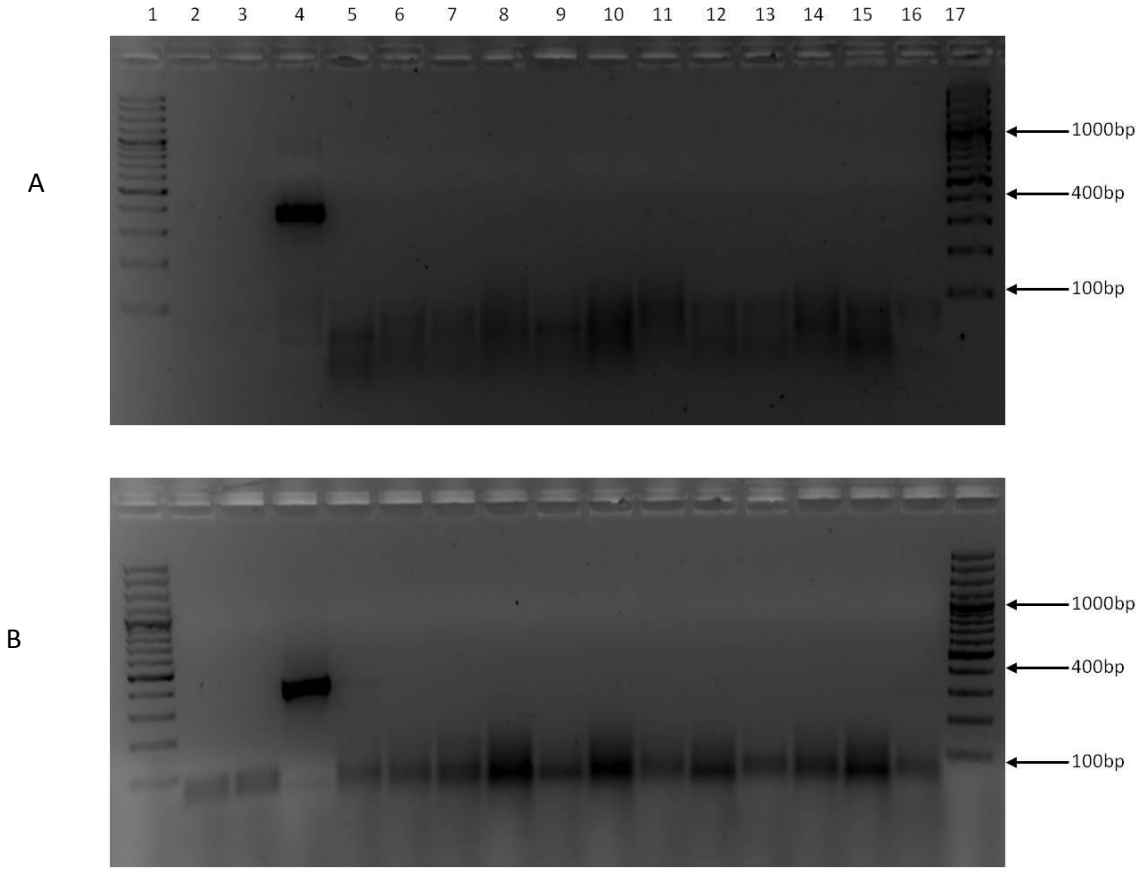


Figure 3.35: A 2% (w/v) agarose gel showing purified total RNA samples amplified with *AtACT2* primers. Lanes 1 and 17 represent the 100bp ladder (Fermentas). Lane 2-3 represents a no template control and lane 4 represents the gDNA template control. Lanes 5-16 represents three successive replicates of RNA templates at 0dpi, 4dpi, 7dpi and 10dpi respectively.

3.5.1 RT-PCR

Figure 3.36 shows agarose gels indicating the integrity of cDNA. The cDNA was amplified with *AtACT2* primers. The bands on the gels show the correct 242bp amplicon of *AtACT2* when amplifying cDNA. Lane 3 and 4 indicate positive control of the 372bp amlpicon amplified from gDNA. This indicates the purity and integrity of the RNA samples together with the quality of the cDNA.

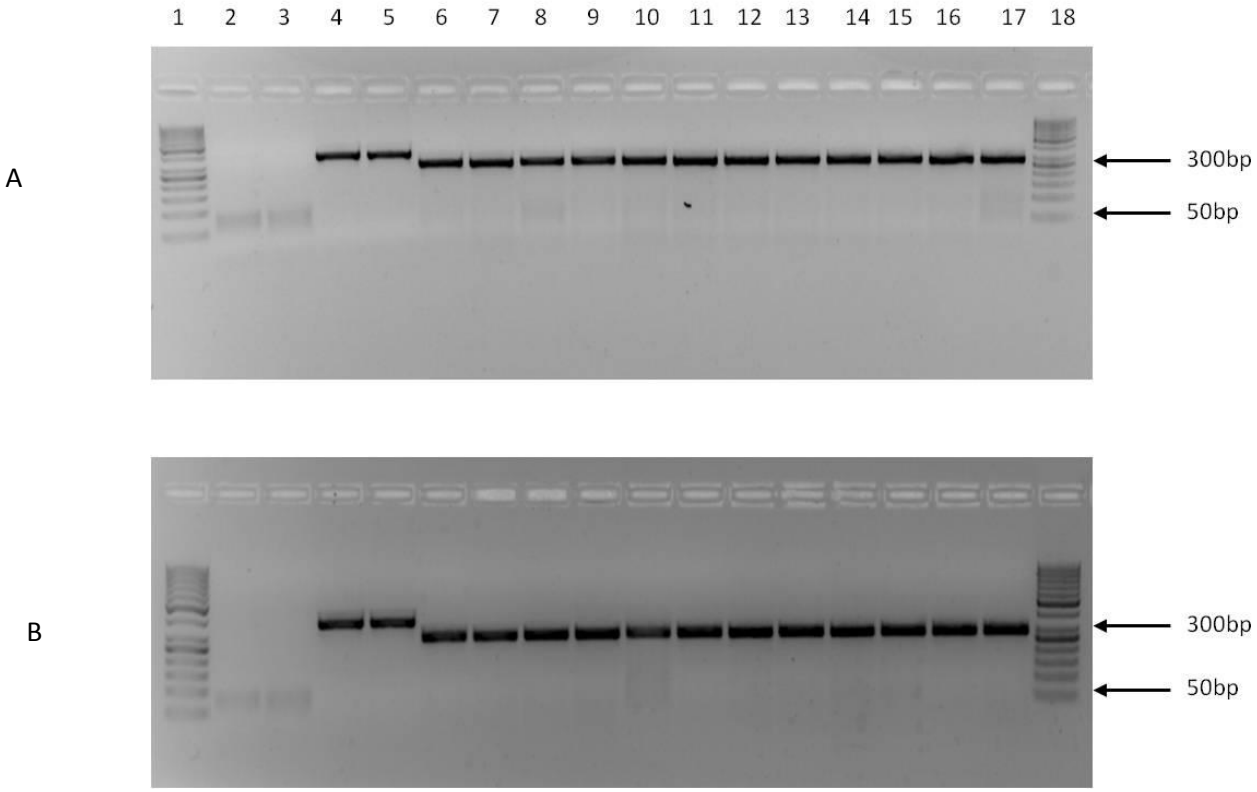
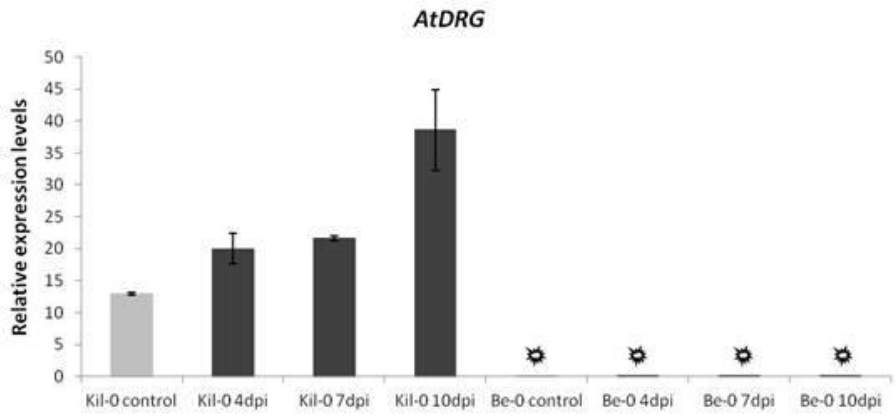


Figure 3.36: A 2 % (w/v) agarose gel illustrating RT-PCR products of Be-0 (A) and Kil-0 (B) control and inoculated cDNA samples. Lanes 1 and 18: 50bp ladder, lanes 2: Water template PCR control, lanes 3-4: no template control, lanes 4-5: gDNA template of 32ng/µl and 129ng/µl respectively. Lanes 6-8: 0dpi control cDNA samples, lanes 9-11: 4dpi cDNA samples, lanes 12-14: 7dpi cDNA samples and lanes 15-17: 10dpi cDNA samples.

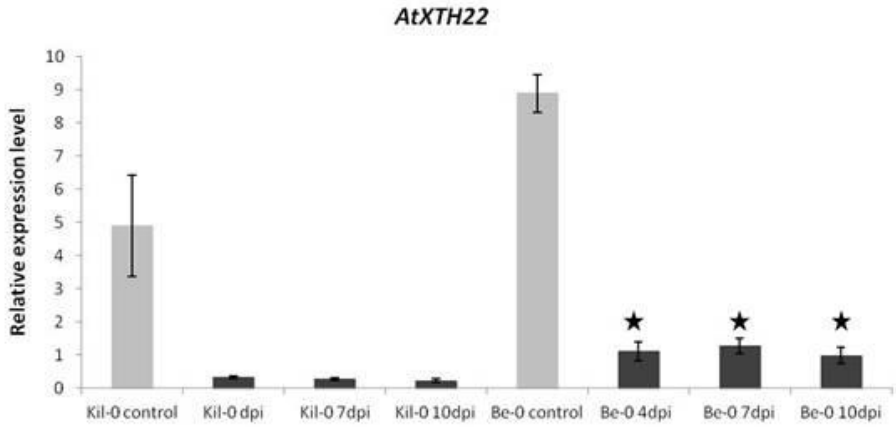
3.5.2 RT-qPCR

Figure 3.37 showed the relative expression levels of the selected candidate defence response genes from Trial 1 on to Trial 2 plant tissue, *AtDRG*, *AtXTH22* and *AtXTH24* respectively. The relative expression levels of these genes were normalised to reference genes, *AtACT2* and *AtUBQ5*. *AtDRG* appeared to be up regulated at 4, 7 and 10dpi. Significance in comparison to control tissue is illustrated at 7dpi. However, the level of expression of *AtDRG* is low in Be-0 and is more or less the same in control plants and inoculated plants. Again with reference from Fig 3.27A and B expression of *AtDRG* in Be-0 is very low. *AtXTH22* appeared to be down regulated in Kil-0 in all the time points. In Be-0 it is significantly down regulated. *AtXTH24* in both Be-0 and Kil-0 showed no differential expression.

A



B



C

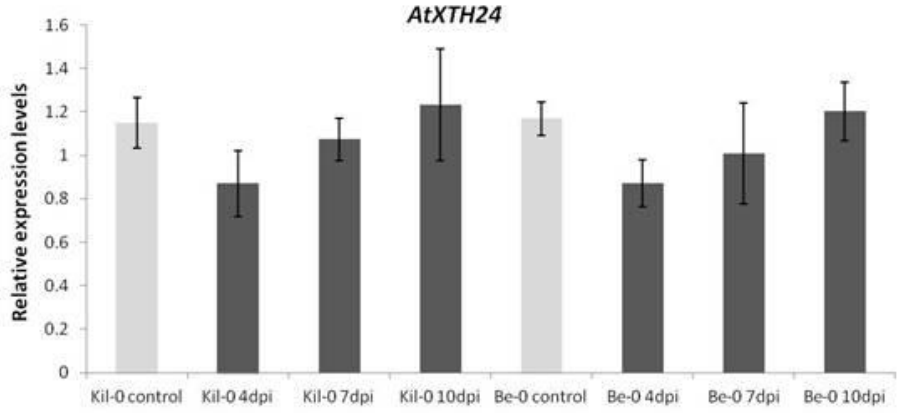


Figure 3.37. The relative expression levels of *AtDRG (A)*, *AtXTH22 (B)* and *AtXTH24 (C)* in Kil-0 and Be-0 following inoculation with *R. solanacearum,* normalized with *AtACT2* and *AtUBQ5*. The expression levels are at 0 (control), 7 and 10 dpi. Error bars show the standard error of the mean of the biological replicates (n=3). Significance is relative to the control in each graph and was calculated by the Student's t-test which is indicated by the star ★ $p < 0.05$. ✡ indicates levels less than 0.03.

3.5.3 Verification of RT-qPCR amplicons

Figure 3.38 shows the RT-PCR and RT-qPCR amplicons. Lanes 2 and 6 represent the RT-PCR products of Kil-0 and Be-0 *AtDRG* respectively (Fig 3.38A) and lanes 3-4 and lanes 7-9 represent the Kil-0 and Be-0 products obtained from the Lightcycler 384 well plate. The three successive lanes represent biological replicates for each ecotype respectively. As per settings of Fig. 3.38A, Fig 3.39B is set in a similar manner (RT-PCR followed by RT-qPCR). The amplicons were purified and sequenced.

Table 3.8 show the BLASTN results of the sequenced RT-qPCR products. This illustrates that the target genes under investigation were indeed the target genes originally identified as in section 3.1. *AtDRG* was sequenced both from Be-0 and Kil-0 backgrounds. All *AtDRG* products from both Kil-0 and Be-0 gave the correct 119bp product size from all selected plate wells (Fig 3.38A). *AtGPX1*, *AtLTP3*, *AtPAH2*, *AtPNT*, *AtXTH22* and *AtXTH24* also give the correct product sizes for all selected plate wells respectively (Fig 3.38B). Lanes 10-13 representing *AtPAH2* products also show primer dimers, however this was not a concern as there was a single peak for each of the melting curves (Fig 3.29).

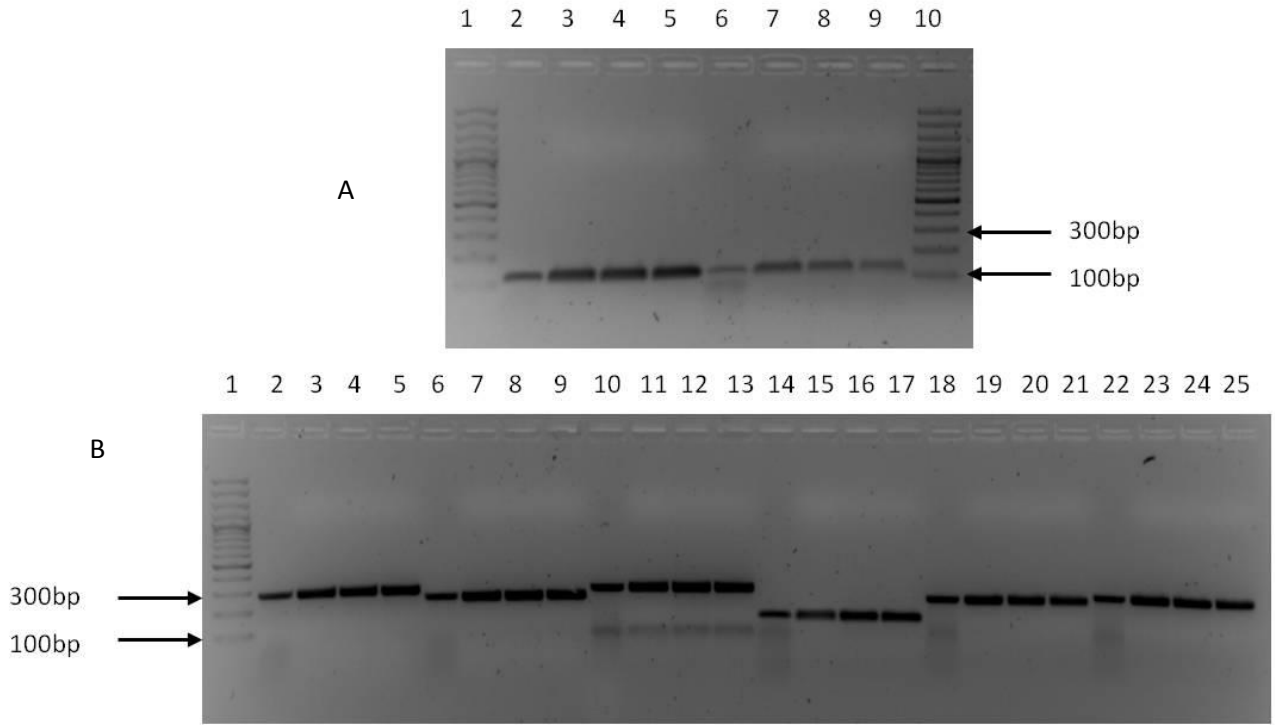


Figure 3.38. A 2% (w/v) agarose gel illustrating RT-PCR and RT-qPCR products of target genes. A) *AtDRG* amplicons of both Kil-0 and Be-0 inoculated samples, respectively. Lanes 1 and 10

represent a 100bp ladder; lanes 2 and 6 represent RT-PCR products of Kil-0 and Be-0 *AtDRG* respectively. Lanes 3-5 represents Kil-0 *AtDRG* RT-qPCR amplicons from three replicates and lanes 7-9 represent Be-0 *AtDRG* RT-qPCR amplicons from three replicates B) *AtGPX1*, *AtLTP3*, *AtPAH2*, *AtPNT*, *AtXTH22* and *AtXTH24* amplicons. Lane 1 represents the 100bp ladder and lanes 2, 6, 10, 14, 18 and 22 represents the RT-PCR amplicons of the respective genes. Lanes 3-5 represent *AtGPX1* (284bp), lanes 7-9 represent *AtLTP3* (239bp), lanes 11-13 represent *AtPAH2* (287bp), lanes 15-17 represent *AtPNT* (134bp), lanes 19-21 represent *AtXTH22* (232bp) and lanes 23-25 represent *AtXTH24* (252bp). All amplicons were amplified from Kil-0 at 4dpi.

Table 3.8: TAIR BLASTN results of sequenced RT-qPCR products from Kil-0 and Be-0

Gene name	Ecotype sequenced	ATG#	Score (bits)	E-value
AtDRG	Be-0	AT5G48620	105	5e-23
AtDRG	Kil-0	AT5G48620	131	9e-31
AtGPX1	Kil-0	AT2G25080	404	e-112
AtLTP3	Kil-0	AT5G59320	325	1e-88
AtPAH2	Kil-0	AT5G42870	416	e-116
AtPNT	Kil-0	AT3G11770	141	1e-33
AtXTH22	Kil-0	AT5G57560	313	4e-85
AtXTH24	Kil-0	AT4G30270	349	7e-96

Figures 3.39-3.46 illustrate the nucleotide sequence alignments of the sequenced RT-qPCR products with the Kil-0 and Col-0 gene sequences. In all sequence alignments the arrows show where the forward and the reverse primers are situated on the gene sequences. *AtDRG* was sequenced both in Be-0 and in Kil-0 with both the forward and the reverse primers. However, for Be-0 only the forward sequence alignment is shown (Fig 3.39) and for Kil-0 only the reverse sequence is shown (Fig 3.40). In the case of *AtGPX1*, *AtLTP3*, *AtPAH2*, *AtPNT*, *AtXTH22* and *AtXTH24* only the forward sequences are shown from the Kil-0 background (Fig 3.41- 3.46).

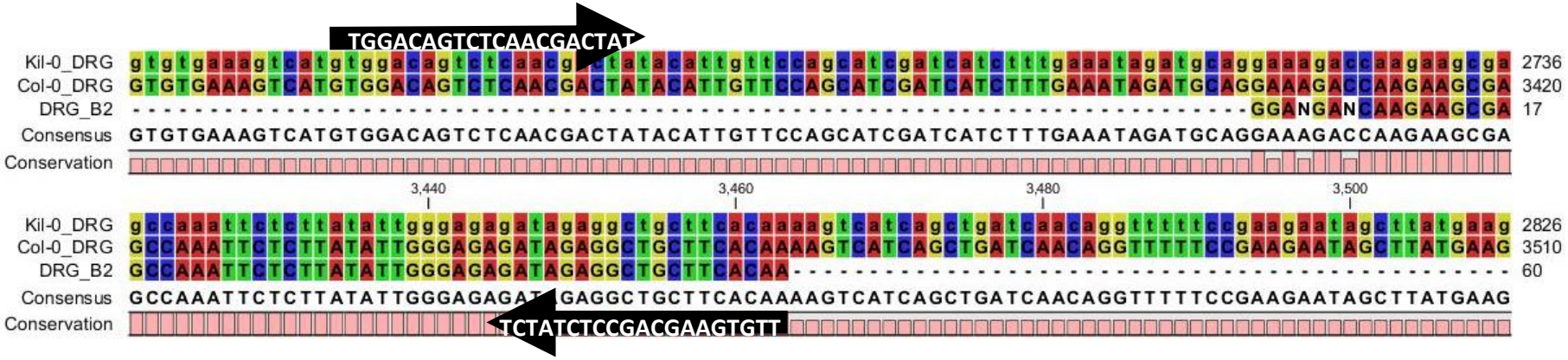


Figure 3.39: Alignments of nucleotide sequences of Kil-0 and Col-0 full cDNA sequences to RT-qPCR amplicon sequence of *AtDRG* gene. Kil-0 full cDNA sequences obtained from (http://www.signal.salk.edu/atg1001/3.0/gebrowser.php). Col-0 full cDNA sequences obtained from TAIR (www.arabidopsis.org). Primer sequences are indicated by the arrows.

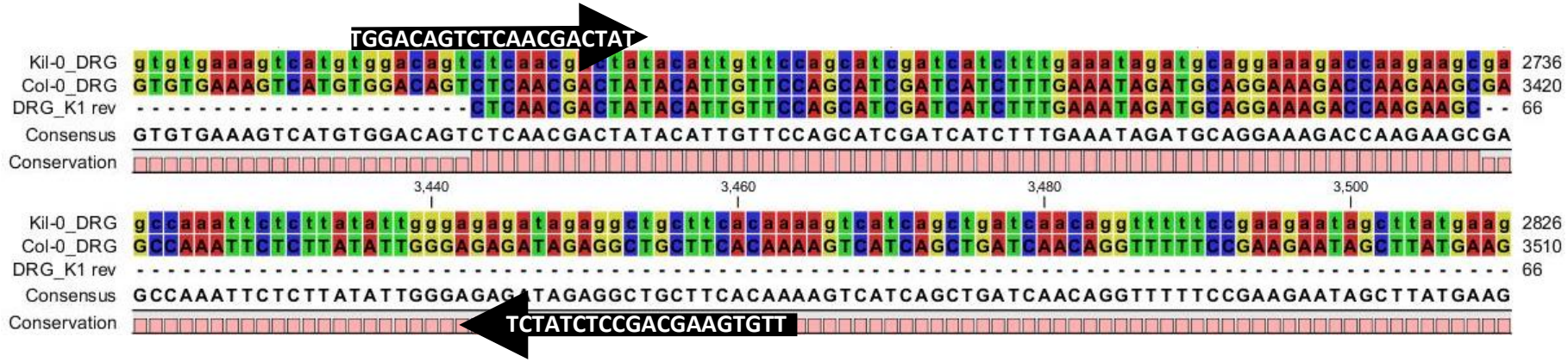


Figure 3.40: Alignments of nucleotide sequences of Kil-0 and Col-0 full cDNA sequences to RT-qPCR amplicon sequence of *AtDRG* gene. Kil-0 full cDNA sequences obtained from (http://www.signal.salk.edu/atg1001/3.0/gebrowser.php). Col-0 full cDNA sequences obtained from TAIR (www.arabidopsis.org). Primer sequences are indicated by the arrows.

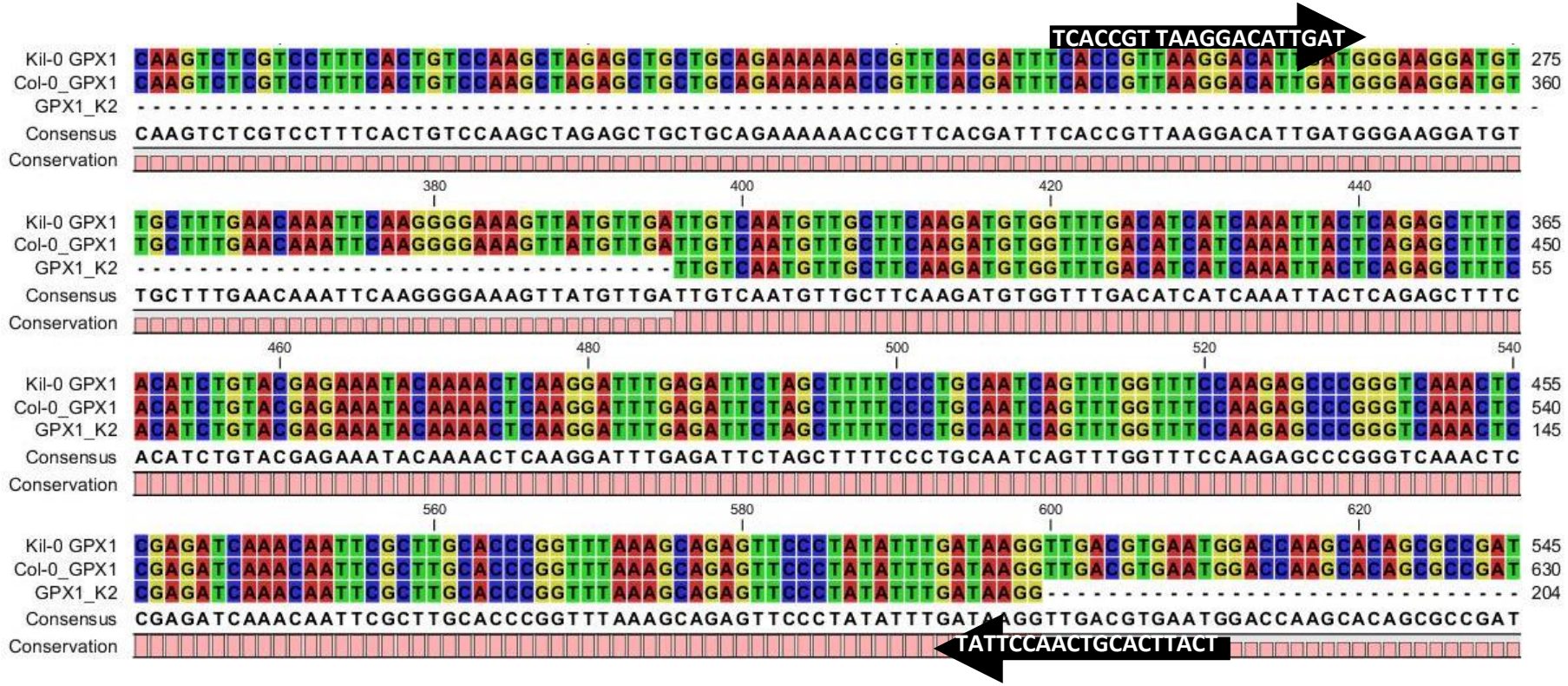


Figure 3.41: Alignments of nucleotide sequences of Kil-0 and Col-0 full cDNA sequences to RT-qPCR amplicon sequence of *AtGPX1* gene. Kil-0 full cDNA sequences obtained from (http://www.signal.salk.edu/atg1001/3.0/gebrowser.php). Col-0 full cDNA sequences obtained from TAIR (www.arabidopsis.org). Primer sequences are indicated by the arrows.

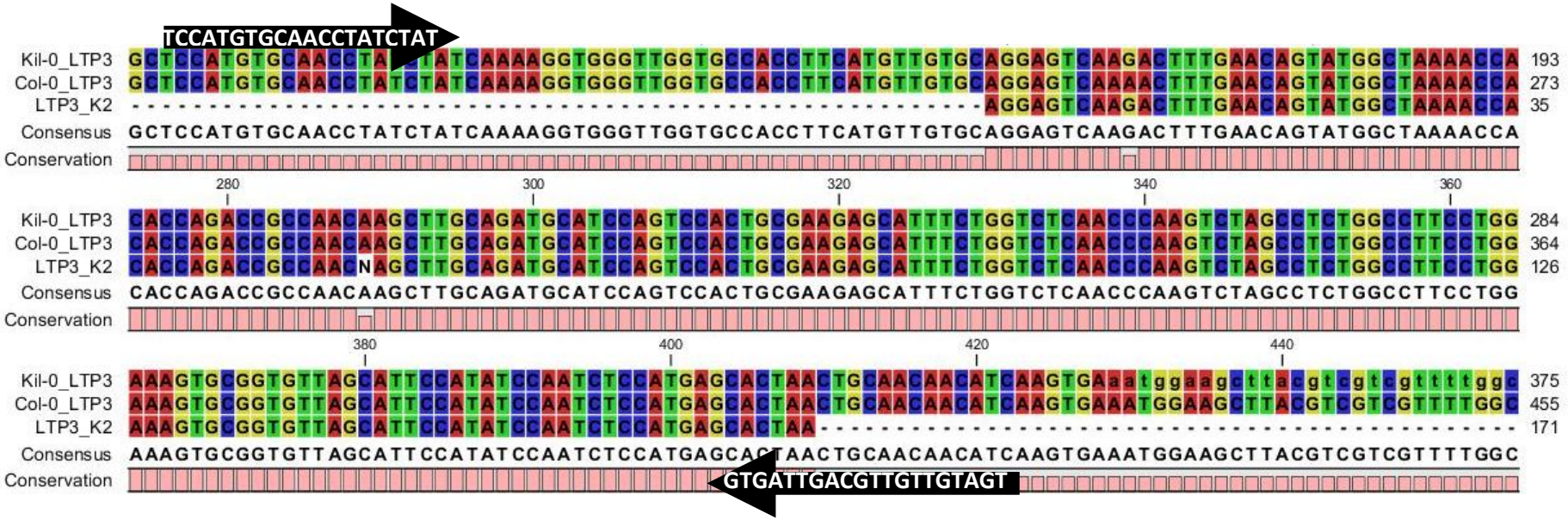


Figure 3.42: Alignments of nucleotide sequences of Kil-0 and Col-0 full cDNA sequences to RT-qPCR amplicon sequence of *AtLTP3* gene. Kil-0 full cDNA sequences obtained from (http://www.signal.salk.edu/atg1001/3.0/gebrowser.php). Col-0 full cDNA sequences obtained from TAIR (www.arabidopsis.org). Primer sequences are indicated by the arrows.

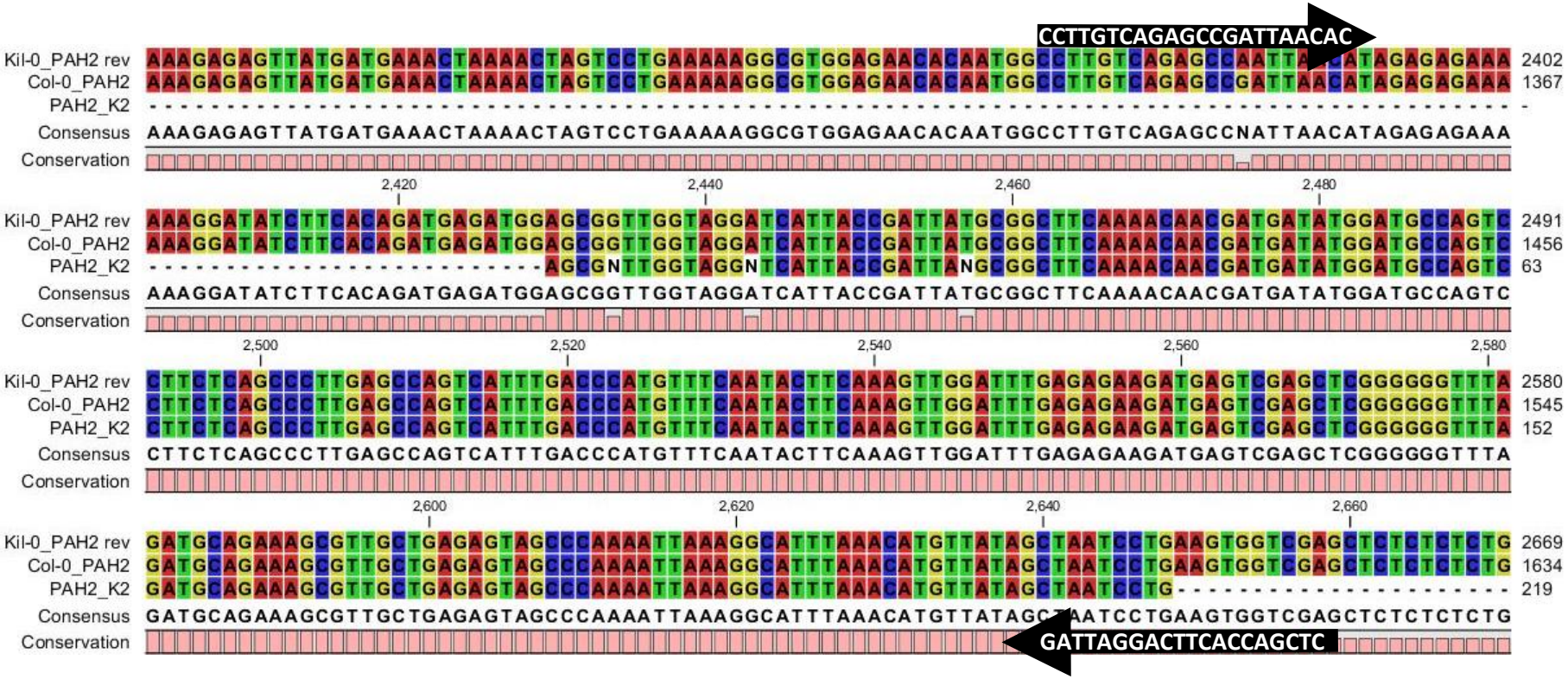


Figure 3.43: Alignments of nucleotide sequences of Kil-0 and Col-0 full cDNA sequences to RT-qPCR amplicon sequence of *AtPAH2* gene. Kil-0 full cDNA sequences obtained from (http://www.signal.salk.edu/atg1001/3.0/gebrowser.php). Col-0 full cDNA sequences obtained from TAIR (www.arabidopsis.org). Primer sequences are indicated by the arrows.

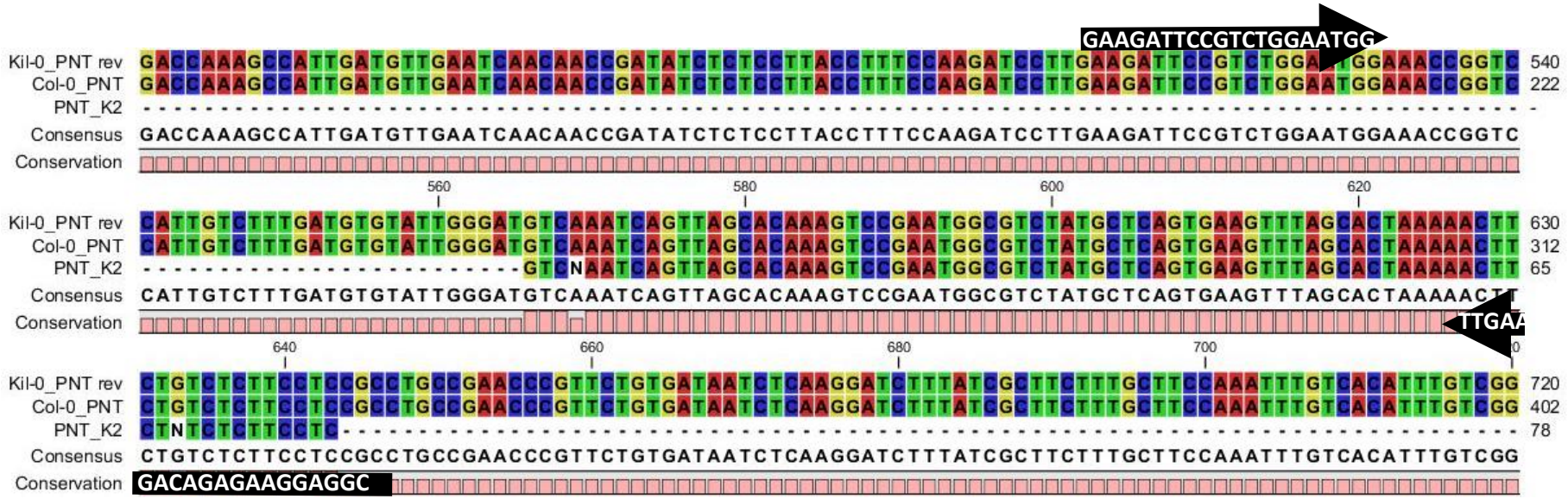


Figure 3.44: Alignments of nucleotide sequences of Kil-0 and Col-0 full cDNA sequences to RT-qPCR amplicon sequence of *AtPNT* gene. Kil-0 full cDNA sequences obtained from (http://www.signal.salk.edu/atg1001/3.0/gebrowser.php). Col-0 full cDNA sequences obtained from TAIR (www.arabidopsis.org). Primer sequences are indicated by the arrows.

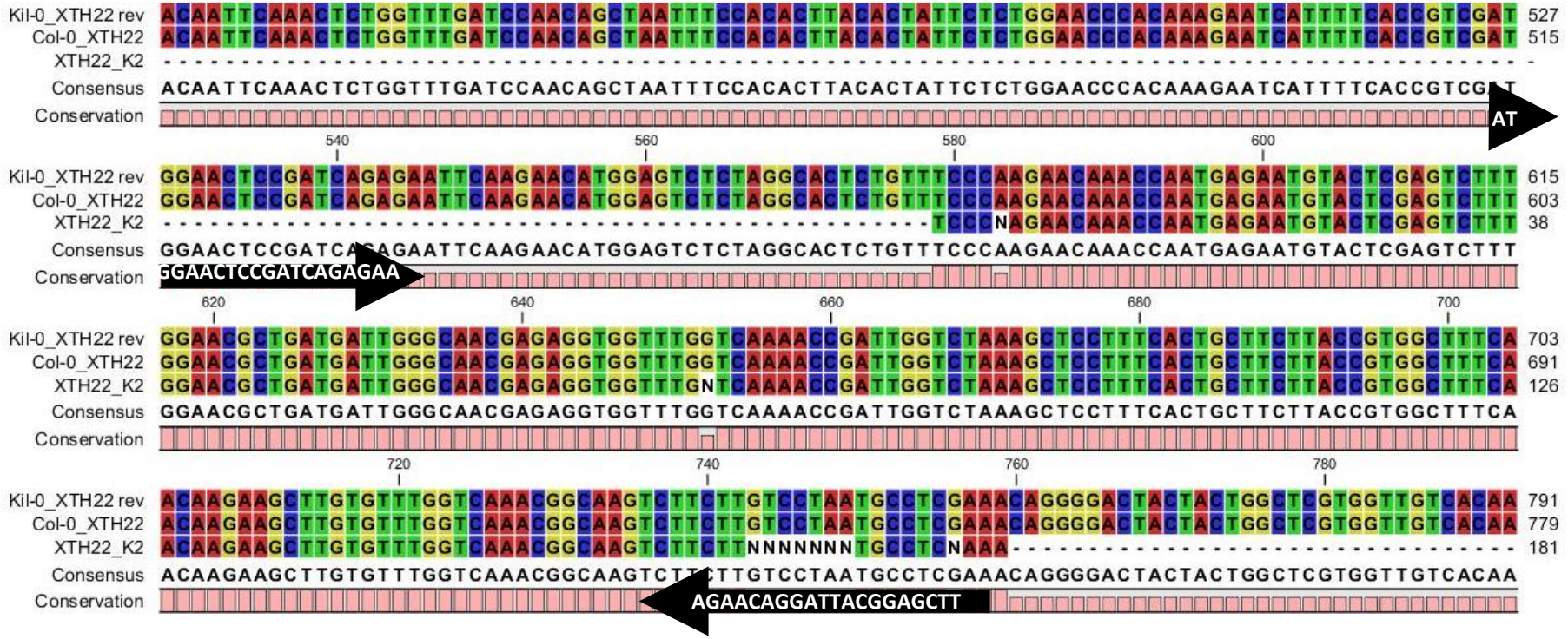


Figure 3.45: Alignments of nucleotide sequences of Kil-0 and Col-0 full cDNA sequences to RT-qPCR amplicon sequence of *AtXTH22* gene. Kil-0 full cDNA sequences obtained from (http://www.signal.salk.edu/atg1001/3.0/gebrowser.php). Col-0 full cDNA sequences obtained from TAIR (www.arabidopsis.org). Primer sequences are indicated by the arrows.

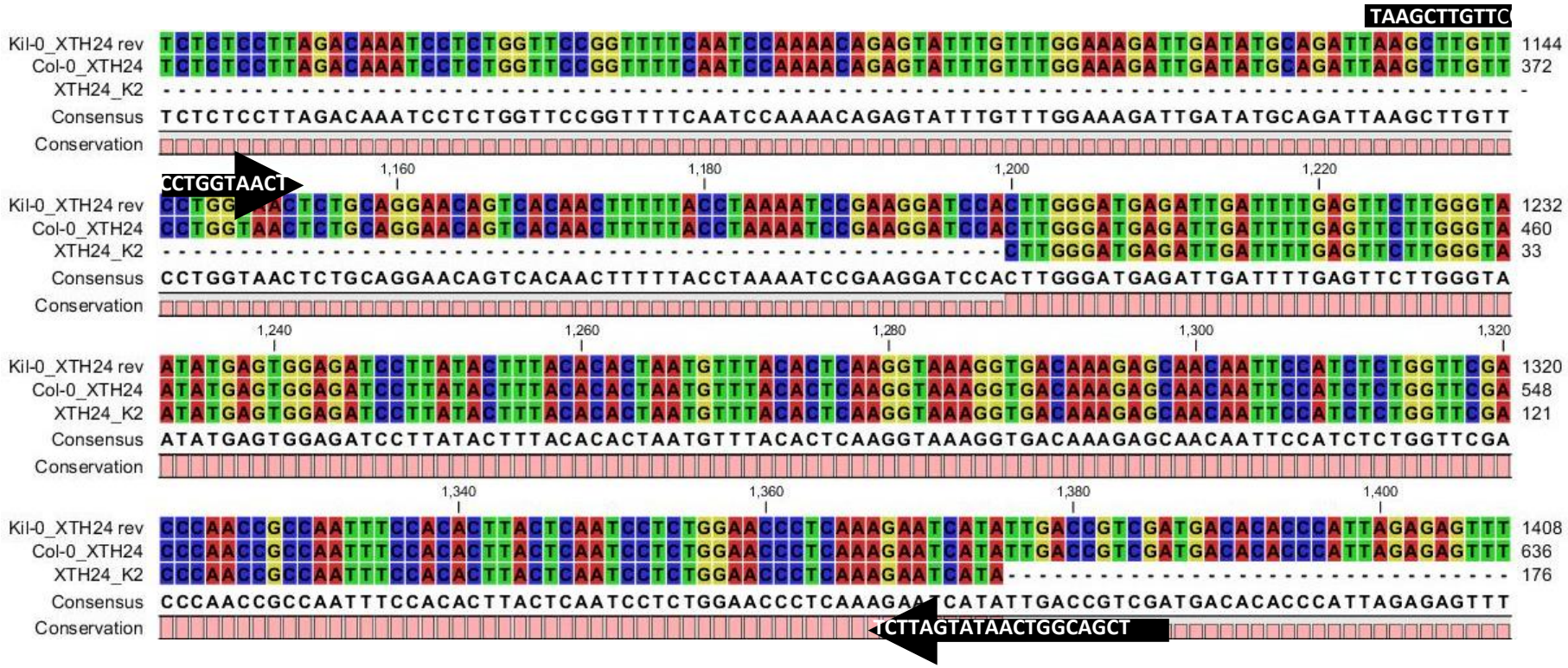


Figure 3.46: Alignments of nucleotide sequences of Kil-0 and Col-0 full cDNA sequences to RT-qPCR amplicon sequence of *AtXTH24* gene. Kil-0 full cDNA sequences obtained from (http://www.signal.salk.edu/atg1001/3.0/gebrowser.php). Col-0 full cDNA sequences obtained from TAIR (www.arabidopsis.org). Primer sequences are indicated by the arrows.

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Chapter 4

Discussions and Future work

4.1 The *Arabidopsis thaliana - Ralstonia solanacearum* pathosystem

Bacterial wilt disease caused by *R. solanacearum* is a worldwide predicament affecting many plant species. The established pathosystem between *A. thaliana* ecotypes (Be-0 and Kil-0) and *R. solanacearum* BCCF402 has provided a model system to dissect defence responses on a molecular level. Tolerant ecotype Kil-0 showed no wilting symptoms while susceptible ecotype, Be-0 was completely wilted at 14dpi (Fouché-Weich et al., 2006; Van der Linden et al., 2013). The pathosystem was subjugated for further studies of defence gene discovery and gene expression profiling. Previous expression profiling studies involved: (i) the construction of the Suppressive Subtractive Hybridization (SSH) libraries from Kil-0 inoculated with *R. solanacearum* BCCF402 and control plants, (ii) microarray hybridization experiments prepared from SSH libraries, and (iii) a whole genome microarray experiment of Kil-0 challenged with BCCF402 and control plants. This has aided further studies of gene expression profiling of candidate defence response genes in the pathosytem. The aims of this study were to select *A. thaliana* candidate defence response genes based on previous microarray data from *A. thaliana* plants inoculated with *R. solanacearum* BCCF402, to determine the differential expression trends of the candidate defence response genes in *A. thaliana* ecotype Be-0, that shows susceptibility to BCCF402 and Kil-0, that is tolerant to BCCF402. Furthermore, this study aimed to establish the time points at which the selected candidate defence response genes are up-regulated in Kil-0 in comparison with Be-0.

4.2 Candidate defence genes revealed in the SSH libraries and the SSHdb

SSH technology was used previously to identify candidate defence response genes in the *A. thaliana* tolerant ecotype, Kil-0 inoculated with *R. solanacearum* BCCF402. Sequencing of a few hundred clones from the SSH libraries led to the discovery of genes encoding proteins that span different compartments of the host cells, i.e. the nucleus, plasma membrane, cytosolic components, cell wall receptors, mitochondria, chloroplast and the nucleus. The encoded proteins include catalytic enzymes, regulatory proteins, transferases, reductases, resistance proteins, pathogenesis related proteins, kinases, membrane receptors, putative and unknown proteins (Beyene, 2007). This suggests that plant defence responses against pathogens involves multiple physiological processes involving dynamic signalling across many compartments of a host cell (Eulgem, 2005). Most of the uncovered genes have been placed in a database, the *A. thaliana* Suppression Subtraction Hybridization database which is denoted the SSHdb (http://www.sshdb.bi.up.ac.za). The SSHdb is a web-based sequence database which facilitates

the management and annotation of the SSH cDNA library clones (Coetzer, 2009). Appendix A shows to date, non-redundant sequences and their annotations currently documented in the SSHdb.

4.2.1 Selection of candidate defence response genes: The SSH libraries and SSHdb

There are four SSH cDNA libraries, comprising of two forward and two reverse libraries. The forward libraries were derived from tester samples as the inoculated samples and driver samples were derived from control samples. The reverse libraries denote the tester samples as the control samples and the driver samples as the inoculated samples, i.e. the inverse of the forward libraries. The libraries were constructed from two independent plant-pathogen trials where *A. thaliana* tolerant ecotype Kil-0 was inoculated with *R. solanacearum* BCCF402 and control plants, mock inoculated. The libraries consist of pooled transcripts from different time points. In one library set, the forward and reverse libraries were denoted AF_ and AR_ (respectively), constructed by A. McLeod (McLeod & Naidoo, 2005) (unpublished). The other set were denoted SF_ and SR_, constructed by S. Naidoo (McLeod & Naidoo, 2005). The AF_ and AR_ libraries were constructed from time points 4h, 24h, 32h, 48h & 96h and the SF_ and SR_ libraries, from time points 30 min, 2h, 8h, 24h, & 7days (McLeod & Naidoo, 2005). The four SSH libraries each have 578 clones, making up a total of 2304 clones. SSH library screening and microarray analysis led to the development of the *A. thaliana* SSHdb, which currently holds approximately 260 ESTs containing 151 non-redundant ESTs (Coetzer, 2009). In this study, only four genes were selected from the non-redundant ESTs documented in the SSHdb (Appendix A). Generally selection was based on the obtained BLAST (Basic Local Alignment Search Tool) Hit Definition as set in Table 3.1, indicating interesting annotations of the ESTs with confidence of the transcripts proposing potential involvement in defence responses. The SSHdb seems to be a very useful platform for selecting differentially expressed genes for further characterisation.

The SSHdb was constructed from the global BLAST analyses of the SSH library sequences as analysed in the GeneBank® database. This indicating that the documented SSHdb ESTs were defined on a global tool across all publicly available genetic sequences as annotated in National Centre of Biotechnological Information (NCBI). Thus SSHdb affirms identity of the ESTs as *A. thaliana* transcripts. The selected transcripts include *AtDRG*, *AtGPX1*, *AtPAH2* and *AtXTH22* (Table3.1). However, this ought to be confirmed in the *A. thaliana* database on TAIR.

AtDRG was selected for the reason that it gives a complete identity as signified with an E-value 0.00 in SSHdb (Table 3.1). It was the only EST with a complete sequence identity in GeneBank®. Further analysis of the EST in TAIR validates the annotation as a putative *R*-gene (Table 3.2). From the literature survey, *R* genes are important in defence. They target effector proteins injected through the T3SS from the pathogen. This places the transcript as a potential gene involved in the Effector Triggered Immunity, considering that bacterial pathogens are able to inject multiple effectors into a host to enhance disease symptoms (Lewis et al., 2009). This could very well be the case within the interaction between Kil-0 and BCCF402. Furthermore, *AtDRG* seems to be a novel gene in *A. thaliana* since no additional data is available for the gene except for its TAIR annotation.

AtGPX1 is considered a free-radical scavenger in plants, suggesting that it is signalled in defence responses against toxic Reactive Oxygen Species (ROS) created when the pathogen integrates into the host cells (Glazebrook, 2005). Moreover, a peroxidase can be considered as a stress marker resulting from both biotic and abiotic factors (Rosa et al., 2010). AtGPX1 is localised in the chloroplast and has been implicated in basal defence (Chang et al., 2009, Kim et al., 2014). The implication instils an interest in uncovering the role of the *AtGPX1* gene in immune responses to bacterial wilt.

AtPAH2 was selected due to its induction upon *R. solanacearum* inoculation which could be linked with the recent suggestion indicating a possible role in defence signalling uncovered in cotton against *Verticillium dahliae* (Phillips et al., 2013).

AtXTH22 encodes a cell wall repair protein involved in restructuring (Becnel et al., 2006). Its induced expression is likely to be related to defence response. It probably localises at the site of cell wall damage from the bacterial attack, as they attempt to pierce through host cell walls in order to gain entry into the cells. In thought, based on localisation of the protein in the cell wall region, it could possibly be involved in the front-line defence responses against bacterial wilt.

4.2.2 Selection of candidate defence response genes: SSH library clones on microarray-based studies

The SSH cDNA libraries were printed on a glass slide microarray to aid gene expression studies. This was to basically elucidate the regulation of each gene with regard to expression level analysis *in planta* post inoculation with the pathogen. In this manner, candidate defence response genes in Kil-0 were identified. A similar approach was undertaken by the authors of “High-throughput

screening of suppression subtractive hybridization cDNA libraries using DNA microarray analysis" (van den Berg et al., 2004). They showed the effectiveness of microarray-based screening of SSH library clones using cDNA microarrays, when exploiting a plant-pathogen interaction of Pearl millet (*Pennisetum glaucum*) with a flagellin elicitor from *Bacillus spp.* and banana (*Musa acuminata*) with *Fusarium oxysporum* (Van den Berg et al., 2004). Naidoo, 2008a looked at microarray-based analysis of the *A. thaliana* SSH libraries to exploit the expression of defence response genes in Kil-0 at 4dpi. At this time point Be-0 (susceptible) develops wilting symptoms but Kil-0 (tolerant) did not (Appendix C). Appendix D demonstrates the eight genes that showed differential expression upon BCCF402 inoculation in Kil-0. *AtWAK1* and *AtXTH24* were selected from this data for further analysis despite non-significant p-values (p>0.05). The selection of the genes was based on whether the genes encode proteins known to locate on the cell wall or cell membrane of plant. It could be speculated that besides physical structural barriers on the cell wall (e.g. the cuticle), cell wall or cell membrane associated coded proteins could potentially portray as front-line molecules of basal defence signalling. This based on their localisation implants likelihood that all early virulence factors have to pass through the cell wall and cell membrane of the host plant cells to enhance disease symptoms. Thus could be thought that abundant expressions of such proteins have the potential to alert signalling networks to activate disease responsive genes. Moreover, the interest also lies in exploiting whether these genes have a similar behavioural pattern in Be-0 against BCCF402.

4.2.3 Selection of candidate defence response genes: Whole genome microarray experiment

Naidoo, 2008b employed a whole genome microarray (70-mer oligonucleotide microarray, University of Arizona) to determine the genome wide expression of *A. thaliana* genes upon *R. solanacearum* BCCF402 inoculation in Kil-0 at 1 dpi, 4 dpi and 7dpi. Appendix E shows the genes that showed significant differential regulation in Kil-0 at 1 dpi and 7 dpi. The genes were further profiled in Kil-0 and Be-0 using RT-qPCR at 1, 4, 7 dpi (Naidoo, 2008b). *AtLTP3* and *AtPNT* were selected from the mentioned study because in the case of *AtPNT*, the gene was significantly up regulated as early as 1 dpi indicating a possible role in early defence responses. Furthermore, its set localisation is in the nucleus. Effector PopP2 has been implicated in nuclear locations where it interacts with RD19 and RRS-1 (Deslandes et al., 2003; Rivas, 2012). This association also suggest likeliness of other pathogen virulence factors possibly targeting nuclear organelles and residing in

the nucleus. The potential of the AtPNT to target such pathogen induced factors and destroy them is based on assumption that the predicted enzymatic properties of AtPNT, an exonuclease, could likely be associated with targeting and cleaving-off of the foreign factors in the nucleus secreted by the pathogen.

AtLTP3 showed a significant up regulation in Kil-0 at 4dpi, and not in Be-0. It is also known that *AtLTP3* is a *PR-14* family gene as annotated in TAIR. It has also been shown to confer growth inhibition against *R. solanacearum*, *Clavibacter michiganensis* and *Fusarium solani* (Segura et al., 1993); and an over expression of the gene enhances resistance to bacterial pathogen, *Pseudomona syringae*. *PR* genes potentially play a crucial role as factors of defence related responses. Their accumulations is closely linked with SA, ET/JA-dependent defence responses which may contribute to resistance against the pathogen (Sels et al., 2008).

In summary, a total of eight candidate defence response genes were selected for further gene expression profiling in *A. thaliana* upon a challenge with *R. solanacearum* BCCF402 at early time points. These were *AtDRG*, *AtGPX1*, *AtLTP3*, *AtPAH2*, *AtPNT*, *AtXTH22*, *AtXTH24* and *AtWAK1*.

4.3 SSH library clone confirmation

The identity of the SSHdb ESTs (Table 3.1) as identified through GeneBank, were determined by BLASTN or BLASTX analysis to the *A. thaliana* database which is based on the Col-0 genome. An E-value of 0.00 indicates that the EST is completely identical to the sequence of the specific gene in the database, as illustrated by *AtDRG* gene (Table 3.2). In that case, the corresponding ATG number is assigned to the EST or clone. Furthermore, in the case of those SSHdb clones that showed E-values close to but not 0.00 (Table 3.2), the ATG numbers can also be taken as valid identity numbers to be assigned to the ESTs or clones. Keeping in consideration that the EST sequences were from the Kil-0 genome, the sequences are expected not to be completely identical to the sequences of the Col-0 genes due to possibilities of single nucleotide polymorphisms (SNPs) between the genotypes. Indeed this is true as indicated by the differences observed in gene length sequences and the number of SNPs among the two genotypes, represented in the alignments from the 1001 Genome Project (http://www.1001genomes.org) (data not shown) and CLCBio Main Workbench 6.0 (Qiagen, Valencia CA, USA).

Probe sequences extracted from the microarray study (Naidoo, 2008b) matched the gene identities that were initially assigned to them with significant E-values corresponding to the

correct ATG number. However, *AtPNT* was originally annotated as an expressed protein (At3g11770) (Naidoo, 2008b). Currently it has been re-annotated as Polynucleaotidyl transferase (TAIR).

Overall, most of the BLASTN and BLASTX results of the ESTs carried out for the MSc study matched the unique ATG annotations of the genes from former studies indicating efficiency in former studies. Exceptionally, AtPNT was originally annotated as an expressed protein, currently it has been re-annotated. This shows that genomic-based studies linked with similar previous studies should always be validated as new data is always being updated in widely studied areas as the model plant.

For further validation, the frozen clones, sequenced from the SSH libraries frozen stocks also yielded good match identities to the ESTs in the SSHdb. *AtDRG*, *AtGPX1*, *AtLTP3*, *AtPAH2*, *AtPNT*, *AtXTH24* and *AtWAK1* (Fig. 3.9-3.16), excluding *AtXTH22* (Fig 3.13) corresponds directly with the original identities assigned to the genes. However, in the case of *AtXTH22* (Fig. 3.13), the obtained sequence of clone SF5_G1_F from -80°C did not match the EST reported in SSHdb which was *AtXTH22*. This did not affect the feasibility of this study. The MSc study did not rely on using the SSH library clone cDNA sequence for designing primers, only for validation purposes. RT-PCR primers were designed based on the *AtXTH22* EST sequence reported in the SSHdb, which had a BLASTX E value of 8e-68 with the AtXTH22 gene from *A. thaliana* (Table 3.2).

In brief, the above illustrates the importance of ensuring that clones from established databases and freezer stocks should be identical for any future studies that might be needed on the specific cDNA clones. Also clones in the database should always be traced to the correct ATG number in the *A. thaliana* database for correct annotation of the specified gene. Thus one would have to find the correct clone in the frozen stocks corresponding to the *AtXTH22* EST in the SSHdb in the future. Thus, for future work on the SSH library, it is important to identify the freezer clones with the correct EST in the SSHdb and the correct ATG numbers in the *A. thaliana* database.

Considering that the *A. thaliana* database in TAIR is based on the Col-0 genome, and the previous experiments were conducted in Kil-0, the ESTs and the clone sequences were further analysed based on the assigned ATG numbers corresponding to the identity descriptions of the specified genes on the available Kil-0 genome at 1001 Genome projects (http://www.1001genomes.org). As expected, the SSHdb EST sequences and the re-sequenced SSH library clones matched the

sequences of the correct Kil-0 ATG gene in the 1001 genomes project. This is illustrated by DRG, GPX1, PAH2, WAK1 and XTH24 (Fig. 3.1-3.4 and Fig. 3.6) with the exception of XTH22 (Fig. 3.5) where the frozen clone was identified as an unknown protein (AT1G73885; Fig.3.13).

The identities of the probes from the whole genome microarray study were also confirmed. The corresponding probes on the *A. thaliana* whole genome microarray matched the correct ATG number in the *A. thaliana* database (Table3.2) and aligned to the corresponding Kil-0 specified gene sequences (Fig 3.7-Fig 3.8). Obtaining ATG numbers was crucial for further *in silico* studies of the specific gene carried out in Genevestigator analysis.

4.4 The Genevestigator database revealed potentiality to defence responses

Genevestigator is a tool that is used to observe expression of genes across various datasets of previous microarray expression profiling experiments. This gives clues to the possible roles of the specified genes across various treatments, stress responses and tissue specific expression corresponding to growth and development (Zimmermann et al., 2005).

The expression patterns of the eight candidate defence response genes were analysed with reference to five well known Pathogenesis Related (PR) genes (*AtPR1*, *AtPR2*, *AtPR3*, *AtPR4* and *AtPDF1.2*) and *AtRRS1 (Resistance to Ralstonia solanacearum 1)* in the Genevestigator database (http://www.genevestigator.com), illustrated in Fig 3.17- Fig 3.24. It is always important to support unfamiliar expression of genes with familiar (control) genes such as *PR* genes which are detected at basal concentrations in healthy tissues and drastically induced upon stressful and pathological conditions (Sels et al., 2008). The *PR* genes were selected with respect for their classical involvement as key elements in the Salicylic acid (SA) pathway and the Ethylene (ET)/Jasmonic acid (JA) pathways. SA, ET and JA are important signalling molecules in the regulation of immune defence responses against biotic and abiotic stresses in plants (Pieterse et al., 2009, Sels et al., 2008). SA signalling positively regulates plant defences against biotrophic pathogens that require living tissue to complete their life cycle. ET/JA pathways are required for the regulation of defence responses against necrotrophic pathogens that generally destroy the host plant and feed on the dead cells (Pieterse et al., 2009). Thus increased levels of these molecules in plants challenged with pathogens correlate with elevated expression of *PR* genes and activation of disease resistance (Kim et al., 2008). *AtPR1*, *AtPR2*, *AtPR3* are SA-responsive genes and *AtPR4* and *AtPDF1.2* are ET/JA-responsive genes upon stressful conditions (Pieterse et al., 2009; Sels et al., 2008). *AtRRS-1* is the key disease resistance/tolerance gene against *R.*

solanacearum in the *A. thaliana* – *R. solanacearum* pathosystem (Deslandes et al., 2002; Van der Linden et al., 2013).

The expression profiles posted in the Genevestigator database, represented in Fig 3.17, as expected, showed induced expression of *AtPR1* and *AtPR2* after treatment with SA. However *AtPR3* appeared to be down regulated. This supports suggestions that *AtPR3* is more a responsive gene induced by JA than SA (Rosa et al., 2010). *AtPR4* and *AtPDF1.2* as expected are not induced by SA since they are JA responsive genes. *AtRRS-1* is induced by SA only at 4h after treatment with SA. This would mean that if SA is induced immediately upon a pathogen challenge, *AtRRS-1* is induced. However, the effect is not effective in Col-0 as it is susceptible to *R. solanacearum* and does not possess the *AtRSS1_R* alleles. Possibly an early induction of SA signals the activation of AtRSS-1 to the recognition of PopP2. All the target candidate defence response genes show slight induction upon treatment with SA at least collectively at 1 day post treatment with SA. This is likely associated with a potential role in defence responses against pathogens.

Plants treated with MeJA (Fig 3.18) show a down regulation at 1h post treatment of all classic *PR* genes, most surprisingly including *AtPR4* and *AtPDF1.2* which should be induced by MeJA. However, the results obtained were only accounted for at 1h after treatment, suggesting that the genes could be up regulated at time points later than 1h post treatment. There was no data available for later time points. *AtRRS-1* on the other hand was induced at low levels, posing the gene as a general biotic responsive gene. Interestingly *AtLTP3* and *AtXTH22* were up regulated by MeJA treatment. This was expected because *AtLTP3* is a *PR* gene and an accumulation of JA induces the expression of *PR* genes, which in this case, *AtLTP3*. In the case of *AtXTH22*, the gene is responsive to a wide range of environmental stimuli (TAIR). Thus, *AtXTH22* also most likely is induced by the expression of JA in plants.

AtPR1 and *AtPR2* were up regulated in plants inoculated with *P. syringae* which is a hemibiotrophic pathogen (Fig 3. 3.23). These types of pathogens are commonly deterred by defences that are controlled by both SA and ET/JA pathway (Pieterse et al., 2009). Thus the two *PR* genes show their plausible role in biotic stresses. *AtLTP3* as well, appeared induced by *P. syringae* indicating characteristics of *PR* genes. *AtWAK1* also was up regulated by *P. syringae*, indicating that cell wall associated molecules could potentially play a role detecting bacterial pathogen A range of time points from the Genevestigator would be useful in determining the behaviour of the candidate defence responsive genes, however there was limited data. *Botrytis cinerea* is a

necrotrophic fungus (Pieterse et al., 2009). An inoculation of plants with *B. cinerea*, as expected, induced all the classic *PR* genes including *AtLTP3*, *AtPAH2*, *AtPNT*, *AtXTH24* and *AtWAK1* (Fig.3.21). However, *AtGPX1* and *AtXTH22* appeared down regulated. This could mean that *B. cinerea* does not induce the early production of ROS in a susceptible interaction, hence *AtGPX1* was not induced. A possible explanation of why *AtXTH22* was not induced could be because the fungal virulence factors may be able to suppress its regulation, possibly targeting other plant cell wall components instead of xyloglucan in the plant cell wall.

The *PR* genes selected for this study were up regulated by the oomycete pathogen, *P. infestans* including *AtRRS-1*. *AtXTH22*, *AtXTH24* and *AtWAK1* also appeared up regulated by *P. infestans* suggesting that the genes are responsive to biotic stresses (Fig 3. 3.22). Contrastingly *AtGPX1* was down regulated, suggesting that *P. infestans* does not induce early ROS production in a susceptible interaction. *AtLTP3* also appeared suppressed by *P. infestans.* This is strange because LTPs share sequence similarity with elicitins from *Phytophthora spp*. which are elicitors of plant defence responses (Durrant & Dong, 2004). Thus, ideally *AtLTP3* should be up regulated as in the case of wheat LTP1 that binds to the same plasma membrane receptor as the *Phytophthora* elicitin crytogein (Buhot et al., 2001) which signals defence responses in wheat. It could be that the oomycetes elicitins are able to suppress their kind in a susceptible interaction.

Generally, *R. solanacearum* shows the life style of a necrotrophic pathogen which is commonly deterred by defences that are controlled by JA and ET pathways. As expected, *AtPR1* and *AtPR2* were down regulated by *R. solanacearum* (Fig 3.24). Surprisingly, *AtPDF1.2* the JA-responsive marker gene (Pieterse et al., 2009) was down regulated. This was unexpected considering that *R. solanacearum* is a necrotroph and plant defences against necrotrops should be associated with the induction of the JA pathway. However, the available results account for only one time point which is insufficient to make conclusive estimations. At this time point, it could be that *R. solanacearum* suppresses the expression of *AtPDF1.2* to delay the signalling of defence responses in the susceptible interaction. However, *AtPR3* and *AtPR4* appeared up regulated which are also JA/ET marker genes. *AtRRS-1* shows no informative data. In the case of the target candidate defence response genes, *AtLTP3*, *AtPAH2*, *AtXTH22*, *AtXTH24* and *AtWAK1* appeared induced by the *R. solanacearum* in the susceptible interaction (Fig 3.24). Clearly the effect of induced genes has no significance in resistance or tolerance against *R. solanacearum* since Col-0 plants wilt and die anyway. This suggests that they may be involved in effective immune responses against *R. solanacearum* in the tolerant background or maybe it could be explained in terms of differential

expression patterns across different genotypes. Possibly these genes might be relatively abundant in tolerant and resistant species. More so, the above mentioned genes pose as defence responsive genes in support of previous experiments conducted on the plant-pathogen interactions. *AtGPX1* and *AtPNT* show no informative data.

Oligogalacturonides (OGs) are regarded as Damage Associated Molecular Patterns (DAMPs) or Host-Associated Molecular Patterns (HAMPs) (Brutus et al., 2010). They activate the plant immune responses and regulate plant growth and development. *AtPR2*, *AtPR3* and *AtPR4* appeared to have a responsive role against pathogen associated OGs (Fig 3.20). *AtRRS-1* also appeared induced by OGs at 1h after treatment. In the case of the target candidate defence response genes, only *AtWAK1* is up regulated. This supports that *AtWAK1* is a receptor of OGs as it has been shown to bind to oligosaccharides *in vitro* (Brutus et al., 2010).

Treatment of plants with EF-Tu shows a down regulation of *PR* genes, excluding *AtPR2* and *AtPDF1.2* which were very slightly induced by the elicitor (Fig 3.19). *AtRRS1* as well was slightly induced. *AtLTP3* showed the properties of a *PR* gene and was as well, slightly induced by EF-Tu. *AtXTH22* appeared up regulated by EF-Tu indicating that it plays a role in immune defence responses where the heat-shock protein may serve as a PAMP (Fig 3.19).

In summary, phytohormonal treatments, pathogen challenges and PAMP treatments are good indicators of gene profiling conditions to test if genes behave as expected in relation to their physiological or biological annotations. Although the data from Genevestigator only illustrated susceptible interactions and provided limited time course experiments, the little information gathered, suggests that the candidate defence response genes do play a role in defence responses against biotic stresses.

4.5 Trial 1 reveals the relative expression of the seven candidate defence response genes

Eight candidate defence response genes in *A. thaliana*, tolerant ecotype, Kil-0 were selected for gene expression profiling. The expression of the genes was analysed at 4dpi in trial 1. This time point represents an early day post inoculation especially in Kil-0, showing little to no symptoms against the pathogen (Appendix C). However Be-0 at 4dpi shows clear wilting symptoms (Appendix C). *AtWAK1* expression profiling could not be conducted as optimal conditions for the RT-qPCR were not met. The primer pair for *AtWAK1* amplifies the gene very well under RT-PCR (Fig

3.2: lane 9), but the same conditions produces double melting peaks in RT-qPCR, indicating a possibility of two amplicons being produced or too long primers dimers (Appendix B). Furthermore a significant standard curve could not be accomplished (Appendix F). Therefore budget constrains dictated that the gene be left out from further investigations. The expression of *AtGPX1* was significantly up-regulated in Kil-0 inoculated with BCCF402, but not in Be-0 (Fig 3.30A). Glutathione peroxidases are a group of enzymes that protects cells against oxidative damage created by Reactive Oxygen Species (ROS) (Milla et al., 2003). *ATGPX1* in *A. thaliana* probably plays a role in protection against ROS intoxication within aerial parts of the plants, because *AtGPX1* is absent in roots (Milla et al., 2003). AtGPXs are also known to catalyse ROS in the chloroplast generated by photosynthesis (Milla et al., 2003). These enzymes have been shown to increase steadily over time under pathogen attack in tobacco plants (Criqui et al., 1992). Hence *AtGPX1* was up regulated in Kil-0 upon pathogen attack. It is well known that pathogen attack *in planta* results in ROS generation to signal defence response genes. The glutathione acts as a redox sensor of environmental cues and in turn forms part of the regulatory circuit that coordinates defence gene expression (Grene, 2002). Thus AtGXP1 is most likely recruited to scavenge off the generated ROS *in planta* and signal defence response genes against pathogens. The gene is not differentially expressed in Be-0 inoculated plants and control plants. This suggests that ROS production is limited in susceptible interactions. The observed data supports that of Genevestigator in the susceptible interaction.

In *A. thaliana AtLTP3* is predicted to encode a pathogenesis-related protein belonging to the lipid transfer protein (PR-14) family, consisting of 14 members. *AtLTP3* specifically encodes the Lipid Transfer Protein 3 (TAIR). The RT-qPCR expression of *AtLTP3* does not correspond with the data obtained from the whole genome microarray study, where it was significantly up-regulated in Kil-0 at 4dpi. It is more highly expressed in Be-0 than in Kil-0. However, the expression is the same in inoculated and control plants, indicating no differential expression in inoculated and control plants (Fig 3.30B). The inconsistency of these results could possibly have arisen from the differences in fitness of the plants from the different independent trials or the variation in the amount of pathogen that was able to proliferate through the plant from trial to trial. Furthermore, the data is not supported by the Genevestigator analysis. *AtLTP3* is a *PR* gene and should ideally be induced upon pathogen inoculation (Sels et al., 2008). Subsequently it has been identified to play a role in defence against the bacterial pathogen, *P. syringae* in tobacco and *A. thaliana,* where a barley *AtLTP2* was over expressed in the plants and the transgenics inoculated with *P. syringae* (Molina &

Garcia-Olmedo, 1997). Furthermore, a crude extract of LTPs showed 50% inhibitory action against bacterial and fungal pathogens (Jung et al., 2005; Molina & Garcia-Olmedo, 1997).

AtPAH2 encoding the Phosphatidate phosphohydrolase 2, shows no significant expression differences *in planta*. The expression of the gene does not change between inoculated plants and control plants. The expression in Be-0 is more than in Kil-0 (Fig 3.30C). However, cotton homologue of *AtPAH2* has been shown to be involved in defence responses against *V. dahlia* that causes wilting and eventually cotton crop losses (Phillips et al., 2013). From the *in silico* studies, it was shown that it is slightly induced by *P. infestans* and *R. solanacearum*.

The expression of *AtPNT* which encodes the Polynucleotidyl transferase, ribonuclease H-like superfamily protein (TAIR) was the same in both Be-0 and Kil-0 inoculated and control plants (Fig 3.30D). This gene was shown to be significantly up regulated in Kil-0 inoculated with BCCF402 at 1dpi (Naidoo, 2008b). In this case, the inconsistency of the results could be due to delayed responses to the pathogen in Trial 1 relative to the trial from Naidoo 2008b. The virulence of the pathogen could also account for the delay in the induction of the gene upon a pathogen challenge. It can be assumed that a less virulent pathogen injects its virulence factors at a delayed pace compared to a more virulent pathogen. Long term bacteria from frozen glycerol stocks are more likely less virulent than bacterial isolates from *in planta* isolations. Frozen stocks are possibly in a dormant state whereas *in planta* isolated bacteria are likely to be actively virulent. Bacterial virulence is likely influenced by host-mediated pathogenesis, which activates the secretion of virulence factors in order to counteract host immunity.

AtXTH22 encoding the Xyloglucan endotransferase protein 22 (a cell wall modifying enzyme) was significantly up regulated in Kil-0 inoculated plants and is highly expressed in Be-0 but not differentially regulated at 4dpi (Fig 3.30E). This gene is involved in cell wall repair functions and restructuring (Becnel et al., 2006). However, in tomato, *LeXTH1* has been shown to be involved in defence responses against *Cuscuta reflexa* (Albert et al., 2004). Moreover, it has been suggested that XTHs play a role in facilitating fungal colonization and disease progression in tomato plants by disassembling of the cell wall (Miedes & Lorences, 2007). In *A. thaliana* it is most likely recruited from the signalling of damaged cell wall caused by *R. solanacearum* BCCF402 when penetrating the host cells in the roots. Furthermore, the defence response role of *AtXTH22* is also supported in Fig 3.24.

AtXTH24 encoding the Xyloglucan endotransferase protein 24 was significantly up regulated in Kil-0 inoculated plants (Fig 3.30F). It showed similar expression patterns as its family member, *AtXTH22* indicating that they are probably both involved in similar defence response mechanisms in *A. thaliana* against *R. solanacearum* BCCF402. This is supported since the two genes are assigned to the same group (Group II) with a bootstrap confidence of >95% in tomato (Muñoz-Bertomeu & Lorences, 2014). Furthermore, *XTHs* have been suggested to be representatives of pathogenecity factors in *Malus domestica* challenged with *Penicillium expansum* (Muñoz-Bertomeu & Lorences, 2014) in a compatible plant-pathogen interaction. Their role in defence responses is likely associated with early defence responses, possibly associated in parallel with PTI. This is also supported in *in silico* studies (Fig 3.24).

AtDRG is annotated as an *R* gene, denoted the disease resistance protein (CC-NBS-LRR class) family. This suggests that it targets an effector from the pathogen and it possibly plays a role in Effector Triggered Immunity (ETI) in plant defence. The expression of the gene in Kil-0 and Be-0 inoculated with *R. solanacearum* BCCF402 showed high expression of the gene in Kil-0 and very low levels in Be-0. However, the gene was down regulated in Kil-0 inoculated with *R. solanacearum* when compared to Kil-0 control plants (Fig 3.31). This is perhaps due to suppression of the gene by the pathogen effectors. It is well estimated that *R. solanacearum* has a great number of effector proteins which serve as pathogenicity factors that subvert host plant cellular pathways to cause disease in plants (Poueymiro & Genin, 2009). However, it is mostly unknown which effectors from the pathogen interact with which *R* gene in host plants, as in the case of *AtRRS1-PopP2* interaction in the nucleus and cytosol of Nd-1 plants (Deslandes et al., 2003) and Kil-0 plants (Van der Linden et al., 2012). *AtDRG* could possibly be vital for detection of a specific effector from *R. solanacearum* in Kil-0 since it was highly expressed. In the case of Be-0, *AtDRG* could be greatly subverted by the effector due to the differences in single nucleotide polymorphisms (SNPs) that probably exists among *A. thaliana* ecotypes.

4.6 Trial 2 reveals the relative expression of three candidate defence response genes across three time points

Based on Trial 1, *AtDRG* was more expressed in Kil-0 than in Be-0. This posed *AtDRG* an exciting gene to further investigate at different time points. Furthermore, not much is known about the gene, which flags it as an interesting novel gene to be fully characterized. *AtXTH22* on the other hand, was also significantly up regulated in Kil-0 but it was more expressed in Be-0 and seemingly

down regulated, thus it was of interest to investigate it further, looking at its expression across different time points. *AtXTH24* was also of interest as it is within the same family as *AtXTH22* and was up regulated in Kil-0. More so, in Genevestigator it seems to be induced by biotic stresses, *B. cinerea* and *P. infestans.*

In Trial 2, the three genes *AtDRG*, *AtXTH22* and *AtXTH24* were chosen for a detailed time course study. An inconsistency of results in Trial 1 is observed in Trial 2, where the expression of *AtDRG* was up-regulated in Kil-0 in all three time points. The differences between Trial 1 and Trial 2 is that the control material in Trial 2 was material harvested at day 0, which represents 30min after wounding the plants and inoculated with sterile B-media. Furthermore, the fitness of Kil-0 plants was questionable as the plants developed wilting symptoms as illustrated in Appendix C. Contradictory to Fig 3.32, Kil-0 shows less tolerance to bacterial wilt. The 0dpi was set as the control time point and was accounted for all time points, considering the wounding effect of the plants before inoculation. Basically, mock inoculated material never show disease symptoms (data not shown) for the duration of any given trial. However, the results confirm that *AtDRG* was expressed at very low levels in Be-0 and high levels in Kil-0 as observed in Trial 1, across all three time points (Fig 3.37A). In Be-0 there is no differential expression of the gene (Appendix G). However, it seems that *AtDRG* in Kil-0 was more induced at a later time point, 10dpi, although not significant (p<0.05).

Another inconsistency was observed with *AtXTH22* which appeared down regulated in Trial 2 in both Be-0 and Kil-0 (Fig 3.37B). The suppression in Be-0 was significant across all three time points (Fig 3.37B). The results do support that *AtXTH22* expression was more in Be-0 than in Kil-0 in relation to Trial 1. This suggests a viewpoint that suppression of *AtXTH22* is likely induced by biotic stresses in a compatible plant-pathogen interaction. This is also supported by Fig 3.21 and Fig 3.22 where *B. cinerea* and *P. infestans* (biotic stresses) challenges induced a down regulation of *AtXTH22* transcripts in Col-0. In the case of *AtXTH24*, there was no conclusive difference in expression levels across all three time points (Fig 3.37C). The differences within the datasets obtained in both Trial 1 and Trial 2 could possibly be based on the virulence phenotype of BBCF402 between the trials or possibly the time frame for tissue harvesting between the trials. In Trail 2 may be delayed responses were attributed by the pathogen's virulence or the plant fitness before inoculation as indicated by the disease index variation between Fig 3.32 and Appendix C.

4.7 Conclusion

This study brought forward the emphasis of synergistic participation of different genes across different compartments in host cells to confer immunity against invading pathogens (Fig 3.30 and 3.31). Although the exact role of each gene in a plant-pathogen interaction is unknown, the speculations put forward reasonable predictions of their involvement in defence responses against biological matter. Also, the results illustrate that differential genotypes among the same species perceive biological stress contrarily; hence there is susceptibility, tolerance and resistance to the same pathogen. Some genes are highly induced by pathogen invasions whereas some genes are suppressed. This is mostly like due to the functionality of each transcript in the aspect of its size and structure. In the case of *AtDRG*, its high expression upon bacterial wilt poses the gene as a novel gene for further characterization. Thus this study showed the significance of gene expression profiling in plants against biotic stresses and the validation thereof of transcript levels across different time points to select the most crucial gene for further characterization in plant-pathogen interactions.

4.8 Future work

AtDRG appears to be a very novel and interesting *R* gene *in A. thaliana*. Furthermore it is not a well-known gene as there is no information for this gene on Genevestigator as no probes for the gene were designed. The identification of this gene from this study suggests that future work on the *AtDRG* gene should be performed. The relative expression of *AtDRG* observed in Kil-0 and Be-0 in this study indicates that the gene seemed to be expressed in high levels in Kil-0 which is tolerant to *R. solanacearum* and is expressed at very low levels in Be-0, which is susceptible. Future work on the *AtDRG* gene instigates a careful look at the expression of this gene in Col-0 which is also susceptible to *R. solanacearum*. Gene specific primers are already available (designed from this study) because they were formally designed based on the available Col-0 gene sequence from TAIR. The sequenced amplicon from both Be-0 and Kil-0 from this study, confirms the alignment of *AtDRG* with the Col-0 gene, when a BLAST function was analysed on TAIR (data not shown). This provides support that the *AtDRG* gene primers are viable to work with across different ecotypes.

The most important characterisation of the *AtDRG* gene would have to be carried out in Kil-0. This will involve the construction of *AtDRG* knockout lines in the Kil-0 background. The suggested technology for the knockout lines would be the use of RNA interference (RNAi) technology. RNA interference is a method used for gene silencing in order to create phenotypes that would give

plausible clues to the specific function of an inactivated gene (Agrawal et al., 2003). The technology makes use of the fact that double stranded RNA (dsRNA) is easily degraded into small interfering RNA (siRNA) within cells by the natural mechanism of a dicer enzyme. siRNAs in most eukaryotic cells are homology dependent (Matthew, 2004), they bind complementary to their corresponding mRNA sequences. The catalytic protein component called the RNA Induced Silencing Complex (RISC complex) (which serves to cleave dsRNA) recognises the complex and therefore degrades the complex and render termination of mRNA synthesis for the target gene. For the construction of *AtDRG* knockout lines, it is expected that if the gene is silenced in Kil-0 and the plants are inoculated with *R. solanacearum*, the plant would show a great deal of enhanced susceptibility to *R. solanacearum* as compared to wildtype plants. If this is conveyed, this would illustrate that the gene is indeed an important *R* gene against *R. solanacearum*.

The knockout lines can be re-complemented with the *AtDRG* gene to check if they would gain tolerance and validate the role of the gene in the plant. Full length primers of the *AtDRG* gene should be designed to specifically amplify the full coding region of the gene. The Gateway® approach would be ideal for the transformation process. It is a two-way, two-step cloning process used in building a construct for transformation. Firstly *AtDRG* will be cloned into an entry vector (eg. A pCR8®/GW/TOPO) flanked by the *attL* sites which allows recombinant Gateway® enabled cloning. The recombinant vector will therefore be transformed into *E. coli* cells. This will enable the *E.coli* cells resistance to kanamycin. Secondly, the *AtDRG* recombinant vector will be used to transfer the *AtDRG* gene from the Gateway® entry vector to the destination vector (e.g. pMDC32) through the *attR* sites. The destination vector will have the 35S promoter for the transcription of the *AtDRG* gene and a termination site (e.g. the NOS terminator). pMDC32 has the hygromycin resistance gene and the kanamycin resistance gene which will allow selection of transformed plants and *E.coli* cells respectively. *Agrobacteruim tumefaciens* will be inoculated with transformed *E.coli* cells for transfection of the *AtDRG* gene. An *A. tumefaciens* mediated transformation will be carried to transform the AtDRG-knockout lines with *AtDRG* (i.e. re-complement the *AtDRG* into the knockout lines). It is expected that after inoculation with *R. solanacearum*, the *AtDRG* re-complemented lines would gain tolerance against *R. solanacearum*. This would indicate the role of *AtDRG* in *A. thaliana* against bacterial wilt.

In addition, Kil-0 lines over expressing *AtDRG* would also be generated. As in the case of *AtDRG* re-complementation in knockout lines using the Gateway® approach, the same procedure would be utilized. The full length coding region of the *AtDRG* gene will be transferred through *A.*

tumefaciens mediated transformation into wildtype Kil-0 plants for over-expression of the gene. Hygromcyin resistance will be used for selection of transformed over-expression lines. Homozygosity tests for the T3 generation will also be undertaken. It is therefore expected that the homozygous T3 generation of the *AtDRG* Kil-0 over-expression lines, when inoculated with *R. solanacearum*, would confer resistance to bacterial wilt. The over expression lines should show little to null bacterial cells within the inoculated plants as compared to wild-type Kil-0 plants that show same magnitude of bacterial bodies within the plants in susceptible Be-0 plants and tolerant Kil-0 plants (Van der Linden et al., 2013). Thus, if resistance is conveyed in the Kil-0 over expression lines, this would give evidence of the biotechnological importance of the *AtDRG* gene. As in the case of Chen et al., 2006 who over expressed Glucanase and Defesin genes to enhance resistance against *R. solanacearum* in tomato plants (Chen et al., 2006).

The Kil-0 *AtDRG* gene would also be over-expressed in Be-0 using the Gateway® approach as described above. Then inoculate Be-0 over-expression lines with *R. solanacearum*. It is expected that Be-0 will show delayed symptom development and tolerance as compared to wild-type susceptible lines. Again this should give evidence of the biotechnological importance of the *AtDRG* gene.

AtDRG is expected to be expressed at low levels in Col-0 corresponding with the observation witnessed in Be-0 because like Be-0, Col-0 is susceptible to *R. solanacearum* (Van der Linden et al., 2013). The next step would be to perform a pathogen trial in Col-0 with BCCF402. Then from the trial extract total RNA for RT-PCR and RT-qPCR. The expected results for both inoculated and mock-inoculated plants should convey similar results to those observed in Be-0 (Fig. 3.27 A and B), where a faint band would be observed on a 2% (v/v) agarose gel indicating low expression of the transcript, as indicated through RT-PCR (Fig. 3.27 A and B) and very low relative expression levels as indicated though RT-qPCR (Fig 3.31 and Fig 3. 37A). However, should an up regulation of the gene be observed in inoculated Col-0 plants, as observed in Be-0, therefore this would indicate that the gene is responsive to the pathogen and thus worthwhile to further characterize the gene in Col-0 through functional genomic characterization, i.e. over expressing *AtDRG* in Col-0.

The MPPI group have available the *AtDRG* knock out line in the Col-0 background purchased from the Arabidopsis Biological Resource Center (ABRC) (https://abrc.osu.edu). This knock out line was constructed by the insertion of a T-DNA sequence harbouring a kanamycin resistance gene within the gene sequence using the vacuum infiltration method with *Agrobacterium tumefaciens* vector

pROK2 consisting of a kanamycin resistance gene (Alonso et al., 2003). A plant-pathogen trial can be conducted to illustrate the importance of the *AtDRG* gene in *A. thaliana* against *R. solanacearum*. It is expected that the knockout line will convey enhanced susceptibility against *R. solanacearum* as compared to the wild-type Col-0 and Be-0 plants. This would give further support to the suggestion that the *AtDRG* gene is important in defence against *R. solanacearum.* As in the case of Hu et al., 2008 which showed that T-DNA knockout of defence response genes can confer resistance to *R. solanacearum* in the susceptible Col-0, suggesting the role of the *WRKY 53* and *N25152* genes upon *R. solanacearum* inoculation in Col-0 plants (Hu et al., 2008). The Col-0 SALK line could also be re-complemented with the Kil-0 *AtDRG* gene as in the case of Kil-0 and Be-0 described above. Furthermore, the Kil-0 *AtDRG* gene can be over expressed in the Col-0. If after inoculation with *R. solanacearum* in the lines, tolerance is conveyed, then the *AtDRG* gene would be an important *R* gene in the Biotechnology and Agricultural industries for resistance against pathogens.

4.9 Reference

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Appendix

Appendix A

Table of *A. thaliana* non- redundant ESTs and their annotations in the SSHdb (http://www.sshdb.bi.up.ac.za)

SSH clone name	Sequence length (bp). Vector sequences removed.	blastX / blastN priority	BLASTX or BLASTN hit in the non redundant (nr) database of Genbank	Genbank accession number of BLAST hit	E_value of BLAST hit
VI64_AR1-G2_R	593	X	glutathione S-transferase [Brassica napus]	ABD36807	2.08E-72
VI64_AR4-F1_R	712	X	carbonic anhydrase chloroplast precursor [*Arabidopsis thaliana*]	BAD93915	5.83E-95
VI64_AR1-E2_R	737	X	FRO2-like protein; NADPH oxidase-like [*Arabidopsis thaliana*]	BAA98161	1.64E-42
VI64_AR4-E9_R	**582**	**N**	**Plant expression vector pDuExP (pDuExAn6) complete sequence**	**EF565883**	**5.88E-51**
VI64_SR4-F8_R	884	X	hypothetical protein [*Arabidopsis thaliana*]	BAE99015	8.68473
VI64_AF1-B10_F	415	X	ribosomal protein putative [*Arabidopsis thaliana*]	AAM64753	5.29E-65
VI64_SF1-F8_F	427	X	putative senescence-associated protein [Pisum sativum]	BAB33422	2.33E-15
VI64_AF1-B11_F	**286**	**X**	**glutathione peroxidase [*Arabidopsis thaliana*]**	**CAA61965**	**1.22E-48**
VI64_AF1-B5_F	**701**	**X**	**PLDALPHA1 (PHOSPHOLIPASE D ALPHA 1); phospholipase D [*Arabidopsis thaliana*] >gi|13124800|sp|Q38882.2|PLDA1_ARATH RecName: Full=Phospholipase D alpha 1; Short=PLD alpha 1; Short=AtPLDalpha1; AltName: Full=Choline phosphatase 1; AltName: Full=Phosphatidylcholine-hydrolyzing phospholipase D 1; AltName: Full=PLDalpha >gi|11994345|dbj|BAB02304.1| phospholipase D [*Arabidopsis thaliana*] >gi|110742066|dbj|BAE98964.1| phospholipase D [*Arabidopsis thaliana*]**	**NP_188194**	**1.38E-133**
VI64_AF1-E2_F	211	X	lipin family protein [*Arabidopsis thaliana*] >gi|9758575|dbj|BAB09188.1| unnamed protein product [*Arabidopsis thaliana*]	NP_199101	5.62E-29
VI64_AF1-G6_F	559	X	Highly similar to auxin-induced protein (aldo/keto reductase family) [*Arabidopsis thaliana*]	AAB71969	1.34E-40
VI64_AF2-A1_F	631	X	ADF1 (ACTIN DEPOLYMERIZING FACTOR 1) [*Arabidopsis thaliana*] >gi|17366511|sp|Q39250.1|ADF1_ARATH RecName: Full=Actin-depolymerizing factor 1; Short=AtADF1; Short=ADF-1 >gi|11513711|pdb|1F7S|A Chain A Crystal Structure Of Adf1 From *Arabidopsis thaliana* >gi|1408471|gb|AAB03696.1| actin depolymerizing factor 1 >gi|3851707|gb|AAC72407.1| actin depolymerizing factor 1 [*Arabidopsis thaliana*] >gi|7630029|emb|CAB88325.1| actin depolymerizing factor 1 (ADF1) [*Arabidopsis thaliana*] >gi|14334962|gb|AAK59658.1| putative actin depolymerizing factor ADF1 [*Arabidopsis thaliana*] >gi|17065584|gb|AAL33770.1| putative actin depolymerizing factor 1 [*Arabidopsis thaliana*] >gi|21553985|gb|AAM63066.1| actin-depolymerizing factor ADF-1 (AtADF1) [*Arabidopsis thaliana*] >gi|195604826|gb|ACG24243.1| hypothetical protein [Zea mays]	NP_190187	1.08E-64
VI64_AF5-E12_F	361	X	ATEYA (*ARABIDOPSIS THALIANA* EYES ABSENT HOMOLOG); protein tyrosine phosphatase metal-dependent [*Arabidopsis thaliana*] >gi|15294282|gb|AAK95318.1|AF410332_1 At2g35320/T4C15.1 [*Arabidopsis thaliana*] >gi|15450950|gb|AAK96746.1| EYA-like protein [*Arabidopsis thaliana*] >gi|17978677|gb|AAL47332.1| EYA-like protein [*Arabidopsis thaliana*] >gi|20197216|gb|AAC61806.2| similar to eyes absent protein [*Arabidopsis thaliana*] >gi|23506145|gb|AAN31084.1| At2g35320/T4C15.1 [*Arabidopsis thaliana*]	NP_565803	4.36E-59
VI64_AF2-F7_F	628	X	alanine aminotransferase 2 [Glycine max]	ABW17197	2.37E-81
VI64_AF2-F9_F	**571**	**N**	***Arabidopsis thaliana* disease resistance protein (CC-NBS-LRR class) putative (AT5G48620) mRNA complete cds**	**NM_124238**	**3.87E-31**
VI64_AF2-G8_F	502	X	hypothetical protein [Homo sapiens]	CAD91136	1.08E-50

Table of *A. thaliana* non- redundant ESTs and their annotations in the SSHdb (http://www.sshdb.bi.up.ac.za)

SSH clone name	Sequence length (bp). Vector sequences removed.	blastX / blastN priority	BLASTX or BLASTN hit in the non redundant (nr) database of Genbank	Genbank accession number of BLAST hit	E_value of BLAST hit
VI64_AF2-H2_F	548	X	putative tubulin alpha-2/alpha-4 chain [Brassica napus]	AAQ81585	2.07E-86
VI64_AF3-A9_F	361	X	C2 domain-containing protein [*Arabidopsis thaliana*] >gi|12321680|gb|AAG50882.1|AC025294_20 unknown protein [*Arabidopsis thaliana*]	NP_175568	2.09E-58
VI64_AR6-B11_R	726	X	RecName: Full=Serine carboxypeptidase-like 20; Flags: Precursor	Q8L7B2	2.61E-118
VI64_AF3-D1_F	464	X	basic helix-loop-helix (bHLH) family protein [*Arabidopsis thaliana*] >gi|75308807|sp|Q9C690.1|BH122_ARATH RecName: Full=Transcription factor bHLH122; AltName: Full=Transcription factor EN 70; AltName: Full=bHLH transcription factor bHLH122; AltName: Full=Basic helix-loop-helix protein 122; Short=bHLH 122; Short=AtbHLH122 >gi|12320788|gb|AAG50543.1|AC079828_14 unknown protein [*Arabidopsis thaliana*] >gi|14334500|gb|AAK59447.1| unknown protein [*Arabidopsis thaliana*] >gi|17104811|gb|AAL34294.1| unknown protein [*Arabidopsis thaliana*]	NP_564583	3.18E-78
VI64_AF3-E4_F	696	X	unnamed protein product [Homo sapiens]	BAG54610	6.01E-57
VI64_AF5-G7_F	666	X	putative receptor protein kinase [*Arabidopsis thaliana*]	AAK92807	1.01E-18
VI64_AF3-G4_F	211	X	binding / catalytic/ coenzyme binding [*Arabidopsis thaliana*] >gi|25083201|gb|AAN72050.1| Unknown protein [*Arabidopsis thaliana*] >gi|30725480|gb|AAP37762.1| At3g18890 [*Arabidopsis thaliana*]	NP_188519	7.77E-24
VI64_AF4-B4_F	655	X	protein A [Enterobacteria phage phiX174]	ABN49677	4.51E-48
VI64_SR4-E8_R	607	X	NCED4 (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 4) [*Arabidopsis thaliana*] >gi|75318399|sp|O49675.1|CCD4_ARATH RecName: Full=Probable carotenoid cleavage dioxygenase 4 chloroplastic; Short=AtCCD4; Short=AtNCED4; Flags: Precursor >gi|2828292|emb|CAA16706.1| neoxanthin cleavage enzyme-like protein [*Arabidopsis thaliana*] >gi|7268712|emb|CAB78919.1| neoxanthin cleavage enzyme-like protein [*Arabidopsis thaliana*] >gi|15983767|gb|AAL10480.1| AT4g19170/T18B16_140 [*Arabidopsis thaliana*] >gi|22531030|gb|AAM97019.1| neoxanthin cleavage enzyme-like protein [*Arabidopsis thaliana*] >gi|31711736|gb|AAP68224.1| At4g19170 [*Arabidopsis thaliana*] >gi|110742605|dbj|BAE99215.1| neoxanthin cleavage enzyme-like protein [*Arabidopsis thaliana*]	NP_193652	2.60E-113
VI64_AF4-E10_F	224	X	PREDICTED: hypothetical protein isoform 3 [Pan troglodytes]	XP_520622	6.27E-29
VI64_AF4-F1_F	459	X	hypothetical protein [*Arabidopsis thaliana*]	BAD94458	7.46E-43
VI64_AF4-G3_F	605	X	LHCA4 (Photosystem I light harvesting complex gene 4); chlorophyll binding [*Arabidopsis thaliana*] >gi|115385|sp|P27521.1|CB24_ARATH RecName: Full=Chlorophyll a-b binding protein 4 chloroplastic; AltName: Full=LHCI type III CAB-4; Short=LHCP; Flags: Precursor >gi|166646|gb|AAA32760.1| light-harvesting chlorophyll a/b binding protein >gi|6522530|emb|CAB61973.1| CHLOROPHYLL A-B BINDING PROTEIN 4 PRECURSOR homolog [*Arabidopsis thaliana*] >gi|20260362|gb|AAM13079.1| chlorophyll A-B binding protein 4 precursor homolog [*Arabidopsis thaliana*] >gi|21554365|gb|AAM63472.1| chlorophyll a-b binding protein 4 precursor homolog [*Arabidopsis thaliana*] >gi|23197770|gb|AAN15412.1| chlorophyll A-B binding protein 4 precursor homolog [*Arabidopsis thaliana*]	NP_190331	7.94E-14
VI64_SF2-G4_F	720	X	PSBP-1 (OXYGEN-EVOLVING ENHANCER PROTEIN 2); poly(U) binding [*Arabidopsis thaliana*] >gi|18206371|sp|Q42029.2|PSBP1_ARATH RecName: Full=Oxygen-evolving enhancer protein 2-1 chloroplastic;	NP_172153	5.5813

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SSH clone name	Sequence length (bp). Vector sequences removed.	blastX / blastN priority	BLASTX or BLASTN hit in the non redundant (nr) database of Genbank	Genbank accession number of BLAST hit	E_value of BLAST hit
			Short=OEE2; AltName: Full=23 kDa subunit of oxygen evolving system of photosystem II; AltName: Full=OEC 23 kDa subunit; Short=OEC23; AltName: Full=23 kDa thylakoid membrane protein; Flags: Precursor >gi|6692695|gb|AAF24829.1|AC007592_22 F12K11.3 [*Arabidopsis thaliana*] >gi|1769905|emb|CAA66785.1| 23 kDa polypeptide of oxygen-evolving comlex (OEC) [*Arabidopsis thaliana*] >gi|15912277|gb|AAL08272.1| At1g06680/F4H5_18 [*Arabidopsis thaliana*] >gi|17979237|gb|AAL49935.1| At1g06680/F4H5_18 [*Arabidopsis thaliana*] >gi|18377710|gb|AAL67005.1| putative 23 kDa polypeptide of oxygen-evolving comlex protein [*Arabidopsis thaliana*] >gi|20465405|gb|AAM20127.1| putative 23 kDa polypeptide of oxygen-evolving complex (OEC) [*Arabidopsis thaliana*] >gi|21592906|gb|AAM64856.1| 23 kDa polypeptide of oxygen-evolving comlex (OEC) [*Arabidopsis thaliana*]		
VI64_AF6-A5_F	404	X	cyclin family protein [*Arabidopsis thaliana*] >gi|147636402|sp|Q9FJK6.2|CCC11_ARATH RecName: Full=Cyclin-C1-1; Short=CycC1;1	NP_199675	5.42E-33
VI64_AF6-E12_F	749	X	MLP31 (MLP-LIKE PROTEIN 31) [*Arabidopsis thaliana*] >gi|148872485|sp|Q941R6.2|MLP31_ARATH RecName: Full=MLP-like protein 31 >gi|19424013|gb|AAL87294.1| unknown protein [*Arabidopsis thaliana*] >gi|21689799|gb|AAM67543.1| unknown protein [*Arabidopsis thaliana*] >gi|110740998|dbj|BAE98593.1| hypothetical protein [*Arabidopsis thaliana*]	NP_177241	2.65E-36
VI64_AF6-H10_F	596	X	zinc finger-like protein [*Arabidopsis thaliana*] >gi|18377546|gb|AAL66939.1| zinc finger-like protein [*Arabidopsis thaliana*]	AAK68811	1.03E-10
VI64_AR1-A1_R	223	X	aspartate aminotransferase Asp2 [*Arabidopsis thaliana*]	AAM91546	8.46E-34
VI64_AR2-A7_R	759	X	ankyrin repeat family protein [*Arabidopsis thaliana*]	NP_001118406	1.72E-143
VI64_AR5-A9_R	689	X	cytochrome b6f complex subunit (petM) putative [*Arabidopsis thaliana*] >gi|30683145|ref|NP_850079.1| cytochrome b6f complex subunit (petM) putative [*Arabidopsis thaliana*] >gi|14030737|gb|AAK53043.1|AF375459_1 At2g26500 [*Arabidopsis thaliana*] >gi|16974525|gb|AAL31172.1| At2g26500/T9J22.17 [*Arabidopsis thaliana*] >gi|20196942|gb|AAM14841.1| expressed protein [*Arabidopsis thaliana*] >gi|21618005|gb|AAM67055.1| unknown [*Arabidopsis thaliana*]	NP_565623	2.87E-59
VI64_AR3-E11_R	563	X	unknown protein [*Arabidopsis thaliana*] >gi|8778707|gb|AAF79715.1|AC020889_23 T1N15.6 [*Arabidopsis thaliana*] >gi|18086465|gb|AAL57686.1| At1g48450/T1N15_5 [*Arabidopsis thaliana*] >gi|24030356|gb|AAN41342.1| unknown protein [*Arabidopsis thaliana*]	NP_175278	7.33E-90
VI64_AR5-A1_R	629	X	26-kD peroxisomal membrane protein [*Arabidopsis thaliana*]	BAF80148	1.20E-31
VI64_SF1-E2_F	874	X	Lhcb2 protein [*Arabidopsis thaliana*]	AAD28771	1.28E-70
VI64_AR4-C10_R	534	X	ribonucleoprotein like protein [*Arabidopsis thaliana*] >gi|7268135|emb|CAB78472.1| ribonucleoprotein like protein [*Arabidopsis thaliana*]	CAB10209	8.86E-44
VI64_AR5-F2_R	668	X	ribosomal protein L35 family protein [*Arabidopsis thaliana*] >gi|17529002|gb|AAL38711.1| putative chloroplast ribosomal protein L35 [*Arabidopsis thaliana*] >gi|20465445|gb|AAM20182.1| putative chloroplast ribosomal protein L35 [*Arabidopsis thaliana*]	NP_850047	1.16E-46
VI64_AR5-F4_R	401	X	Mandelate racemase / muconate lactonizing enzyme C-terminal domain protein [Brevundimonas sp. BAL3]	EDX80071	2.79E-37

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SSH clone name	Sequence length (bp). Vector sequences removed.	blastX / blastN priority	BLASTX or BLASTN hit in the non redundant (nr) database of Genbank	Genbank accession number of BLAST hit	E_value of BLAST hit
VI64_AR6-A2_R	456	X	omega-3 fatty acid desaturase chloroplast precursor [*Arabidopsis thaliana*]	BAD94215	6.81E-12
VI64_SF1-A1_F	434	X	sodium-dicarboxylate cotransporter-like [*Arabidopsis thaliana*]	BAF02012	4.65E-69
VI64_SF1-B1_F	454	X	troponin T type 1 (skeletal slow) isoform CRA_a [Homo sapiens]	EAW72336	2.41E-41
VI64_SF1-E10_F	724	N	Synthetic construct Homo sapiens gateway clone IMAGE:100023435 5' read SEPHS2 mRNA	CU677936	7.65E-35
VI64_SF1-G2_F	475	X	nucellin-like protein [*Arabidopsis thaliana*]	AAM64443	1.66E-58
VI64_SF2-C1_F	234	X	protein D [Enterobacteria phage phiX174] >gi|125661507|gb|ABN49759.1| protein D [Enterobacteria phage phiX174] >gi|125661591|gb|ABN49836.1| protein D [Enterobacteria phage phiX174] >gi|125661615|gb|ABN49858.1| protein D [Enterobacteria phage phiX174]	ABN49748	6.21E-29
VI64_SF2-D9_F	736	X	KEU (KEULE); protein transporter [*Arabidopsis thaliana*] >gi|150421587|sp|Q9C5X3.2|KEULE_ARATH RecName: Full=SNARE-interacting protein KEULE >gi|110743380|dbj|BAE99577.1| hypothetical protein [*Arabidopsis thaliana*]	NP_563905	1.70E-31
VI64_SR2-H8_R	354	N	*Arabidopsis thaliana* ATVAMP714 (Vesicle-associated membrane protein 714) (ATVAMP714) mRNA complete cds	NM_122141	0.00117951
VI64_SF2-H9_F	592	X	aldo/keto reductase family protein [*Arabidopsis thaliana*] >gi|14326473|gb|AAK60282.1|AF385689_1 At2g27680/F15K20.22 [*Arabidopsis thaliana*] >gi|3860264|gb|AAC73032.1| expressed protein [*Arabidopsis thaliana*] >gi|18700198|gb|AAL77709.1| At2g27680/F15K20.22 [*Arabidopsis thaliana*]	NP_565656	5.51E-110
VI64_SF3-B11_F	360	X	ribulose bisphosphate carboxylase small chain 3B / RuBisCO small subunit 3B (RBCS-3B) (ATS3B) [*Arabidopsis thaliana*] >gi|20141686|sp|P10798.2|RBS3B_ARATH RecName: Full=Ribulose bisphosphate carboxylase small chain 3B chloroplastic; Short=RuBisCO small subunit 3B; Flags: Precursor >gi|13430424|gb|AAK25834.1|AF360124_1 putative ribulose bisphosphate carboxylase small chain 3b precursor [*Arabidopsis thaliana*] >gi|15294246|gb|AAK95300.1|AF410314_1 F1O19.10/F1O19.10 [*Arabidopsis thaliana*] >gi|18087561|gb|AAL58912.1|AF462822_1 At5g38410/F1O19.10 [*Arabidopsis thaliana*] >gi|9758819|dbj|BAB09353.1| ribulose bisphosphate carboxylase small chain 3b precursor (RuBisCO small subunit 3b) [*Arabidopsis thaliana*] >gi|15293183|gb|AAK93702.1| putative RuBisCO small 3b subunit precursor [*Arabidopsis thaliana*] >gi|15450944|gb|AAK96743.1| ribulose bisphosphate carboxylase small chain 3b precursor (RuBisCO small subunit 3b) [*Arabidopsis thaliana*] >gi|17978793|gb|AAL47390.1| ribulose bisphosphate carboxylase small chain 3b precursor (RuBisCO small subunit 3b) [*Arabidopsis thaliana*] >gi|20466117|gb|AAM19980.1| At5g38410/F1O19.10 [*Arabidopsis thaliana*] >gi|23397160|gb|AAN31863.1| putative ribulose bisphosphate carboxylase small chain 3b precursor (RuBisCO small subunit 3b) [*Arabidopsis thaliana*]	NP_198657	2.04E-48
VI64_SF3-G1_F	407	X	phosphoribulokinase/uridine kinase-related [*Arabidopsis thaliana*]	NP_001077855	7.52E-43
VI64_SF5-B2_F	664	X	contains similarity to plastid ribosomal protein L19 [*Arabidopsis thaliana*]	AAM64533	5.88E-51
VI64_SF5-C4_F	687	X	nascent polypeptide-associated complex alpha subunit [Pongo abelii] >gi|71152000|sp|Q5R9I9.1|NACA_PONAB RecName: Full=Nascent polypeptide-associated complex subunit alpha; Short=NAC-alpha; AltName: Full=Alpha-	NP_001125923	4.93E-48

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SSH clone name	Sequence length (bp). Vector sequences removed.	blastX / blastN priority	BLASTX or BLASTN hit in the non redundant (nr) database of Genbank	Genbank accession number of BLAST hit	E_value of BLAST hit
			NAC >gi|55729684|emb|CAH91571.1| hypothetical protein [Pongo abelii]		
VI64_SF5-G1_F	**357**	**X**	**TCH4 (TOUCH 4); hydrolase acting on glycosyl bonds / xyloglucan:xyloglucosyl transferase [*Arabidopsis thaliana*] >gi|38605148|sp|Q38857.1|XTH22_ARATH RecName: Full=Xyloglucan endotransglucosylase/hydrolase protein 22; Short=At-XTH22; Short=XTH-22; AltName: Full=Touch protein 4; Flags: Precursor >gi|14194113|gb|AAK56251.1|AF367262_1 AT5g57560/MUA2_13 [*Arabidopsis thaliana*] >gi|17386136|gb|AAL38614.1|AF446881_1 AT5g57560/MUA2_13 [*Arabidopsis thaliana*] >gi|886116|gb|AAA92363.1| TCH4 protein >gi|2952473|gb|AAC05572.1| xyloglucan endotransglycosylase related protein [*Arabidopsis thaliana*] >gi|9758317|dbj|BAB08791.1| TCH4 protein [*Arabidopsis thaliana*] >gi|15450689|gb|AAK96616.1| AT5g57560/MUA2_13 [*Arabidopsis thaliana*] >gi|15777883|gb|AAL05902.1| AT5g57560/MUA2_13 [*Arabidopsis thaliana*]**	**NP_200564**	**1.41E-65**
VI64_SF6-C11_F	539	X	ferredoxin-dependent glutamate synthase [*Arabidopsis thaliana*]	BAE98673	1.08E-60
VI64_SF6-C3_F	425	X	POM1 (POM-POM1); chitinase [*Arabidopsis thaliana*] >gi|6850314|gb|AAF29391.1|AC009999_11 Contains similarity to a basic endochitinase from Arabidopis thaliana gb|AB023448 and contains a Chitinases class I PF|00182 domain. ESTs gb|AI995747 gb|AA728545 gb|Z26222 gb|Z25683 gb|T88386 gb|T14122 gb|T04241 gb|N38122 come from this gene. [*Arabidopsis thaliana*] >gi|12083324|gb|AAG48821.1|AF332458_1 putative class I chitinase [*Arabidopsis thaliana*] >gi|17226329|gb|AAL37736.1|AF422178_1 chitinase-like protein 1 [*Arabidopsis thaliana*] >gi|17226331|gb|AAL37737.1|AF422179_1 chitinase-like protein 1 [*Arabidopsis thaliana*] >gi|14334488|gb|AAK59442.1| putative class I chitinase [*Arabidopsis thaliana*] >gi|21280935|gb|AAM44973.1| putative class I chitinase [*Arabidopsis thaliana*]	NP_172076	3.29E-46
VI64_SF6-H9_F	540	X	CPN60B (CHAPERONIN 60 BETA); ATP binding / protein binding / unfolded protein binding [*Arabidopsis thaliana*] >gi|30695947|ref|NP_849811.1| CPN60B (CHAPERONIN 60 BETA); ATP binding / protein binding / unfolded protein binding [*Arabidopsis thaliana*] >gi|27735252|sp|P21240.3|RUBB_ARATH RecName: Full=RuBisCO large subunit-binding protein subunit beta chloroplastic; AltName: Full=60 kDa chaperonin subunit beta; AltName: Full=CPN-60 beta; Flags: Precursor >gi|14423416|gb|AAK62390.1|AF386945_1 Rubisco subunit binding-protein beta subunit [*Arabidopsis thaliana*] >gi|4204266|gb|AAD10647.1| Rubisco subunit binding-protein beta subunit [*Arabidopsis thaliana*] >gi|20148345|gb|AAM10063.1| Rubisco subunit binding-protein beta subunit [*Arabidopsis thaliana*]	NP_175945	6.40E-93
VI64_SR6-B1_R	366	X	glyceraldehyde 3-phosphate dehydrogenase B subunit [*Arabidopsis thaliana*]	AAD10210	1.52E-20
VI64_SR1-C4_R	622	X	cytochrome P450-like TBP protein [Lilium longiflorum]	ABO20848	1.01E-53
VI64_SR1-D12_R	1094	N	Homo sapiens oxysterol-binding protein-related protein 1 (OSBPL1A) gene complete cds alternatively spliced	EF445004	9.30E-94
VI64_SR2-D2_R	356	X	chlorophyll a/b-binding protein CP29 [*Arabidopsis thaliana*]	AAM12979	5.78E-35
VI64_SR2-G10_R	564	X	ATP synthase delta chain chloroplast putative / H(+)-transporting two-sector ATPase delta (OSCP) subunit putative [*Arabidopsis thaliana*] >gi|7267660|emb|CAB78088.1| H+-transporting ATP synthase-like protein [*Arabidopsis thaliana*] >gi|7321084|emb|CAB82132.1| H+-transporting ATP synthase-like protein [*Arabidopsis thaliana*] >gi|17473800|gb|AAL38334.1| H+-transporting ATP synthase-like protein [*Arabidopsis thaliana*] >gi|21386985|gb|AAM47896.1| H+-transporting ATP synthase-like protein [*Arabidopsis thaliana*]	NP_192703	9.00E-72

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SSH clone name	Sequence length (bp). Vector sequences removed.	blastX / blastN priority	BLASTX or BLASTN hit in the non redundant (nr) database of Genbank	Genbank accession number of BLAST hit	E_value of BLAST hit
			>gi|21593484|gb|AAM65451.1| H+-transporting ATP synthase-like protein [*Arabidopsis thaliana*] >gi|21689783|gb|AAM67535.1| putative H+-transporting ATP synthase [*Arabidopsis thaliana*]		
VI64_SR3-A10_R	734	N	*Arabidopsis thaliana* unknown protein mRNA complete cds	AY136474	6.14E-29
VI64_SR2-H2_R	432	X	ATCYSD2 (*Arabidopsis thaliana* cysteine synthase D2); cysteine synthase >gi|30690901|ref|NP_851087.1| ATCYSD2 (*Arabidopsis thaliana* cysteine synthase D2); cysteine synthase >gi|79328865|ref|NP_001031956.1| ATCYSD2 (*Arabidopsis thaliana* cysteine synthase D2); cysteine synthase >gi|79328884|ref|NP_001031957.1| ATCYSD2 (*Arabidopsis thaliana* cysteine synthase D2); cysteine synthase >gi|145334565|ref|NP_001078628.1| ATCYSD2 (*Arabidopsis thaliana* cysteine synthase D2) >gi|15983448|gb|AAL11592.1|AF424598_1 AT5g28020/F15F15_90 [*Arabidopsis thaliana*] >gi|4996618|dbj|BAA78561.1| cysteine synthase [*Arabidopsis thaliana*] >gi|21700833|gb|AAM70540.1| AT5g28020/F15F15_90 [*Arabidopsis thaliana*]	NP_198154	3.70E-66
VI64_SR2-H5_R	444	X	cystatin-like protein [*Arabidopsis thaliana*]	AAM64661	2.38E-20
VI64_SR3-B10_R	114	N	*Arabidopsis thaliana* mRNA for hypothetical protein complete cds clone: RAFL06-07-G22	AK226407	1.66E-26
VI64_SR3-B11_R	751	X	F4N2.21 [*Arabidopsis thaliana*]	AAF27061	4.01E-20
VI64_SR3-D3_R	562	X	unknown protein [*Arabidopsis thaliana*] >gi|79325273|ref|NP_001031724.1| unknown protein [*Arabidopsis thaliana*] >gi|4938501|emb|CAB43859.1| putative protein [*Arabidopsis thaliana*] >gi|7269515|emb|CAB79518.1| putative protein [*Arabidopsis thaliana*]	NP_194393	1.85E-08
VI64_SR4-A11_R	489	X	alpha 1 4-glycosyltransferase family protein / glycosyltransferase sugar-binding DXD motif-containing protein [*Arabidopsis thaliana*] >gi|5923667|gb|AAD56318.1|AC009326_5 hypothetical protein [*Arabidopsis thaliana*] >gi|6403486|gb|AAF07826.1|AC010871_2 unknown protein [*Arabidopsis thaliana*]	NP_187514	3.10E-20
VI64_SR4-D11_R	490	X	chlorophyll a/b binding protein [Brassica oleracea]	AAP44089	5.44E-25
VI64_SR6-E10_R	461	X	unknown protein [*Arabidopsis thaliana*]	AAL49886	1.14E-59
VI64_SR6-G1_R	403	X	APE1 (ACCLIMATION OF PHOTOSYNTHESIS TO ENVIRONMENT) [*Arabidopsis thaliana*] >gi|87116662|gb|ABD19695.1| At5g38660 [*Arabidopsis thaliana*]	NP_198682	9.33E-42
VI64_SR3-B9_R	380	X	leucine-rich repeat transmembrane protein kinase putative [*Arabidopsis thaliana*]	NP_172244	1.41E-65
VI64_SR5-H12_R	330	X	TIF3B1 (EUKARYOTIC TRANSLATION INITIATION FACTOR 3B); nucleic acid binding / translation initiation factor [*Arabidopsis thaliana*]	NP_001031954	1.08E-57
VI64_SR3-B7_R	247	N	*Arabidopsis thaliana* SHM4 (SERINE HYDROXYMETHYLTRANSFERASE 4); glycine hydroxymethyltransferase (SHM4) mRNA complete cds	NM_117467	6.55E-15
VI64_SR6-E7_R	352	X	PREDICTED: similar to colonic and hepatic tumor over-expressed protein isoform 1 [Bos taurus]	XP_001790297	2.58E-59
VI64_SR6-F1_R	869	X	heat shock protein binding / unfolded protein binding [*Arabidopsis thaliana*] >gi|186496567|ref|NP_001031306.2| heat shock protein binding / unfolded protein binding [*Arabidopsis thaliana*] >gi|186496571|ref|NP_001117623.1| heat shock protein binding / unfolded protein binding [*Arabidopsis thaliana*] >gi|110743727|dbj|BAE99700.1| putative DnaJ protein [*Arabidopsis thaliana*]	NP_178112	1.09E-142
VI64_AF3-B8_F	412	X	alpha-L-arabinofuranosidase [*Arabidopsis thaliana*]	AAO92261	6.59E-07
VI64_AF1-C2_F	245	X	SIGB (SIGMA FACTOR B); DNA binding / DNA-directed RNA polymerase/ transcription factor [*Arabidopsis thaliana*]	NP_172330	5.53E-38

Table of *A. thaliana* non- redundant ESTs and their annotations in the SSHdb (http://www.sshdb.bi.up.ac.za)

SSH clone name	Sequence length (bp). Vector sequences removed.	blastX / blastN priority	BLASTX or BLASTN hit in the non redundant (nr) database of Genbank	Genbank accession number of BLAST hit	E_value of BLAST hit
			>gi|6664315|gb|AAF22897.1|AC006932_14 T27G7.22 [*Arabidopsis thaliana*] >gi|9802550|gb|AAF99752.1|AC003981_2 F22O13.2 [*Arabidopsis thaliana*] >gi|2443357|dbj|BAA22427.1| SigB [*Arabidopsis thaliana*] >gi|2597831|emb|CAA75584.1| sigma factor [*Arabidopsis thaliana*] >gi|2879922|dbj|BAA24825.1| plastid RNA polymerase sigma-subunit [*Arabidopsis thaliana*] >gi|5478446|dbj|BAA82449.1| sigma factor SigB [*Arabidopsis thaliana*] >gi|133778838|gb|ABO38759.1| At1g08540 [*Arabidopsis thaliana*]		
VI64_AF4-A1_F	421	X	ATNHD1 (*Arabidopsis thaliana* Na/H antiporter 1); sodium:hydrogen antiporter >gi|11994472|dbj|BAB02474.1| unnamed protein product [*Arabidopsis thaliana*] >gi|21537143|gb|AAM61484.1| unknown [*Arabidopsis thaliana*]	NP_566638	6.05E-45
VI64_AR3-H2_R	409	X	hydroxypyruvate reductase [*Arabidopsis thaliana*]	BAA19751	6.10E-61
VI64_SF4-B9_F	361	X	ARC5 (ACCUMULATION AND REPLICATION OF CHLOROPLAST 5); GTP binding / GTPase [*Arabidopsis thaliana*]	NP_850615	9.46E-46
VI64_AR1-H10_R	168	X	RecName: Full=Pentatricopeptide repeat-containing protein At1g47580 chloroplastic; Flags: Precursor	P0C7R1	1.74E-23
VI64_AF3-C2_F	449	X	protein binding / protein transporter [*Arabidopsis thaliana*] >gi|9758308|dbj|BAB08782.1| unnamed protein product [*Arabidopsis thaliana*] >gi|44917457|gb|AAS49053.1| At5g57460 [*Arabidopsis thaliana*] >gi|56381939|gb|AAV85688.1| At5g57460 [*Arabidopsis thaliana*] >gi|110737819|dbj|BAF00848.1| hypothetical protein [*Arabidopsis thaliana*]	NP_200555	2.25E-79
VI64_SF6-D7_F	681	X	SAMDC (S-ADENOSYLMETHIONINE DECARBOXYLASE); adenosylmethionine decarboxylase [*Arabidopsis thaliana*] >gi|145331732|ref|NP_001078093.1| SAMDC (S-ADENOSYLMETHIONINE DECARBOXYLASE) [*Arabidopsis thaliana*] >gi|19864681|sp|Q96286.2|DCAM1_ARATH RecName: Full=S-adenosylmethionine decarboxylase proenzyme 1; Short=AdoMetDC 1; Short=SamDC 1; Contains: RecName: Full=S-adenosylmethionine decarboxylase 1 alpha chain; Contains: RecName: Full=S-adenosylmethionine decarboxylase 1 beta chain >gi|16226705|gb|AAL16237.1|AF428468_1 AT3g02470/F16B3_10 [*Arabidopsis thaliana*] >gi|6957710|gb|AAF32454.1| S-adenosylmethionine decarboxylase [*Arabidopsis thaliana*] >gi|14596073|gb|AAK68764.1| S-adenosylmethionine decarboxylase [*Arabidopsis thaliana*] >gi|20148235|gb|AAM10008.1| S-adenosylmethionine decarboxylase [*Arabidopsis thaliana*]	NP_186896	6.70E-130
VI64_SF4-D1_F	421	X	Chain A Crystal Structure Of The Allene Oxide Cyclase 2 With Bound Inhibitor Vernolic Acid >gi|119389187|pdb|2DIO|B Chain B Crystal Structure Of The Allene Oxide Cyclase 2 With Bound Inhibitor Vernolic Acid >gi|119389188|pdb|2DIO|C Chain C Crystal Structure Of The Allene Oxide Cyclase 2 With Bound Inhibitor Vernolic Acid	2DIO_A	2.94E-07
VI64_SR4-G4_R	406	X	Hypothetical protein [*Arabidopsis thaliana*]	AAD39273	7.60E-19
VI64_SF1-H3_F	479	X	SRZ-22 (SERINE/ARGININE-RICH 22) [*Arabidopsis thaliana*] >gi|145334187|ref|NP_001078474.1| SRZ-22 (SERINE/ARGININE-RICH 22) [*Arabidopsis thaliana*] >gi|3281869|emb|CAA19765.1| RSZp22 splicing factor [*Arabidopsis thaliana*] >gi|3435094|gb|AAD12769.1| 9G8-like SR protein [*Arabidopsis thaliana*] >gi|7270061|emb|CAB79876.1| RSZp22 splicing factor [*Arabidopsis thaliana*] >gi|17529204|gb|AAL38828.1| putative RSZp22 splicing factor [*Arabidopsis thaliana*] >gi|21436285|gb|AAM51281.1| putative RSZp22 splicing factor [*Arabidopsis thaliana*] >gi|21554419|gb|AAM63524.1| RSZp22 splicing factor [*Arabidopsis thaliana*]	NP_194886	1.90E-46

Table of *A. thaliana* non- redundant ESTs and their annotations in the SSHdb (http://www.sshdb.bi.up.ac.za)

SSH clone name	Sequence length (bp). Vector sequences removed.	blastX / blastN priority	BLASTX or BLASTN hit in the non redundant (nr) database of Genbank	Genbank accession number of BLAST hit	E_value of BLAST hit
VI64_SF3-A1_F	349	X	photosystem I subunit III precursor [*Arabidopsis thaliana*] >gi|21593588|gb|AAM65555.1| photosystem I subunit III precursor putative [*Arabidopsis thaliana*]	CAB52747	2.89E-50
VI64_SF5-B1_F	621	X	calmodulin-like calcium-binding protein 22 kDa (CaBP-22) [*Arabidopsis thaliana*] >gi|231700|sp|P30187.1|CML10_ARATH RecName: Full=Calmodulin-like protein 10; AltName: Full=22 kDa calmodulin-like calcium-binding protein; AltName: Full=CABP-22 >gi|16209|emb|CAA78124.1| calcium binding protein [*Arabidopsis thaliana*] >gi|3402708|gb|AAD12002.1| calcium binding protein (CaBP-22) [*Arabidopsis thaliana*] >gi|15028259|gb|AAK76718.1| putative calcium binding protein CaBP-22 [*Arabidopsis thaliana*] >gi|21537205|gb|AAM61546.1| calcium binding protein CaBP-22 [*Arabidopsis thaliana*]	NP_181642	7.95E-105
VI64_AR4-A7_R	311	X	unknown protein [*Arabidopsis thaliana*] >gi|105829364|gb|ABF74691.1| At1g52827 [*Arabidopsis thaliana*]	NP_974010	1.46E-06
VI64_AR4-E12_R	180	X	At4g10340-like protein [Arabidopsis lyrata subsp. petraea]	ABS78806	1.27E-18
VI64_AR4-F7_R	68	N	*Arabidopsis thaliana* ATPRX Q; antioxidant/ peroxiredoxin (ATPRX Q) mRNA complete cds	NM_113510	1.67E-10
VI64_AR4-G10_R	463	X	mitochondrial ribosomal protein L51/S25/CI-B8 family protein [*Arabidopsis thaliana*] >gi|6996301|emb|CAB75462.1| putative protein [*Arabidopsis thaliana*] >gi|21617971|gb|AAM67021.1| unknown [*Arabidopsis thaliana*] >gi|27808540|gb|AAO24550.1| At3g59650 [*Arabidopsis thaliana*] >gi|110743592|dbj|BAE99633.1| hypothetical protein [*Arabidopsis thaliana*]	NP_191524	5.45E-62
VI64_AR5-C9_R	748	X	sigma factorB [*Arabidopsis thaliana*]	CAA74896	3.40E-128
VI64_AR5-E3_R	237	X	Chain A Crystal Structure Of O-Acetylserine Sulfhydrylase From *Arabidopsis thaliana* In Complex With C-Terminal Peptide From Arabidopsis Serine Acetyltransferase	2ISQ_A	5.03E-39
VI64_AR5-F9_R	534	N	*Arabidopsis thaliana* mRNA for hypothetical protein complete cds clone: RAFL24-16-G14	AK230328	6.15E-13
VI64_AR5-H6_R	172	N	*Arabidopsis thaliana* LP1 (nonspecific lipid transfer protein 1) (LP1) mRNA complete cds	NM_129411	3.25E-22
VI64_AR6-B6_R	302	X	BRI1 (BRASSINOSTEROID INSENSITIVE 1); kinase [*Arabidopsis thaliana*] >gi|29427562|sp|O22476.1|BRI1_ARATH RecName: Full=Protein BRASSINOSTEROID INSENSITIVE 1; Short=AtBRI1; AltName: Full=Brassinosteroid LRR receptor kinase; Flags: Precursor >gi|2392895|gb|AAC49810.1| brassinosteroid insensitive 1 [*Arabidopsis thaliana*] >gi|5042156|emb|CAB44675.1| brassinosteroid insensitive 1 gene (BRI1) [*Arabidopsis thaliana*] >gi|7270924|emb|CAB80603.1| brassinosteroid insensitive 1 gene (BRI1) [*Arabidopsis thaliana*]	NP_195650	9.45E-54
VI64_AR6-E10_R	223	X	plasma membrane proton pump H+ ATPase	AAA32813	1.82E-36
VI64_AR6-F1_R	257	X	unknown protein [*Arabidopsis thaliana*] >gi|6648215|gb|AAF21213.1|AC013483_37 unknown protein [*Arabidopsis thaliana*] >gi|18252185|gb|AAL61925.1| unknown protein [*Arabidopsis thaliana*] >gi|21555252|gb|AAM63815.1| unknown [*Arabidopsis thaliana*] >gi|23397197|gb|AAN31881.1| unknown protein [*Arabidopsis thaliana*] >gi|27311877|gb|AAO00904.1| unknown protein [*Arabidopsis thaliana*]	NP_566328	1.23E-16
VI64_AR6-G10_R	214	X	potassium channel tetramerisation domain-containing protein [*Arabidopsis thaliana*] >gi|16226415|gb|AAL16162.1|AF428394_1 AT4g30940/F6I18_150 [*Arabidopsis thaliana*] >gi|2980772|emb|CAA18199.1| putative protein [*Arabidopsis thaliana*] >gi|7269996|emb|CAB79812.1| putative protein [*Arabidopsis thaliana*] >gi|22137122|gb|AAM91406.1| At4g30940/F6I18_150 [*Arabidopsis thaliana*]	NP_194823	1.66E-34

Appendix A

Table of *A. thaliana* non- redundant ESTs and their annotations in the SSHdb (http://www.sshdb.bi.up.ac.za)

SSH clone name	Sequence length (bp). Vector sequences removed.	blastX / blastN priority	BLASTX or BLASTN hit in the non redundant (nr) database of Genbank	Genbank accession number of BLAST hit	E_value of BLAST hit
VI64_AR2-F2_R	626	X	RecName: Full=Probable fructose-bisphosphate aldolase 2 chloroplastic; Flags: Precursor >gi|16226653|gb|AAL16224.1|AF428455_1 AT4g38970/F19H22_70 [*Arabidopsis thaliana*]	Q944G9	2.12E-113
VI64_AR6-H2_R	533	X	CbbY protein-related [*Arabidopsis thaliana*] >gi|44917581|gb|AAS49115.1| At5g45170 [*Arabidopsis thaliana*] >gi|62321581|dbj|BAD95125.1| putative protein [*Arabidopsis thaliana*]	NP_199330	1.70E-95
VI64_SF1-H2_F	262	N	*Arabidopsis thaliana* EMB2386 (EMBRYO DEFECTIVE 2386); structural constituent of ribosome (EMB2386) mRNA complete cds	NM_100157	3.63E-99
VI64_SF3-F4_F	726	X	putative RNA-binding protein [*Arabidopsis thaliana*]	AAM66970	4.38E-93
VI64_SF3-B7_F	294	X	elongation factor 1B alpha-subunit 2 (eEF1Balpha2) [*Arabidopsis thaliana*] >gi|75313298|sp|Q9SCX3.1|EF1B2_ARATH RecName: Full=Elongation factor 1-beta 2; Short=EF-1-beta 2; AltName: Full=Elongation factor 1B-alpha 2; AltName: Full=eEF-1B alpha 2; AltName: Full=Elongation factor 1-beta' 2; Short=EF-1-beta' 2 >gi|13430784|gb|AAK26014.1|AF360304_1 putative elongation factor 1B alpha-subunit [*Arabidopsis thaliana*] >gi|6686821|emb|CAB64730.1| elongation factor 1B alpha-subunit [*Arabidopsis thaliana*] >gi|15810631|gb|AAL07240.1| putative elongation factor 1B alpha-subunit [*Arabidopsis thaliana*]	NP_568375	1.54E-19
VI64_SF3-B10_F	755	X	lipoxygenase AtLOX2 [*Arabidopsis thaliana*]	AAL32689	6.72E-140
VI64_SF3-D10_F	357	X	IAA16 (indoleacetic acid-induced protein 16); transcription factor [*Arabidopsis thaliana*] >gi|11131089|sp|O24407.1|IAA16_ARATH RecName: Full=Auxin-responsive protein IAA16; AltName: Full=Indoleacetic acid-induced protein 16 >gi|6175173|gb|AAF04899.1|AC011437_14 auxin-induced protein [*Arabidopsis thaliana*] >gi|12083210|gb|AAG48764.1|AF332400_1 auxin-induced protein IAA16 [*Arabidopsis thaliana*] >gi|14030659|gb|AAK53004.1|AF375420_1 AT3g04730/F7O18_22 [*Arabidopsis thaliana*] >gi|2618721|gb|AAB84353.1| IAA16 [*Arabidopsis thaliana*] >gi|21592802|gb|AAM64751.1| auxin-induced protein [*Arabidopsis thaliana*] >gi|23507781|gb|AAN38694.1| At3g04730/F7O18_22 [*Arabidopsis thaliana*] >gi|110738766|dbj|BAF01307.1| auxin-induced protein [*Arabidopsis thaliana*]	NP_187124	1.99E-27
VI64_SF5-A10_F	249	X	PAP1 (PURPLE ACID PHOSPHATASE 1); acid phosphatase/ protein serine/threonine phosphatase [*Arabidopsis thaliana*] >gi|18086494|gb|AAL57700.1| At2g27190/T22O13.4 [*Arabidopsis thaliana*] >gi|22137168|gb|AAM91429.1| At2g27190/T22O13.4 [*Arabidopsis thaliana*]	NP_180287	6.29E-42
VI64_SF5-H7_F	214	N	No significant hit	NULL	
VI64_SF6-F2_F	374	X	myoglobin isoform CRA_a [Homo sapiens] >gi|119580471|gb|EAW60067.1| myoglobin isoform CRA_a [Homo sapiens]	EAW60065	7.83E-56
VI64_SR1-E10_R	477	X	myosin heavy chain 1 skeletal muscle adult [Homo sapiens] >gi|119610411|gb|EAW90005.1| hCG1986604 isoform CRA_b [Homo sapiens]	NP_005954	5.98172
VI64_SR1-F9_R	387	N	*Arabidopsis thaliana* zinc finger (C3HC4-type RING finger) family protein (AT5G15790) mRNA complete cds	NM_203056	2.39E-12
VI64_AF1-C7_F	287	X	40S ribosomal protein S3a [Zea mays]	ACG48293	1.44E-41
VI64_AF1-D2_F	244	X	geranylgeranyl pyrophosphate synthase [*Arabidopsis thaliana*]	AAM65107	1.73E-15
VI64_AF1-O8_F	385	X	At2g42380/MHK10.10 [*Arabidopsis thaliana*]	AAL69473	9.77E-43
VI64_AF1-G11_F	384	X	CYP83A1 (CYTOCHROME P450 83A1); oxygen binding [*Arabidopsis thaliana*]	NP_193113	3.36E-59

Table of *A. thaliana* non- redundant ESTs and their annotations in the SSHdb (http://www.sshdb.bi.up.ac.za)

SSH clone name	Sequence length (bp). Vector sequences removed.	blastX / blastN priority	BLASTX or BLASTN hit in the non redundant (nr) database of Genbank	Genbank accession number of BLAST hit	E_value of BLAST hit
			>gi|6166038|sp|P48421.2|C83A1_ARATH RecName: Full=Cytochrome P450 83A1; AltName: Full=CYPLXXXIII >gi|16226709|gb|AAL16238.1|AF428469_1 AT4g13770/F18A5_160 [*Arabidopsis thaliana*] >gi|2454176|gb|AAB71623.1| cytochrome P450 monooxygenase [*Arabidopsis thaliana*] >gi|3164128|dbj|BAA28532.1| cytochrome P450 monooxygenase [*Arabidopsis thaliana*] >gi|4455306|emb|CAB36841.1| cytochrome P450 monooxygenase (CYP83A1) [*Arabidopsis thaliana*] >gi|7268081|emb|CAB78419.1| cytochrome P450 monooxygenase (CYP83A1) [*Arabidopsis thaliana*] >gi|18700184|gb|AAL77703.1| AT4g13770/F18A5_160 [*Arabidopsis thaliana*] >gi|20857340|gb|AAM26713.1| AT4g13770/F18A5_160 [*Arabidopsis thaliana*]		
VI64_AF1-G3_F	398	X	unknown protein [*Arabidopsis thaliana*] >gi|9280221|dbj|BAB01711.1| unnamed protein product [*Arabidopsis thaliana*] >gi|17065156|gb|AAL32732.1| Unknown protein [*Arabidopsis thaliana*] >gi|27311937|gb|AAO00934.1| Unknown protein [*Arabidopsis thaliana*]	NP_566678	7.30E-70
VI64_AF2-A2_F	164	X	glutathione peroxidase putative [*Arabidopsis thaliana*] >gi|75155056|sp|Q8LBU2.1|GPX8_ARATH RecName: Full=Probable glutathione peroxidase 8 >gi|21592603|gb|AAM64552.1| unknown [*Arabidopsis thaliana*] >gi|27765006|gb|AAO23624.1| At1g63460 [*Arabidopsis thaliana*] >gi|110743432|dbj|BAE99602.1| glutathione peroxidase like protein [*Arabidopsis thaliana*]	NP_564813	2.44E-25
VI64_AF2-A3_F	283	X	hCG2038983 [Homo sapiens]	EAW54940	6.99E-09
VI64_AF2-B8_F	128	N	Homo sapiens RAP2A member of RAS oncogene family (RAP2A) Mrna	NM_021033	6.78E-63
VI64_AF2-G3_F	137	X	60S acidic ribosomal protein P0 (RPP0B) [*Arabidopsis thaliana*]	NP_001078125	8.28E-18
VI64_AF2-G6_F	587	X	ATRAB7; GTP binding [*Arabidopsis thaliana*] >gi|79319588|ref|NP_001031161.1| ATRAB7; GTP binding [*Arabidopsis thaliana*] >gi|5430767|gb|AAD43167.1|AC007504_22 Putative RAB7 GTP-binding Protein [*Arabidopsis thaliana*] >gi|15718414|dbj|BAB68374.1| AtRab74 [*Arabidopsis thaliana*] >gi|28416619|gb|AAO42840.1| At1g49300 [*Arabidopsis thaliana*] >gi|110743311|dbj|BAE99544.1| hypothetical protein [*Arabidopsis thaliana*]	NP_175355	1.10E-102
VI64_AF3-B6_F	256	X	LHCA1; chlorophyll binding [*Arabidopsis thaliana*] >gi|11762180|gb|AAG40368.1|AF325016_1 AT3g54890 [*Arabidopsis thaliana*] >gi|11908038|gb|AAG41448.1|AF326866_1 putative chlorophyll a/b-binding protein [*Arabidopsis thaliana*] >gi|12642856|gb|AAK00370.1|AF339688_1 putative chlorophyll a/b-binding protein [*Arabidopsis thaliana*] >gi|13605732|gb|AAK32859.1|AF361847_1 AT3g54890/F28P10_130 [*Arabidopsis thaliana*] >gi|16207|emb|CAA39534.1| chlorophyll A/B-binding protein [*Arabidopsis thaliana*] >gi|166644|gb|AAA32759.1| chlorophyll a/b-binding protein >gi|4678304|emb|CAB41095.1| chlorophyll a/b-binding protein [*Arabidopsis thaliana*] >gi|17979245|gb|AAL49939.1| AT3g54890/F28P10_130 [*Arabidopsis thaliana*] >gi|20453135|gb|AAM19809.1| AT3g54890/F28P10_130 [*Arabidopsis thaliana*] >gi|23507771|gb|AAN38689.1| At3g54890/F28P10_130 [*Arabidopsis thaliana*]	NP_191049	2.38E-28
VI64_AF3-B11_F	601	X	phox (PX) domain-containing protein [*Arabidopsis thaliana*] >gi|10257486|dbj|BAB10207.1| sorting nexin-like protein [*Arabidopsis thaliana*] >gi|119935967|gb|ABM06047.1| At5g06140 [*Arabidopsis thaliana*]	NP_196232	1.73E-106
VI64_AF5-C2_F	536	X	hypothetical protein OsI_18129 [Oryza sativa Indica Group]	EEC78366	2.87E-82

Appendix A

Table of *A. thaliana* non- redundant ESTs and their annotations in the SSHdb (http://www.sshdb.bi.up.ac.za)

SSH clone name	Sequence length (bp). Vector sequences removed.	blastX / blastN priority	BLASTX or BLASTN hit in the non redundant (nr) database of Genbank	Genbank accession number of BLAST hit	E_value of BLAST hit
VI64_AF3-G3_F	473	X	CLB6 (CHLOROPLAST BIOGENESIS 6); 4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase [*Arabidopsis thaliana*] >gi|14596179|gb|AAK68817.1| putative protein [*Arabidopsis thaliana*] >gi|20148251|gb|AAM10016.1| putative protein [*Arabidopsis thaliana*] >gi|27368105|gb|AAN87171.1| ISPH [*Arabidopsis thaliana*]	NP_567965	1.78E-68
VI64_AF4-F3_F	118	N	*Arabidopsis thaliana* mRNA for ribulose bisphosphate carboxylase like protein small subunit complete cds clone: RAFL07-10-M24	AK226546	6.31E-56
VI64_AF4-F7_F	425	X	Chain A Crystal Structure Of The Pyruvate Dehydrogenase (E1p) Component Of Human Pyruvate Dehydrogenase Complex With The Subunit-Binding Domain (Sbd) Of E2p But Sbd Cannot Be Modeled Into The Electron Density	3EXI_A	2.20E-42
VI64_AF4-G6_F	238	N	*Arabidopsis thaliana* FQR1 (FLAVODOXIN-LIKE QUINONE REDUCTASE 1) (FQR1) mRNA complete cds	NM_124830	5.81E-11
VI64_AF4-H7_F	396	X	Similar to NAM protein [*Arabidopsis thaliana*]	AAF02847	5.87E-32
VI64_AF5-E9_F	234	N	*Arabidopsis thaliana* HSC70-1 (heat shock cognate 70 kDa protein 1); ATP binding (HSC70-1) mRNA complete cds	NM_001125684	5.02E-15
VI64_AF6-B7_F	367	X	unknown protein [*Arabidopsis thaliana*] >gi|98961813|gb|ABF59236.1| unknown protein [*Arabidopsis thaliana*] >gi|110737180|dbj|BAF00539.1| hypothetical protein [*Arabidopsis thaliana*]	NP_001031562	4.83E-34
VI64_AF6-B9_F	355	X	ATNOA1/ATNOS1/NOA1/NOS1 (NO ASSOCIATED 1); nitric-oxide synthase [*Arabidopsis thaliana*]	NP_190329	2.11E-61
VI64_AF6-F1_F	234	N	*Arabidopsis thaliana* ATGRP7 (COLD CIRCADIAN RHYTHM AND RNA BINDING 2); RNA binding (ATGRP7) mRNA complete cds	NM_179686	3.93E-34
VI64_AR2-B8_R	224	X	protein kinase family protein [*Arabidopsis thaliana*] >gi|4646200|gb|AAD26873.1|AC007230_7 Contains PF|00069 Eukaryotic protein kinase domain. [*Arabidopsis thaliana*] >gi|14334554|gb|AAK59685.1| unknown protein [*Arabidopsis thaliana*] >gi|23296681|gb|AAN13145.1| unknown protein [*Arabidopsis thaliana*]	NP_564844	3.95E-15
VI64_AR1-H2_R	410	X	ATCS (CITRATE SYNTHASE 4); citrate (SI)-synthase [*Arabidopsis thaliana*] >gi|14423562|gb|AAK62463.1|AF387018_1 citrate synthase [*Arabidopsis thaliana*] >gi|20197191|gb|AAC16084.2| citrate synthase [*Arabidopsis thaliana*] >gi|30387583|gb|AAP31957.1| At2g44350 [*Arabidopsis thaliana*]	NP_566016	1.25E-13
VI64_AR1-G10_R	497	X	putative chloroplast translation elongation factor EF-Tu precursor [*Arabidopsis thaliana*]	AAN31832	5.45E-76
VI64_AR1-G6_R	415	X	putative thioredoxin-like U5 small ribonucleoprotein particle protein [*Arabidopsis thaliana*]	AAM61612	4.54E-32
VI64_AR1-E10_R	173	X	unknown [Picea sitchensis] >gi|116782328|gb|ABK22466.1| unknown [Picea sitchensis]	ABK22391	6.60E-07
VI64_AR2-A2_R	893	X	unknown protein [*Arabidopsis thaliana*] >gi|9759554|dbj|BAB11156.1| unnamed protein product [*Arabidopsis thaliana*] >gi|17380768|gb|AAL36214.1| unknown protein [*Arabidopsis thaliana*] >gi|20259067|gb|AAM14249.1| unknown protein [*Arabidopsis thaliana*]	NP_850785	6.41E-120
VI64_AR3-E9_R	354	X	unknown protein [*Arabidopsis thaliana*] >gi|5823570|emb|CAB53752.1| putative protein [*Arabidopsis thaliana*] >gi|7267961|emb|CAB78302.1| putative protein [*Arabidopsis thaliana*] >gi|21592847|gb|AAM64797.1| unknown [*Arabidopsis thaliana*] >gi|27311749|gb|AAO00840.1| putative protein [*Arabidopsis thaliana*] >gi|32189305|gb|AAP75807.1| At4g12590 [*Arabidopsis thaliana*]	NP_192996	5.21E-60
VI64_AR2-A1_R	**376**	**X**	**Identical to wall-associated kinase 1 from *Arabidopsis thaliana* gb|AJ009696 and contains Eukaryotic protein**	**AAF81356**	**2.28E-47**

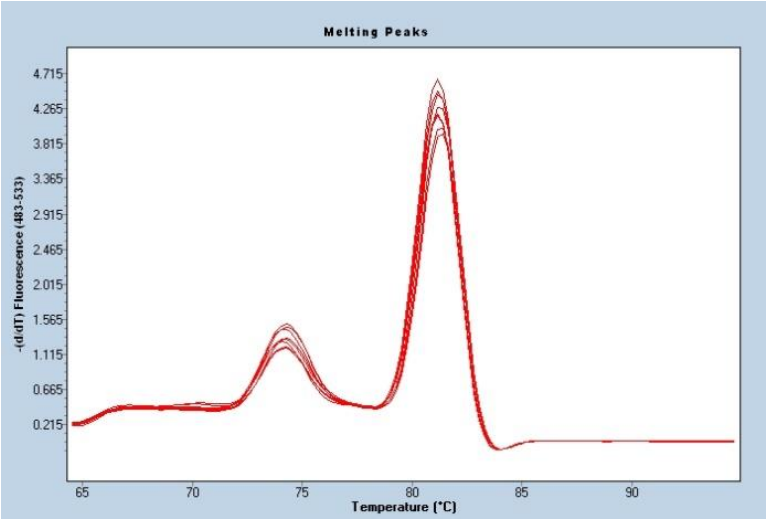
Appendix A

Table of *A. thaliana* non- redundant ESTs and their annotations in the SSHdb (http://www.sshdb.bi.up.ac.za)

SSH clone name	Sequence length (bp). Vector sequences removed.	blastX / blastN priority	BLASTX or BLASTN hit in the non redundant (nr) database of Genbank	Genbank accession number of BLAST hit	E_value of BLAST hit
			kinase PF|00069 and EGF-like PF|00008 domains. ESTs gb|T04358 gb|AI998376 gb|AW004557 come from this gene		
VI64_SF2-E7_F	782	X	squalene epoxidase 3 [*Arabidopsis thaliana*]	NP_568033	3.00E-154
VI64_AF3-C6_F	614	X	hydroperoxide lyase 1 HPL1 [*Arabidopsis thaliana*]	NP_193279	1.00E-16
VI64_SR2-C7_R	540	X	RecName: Full=Chlorophyll a-b binding of LHCII type 1 protein; AltName: Full=Chlorophyll a-b binding of LHCII type I protein; Short=CAB; Short=LHCP	P14584	8.00E-20
VI64_SR1-E2_R	767	X	photosystem II light harvesting complex protein 2.2 [Arabidopsis	NP_178582	6.00E-123
VI64_SF5-C5_F	458	X	photosystem II subunit Q-2 [*Arabidopsis thaliana*]	NP_192427	4.00E-49
VI64_AR3-F2_R	320	X	F1O19.10/F1O19.10 [*Arabidopsis thaliana*] Ribulose bisphosphate carboxylase/oxygenase (Rubisco)	AF372874	3.00E-12

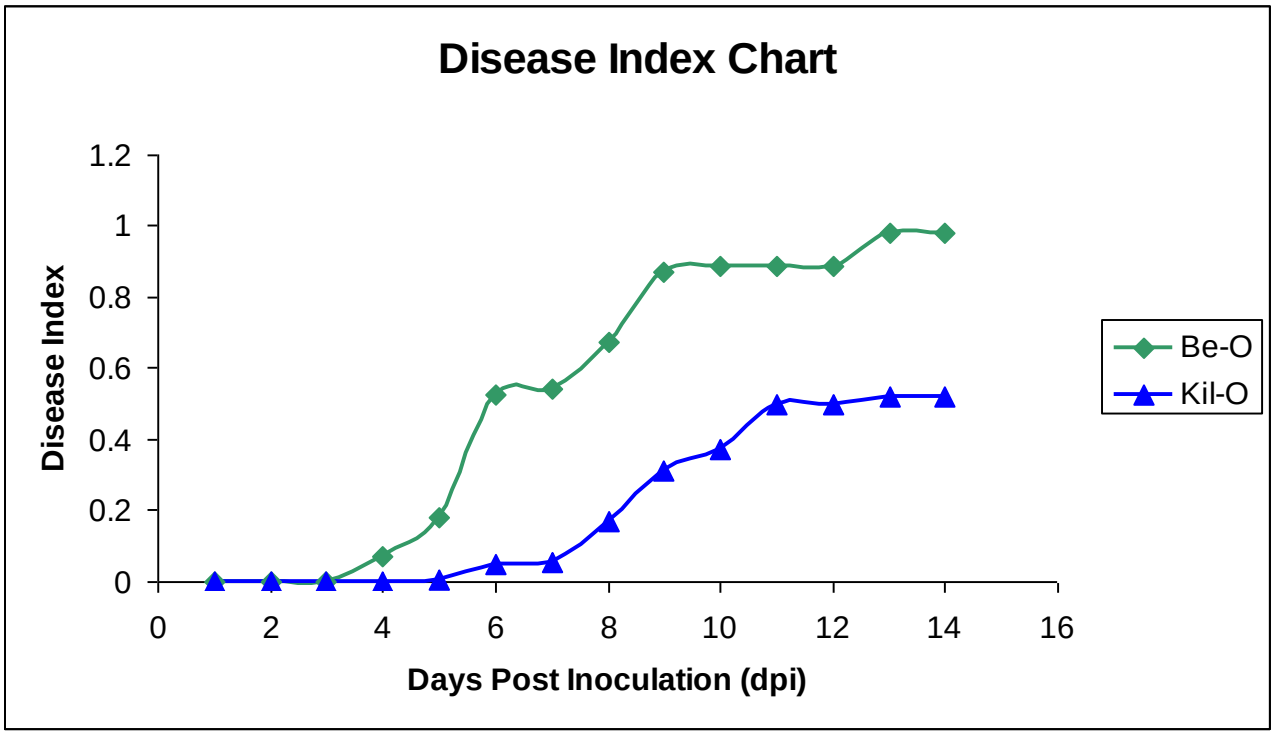
Appendix B

Melting curve of AtWAK1 as analysed with the LightCycler 480 instrument (version 1.2, Roche Diagnostics GmbH, Germany). The X-axis indicates the melting temperature (°C) and the Y-axis indicates the (d/dT) Fluorescence (483-533). The melting curve shows the temperature with which the AtWAK1 amplicon denatures and the fluorescent signal of the MasterPLUS SYBR Green I system (Roche) is detected. Double peaks indicate either large primer dimers, detection of secondary structures or amplification double amplicons.



Appendix C

Disease index chart extracted from Naidoo R (2008) illustrating disease progression *upon R. solanacearum* BCCF402 inoculation. The X-axis indicates Disease Index (DI) and the Y-axis indicates the days post inoculation (dpi). Disease symptoms in Be-0 are visible as early as 4-5 dpi indicating susceptibility. Kil-0 develops diseases symptoms much later than Be-0, around 7dpi.



Appendix D

Table of sequenced and annotated *A. thaliana* SSH cDNA libraries from R Naidoo, 2008

Clone ID	TAIR identity	Regulation	Fold Change	P – Value	Gene Description	Biological Gene Ontology	DRASTIC	
							Regulation	Biotic and/or Abiotic Stress
AR4_E9	AT4G30270	Up	1.69	5.33E-05	Endoxyloglucan transferase	Ageing, Gibberellic acid mediated signalling		None
SF1_F4	AT2G21660	Up	1.67	5.33E-05	Small glycine rich RNA-binding protein, part of negative feedback loop through interaction with GRP7	Circadian rhythum, lignin biosynthetic process, response to cold	Up	*M. persicae*, high light, low oxygen
SR6_B10	AT1G59870	Up	1.55	0.020628	ATP binding cassette transporter	SAR, defense response to fungi,	Up	*M. persicae*, low oxygen, flagellin 22
AR2_A1	AT1G21250	Up	1.48	0.021217	Cell-wall associated kinase, WAK1	Response to salicylic acid stimulus	Up	Salicylic acid, Jasmonic acid, *F. occidentalis*
SR4_F8	AT1G79245	Up	1.48	0.005512	Unknown protein	Unknown		Unknown
AR1_G2	AT1G78370	Down	1.5	0.000721	Glutathione transferase	Toxin catabolic process	Down	*M. persicae,* turnip mosaic potyvirus (TuMV)
AR4_F1	AT3G01500	Down	1.52	0.008269	Carbonic Anhydrase (CA1)	Carbon utilization		None
AR5_C5	AT5G49740	Down	1.6	0.001652	Chloroplast ferric chelate reductase	Electron transport		None

Appendix E

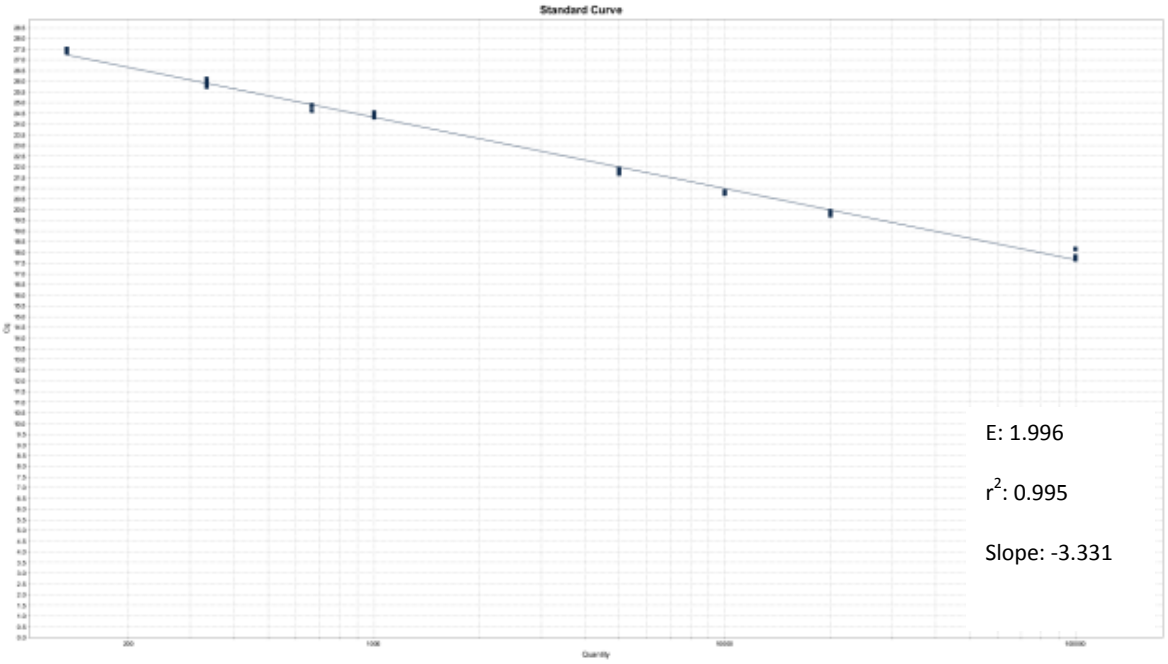
Table of up regulated genes in response to *R. solanacearum* BCCF402 infection in Arabidopsis ecotype Kil-0, 1 and & days after inoculation. The expression data is ordered from most induced to least induced at a significant threshold of p<0.01

AGI number	Description	log2 fold change	p-value	Fold change
At5g59320	lipid transfer protein 3 (LTP3)	2.08	4.31E-03	4.23
At5g59310	lipid transfer protein 4 (LTP4)	1.90	4.86E-03	3.72
At5g59330	LTP family protein pseudogene	1.51	2.09E-02	2.85
At2g12945	hypothetical protein A	1.24	4.86E-03	2.36
At3g49120	peroxidase (PRX34)	1.15	4.31E-03	2.21
At2g29350	tropinone reductase (SAG13)	1.02	2.09E-02	2.03
At1g07590	pentatricopeptide repeat-containing protein (PPR)	0.98	4.86E-03	1.97
At1g56555	hypothetical protein B	0.88	2.09E-02	1.84
At5g43580	serine-type endopeptidase inhibitor	0.85	1.80E-02	1.81
At3g28940	avirulence-responsive protein (AIG)	0.82	4.86E-03	1.77
At5g54940	eukaryotic translation initiation factor SUI1	0.79	2.09E-02	1.72
At3g54480	SKP1 interacting partner 5 (SKIP5)	0.75	2.09E-02	1.68
At3g11770[a]	expressed protein	0.72	5.34E-03	1.65

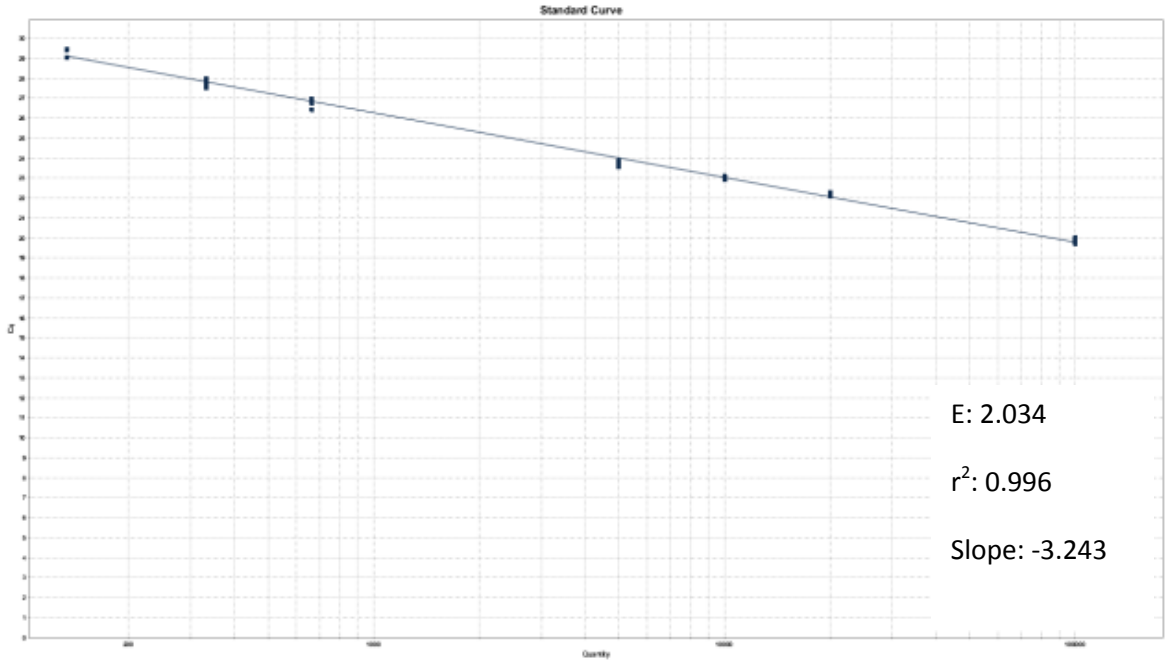
Appendix F

Standard curves of the selected reference genes (*AtACT2, ACPB20*, *AtELFα, AtTUB4, AtUBQ)* and candidate defence response genes *(AtDRG, AtGPX1*, *AtLTP3, AtPAH2, AtPNT, AtXTH22, AtXTH24* and *AtWAK1*), analysed on *qBASE*plus v1.0 constructed from 12 pooled cDNA samples. The X-axis indicates the crossing point (Cq) and the Y-axis indicates the quantity. Standard curves determine the efficiency of the PCR and the viability of the primers. A slope of -3.3 indicates a 100% PCR efficiency (E=1.8-2). R^2 reflects the linearity of the standard curve, illustrating how well the data fits the standard curve.

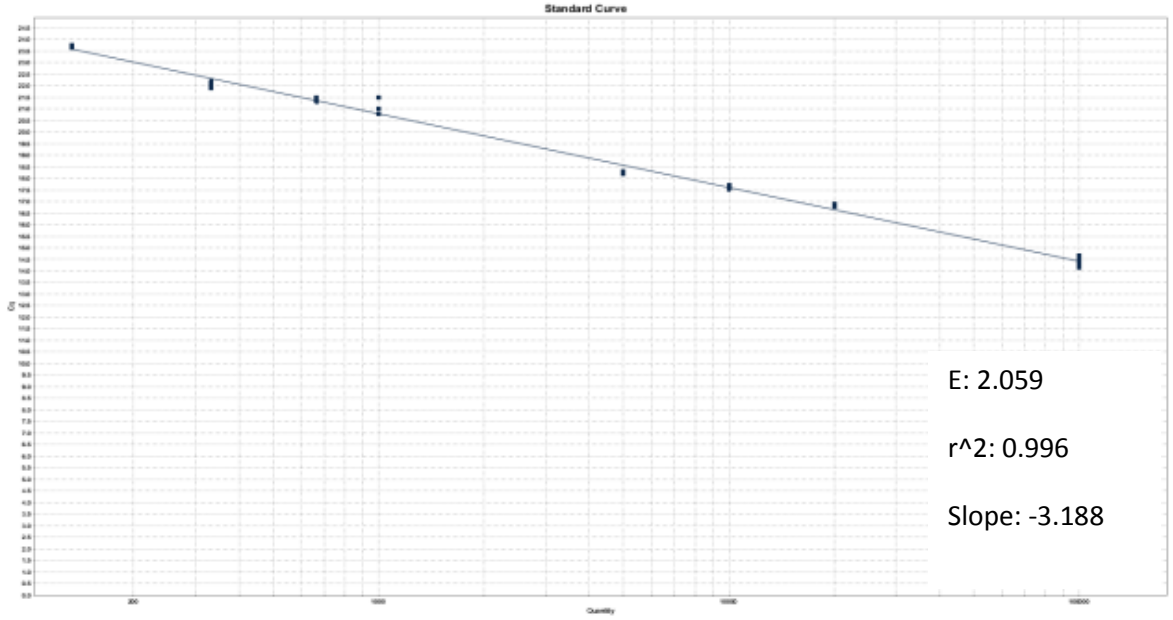
AtACT2



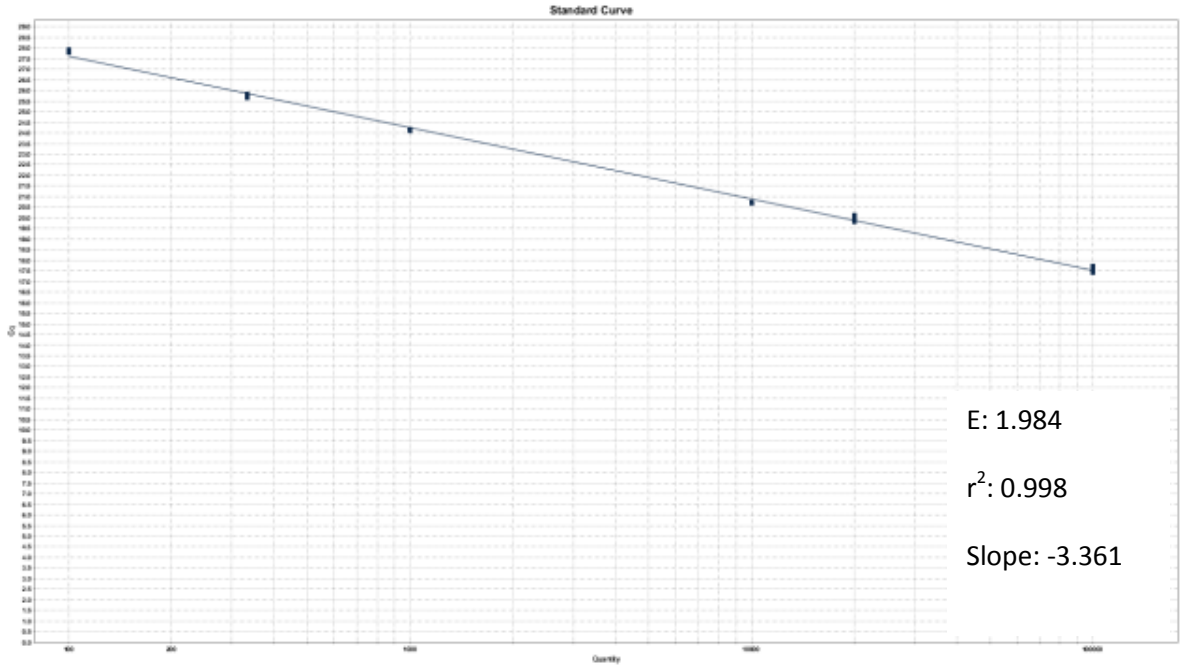
AtCBP20



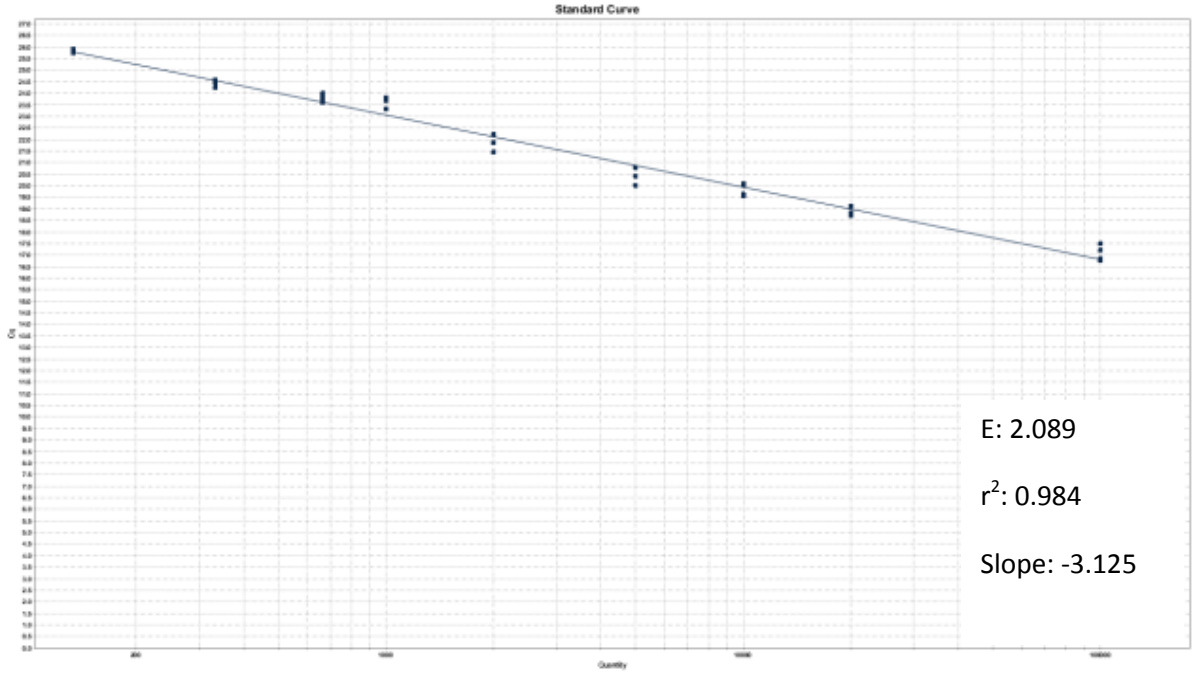
AtELF1α



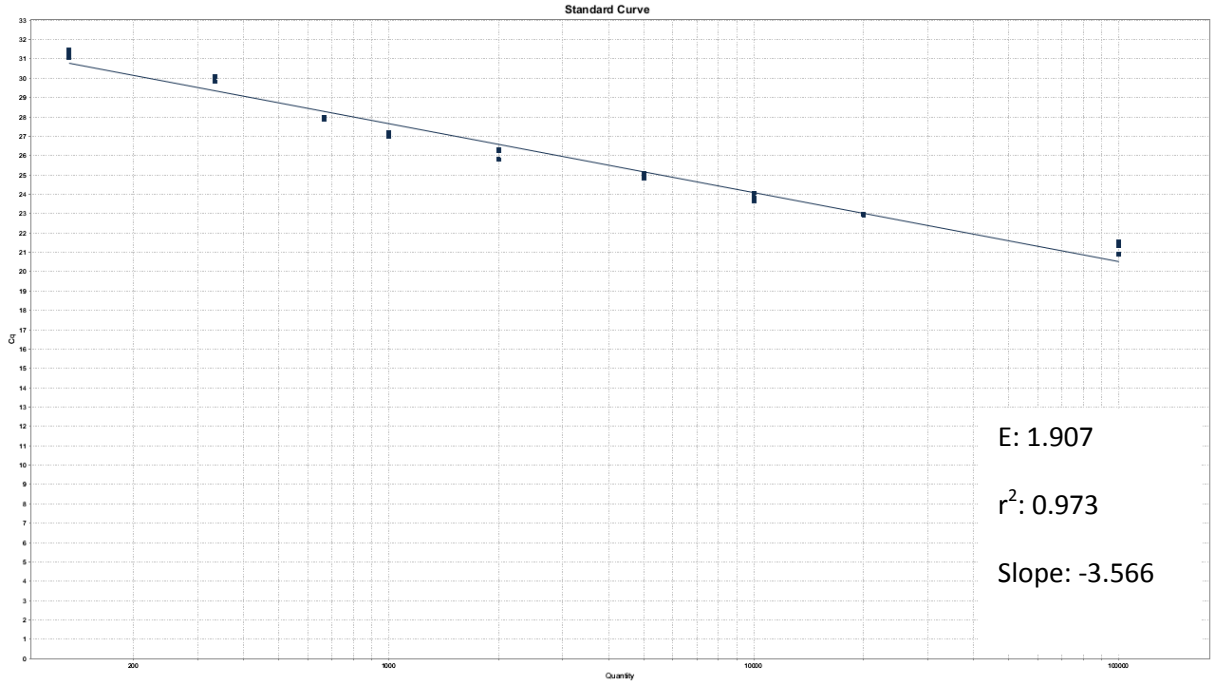
AtTUB4



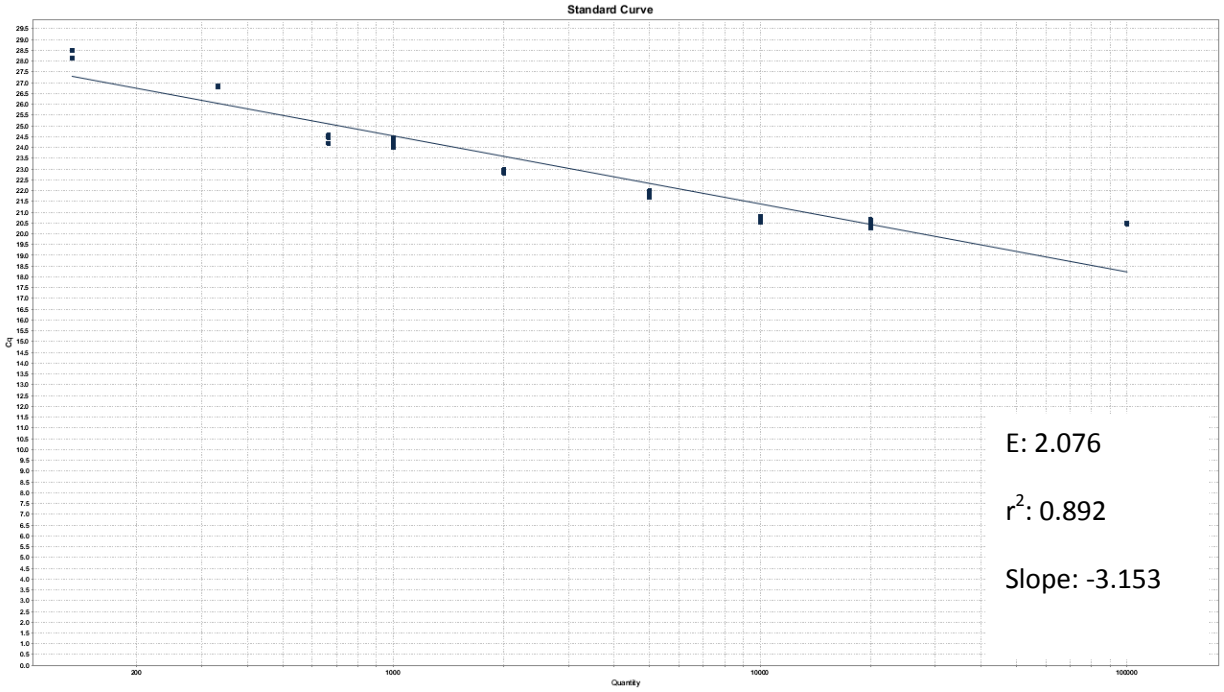
AtUBQ5



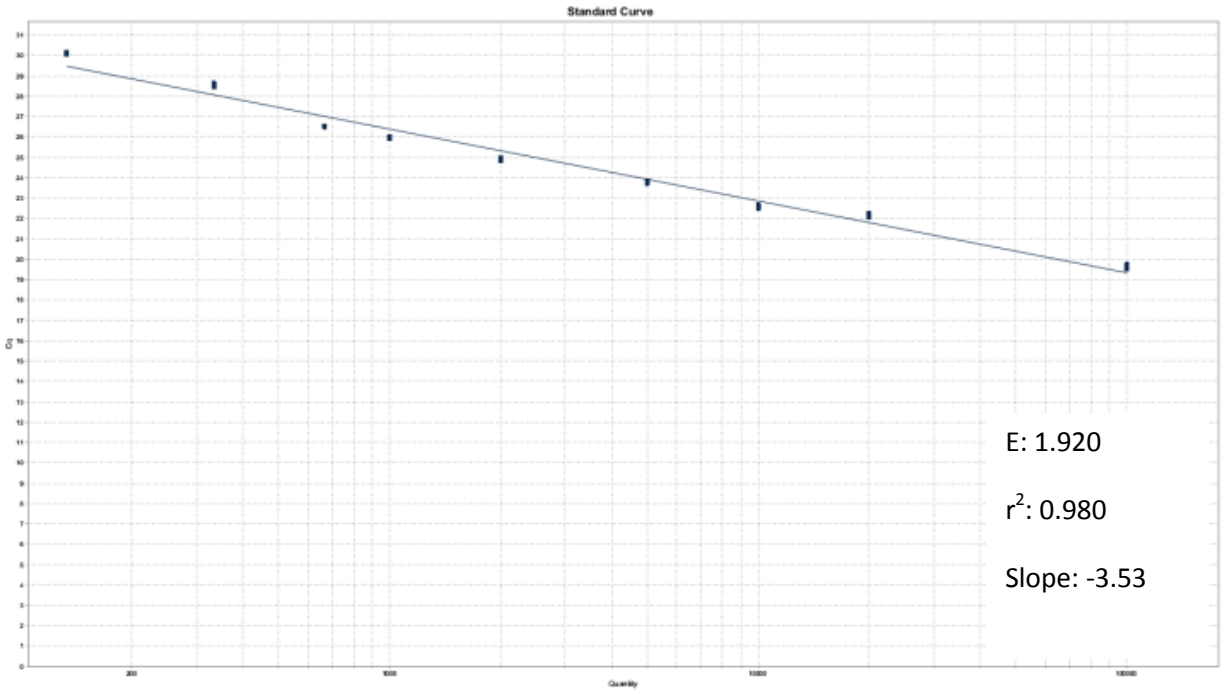
AtDRG



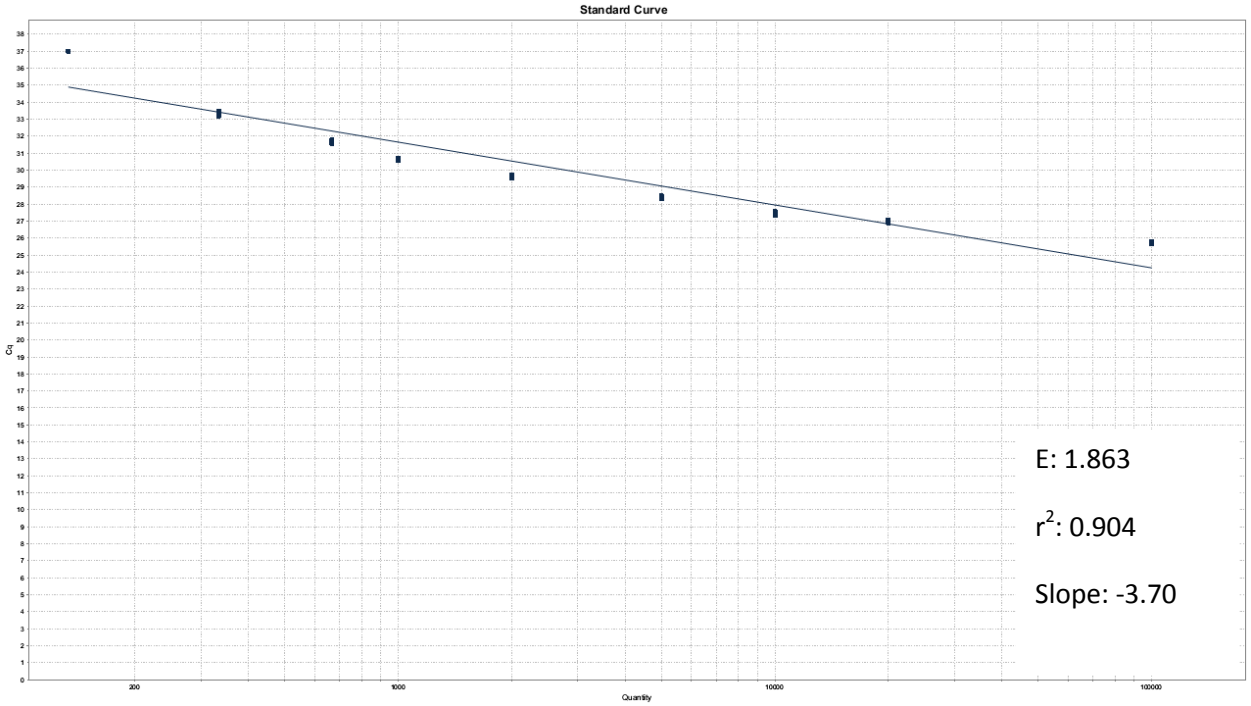
AtGPX1



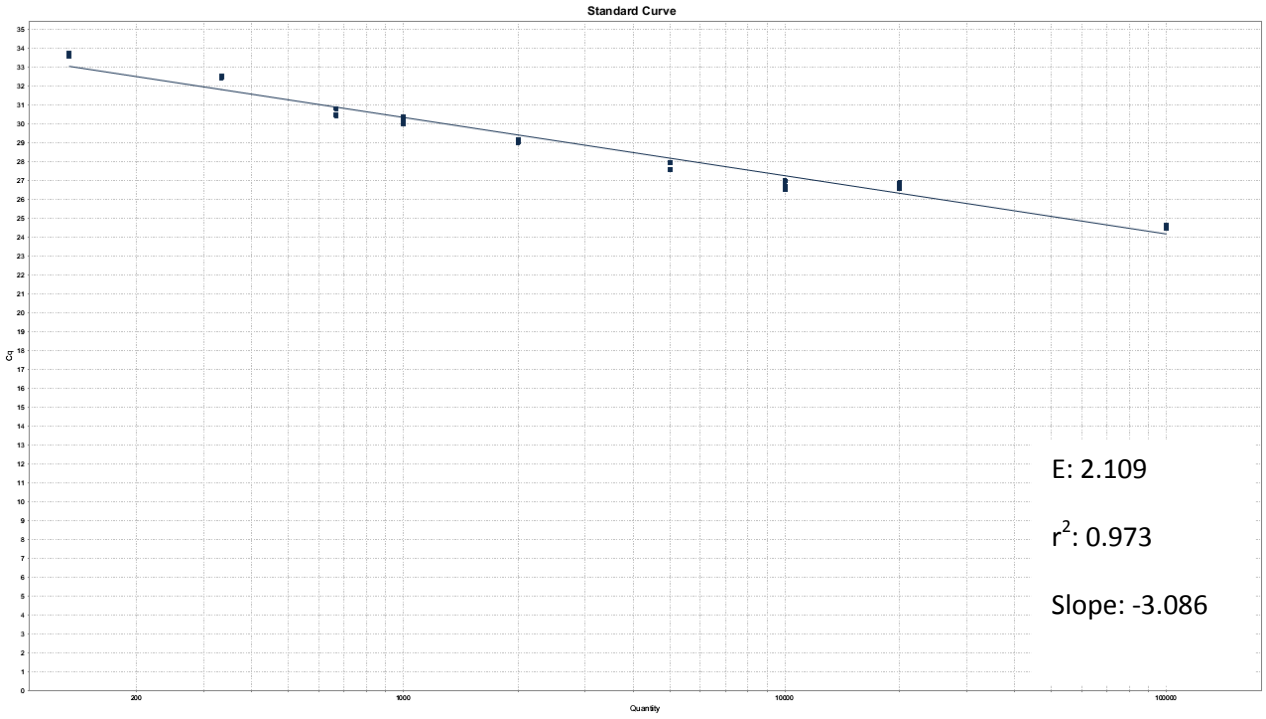
AtLTP3



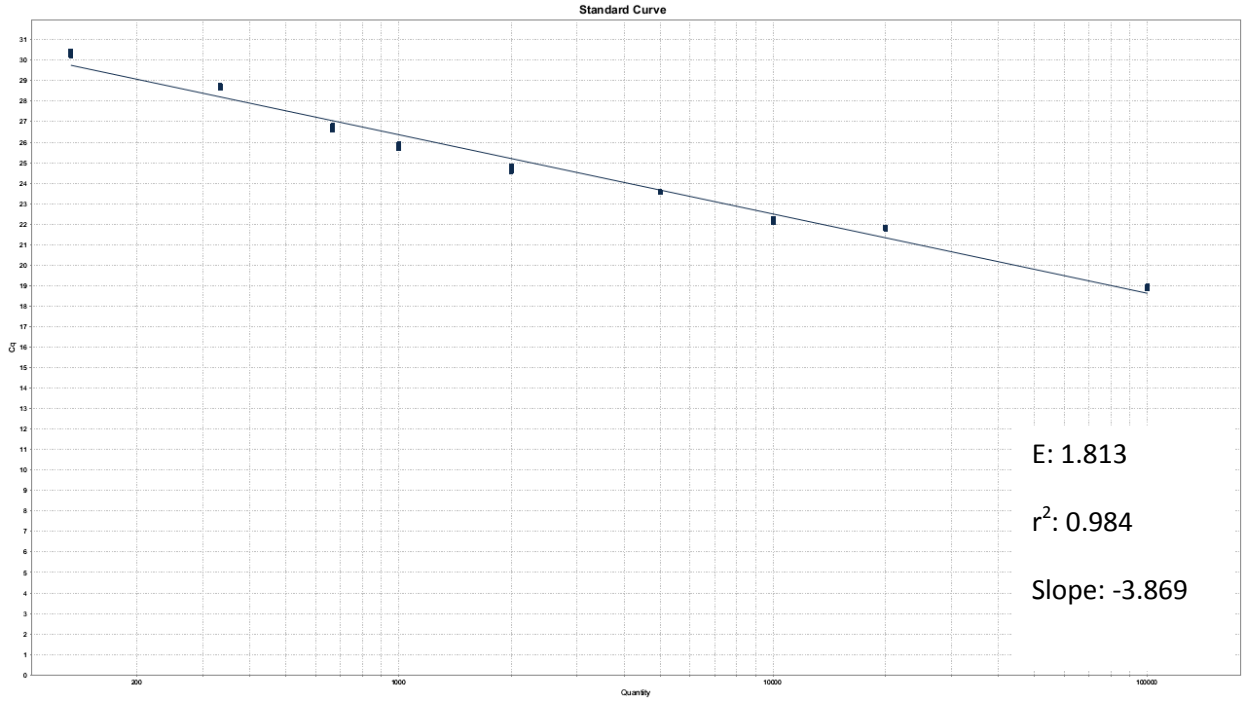
AtPAH2



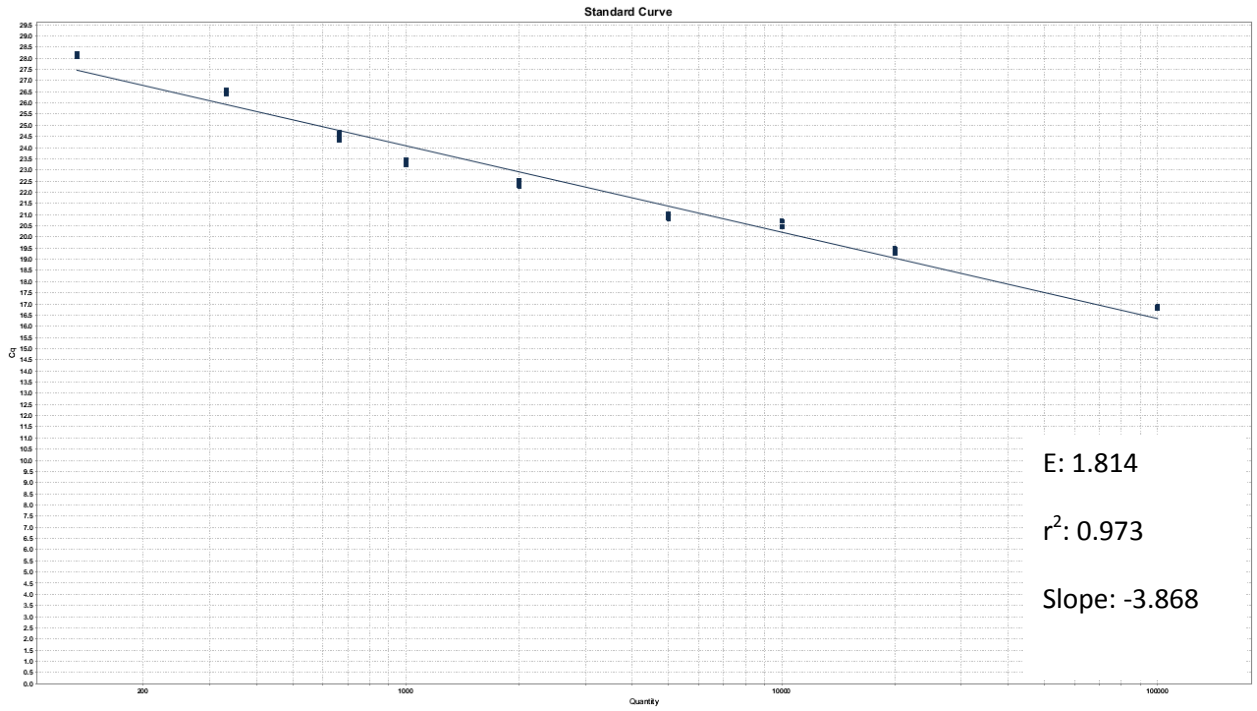
AtPNT



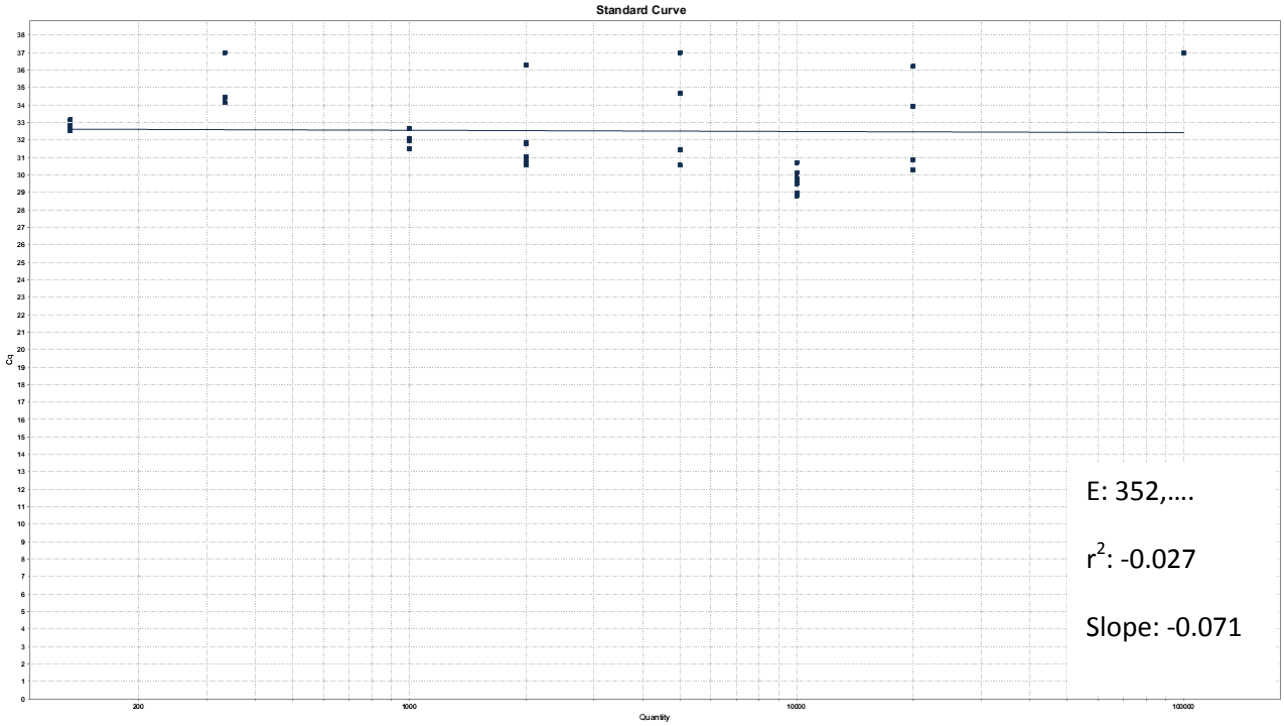
AtXTH22



AtXTH24



AtWAK1


Standard Curve
Cq
Quantity
200
1000
10000
100000
E: 352,….
r2: -0.027
Slope: -0.071

Appendix G

The relative expression profiles of *AtDRG* in Be-0 across three time points. The relative expression of the transcript is less than 0.03 in Be-0. There is no differential expression of the transcript across different time points in control plants and inoculated plants.