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Defense priming and epigenetic mechanisms in regulating resistance against *Botrytis cinerea* in strawberry

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Abstract

Botrytis cinerea is a necrotrophic pathogen that is reported to cause disease in more than 1400 plant species. Each year the fungi cause yield losses of great economic importance and is the most important pathogen in Norwegian strawberry production. Priming has been shown to enhance defense in plants, and epigenetic gene regulations are hypothesized to be part of the underlying mechanisms of the priming effect. Epigenetic gene regulation is mediated by specific enzymes that alter DNA or the associated histones without changing the underlying DNA sequence.

In the first part of this thesis I have investigated the effect of defense priming. Plants of *F. x ananassa* were primed for defense by soil drenching with the chemical agent β-aminobutyric acid (BABA) and leaves were detached and drop-infected with *B. cinerea.* The disease development was scored phenotypically to determine if primed plants were more or less resistant to the pathogen compared to non-primed plants. After phenotypic scoring, candidate defense genes were analyzed by RT-qPCR. An enhanced susceptibility to *B. cinerea* was observed in the phenotypic scoring of the primed leaves compared to the non-primed leaves, and the increase in gene expression of defense related genes were mainly found to be in response to *B. cinerea* and not due to the priming effect of BABA.

In the second part of the thesis, new constructs for CRISPR/Cas9 gene editing was made to target putative epigenetic modifiers homologous to important epigenetic modifiers in *Arabidopsis thaliana* to identify their role in defense against *B. cinerea* in *F. vesca*. To this end, CRISPR-vectors with different U6-promoters of *F. vesca* were designed and tested in a transient expression system. The vector with the highest U6-promoter-driven sgRNA-expression was then selected to knock out target genes involved in regulation of defense responses.

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Lisa Karine Haugland

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Abbreviations

bp	base pair(s)
cDNA	single-stranded complimentary deoxyribonucleic acid
CRISPR	clustered regularly interspaced palindromic repeat
dH_2O	here: distilled water of Milli-Q quality
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ET	ethylene
ETI	effector triggered immunity
JA	jasmonic acid
kb	kilo base pair(s)
MAMP	microbe-associated molecular pattern
PAM	proto-spacer adjacent motif
PCR	polymerase chain reaction
PR	pathogenesis related
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
SA	salicylic acid
sgRNA	single-guide ribonucleic acid
tRNA	transfer ribonucleic acid
rpm	rotations per minute
RT-qPCR	reverse-transcriptase quantitative polymerase chain reaction
UV	ultra violet

Units of measurements

°C	degree Celsius
µg	microgram (10^{-6} g)
µl	microliter (10^{-6} g)
µM	micromolar (10^{-6} M)
g	gram
l	liter
M	molar
mg	milligram (10^{-3} g)
min	minutes
ml	milliliter (10^{-3} g)
mm	millimeter
mM	millimolar (10^{-3} M)
ng	nanogram (10^{-9} g)
nm	nanometer (10^{-9} m)
pmol	picomole (10^{-12} mol)
V	volt

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1. Introduction

1.1. Background

Plants have evolved mechanisms for a general defense that is effective against a wide range of potential attackers. Because of their sessile nature, plants need to respond and adapt to recurring biotic and abiotic stress that they cannot escape and possess a remarkable capacity to perceive signals that allow them to respond to their surroundings (Bruce et al., 2007). Despite not having an immune system comparable to that of animals, plants are astoundingly resistant to diseases caused by pathogens such as fungi, bacteria and viruses due to chemical or mechanical barriers, basal and inducible defense mechanisms (Taiz et al., 2015).

To increase crop yield and reduce the use of pesticides it is important to continuously improve crops to better withstand a changing environment and damaging pests and pathogens (Niks et al., 2011). Therefore, efficient and durable methods to improve crop resistance in plants are needed.

1.2. Strawberry

Strawberry (*Fragaria x ananassa*) is a non-climacteric fruit in the Rosaceae family (Folta & Davis, 2006), and is an economically important crop with a worldwide production of more than 9 million tons in 2016 (FAOSTAT, 2017). Strawberry plants are susceptible to a range of pathogens that limit fruit production and cause severe crop losses pre- and post-harvest every year. Genetic improvement of most Roseaceous crops by traditional breeding strategies are demanding because of a large genome size, polyploidy, intolerance to inbreeding and a long life cycle (Oosumi et al., 2006). Genetic diversity is considered a critical factor in improving crops because a higher diversity can increase the possibility to possess advantageous alleles or allele combinations. Closely related wild species are therefore considered a valuable source of genetic diversity and novel genes to improve resistance to diseases and environmental stress in plants (Niks et al., 2011).

The cultivated *F. x ananassa* genome harbors 56 chromosomes derived from four diploid ancestors, which makes genetic studies extremely complicated (Shulaev et al., 2010). The woodland strawberry *Fragaria vesca* is a diploid relative of *F. x ananassa*. Because of its

small genome size, short generation time and easy vegetative propagation, *F. vesca* is an attractive and functional plant for crop improvement and gene function studies within the Rosaceae family (Shulaev et al., 2010). Compared to the traditional model plant *Arabidopsis thaliana*, *F. vesca* is a perennial plant. Traits such as disease resistance, fruit flavor and quality can be addressed with the *F. vesca* model system. The system can also more easily be adopted to its cultivated and economically important relatives, such as *F. x ananassa* and crops with longer generation time as apple, peach and cherry (Shulaev et al., 2010).

1.3. Grey mold

Grey mold disease is caused by the necrotic ascomycete *Botrytis cinerea* Pers., and has a host range of over 1400 plant species, causing severe damage in a wide range of important crops (Kan et al., 2017). *B. cinerea* cause severe yield losses in strawberry crops worldwide and is the most important disease in field-grown strawberries in Norway (Strømeng et al., 2009). Pesticides are the most important measures in controlling grey mold and are widely used to maintain a profitable and high-quality strawberry production. In Norwegian commercially grown strawberry fields, pesticides are used several times during the growth season to control *B. cinerea* (Strømeng & Stensvand, 2017). The short life cycle and ability to sporulate abundantly makes *B. cinerea* a pathogen with a high risk to develop resistance against fungicides (Hahn, 2014). Resistance against the active substances in several fungicides have been revealed in Norwegian fields (Strømeng & Stensvand, 2017), as well as in other parts of the world (Fernández-Ortuño et al., 2014; Fernández-Ortuño et al., 2016). Therefore, methods to control *B. cinerea* without using pesticides are of great importance to maintain a sustainable strawberry production in all parts of the world.

1.3.1. Taxonomy and life cycle

B. cinerea is of the genus *Botrytis*, and is an Ascomycete fungi of class Leotiomycetes, order Heliotiales and family Sclerotiniaceae.

The life cycle of *B. cinerea* has various stages; a vegetative mycelial system that produces asexual conidiophores, conidia, and sclerotia, as illustrated in Figure 1.1. The sclerotia consists of a β-glucan and melanized coated layer of mycelium that is initiated under unfavorable conditions, and represents the most important survival mechanism for the fungi.

(Williamson et al., 2007). *B. cinerea* survives through the winter as mycelium on dead or living plant material or as sclerotia on plant debris, on soil surface or in the soil (Williamson et al., 2007). Germination of over-wintering structures of the fungi is initiated in the spring, where mycelium and sclerotia germinate to mycelium that produces long branched conidiophores bearing clusters of conidia resembling grape-like structures for dispersal and spread (Agrios, 2005). *B. cinerea* is a polycyclic fungus and can cause great damage both pre- and post-harvest in strawberry. In perennial crops such as strawberry, infected leaves, flowers and mummified fruits contain masses of spores, and serves as an important sources of infection in the field (Strømeng et al., 2009). *B. cinerea* is considered a heterothallic fungus with two distinct mating types, and sexually produced apothecia of *B. cinerea* can be produced from sclerotia although it has not been observed in the field. Despite of the absence of sexual reproduction *B. cinerea* has a great morphological and genetic diversity that is due to a larger species complex (Hahn et al., 2014).

Conidia serves as the main produced and dispersed inoculum and are predominantly carried by air currents. Sclerotia in Mycelial fragments can also serve as inoculum. (Williamson et al., 2007). Optimal environmental conditions for the fungi to grow, sporulate, release spores and infect is high humidity (> 90% RH) and cool weather with an approximate temperature range of 18 to 26°C (Agrios, 2005). The pathogen is also active at low temperatures with high humity, but the activity will decrease under warm and dry weather.

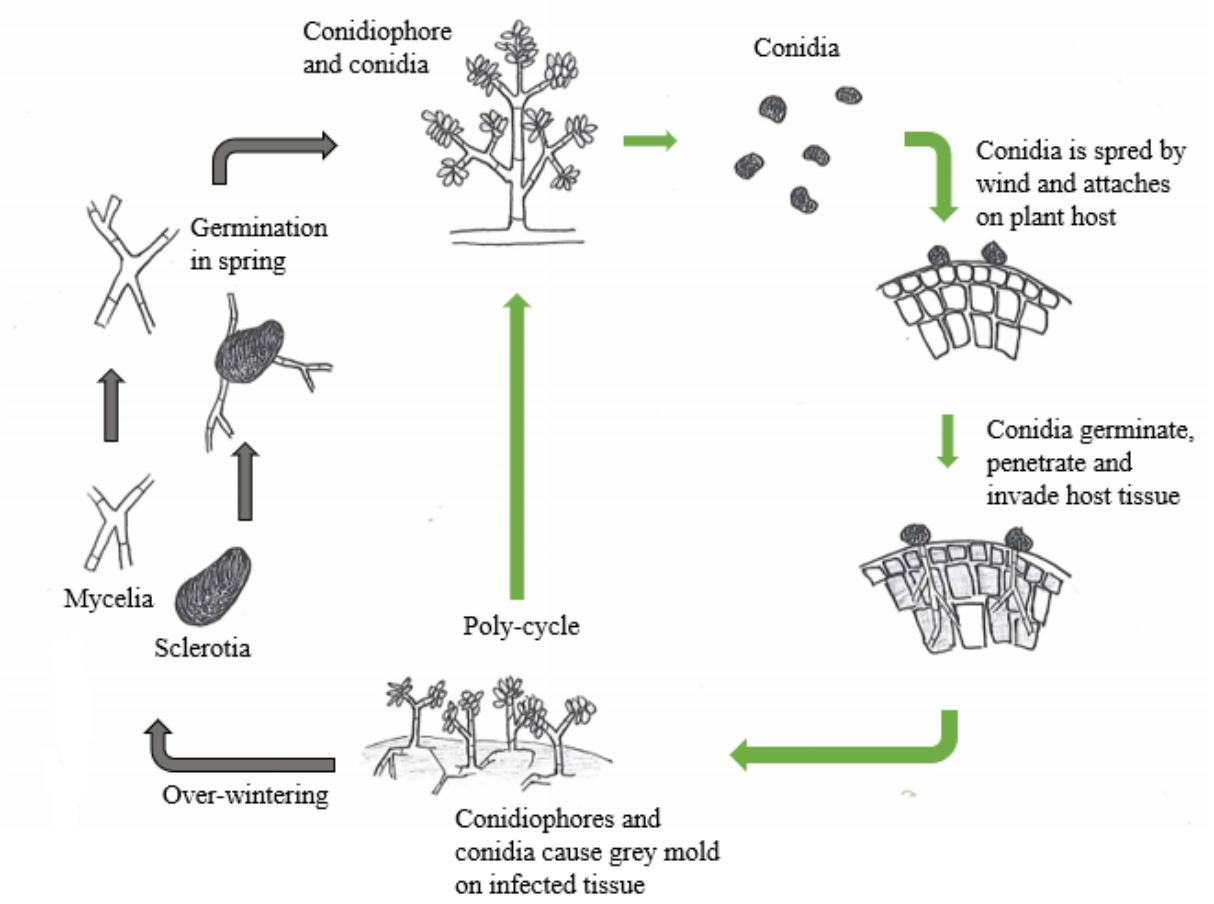


Figure 1.1. Generalized life-cycle of *B. cinerea*. Over-wintering mycelia and sclerotia germinate to mycelium the spring that forms conidiophores and conidia. Conidia are dispersed and land on plant hosts where they germinate, penetrate and invade. Infected cells collapse and cause grey mold disease that develops on infected tissue. The polycyclic part of the lifecycle is marked with green arrows, and is repeated as long as the conditions are favorable. Sexual reproduction is not represented. Figure adapted from Agrios (2005).

1.3.2. Pathogenesis

To be pathogenic, the fungi must enter the plant interior to be able to feed from its nutrients. Successful pathogens have evolved a number of strategies to invade their host plant and acquire nutrition. *B. cinerea* is a necrotrophic pathogen that attack by killing the affected plant cells to colonize the host (Niks et al., 2011). The fungi is considered a weak parasite as it usually need wounds or weakened tissue to be able to penetrate the plant host (Williamson et al., 2007).

B. cinerea possesses multiple tools to initiate cell death in the plant host. When conidia of *B. cinerea* has attached to the cuticula of the host plant and started germinating, the fungi infects the host cells by forming an appressorium to penetrate the host surface (van Kan, 2006). To further colonize the plant host, *B. cinerea* releases several extracellular enzymes capable of degrading cell wall polymers through the appressoria (Choquer et al., 2007). The enzymes break down the tough cell wall of the plant and triggers reactive oxygen species to accumulate in toxic concentrations in the plant tissue. This is followed by further cell collapse, tissue decay and expanding lesions in the plant host that facilitate for the fungi to sporulate (van Kan, 2006).

1.3.3. Symptoms of disease

In the strawberry field, *B. cinerea* usually infects during blossom where it establishes in the flower petals and precedes into the fruit and cause fruit rot (Agrios, 2005). Infected fruits become soft before it rots, and the fungi forms a network of grey cotton-like mycelium with visible dark spores on infected tissue (Williamson et al., 2007) . *B. cinerea* can attack leaves, flowers, berries and the crown of the strawberry plant, but it is fruit rot in the field or in harvested berries that cause the most economic damage. There is also evidence that *B. cinerea* can systemically colonize plants without causing disease symptoms under the appropriate conditions (Kan et al., 2014).

1.4. Plant defense

The ability to detect and respond to damaging pathogens has been crucial to the developmental success of many plants. In general, plants require a broad range of defense mechanisms to defend themselves against pathogen attack. These mechanisms include constitutive barriers such as waxy cuticula and rigid cell walls, as well as inducible defense responses activated upon pathogen attack (Agrios, 2005). Plants have to rely on their innate immunity of each cell and on the systemic signals between cells in their defense against plant pathogens (Jones & Dangl, 2006).

1.4.1. Plant defense responses to pathogen infection

The innate immune system of plants comprises local and systemic responses, and pathogen infection give rise to a variety of molecular signals in the host plant. For the host to be able to distinguish between "self" and "non-self" during an attack, plants possess pattern recognition receptors which are transmembrane protein complexes essential for perceiving the molecular signals associated with pathogen infection (Zipfel, 2009). MAMPs are microbe-associated molecular patterns that are conserved among pathogens, and recognition of MAMPs by the pattern recognition receptors activates what is called MAMP-triggered immunity (MTI) (Reimer-Michalski & Conrath, 2016) However, adapted pathogens express effector proteins that can suppress these basal defenses. Effectors are enzymes, toxins or growth regulators that change the plant structure, metabolism or hormonal activity that benefit the pathogen to sustain growth (Taiz et al., 2015). As a response, plants have evolved resistance proteins that detect the pathogen effectors and activates defense responses in another layer of defense called effector-triggered immunity (ETI) (Jones & Dangl, 2006). ETI is often associated with hypersensitive responses, and some pathogenic fungi have adapted effectors to interfere with ETI to overcome this gene-for-gene resistance in the host (Chisholm et al., 2006). MTI and ETI are both associated with a variety of complex defense signals in the plant, including reactive oxygen species, mitogen activated protein kinases, plant hormone signaling, transcriptional reprogramming and accumulating secondary metabolites (Reimer-Michalski & Conrath, 2016).

1.4.2. Systemic resistance

Induced resistance in plants occur not only at the site of the tissue exposed to the pathogen, but also excites systemic responses in tissues distant from the primary site of attack (Pastor et al., 2014). Localized pathogen attack elicits a broad range of signalized immunity in all parts of the plant, and have been defined by differences in signaling pathways as systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Reimer-Michalski & Conrath, 2016).

The signaling pathways that are induced in systemic resistance varies with the pathogen's mode of action. The plant phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are secondary messengers known to be involved in activating induced defense responses in plants against different pathogens (Jones & Dangl, 2006). SA is generally

involved in activating defense responses against biotrophic and hemi-biotrophic pathogens, and is required for the establishment of SAR (Fu & Dong, 2013). By contrast, JA/ET are associated with defense against necrotic pathogens, and is required for ISR (Pandey et al., 2016). The two signaling defense pathways of SA and JA/ET are partially overlapping and are considered to be antagonistic because both are dependent on the defense regulatory non-expressor of pathogenesis-related proteins 1 (NPR1) (Pieterse & Van Loon, 2004; Spoel et al., 2003). The antagonistic relationship between SA and JA/ET-mediated defense pathways provides plants with the potential of a more precise regulation. The regulation is thought to be an evolutionary answer for the plant to respond and encounter the different strategies of pathogens that attack (Caarls et al., 2015).

Pathogenesis-related proteins (PR proteins) are produced by the plants in induced resistance. PR proteins are effective in inhibiting pathogen growth and have different antifungal functions (Sels et al., 2008). PR1 and PR5 are PR protein families known to interact with the fungal plasma membranes and are accumulated in induced resistance (Amil-Ruiz et al., 2011). Pathogenic fungi produce polygalacturonases (PGs) that degrade cell-walls of the host plant, and as an evolutionary adaptation to this, plants express polygalacturonase-inhibiting proteins (PGIPs) as an effective defense strategy (Kalunke et al., 2015). Recent studies have revealed that strawberry plants that over-expressed the PGIP gene showed less susceptibility to *B. cinerea* than lower expression of the genes (Saavedra et al., 2017). Other PR genes encode β-1,3-glucanases (BGs) that hydrolyses β-1,3-glucans which are cell wall components in many fungi. BGs are one of the most abundant classes of PR proteins in plants along with chitinases (Amil-Ruiz et al., 2011).

1.4.3. Priming for enhanced defense

When a plant is challenged by a pathogen plants are often promoted to a primed state of enhanced defense that enables the plant to be more prepared when exposed to future pathogen attacks (Bruce et al., 2007). The phenomenon of priming establishes a faster and more robust activation of the various defense responses in the systemic parts of the plant that has not been challenged by a pathogen (Conrath et al., 2015). When primed, plants respond to lower pathogen stimulus than non-primed plants, and the reaction is more fast and robust compared to non-primed plants, as illustrated in Figure 1.2.

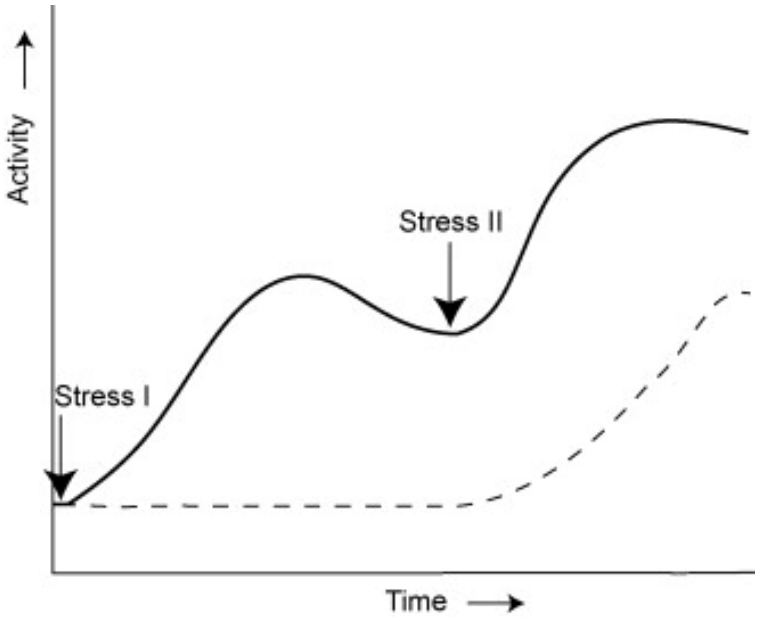


Figure 1.2.: Priming stimuli. A simplified diagram showing the activity of stress responses in a non-primed plant (- -) and a primed plant (-). The primed plant is challenged with two stresses, Stress I and II, and the non-primed plant is challenged with Stress II only. The graph show that the activity level of the primed plant is higher, implying that a primed state generates a plant memory that prepare the plant for future attack by acting in a faster and more robust way. Figure from Bruce et al. (2007).

Priming is a part of all induced immunity responses in plants and requires SA or JA/ET signaling pathways. Besides from biologically priming through SAR and ISR, plants can also be primed by exogenous natural or synthetic chemicals for enhanced defense (Conrath et al., 2015). Exogenous priming agents such as SA, benzothiadiazole, methyl jasmonate, chitosan and β-aminobutyric acid (BABA) have been reported to induce resistance in several species and pathosystems (Baccelli & Mauch-Mani, 2016; Reimer-Michalski & Conrath, 2016). Priming as an overall defense strategy has been known for a long time as reviewed in Kuc (1987), but several recent studies have started to reveal the molecular mechanisms behind priming.

Exogenous priming agents have been shown to cause several responses in plants that mimic those of SAR and ISR. In general, induced priming responses include enhanced levels of pattern recognition receptors (Reimer-Michalski & Conrath, 2016), potentiated levels of reactive oxygen species (Pastor et al., 2013), earlier and stronger expression of defense related genes (Conrath et al., 2015), accumulated callose deposition (Baccelli & Mauch-Mani, 2016),

as well as potentiated hypersensitive responses. Primed plants have also shown increased synthesis of metabolites, amino acids and phytoalexins (Balmer et al., 2015).

Specific defense mechanisms depend strongly on the priming state, and priming has been divided into three different phases depending on the response; the priming phase, a post-challenge primed phase and a transgenerational primed phase, and is illustrated in Figure 1.3. (Pastor et al., 2014).

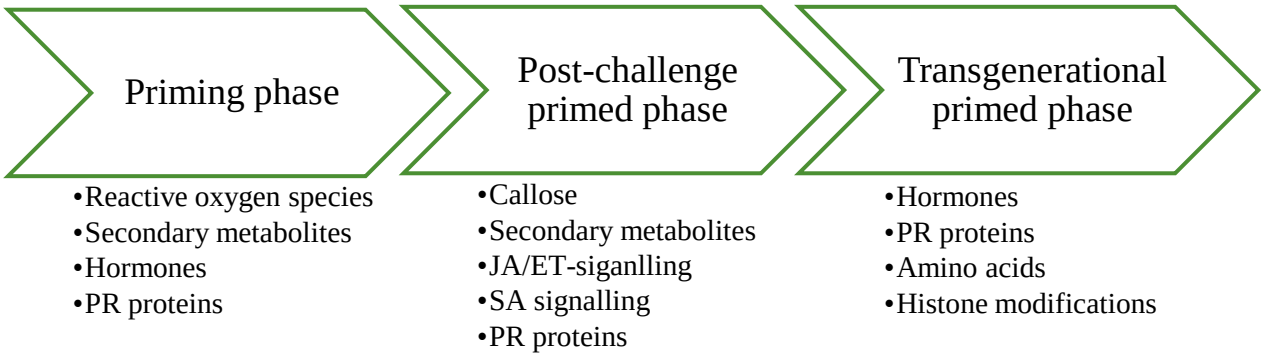


Figure 1.3: General overview of priming phases. The initial priming phase is triggered by a priming stimulus and sets the plant in a primed state by altering the amount of secondary metabolites, enzymes and hormones. The post-challenge primed phase is triggered by an attacking pathogen or other stress and induce the appropriate reactions to combat the given challenge. The third transgenerational primed phase is found in the progeny of primed parental plants and makes the progeny able to react more strong and rapid upon challenge.

1.4.4. Epigenetic mechanisms in plant defense

Priming has been found to cause heritable and reversible changes in gene expression without inducing changes in the underlying DNA, referred to as epigenetics. Within each cell, the genetic information is compacted into chromatin with the fundamental core of nucleosomes. Nucleosomes are composed of segments with approximately 146 bp of DNA wrapped around octamer histone cores consisting of two copies of each histone protein H2A, H2B, H3 and H4 (Pfluger & DorisWagner, 2007). The chromatin acts as a structure for organizing DNA and for regulating access for proteins that need contact with the DNA, including proteins that regulate gene expression. Active genes are organized into loosely compacted euchromatin,

while silenced genes are organized into tightly packed heterochromatin. Genes that are constitutively expressed in plants are often associated with nucleosome-free regions of their promoters (Pfluger & DorisWagner, 2007). The changes in chromatin structure and compaction can ultimately alter several genomic processes such as gene transcription, replication, and recombination. (Mauch-Mani et al., 2017).

All four histones can be modified by acetylation and methylation, among others, and are the two most commonly studied forms (Zhou et al., 2010). Generally, acetylation of histones H3 and H4 is associated with active transcription while methylation is associated with suppressed transcription. Studies that have investigated the role of epigenetic mechanisms in the activation of defense related genes have shown that progeny from disease-exposed *Arabidopsis* plants were primed for defense in a transgenerational manner, in what is described as the transgenerational priming state (Luna et al., 2012; Luna & Ton, 2012; Slaughter et al., 2012). These findings shows that priming can give changes at the epigenetic level that alters the chromatin structure in a way that leaves gene promoters more accessible and easier to activate, and that they are maintained in the next generation of progeny (Mauch-Mani et al., 2017). Acetylation of lysine at histone H3 and H4 has been associated with active genes that gives an open chromatin state and possibility for active transcription of defense related genes in the region (Jaskiewicz et al., 2011).

Epigenetically inherited priming is beneficial for short-generational plant species, especially a perennial and vegetative propagated plant as strawberry with a limited ability to outlive diseases. Epigenetic heritability serves as an excellent evolutionary strategy for plants to adapt to stress, and priming has the potential to make plants more resistant against several abiotic and biotic stresses (Mauch-Mani et al., 2017).

1.5. Gene editing for resistance

An approach in improving crops and making plants more resistant towards pathogens is to use genome editing methods in the desired plant to better withstand biotic and abiotic challenges. Heritable variation in plant phenotypes is in principle not only is caused by variation in DNA sequence, but also in variations of the underlying epigenetics. By using the efficient and

versatile targeted genome editing method of CRISPR/Cas9 to introduce small site-directed mutations in the epigenetic machinery of plants may reveal the role of epigenetics in defense priming (Belhaj et al., 2013).

1.5.1. CRISPR technology

Precise, efficient and versatile gene editing methods are needed to improve crops, and the CRISPR method has emerged as an efficient method to alter genomes. CRISPR is an abbreviation of clustered regulatory interspaces palindromic repeats, and is based on the bacterial CRISPR/Cas type II prokaryotic adaptive immune system with CRISPR associated (Cas) proteins (Cong et al., 2013). CRISPR is a family of DNA sequences that are widespread in bacteria and archaea, and the type II CRISPR system serves as a defense system that degrade foreign genetic elements such as attacking viral and plasmid DNAs (Barrangou et al., 2007).

A simple version of the CRISPR/Cas system has been modified to edit genomes, and the two main components are the Cas protein Cas9 nuclease and a single guide RNA (sgRNA) (Mojica et al., 2009). The sgRNA is a chimera combined by CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) that confers DNA target specificity (Cong et al., 2013). The sgRNA forms a complex with the Cas9 nuclease and guide the Cas9 to recognize and cleavage site-specific double strands of genomic DNA (Shan et al., 2013). The target specificity is governed by the sgRNA and binds directly to a 20 bp sequence on the target DNA. The transport of Cas9 mediated by sgRNA can go anywhere in the genome, but no direct binding can occur without recognition of a certain sequence called the protospacer adjacent motif (PAM) (Mojica et al., 2009). The PAM sequence NGG immediately follows the DNA sequence targeted by the Cas9 nuclease. No binding or cleaving of the DNA sequence will happen if not followed by a PAM sequence (Esvelt et al., 2013). The Cas9 nuclease recognizes two nucleotides of a PAM, and the cut is predominantly three bp upstream from the PAM sequence. This means that any 23 bp spanning with a sequence ending in NGG can be targeted by the CRISPR/Cas9 system (Fauser et al., 2014).

This targeted cleavage of genomic DNA results in a double strand break that is repaired by the cells through non-homologous end joining or homologous recombination, and the repair by these endogenous mechanisms are causing insertions or deletions in the DNA (Nekrasov et al., 2013). By modifying the singe-guide sequence, it is possible to design sgRNA with different target specificity that enables the CRISPR/Cas9 system to be used to perform sequence-specific genome editing in a wide range of organisms (Shan et al., 2013). U6 and U3 are non-coding small nuclear RNA genes that has been identified in most eukaryotic organisms, including plants. The U6 or U3 promoter are transcribed by RNA polymerase III and used in CRISPR-plasmids to express the sgRNA (Belhaj et al., 2013). Several U6 and U3 promoters have also been identified in plants, including *F. vesca* (Cui et al., 2017) in addition to identification of U6-promoter alignments in Figure 3.4. in chapter 3.3.1 by Thorstensen, T. (unpublished).

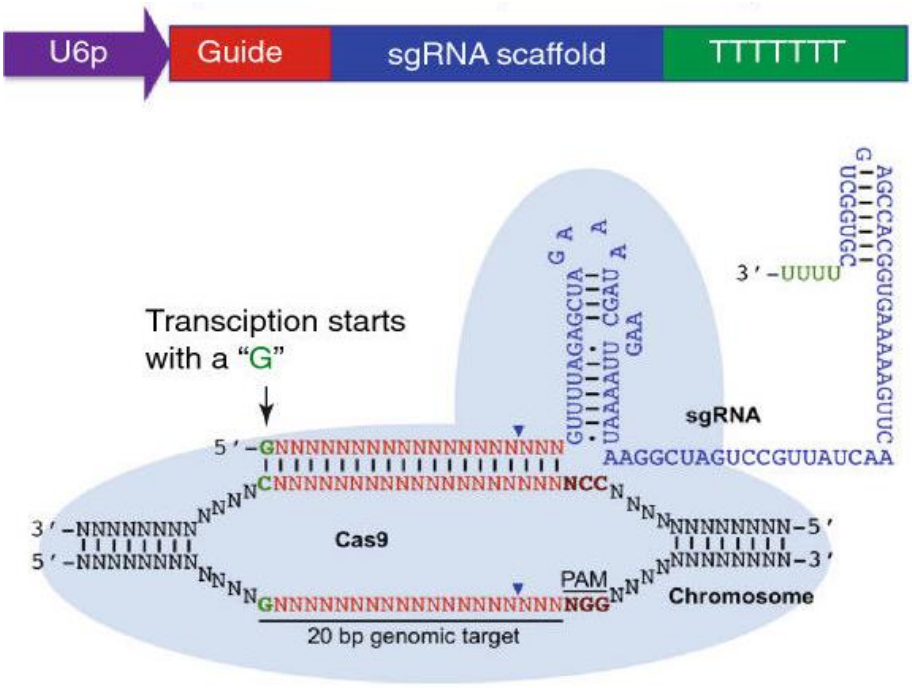


Figure 1.4.: Schematic illustration of the engineered CRISPR/Cas9 system. sgRNA is expressed under the U6 promoter, and transcription starts with a G nucleotide. Cas9 is activated by the sgRNA scaffold and cleavages the DNA strand provided by the presence of the PAM sequence at the 3' end of the 20 bp genomic target. Figure from Belhaj et al. (2013).

1.5.2. Gene regulation in plant defense

By delivering the Cas9 enzyme complexed with a synthetic sgRNA into a plant cell, the cell's genome can be cut at a desired location, allowing genes to be added or removed. In plants, the CRISPR/Cas9 system has been implemented using stable or transient expression methods, and widely used assays in plant research are protoplast transformation and agroinfiltration assay. The agroinfiltration assay, which is performed on intact or detached plant parts, is a system based on infiltration of *Agrobacterium tumefaciens* strains carrying a plasmid with expression cassettes for the sgRNA and the Cas9-endonuclease (Belhaj et al., 2013).

Large deletions can be achieved by introducing two double strand breaks of the DNA guided by two sgRNAs targeting the same locus. By doing this, one sgRNA at each side of the gene of interest induces a cleavage, and a large deletion that is easier targeted is made when the cells own reparation system . This gives the opportunity to knock-out negative regulators of plant defense using the CRISPR/Cas9 system in plants (Belhaj et al., 2013).

Methylation of histone H3 at the lysine position 9 of the histone tail (H3K9) is associated with inhibition of gene expression due to a more closed chromatin state. In plants, DNA methylation of promoter regions usually inhibits transcription and is an important epigenetic mark that functions in a complex web of interactions with histone modifications to change the states of epigenetic gene expression (Chan et al., 2005). In studies with *A. thaliana*, suppression of Methyltransferase 1 (*MET1*) has been shown to drastically reduce methylation of H3K9 because the role of *MET1* in maintaining and directing histone methylation in H3K9 (Espinas et al., 2016; Soppe et al., 2002; Tariq et al., 2003). *MET1* is a major maintenance DNA methyltransferase and is associated with a more closed and tightly wrapped chromatin state (Chan et al., 2005).

Phenotypic plasticity is an important trait in a plant population, and the ability of a genotype to express different phenotypes in different environments allows the plant to adjust to changing surroundings (Pigliucci, 2005). By improving the underlying epigenetic mechanisms, a single plants can potentially be improved to better withstand pathogen attack.

In this way, the plant genome can hypothetically be edited so that the plant is constantly in a primed state, where transcription of defense related genes are induced faster upon pathogen challenge because of a more open chromatin state.

1.6. Hypothesis and aim of the study

1.6.1. Priming in detached leaf assay

To investigate whether priming can be used to increase resistance in strawberry crops, a detached leaf assay of *F. x anansaas* was carried out. The study aims to investigate whether the strawberry plants can be primed with the chemical agent BABA to better withstand a challenge by *B. cinerea*. To investigate the priming effect, strawberry plants was soil drenched with a priming stock of BABA 8d in advance of *B. cinerea* infection. To investigate whether the plants could memorize the priming stimuli, phenotypic scoring and gene expression analysis was done at different time points to determine if the plants were more or less resistant after priming, and to identify some of the genes involved. The hypothesis is that the strawberry plants that are primed with BABA will be more resistant to challenge by *B. cinerea* than strawberry plants that have not been primed.

1.6.2. Epigenetic regulation of defense

Another approach in making plants more resistant to disease, is by changing the underlying epigenetic machinery of the plant. The aim of this experiment was to identify and test different *F. vesca* U6 promoters for directing transient expression of sgRNA in strawberry and use this promoter in specific *F. vesca* CRISPR/Cas9 constructs to knock out the DNA-methyltransferase *MET1*. The hypothesis is that by making a large deletion in the *MET1* gene using the CRISPR/Cas9 system, the plant will be less susceptible to the pathogen *B. cinerea* because of a more open chromatin structure that will activate transcription of defense related genes faster upon pathogen attack.

2. Materials

An overview of the laboratory chemicals and equipment used in the experiments are presented in Table 2.1. – 2.10. in this chapter.

Table 2.1. Chemicals and their suppliers.

Chemical	Supplier
Agarose	Sigma-Aldrich, St. Louis, MO, USA
Boric acid	Sigma-Aldrich, St. Louis, MO, USA
Chlorine	Orkla, Norway
Chloroform:Isloamylalcohol (24:1)	Thermo Fisher Scientific, Waltham, MA, USA
dNTP nucleotides	Thermo Fisher Scientific, Waltham, MA, USA
Ethanol 96%	VWR Chemicals, Radnor, PA, USA
Ethidium bromide (EtBr)	Merck KGaA, Danmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, St. Louis, MO, USA
Glycerol	Merck KGaA, Danmstadt, Germany
Hexadecyltrimethylammonium bromide (CTAB)	Sigma-Aldrich, St. Louis, MO, USA
Nitrogen (liquid)	AGA, Norway
Polyvinylpolypyrollidone (PVPP)	Sigma-Aldrich, St. Louis, MO, USA
SOC Outgrowth Media	New England BioLabs, Ipswich, MS, USA
Sodium chloride (NaCl)	Merck KGaA, Danmstadt, Germany
Tris-base	Sigma-Aldrich, St. Louis, MO, USA
Trizma® hydrochloride (Tris-HCl)	Sigma-Aldrich, St. Louis, MO, USA
Tween® 20	Sigma-Aldrich, St. Louis, MO, USA
β-aminobutyric acid (BABA)	Sigma-Aldrich, St. Louis, MO, USA
β-mercaptoethanol (ME)	Sigma-Aldrich, St. Louis, MO, USA

Table 2.2. Solutions.

Solution	Reagent	Volume
1 X TBE buffer	Tris-base	108 g
	Boric acid	55 g
	EDTA (0.5 M)	40 ml
	Distilled H_2O	Up to 1L
CTAB buffer	CTAB	2 %
	EDTA (pH 8.0)	25 mM
	NaCl	1 M
	PVPP	2 %
	Tris-HCl (pH 8.0)	100 mM
	β-ME	1 %
Luria-Bertani (LB) Broth	Tryptone	10 g
	NaCl	10 g
	Yeast extract	5 g
	Distilled H_2O	Up to 1L
Luria-Bertani (LB) Agar	Tryptone	10 g
	NaCl	10 g
	Yeast extract	5 g
	Agar	15.0 g
	Distilled H_2O	Up to 1L
Potato Dextrose Agar (PDA)	Potato starch	4.0 g
	Dextrose	20.0 g
	Agar	15.0 g
	Distilled H_2O	Up to 1L
Potato Dextrose Broth (PDB)	Potato	4.0 g
	Dextrose	20.0 g
	Distilled H_2O	Up to 1L
1 X SOC Outgrowth Media	Vegetable Peptone	2 %
	Yeast Extract	0.5 %
	NaCl	10 mM
	KCl	2.5 mM
	MgCl2	10 mM
	MgSO4	10 mM
	Glucose	20 mM

Table 2.3. Equipment and their supplier

Equipment	Model	Supplier
Centrifuge	5810 R	Eppendorf, Hamburg, Germany
	CVP-2	Thermo Fisher Scientific, Waltham, MA, USA
	Dupont Sorvall® RC-50 Plus	Kendro Laboratory Products, Newtown, CT, USA
	Heraeus Fresco 21	Thermo Fisher Scientific, Waltham, MA, USA
	Heraeus Pico 21	Thermo Fisher Scientific, Waltham, MA, USA
Electrophorese visualiser	Gel Doc™ EQ	Bio-Rad, Hercules, CA, USA
Fluorometer	Qubit® 2.0	Invitrogen, Carlsbad, CA, USA
Power supply	Power Pac 300	Bio-Rad, Hercules, CA, USA
Heatblock	Thermo-Shaker PSC24	Thermo Fisher Scientific, Waltham, MA, USA
Microscope	DM LS40	Leica, Wetzlar, Germany
PCR machine	T100™ Thermal Cycler	Bio-Rad, Hercules, CA, USA
RT-qPCR machine	CFX96™ Real-Time System	Bio-Rad, Hercules, CA, USA
Spectrophotometer	NanoDrop™ 2000	Thermo Fisher Scientific, Waltham, MA, USA
Water bath	Isotemp® GPD 05	Thermo Fisher Scientific, Waltham, MA, USA

Table 2.4. Kits and their suppliers.

Kits	Supplier
DNase I Amplification Grade Kit	Sigma-Aldrich, St. Louis, MO, USA
DNeasy® Plant Mini Kit	Qiagen, Hilden, Germany
iScript™ Advanced cDNA Synthesis Kit	Bio-Rad, Hercules, CA, USA
Qiagen® Plasmid Midi Kit	Qiagen, Hilden, Germany
QIAprep® Spin Miniprep Kit	Qiagen, Hilden, Germany
Spectrum™ Plant Total RNA Kit	Sigma-Aldrich, St. Louis, MO, USA
Wizard® Gel and PCR Clean-Up System	Promega, Madison, WI, USA

Table 2.5. Primers and primer design.

Name	Sequence 5' to 3'
Bar1 F	GACAAGCACGGTCAACTTCC
Bar1 R	GTCCAGTCGTAGGCGTTGC
FvActin RTF	CTTTTGGATTGAGCCTCGTC
FvActin RTR	ACGAGCTGTTTTCCCTAGCA
FvBG2-1RTF	CCATATTGCTGCTCCTTGTTCTG
FvBG2-1RTR	CCTTCCAATTCCATTGCTTTTGTAC
FvEF1-α RTF	GCCCATGGTTGTTGAAACTTT
FvEF1-α RTR	GGCGCATGTCCCTCACA
FvPGIP1-RTF	CCTAGTTCATACGGGAAATTCGTTG
FvPGIP1-RTR	TTCATGTTAGCAAATGAGGTTGGG
FvPR1-F	CCTCATTTCCCTCGTAGCCTTAGCC
FvPR1-R	CTTTGTGCATAGGCTGCTAGATTGGG
FvPR5.3F	ACCTCCTAATGACACTCCCGAAACA
FvPR5.3R	CGTAGTTAGGTCCACCGAAGCATGTA
gRNA-Ra2	GCACCGACTCGGTGCCAC
Met1 -F2	ATTGGCGATCTCCCAGCTG
pFGC_F-3710	GAATACCCGCGAAATTCAGGCC
pFGC_R	TAGCTGTTTGCCATCGCTAC
RPPL1 gRNA1-F	TTCTCCGGCGTGTAAACCA
RPPL1 gRNA1-R	TGATTTGGTACGCGTTGGAG
sgRNA_F1	TGTTTTAGAGCTAGAAATAGCAAGT
TPC_F	TCTTGAATTGGTTTGTTTCTTCAC
TPC_R	TAGACAAGCGTGTCGTGCTC

Table 2.6. sgRNA and their sequences.

Name	Sequence (5' - 3')
Met_gRNA n1	ATAGCAGTCTTATAATAGGC
Met_gRNA n2	CCAGTTGTGAAGCATGTGCG
RPPL_gRNA n1	GCTCCTCCTCATATTATCAG

Table 2.7. Enzymes and their suppliers.

Enzymes	Supplier
Alkaline phosphatate, Calf Intestinal (CIP)	New England BioLabs, Ipswich, MS, USA
AmpliTaq DNA Polymerase	Applied Biosystems, Foster, CA, USA
iScript Advanced Reverse Transcriptase	Bio-Rad, Hercules, CA, USA
PACI	New England BioLabs, Ipswich, MS, USA
SsoAdvanced™ Universal SYBR® Green	Bio-Rad, Hercules, CA, USA
T4 DNA Ligase	Thermo Fisher Scientific, Waltham, MA, USA

Table 2.8. Competent cells and their suppliers.

Competent cells	Supplier
NEB® 5-alfa Competent *E. coli*	New England BioLabs, Ipswich, MS, USA
One Shot™ Top 10 Chemically Component *E. coli*	Thermo Fisher Scientific, Waltham, MA, USA

Table 2.9. Size marker ladders for gel electrophoresis.

Ladder	Supplier
100 bp	New England BioLabs, Ipswich, MS, USA
1 kb	New England BioLabs, Ipswich, MS, USA

Table 2.10. Software and its manufacturers

Software	Manufacturer
Benchling	Benchling Inc., San Fransisco, CA, USA
Bio-Rad CFX manager	Bio-Rad, Hercules, CA, USA
Excel	Microsoft, Redmond, WA, USA
ImageJ	https://imagej.net/Welcome

3. Methods

3.1. Experiments

All experiments were performed at NIBIO (Norwegian Institute of Bioeconomic Research) in Ås, Akershus, Norway (59°39′37″N10°47′1″E), were all laboratory, greenhouse and growth-chamber facilities are located.

3.2. Detached leaf assay

3.2.1. Priming of plants

16 plants of *Fragaria* x *ananassa* cv. Corona at the vegetative stage were grown in 2 L pots under greenhouse conditions (18°C day/12°C night) at a photoperiod of 16h. Plants were primed for defense against *B. cinerea* with the chemical agent β-aminobutyric acid (BABA). BABA is an isomer of the non-protein amino acid aminobutyric acid, and is known for its ability to induce resistance against plant pathogens and abiotic stress in plants (Baccelli & Mauch-Mani, 2016). BABA is highly water-soluble and has been shown to deploy its action when applied as soil drench, foliar spray or injected into the stems of plants (Conrath et al., 2015). BABA (Sigma-Aldrich®, catalogue number 7574 54-1G) was dissolved in distilled water to a stock concentration of 1,6 mM (164.99 mg / 103.12 MW). 250 ml of BABA stock was added to the given pots, resulting in a final concentration of 200 µM for each pot (250 ml x 1600 µM / 2000 ml).

The 16 plants were divided into two groups of eight. One group was soil drenched with 250 ml of the BABA stock and the second group was soil drenched with 250 ml of dH_2O. Plants were transferred to a growth chamber with room temperature of 16°C and a photoperiod of 18h and maintained for 8 days. Plants were watered every second day with 250 ml tap water to make sure the pots were drained from the BABA treatment to be able to investigate epigenetic mechanisms regulating the defense priming. After 8 days, each of the primed and non-primed plants were subdivided into two new groups of four plants used to either mock inoculate or inoculate with *B. cinerea* (Fig. 3.1).

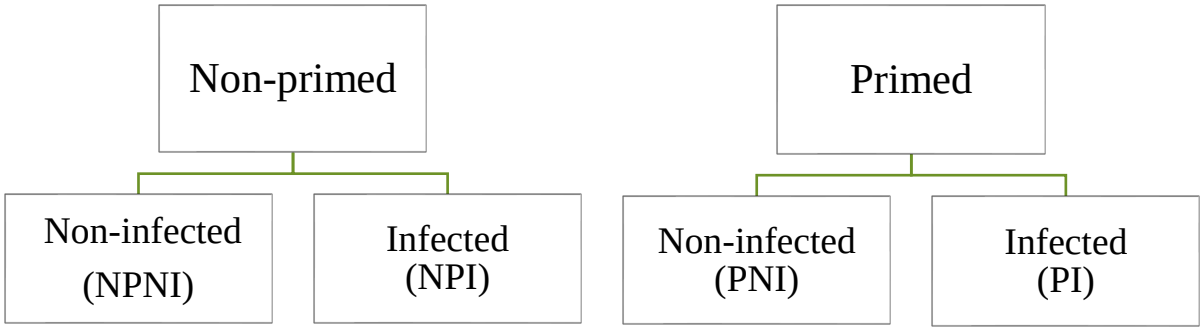


Figure 3.1. Experimental setup for the detached leaf assay. 16 plants were first divided into two groups of eight; non-primed and primed, and then subdivided into four groups of plants; non-primed non-infected (NPNI), non-primed infected (NPI), primed non-infected (PNI) and primed infected (PI).

3.2.2. Cultivation of the pathogen

A strain of *B. cinerea* (isolate B.c101, provided by Abdelhameed Elameen at NIBIO) grown for 3 weeks in room temperature on a petri dish containing PDA was prepared as spore suspension. Preparations were done by flooding the petri dish with PDB and by releasing the spores from the fungal cultures by rubbing a sterilized bacteriological loop on the agar. To remove redundant agar and mycelium fragments from the suspension, the solution was filtered through a sterilized spoon strainer. In the end, spore suspension was determined by using a Bürker hemocytometer, and diluted to 10^6 spores per. ml in PDB. One percent of Tween 20 was added to break the surface tension of water and to make sure that spores were evenly distributed in the spore solution.

3.2.3. Detached leaf assay

After 8 days of priming, leaves were detached and drop-infected with *B. cinerea*. The method was based on previously described detached leaf assays by Audenaert et al. (2002). Three leaflets from each plant of the sub-divided groups were cut off and put in carefully marked sterile glasses of dH_2O. Leaves of approximately same size were chosen to exclude differences in developmental stages. Leaves were surface sterilized in three steps; 1 min of 1% chlorine followed by 1 min of 70% ethanol and 1 min of washing in dH_2O. The third washing step was repeated twice to make sure that leaves were properly cleaned. Two randomly selected leaves from each of the four treatments illustrated in Figure 3.1. was placed on two Whatman™ filter papers (GE Healthcare, Chicago, IL, USA) in petri dishes (100 x 15 mm). For leaves collected from the plants in the NPI and PI groups, a drop of 8 µl spore

suspension was applied on three different spots of each leaf by pipetting. After applying the spore suspension, each leaf was wounded through the drop by stinging a 0.5 mm syringe needle three times to facilitate pathogen infection. For leaves collected from the NPNI and PNI groups, 8 µl PDB medium was added on each spot with the same procedure to serve as controls. 2 ml of nuclease-free H_2O was applied onto the filter papers in the end by pipetting to keep high moisture in the petri dishes. Finally, petri dishes were sealed with plastic film and incubated in a growth chamber at 22°C with a photoperiod of 16h.

48h after infection, four samples from each of the treatments were collected and immediately frozen in liquid nitrogen and stored at -80°C for further RNA isolation and gene expression analysis. The same procedure was done with leaves from each treatment after 5 days of incubation when symptoms of disease development were more developed on the infected leaves.

3.2.4. Phenotypic disease scoring

16 samples from each treatment were observed to score the phenotypic development over time to determine if primed plants were more or less resistant against *B. cinerea* compared to the non-primed plants. The leaves were photographed at time points 48h, 5d, 8d and 10d after infection to follow and document disease development. Lesion area of the disease development were measured from photos using the image measuring software ImageJ (Schneider et al., 2012), and calculated by subtracting healthy leaf area from the total leaf area (Fig. 3.2). Every individual leaf was measured at each time point to make statistical analysis of the infected area.

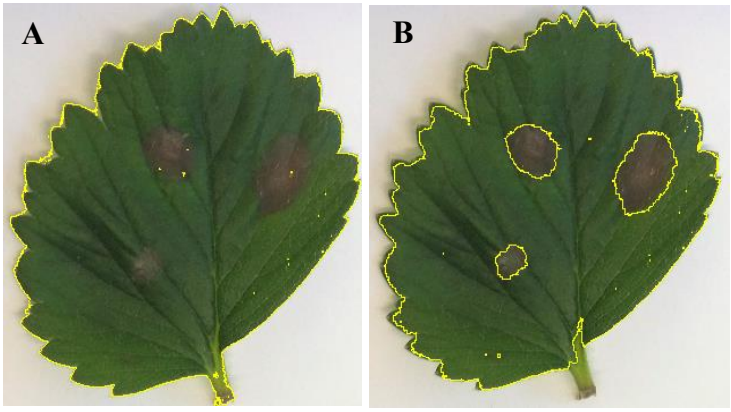


Figure 3.2. Phenotypic measurement of infected leaf. Measurement of infected area was done using ImageJ. (A) Total leaf area and (B) healthy leaf area was compared to determine lesion diameter and to calculate percentage of infected leaf.

3.2.5. RNA isolation and gene expression analysis

Leaves stored at -80°C were disrupted and ground to fine powder in liquid nitrogen using mortar and pistil. Up to 100 mg of ground tissue was used for RNA isolation.

Because of the high content of polysaccharides, polyphenols and other secondary metabolites in strawberry tissues RNA extraction can be particularly challenging. It was therefore necessary to optimize the method of RNA isolation to obtain RNA of best possible quality. Two methods were tested:

1. Spectrum method:
   RNA isolation using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich®, St. Louis, MS, USA), following the manufacturer's protocol.

2. CTAB/Spectrum method:
   Modified method with initial steps involving CTAB extraction buffer followed by the use of Spectrum™ Plant Total RNA Kit (Sigma-Aldrich®, St. Louis, MS, USA).

The CTAB/Spectrum method was based on a method by Chang et al. (1993) that was originally developed for RNA extraction of pine tree tissues. The method has been used in a range of difficult plant tissues with a high content of secondary metabolites such as blackcurrant (Woodhead et al., 1997), apple (Gasic et al., 2004) and grape (Iandolino et al.,

2004), and could potentially increase the quality of isolated RNA from the strawberry tissue compared to the Spectrum Kit method.

3.2.5.1. RNA isolation using Spectrum method

Lysing of cells:

1. 500 µl of Lysis solution/2-ME mixture was prepared by adding 10 µl of 2-ME for every 1 ml of Lysis Solution. The mixture was mixed briefly and incubated at 56°C for 5 min with 30 seconds of vortexing to lyse the cells.
2. Samples were centrifuged at 13000 rpm for 5 min, and lysis supernatant was pipetted into a Filtration column and centrifuged at 1 min.

Binding of RNA:

3. 500 µl of Binding Solution was added to the lysate and mixed immediately by pipetting 6-8 times. 700 µl of the solution was then pipetted into a Binding Column and centrifuged for 1 min to bind nucleic acids, including RNA. Flow-through was decanted, and the procedure was repeated with the rest of the lysate mix.

DNase treatment:

4. To remove DNA from the samples, on-Column DNase Digestion was carried out. RNA bound to the binding column was first washed by pipetting 300 µl of Wash Solution I and centrifuged for 1 min. Thereafter, 10 µl DNase I and 70 µl DNase digestion was combined for each sample and added to the column. Samples were incubated in room temperature for 15 min to digest the DNA.

Washing:

5. To remove digested DNA, 500 µl of Wash Solution 1 was added to each sample and centrifuged for 1 min. Flow through was decanted.
6. A volume of 500 µl Wash Solution 2 was added to the columns and centrifuged for 30 seconds, and residual liquid in the collection tube was discarded. This step was repeated a second time, followed by drying of the column by centrifuging samples for 1 min.

Elution:

7. To elute the RNA, columns were placed in new 1.5 ml tubes, and 50 µl of Elution Buffer was added. The samples were incubated for 1 min, followed by 1 min centrifugation.

3.2.5.2. RNA isolation using CTAB/Spectrum method

Lysing of cells:

1. 600 µl of preheated (65°C) CTAB extraction buffer mixed with PVPP (2%) was added to the ground plant tissue in 2 ml tubes. Tubes were incubated on a heat block at 65°C for 8min, with 1 min of vortexing to lyse the cells. The tubes were inverted 2-3 times by hand during the incubation time to make sure that the extraction buffer got in contact with all the grounded tissue.
2. After incubation, tubes were centrifuged for 10min at 13000 rpm, and supernatant was transferred to new 2 ml tubes. Equal volume of Chloroform:Isloamylalcohol (24:1) was added to the supernatant and inverted 5-6 times by hand.
3. Tubes were centrifuged for 10min at 4°C, and top aqueous layer was transferred into Filtration tubes provided by Sigma-Aldrich® and centrifuged for 1 min at 13000 rpm.

After cell lysing steps, the isolation procedure was done as described in the method of the Spectrum Kit from the DNase treatment in step 4.

After isolating RNA with both methods, RNA was tested with NanoDrop™ 2000 spectrophotometer and Qubit® fluorometer to compare and identify the best method for isolating RNA from the strawberry samples. Spectrophotometric analysis are based on the principles that nucleic acids absorb ultraviolet light at a wavelength of 260 nanometres. By measuring the amount of absorbed light through the sample compared to a blank sample, a quantification of nucleic acid concentration and purity can be done. Fluorometer measurements are based on a fluorescent dye to measure the intensity of the dye that bind to nucleic acid and fluoresce when bound.

3.2.6. cDNA synthesis and real-time expression

Reverse transcription reaction is the process where the isolated mRNA is copied into DNA, and the product is called cDNA. Because the mRNA is derived from genes coding for

proteins, cDNA will consequently represent genes that are expressed from the isolated plant cells of the strawberry leaves.

cDNA synthesis was done using iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA, U.S.A) for RT-qPCR, according to the manufacturer's protocol. For each RNA sample, one reaction was made with reverse transcriptase enzyme and one without to serve as controls for contaminating of DNA. 1 µg RNA/µl was added in each reaction.

Solution:

4 µl	5x iScript Advanced Reaction Mix
1 µl	iScript Advanced Reverse Transcriptase
Variable	RNA template
Up to 20 µl	Nuclease-free H_2O
20 µl	Total volume

The samples were placed in a S100™ Thermal Cycler PCR machine (Bio-Rad, Hercules, CA, USA) with the following synthesis reaction steps:

1. Reverse transcription 20 min at 46°C
2. Inactivation 1 min at 95°C

The synthesized cDNA was used as template for RT-qPCR. cDNA was diluted 10-fold prior to use by adding 10 µl cDNA to 90 µl nuclease-free water.

Gene expression analysis was performed using CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA) in duplicates in a 96-well reaction plate using SsoAdvanced™ Universal SYBR® Green dye system (Bio-Rad, Hercules, CA, USA). The amount of cDNA was calculated so that 1 µg cDNA/µl as added in each reaction.

Solutions:

10 µl	SsoAdvanced TM Universal SYBR® Green Supermix
1 µl	Primer forward / reverse

2 µl	cDNA template
6 µl	Nuclease free H_2O
20 µl	Total volume

Primers:

	Forward	Reverse
Actin	FvActinF	FvActin R
EF1-α	FvEF1-α RTF	FvEF1-α RTR
BG2-1	FvBG2-1 RTF	FvBG2-1 RTR

RT-qPCR is a system used for detection and quantification of a fluorescent dye such as the SYBR® Green, which fluoresces when bound to double stranded DNA of any kind. The fluorescence is measured in each cycle during PCR, and increased amount of DNA product will give an increasing fluorescence that is plotted against the cycle number in the PCR reaction. The threshold cycle (Ct) is defined as the cycle number of where the fluorescence emission exceeds a threshold that is the parameter for quantification. High amount of DNA template in the solution give a more rapid detection in the PCR process which subsequently give a lower Ct-value. High Ct-values are low detection of DNA template. A relative quantification can be found by comparing the expression of the targeted gene with a householding gene.

The RT-qPCR was run with the thermal cycling conditions as following:

Initial denaturation at 95°C for 3min followed by 40 cycles of denaturation at 95°C for 10s, and primer annealing at 60°C for 30s.

Once the most efficient RNA extraction method was established, RNA for all 32 samples were isolated. For all the samples, a second round of DNase treatment was carried out in solution using 16 µl of the purified RNA with DNase I Amplification Grade kit from Sigma-Aldrich® (Catalog number AMPD1) following the manufacturer's protocol. This was to investigate whether the DNase treatment could efficiently remove residual DNA

contamination from the samples. RNA yield was measured before and after DNase treatment using Qubit® fluorometer to compare the two DNase treatments.

cDNA synthesis was done as described earlier in this chapter, and two samples were made without reverse transcriptase enzyme to serve as control. The cDNA was diluted 10-fold and used as template in a second round of RT-qPCR to compare the two DNase treatments. The solutions and cycling conditions was done as described earlier.

Primers:

	Forward	Reverse
BG2-1	FvBG2-1 RTF	FvBG2-1 RTR
PR1	FvPR1 F	FvPR1 R
PR5.3	FvPR5.3 F	FvPR5.3 R
PGIP	FvPGIP RTF	FvPGIP1 RTR

All RT-qPCR reactions were normalized by the Ct value using the Pfaffel method (Pfaffl, 2001) in excel.

3.3. Epigenetic regulation of defense

3.3.1. Quantification of *F. vesca* U6 promoters

The experiment aims to design CRISPR-vectors with three different U6-promoters of *F. vesca* to test in a transient expression system. Transient expression is the result of transcription and translation of a non-integrated transferred DNA vector from *A. tumefaciens* and does not interfere with the stability of the host genome. Transiently transformed plants are expected to show a peak in gene expression 2–4 days after infecting the plant material and the expression will subsequently decline with time (Krenek et al., 2015). In molecular cloning, genetically engineered plasmid vectors are used to carry the expression cassette to the target cell. The plasmid contains multiple cloning sites for insertion of DNA for transformation.

The transient expression experiment in *F. vesca* aims to knock out the DNA-methyltransferase *MET1*. *MET1* plays an important part of the epigenetic map in *F. vesca*, and

can be knocked out by using the CRISPR/Cas9 system for gene editing. Figure 3.3. shows the structure of the *MET1* gene with possible binding sites for sgRNA to mediate cleavage by Cas9 nuclease. Constructs containing designed sgRNA targeting *MET1* was made, in addition to sgRNA targeting *RPPL1* . *RPPL1* is a homolog to the *A. thaliana AT3G14470* gene which is thought to increase resistance.

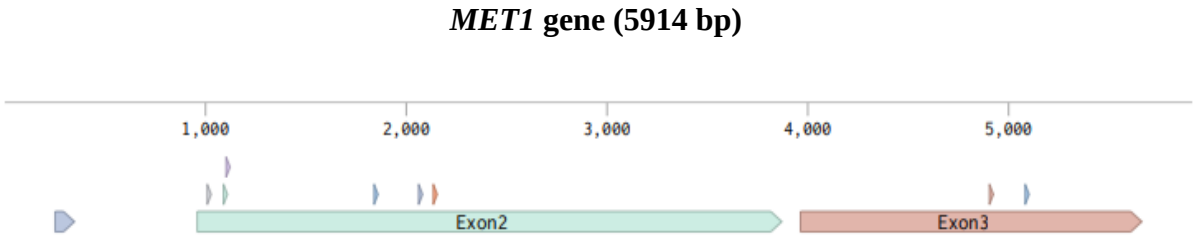


Figure 3.3. DNA-methyltransferase 1 (*MET1*) gene structure. The illustration includes; Exon1 (not annotated), Exon2 (blue) and Exon3 (red). In the open space between exons are non-coding introns. The small arrows above the exons represents different sgRNA sequences to target cleavage by Cas9-nuclease.

Berries of *F. vesca* had previously been infiltrated with *A. tumefaciens* containing CRISPR-constructs with different U6 promoters. Because the promoter used to drive sgRNA expression is dependent upon the host in question, several U6 promoters were identified from *F. vesca* prior to the infiltration and selected for the experiment (Figure 3.4.). The sgRNA spacer sequences in Table 3.1. were designed using the tool CRISPR-P (http://cbi.hzau.edu.cn/crispr/).

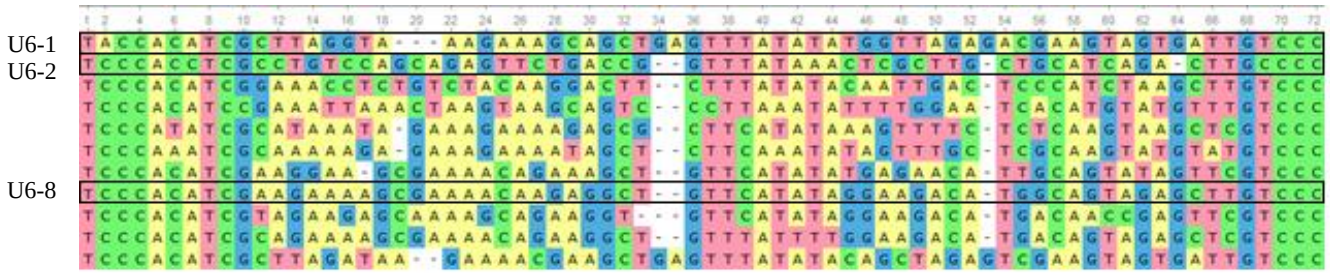


Figure 3.4. Schematic view of potential U6 promoters in *F. vesca*. The promoters selected for testing (U6-1, U6-2 and U6-8) are marked in black boxes. Figure from Thorstensen, T. (unpublished)

Table 3.1. Synthesized U6-1, U6-2 and U6-8 expression cassettes. Synthesizing was done using the GeneArt Gene Synthesis service at Thermo Fisher Scientific (Waltham, MA, USA) for cloning into *F. vesca* CRISPR vector.

Vector	**sgRNA cloning site**	**Expression cassette length**
FvU6-1sgRNAExpression	BsaI	698 bp
FvU6-2sgRNAExpression	BsaI	696 bp
FvU6-8sgRNAExpression	BsaI	707 bp

The *Agrobacterium*-mediated transient transformation of *F. vesca* berries was done with a syringe needle in attached berries with bacterial solutions of *A. tumefaciens* carrying each of the tree expression cassettes for U6 promoters and sgRNA. The CRISPR vector carrying U6-1 promoter for sgRNA expression in *MET1* is shown in Figure 3.5.

Empty vector and sgRNAs:

Name	Abbreviation
pFGC-pcoCas9	pFGC
Met_gRNA n1	MET1
RPPL_gRNA n1	RPPL1

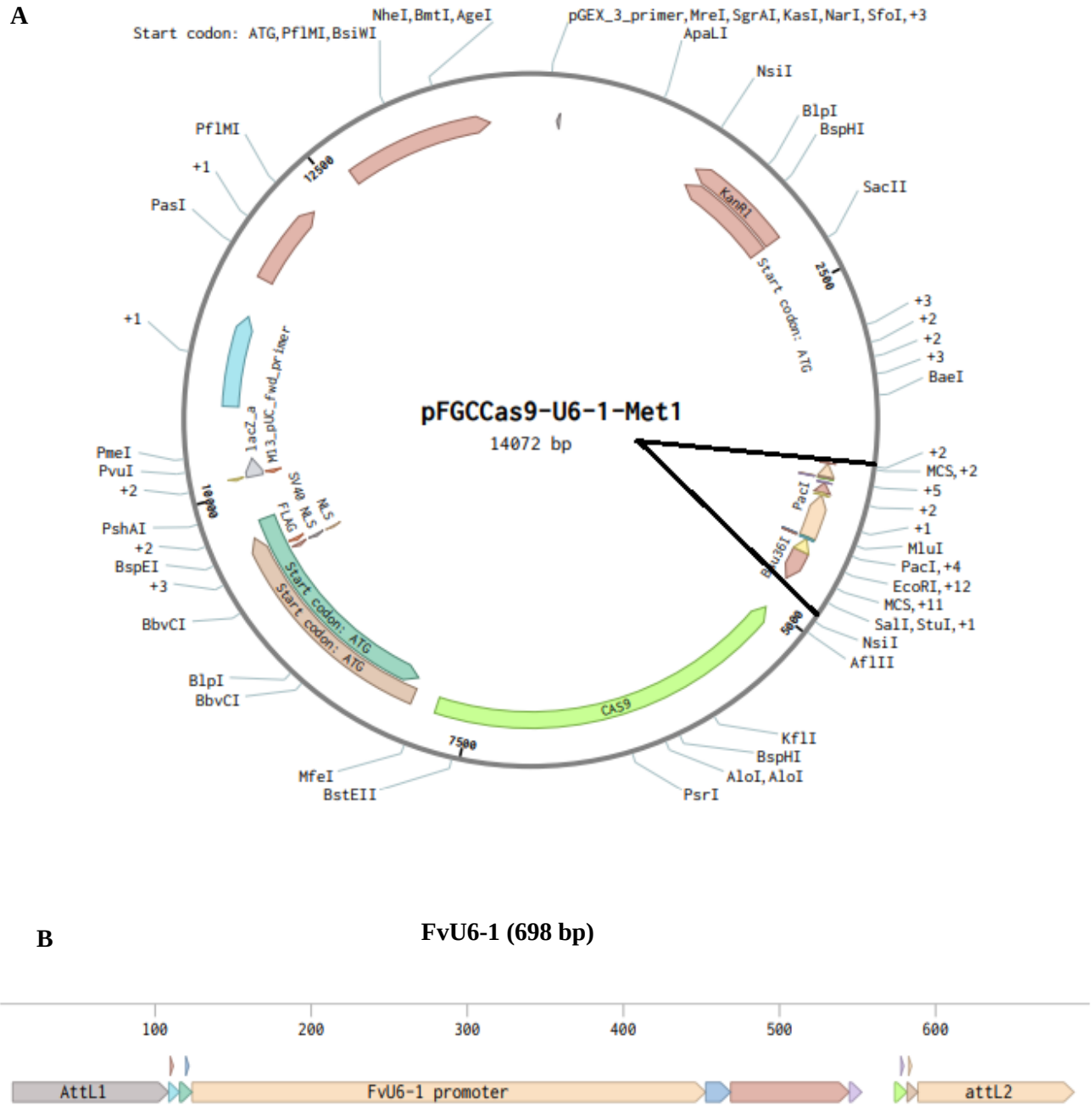


Figure 3.5. pFGC Cas9 plasmid with U6-1 promoter for expression of sgRNA. (A) pFGC binary vector with U6-1 promoter of sgRNA targeting *MET1* (14072 bp) illustrated. The vector has kanamycin resistance gene for bacterial selection (KanR1). The position of the U6 promoters of sgRNA *MET1* construct are between the black dividing lines. (B) FvU6-1 promoter of sgRNA expression. sgRNA (blue) is following the U6-1 promoter and the sgRNA scaffold (purple) is downstream of the sgRNA. On each side are multiple cloning sites. Attl is for gateway cloning (not used in this experiment).

In this study, agroinfiltrated strawberry plants were kept in green-house facilities (18°C day/16°C night) with a photoperiod of 16h and harvested 48 hours after the infiltration. Harvested berries were immediately frozen in liquid nitrogen and stored until RNA isolation and gene expression analysis.

3.2.1.1. RNA and DNA isolation

Two berries from three assays of each sample were disrupted and ground to fine powder in liquid nitrogen using mortar and pistil. Up to 100 mg of ground tissue from each sample was transferred into 2 ml tubes and stored at -80°C until use. RNA was isolated using the CTAB/Spectrum-method. Isolated RNA was stored in 2 ml tubes at -80°C until use. DNA from the same grounded tissue was isolated using DNeasy® Plant Mini Kit (Qiagen®, Hilden, Germany), following the manufacturer's protocol. Isolated DNA was stored in -20°C until use.

3.2.1.2. cDNA synthesis and Real-Time expression

For the RNA samples, a reverse transcription reaction was done using iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA, U.S.A) for RT-qPCR, following the manufacturer's protocol. The amount of RNA was calculated so that 1 µg RNA/µl was added in each reaction, and two of the samples was made without reverse transcriptase enzyme to serve as controls. Samples were combined in PCR plates.

Solution:

4 µl	5x iScript Advanced Reaction Mix
1 µl	iScript Advanced Reverse Transcriptase
Variable	RNA template
Up to 20 µl	Nuclease-free H_2O
20 µl	Total volume

The samples were placed in a S100™ Thermal Cycler PCR machine (Bio-Rad, Hercules, CA, USA) with the previously described synthesis reaction steps.

1. Reverse transcription 20 min at 46°C
2. Inactivation 1 min at 95°C

The synthesized cDNA was used as template for RT-qPCR and diluted 10-folded prior to use. Gene expression analysis was performed in duplicates in a 96-well reaction plate using SsoAdvanced™ Universal SYBR® Green dye system (BIO-RAD, Hercules, CA, USA) in a CFX96^{TM} Real-Time System (BIO-RAD, Hercules, CA, USA).

Solution:

10 µl	SsoAdvanced™ Universal SYBR® Green
1 µl	Primer forward / reverse
2 µl	cDNA template
6 µl	Nuclease free H_2O
20 µl	Total volume

Primers:

	Forward	Reverse
Actin	Actin F	Actin R
Bar1	Bar1 F	Bar1 R
RPPL1	RPPL1 gRNA1-F	RPPL1 gRNA1-R
MET1	Met1 F2	gRNA-Ra2

RT-qPCR was run with the thermal cycling conditions as following:

Initial denaturation at 95°C for 3min, followed by 40 cycles of amplification/denaturation at 95°C for 10s, and primer annealing at 60°C for 30s.

All RT-qPCR reactions were normalized by the Ct value using the Pfaffel method (Pfaffl, 2001) in excel. The same RT-qPCR procedure was done for the DNA samples in order to normalize the RNA Ct values against DNA Ct values to get more reliable results.

3.3.2. Transient expression of CRISPR construct

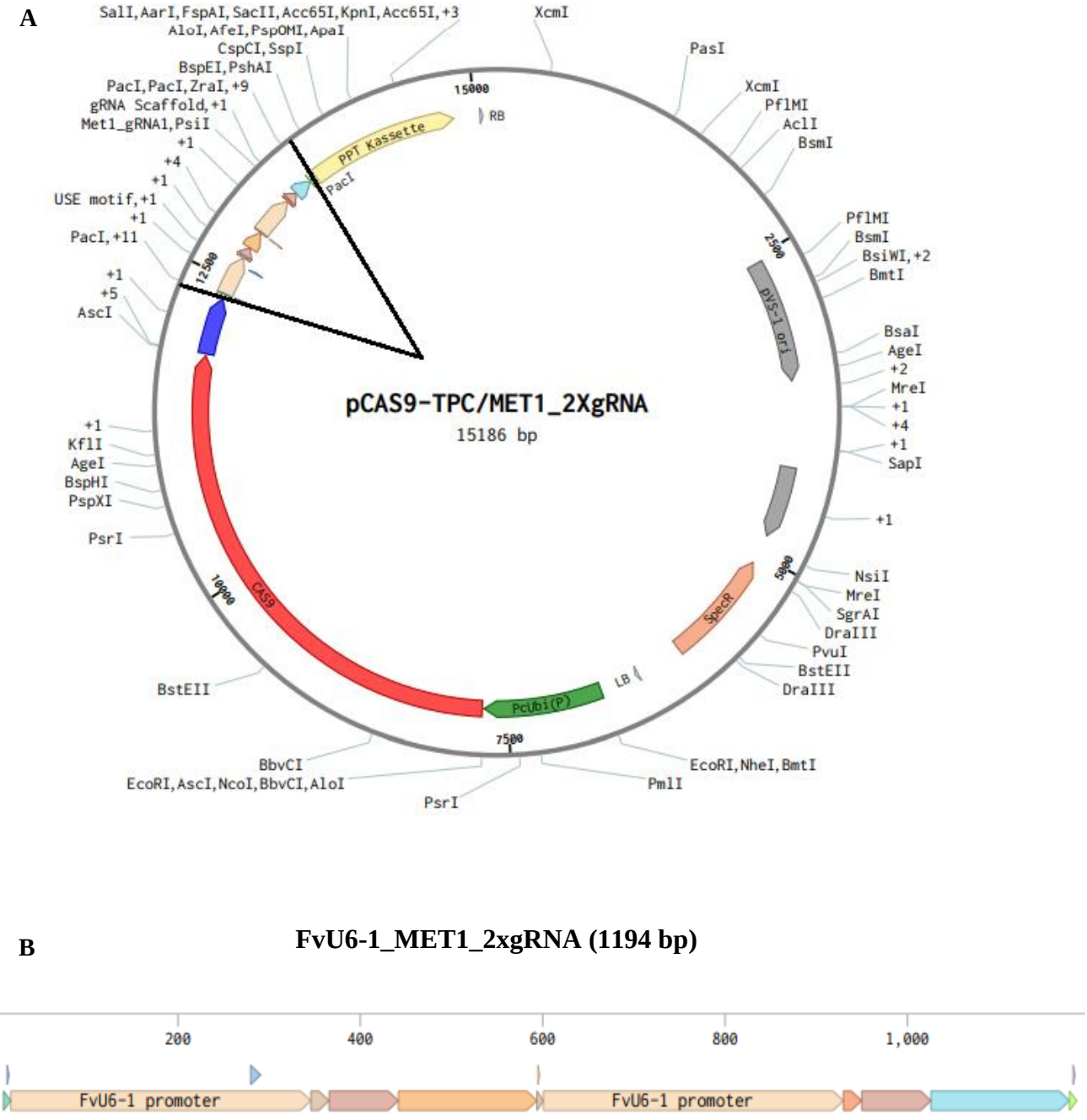
With quantification results of the three different U6 promoters, U6-1 was chosen for further use in the experiment because of high expression for both *MET1* and *RPPL1* sgRNA.

Introducing two CRISPR constructs in this experiment containing two different sgRNA-target sites of the *MET1* gene, will delete the region between the sgRNAs. This will in principle make the mutagenesis more efficient than a single sgRNA because the gRNAs may have different targeting efficiency and because a deletion of a larger region is more likely to make a knock out than a small mutation. A larger deletion is also easier to detect with simple molecular methods like PCR if the transient expression is successful.

Empty vectors:

Name	Abbreviation
pFGC-pcoCas9	pFGC
pCAS9-TPC	pTPC

To limit the extent of the experiment, only the *MET1* construct were chosen for insertion in the plant vectors in the following experiments. Figure 3.6. shows the pCas9-TPC CRISPR vector carrying U6-1 promoter for 2 x sgRNA expression in *MET1*.



B **FvU6-1_MET1_2xgRNA (1194 bp)**

Figure 3.6. pCas9-TPC plasmid with of U6-1 and 2 x sgRNA MET1. (A) pTPC binary vector with the selected U6-1 promoter for expression of 2 x sgRNA targeting *MET1* (15186 bp). The binary vector has spectromycin resistance gene for bacterial selection (SpecR). The position of the 2 x sgRNA construct are between the black dividing lines. (B) Illustration of the 2 x sgRNA construct. The figure shows two FvU6 promoters (pink). Both promoters are followed by a 20 bp MET1_sgRNA n2 and a 20bp MET1_sgRNA n1. Each sgRNA is followed by an identical sgRNA scaffold (purple), and a FvU6 long terminator. On each boarder side of the construct is a PacI cutting site for cloning.

3.3.2.1. Cloning of sgRNA into plant vectors

In order to make a site-specific mutagenesis in *F. vesca* using *Agrobacterium*-mediated transformation, a plasmid transformation was done in *Escherichia coli.* The system consists of *E. coli* for transformation and propagation of the binary vector, and *A. tumefaciens* to deliver the CRISPR construct for transient expression. Both pTCP and pFGC plant vectors are used in the experiment to investigate which one is most effective in transient expression.

Transformation of bacterial cells

First, bacterial cells carrying synthesized 2 x sgRNA constructs from GeneArt Cloning (Thermo Fisher Scientific, Waltham, MA, USA) were transformed for propagation and further isolation of sgRNA by using NEB® 5-alfa competent *E. coli* cells and selective LB-medium containing antibiotics corresponding to the plasmid-encoding resistance genes in pTPC.

Plasmid isolation from *E. coli*

DNA purification was carried out using Qiagen Plasmid Midi Kit (Qiagen®, Hilden, Germany) following the manufacturer's protocol, using 50 ml of starter culture. In this step proteins and chromosomal DNA is denatured while smaller plasmid DNA remains in the lysate. After plasmid isolation, DNA yield for MET1, pFGC and pTPC was measured using NanoDrop™ 2000 Spectrometer.

Total DNA yield:

	ng/µl
MET1 pTPC	263.7
pTPC	187.9
pFGC	100

Cutting

After DNA isolation, cutting with restriction enzymes were set up to open the circular plasmids to linear fragments. 8 µl of plasmid DNA was used in the reaction. Cutting was set up at 37°C for 10h overnight in a S100™ Thermal Cycler PCR machine.

Solution for MET1 and pTPC:

5 µl	Smart Buffer
4 µl	PACI (10 u/µl)
Variable	Plasmid DNA
Up to 50 µl	dH_2O
50 µl	Total

Solution for pFGC:

10 µl	Smart Buffer
4 µl	*PACI* (10 u/µl)
Variable	DNA
Up to 100 µl	dH_2O
100 µl	Total

Gel purification

Followed by the cutting, agarose gel electrophoresis was done to separate the fragments of sgRNA. In gel purification, the volt difference across an agarose gel matrix is used to separate the negatively charged DNA through the gel in a buffer solution. Large fragments of DNA will migrate slower than smaller fragments, making it possible to visualize and identify individual pieces of fragments by comparing it to known bp ladders visualized through UV light and Ethidium-Bromide (EtBr) staining. For preparations, 1% agarose was mixed with 1 X TBE buffer solution and heated in a microwave until completely homogenized. The liquid

was thereafter cooled until approximately 60°C, and 1 drop of EtBr was added per 50 ml of liquid. The solution was carefully mixed and finally added to a prepared gel tray.

25 µl of DNA from each sample was combined with 5 µl of loading dye to a total volume of 30 µl. A gel of 150 ml were run at 70V and visualized under UV light. After 50min, sgRNA fragments from MET1 were clearly separated and cut out from the gel at the expected size of 1171 bp using a clean scalpel. The gel slice was weighted and put in 1.5 ml tubes. The same procedure was done for the pTPC and pFGC vectors after 45 more minutes with electrophoresis at the expected size of 14 kb. After gel slicing, DNA was cleaned with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA).

Dephosphorylation

Before ligation, a phosphatase was used to prevent the binary vectors from re-circularization during ligation. The phosphatase is added to remove the 5’ phosphate ends of the vector DNA and prevents occurrence of vector re-closure in the ligation step. The phosphatase therefore reduces the background during subsequent transformation.

Solutions:

42.5 µl	Plant vector
2.5 µl	CIPI enzyme
5 µl	Smart Buffer
50 µl	Total

The dephosphorylation process was done in a S100™ Thermal Cycler PCR machine (BIO-RAD, Hercules, CA, USA) at 37°C for 30 min.

Ligation

The DNA ligase enzyme catalyzes the formation of covalent phosphodiester linkages at the sticky ends of the vector and the insert, and permanently join the nucleotides together. After this, the complete plasmid can be transformed into bacterial cells for propagation. The ligation reaction was done in a PCR machine for 16h at 16°C.

Solutions:

1 / 2 µl	pTPC / pFGC vector
1 µl	Ligase Buffer
0.1 µl	T4 DNA Ligase enzyme
0.9 µl	dH_2O
Up to 10 µl	Insert sgRNA
10 µl	Total volume

The CIP treatment and ligase reaction were finally verified by gel electrophorese to confirm that the insert had been connected to the binary vectors with the desired orientation. After verifying the transformation of plasmids, transformation of the constructs in *A. tumefaciens* cells could take place.

Transformation of bacterial cells

Transformation:

1. 1 µl of ligase mix was added to 20 µl NEB® 5-alfa competent *E. coli* and One Shot™ *E. coli* cells, respectively, for both pFGC and pTPC binary vectors, and kept on ice for 30min followed by a heat shock at 42°C for 30 s in a water bath.
2. After heat shock, tubes were immediately put on ice for 2 min, and 700 µl of room tempered SOC Outgrowth Medium (New England BioLabs, Ipswich, MS, USA) was added to each tube.
3. Tubes were then incubated at 37°C in a heat chamber with shaking at 225 rpm for 1 hour.

4. After incubation, 20 µl of the culture was stroke out on petri dishes containing LB agar and kanamycin (50 µg/ ml) and spectinomycin (50 µg/ml) for pFGC and pTPC, respectively.
5. The remaining culture was centrifuged for 15 seconds at 10.000 rpm and resuspended in approximately 100 µl of the liquid. 100 µl of the resuspended culture was stroke out on the petri dish. All petri dishes were incubated over night at 37°C for bacterial colony growth.

PCR Screening

Bacterial colonies from the transformation were screened with agarose gel electrophoresis after PCR for presence of the vector with the insert in the desired orientation by using orientation specific primers for the insert.

8 µl of nuclease-free H_2O was added to PCR plates and 8 colonies from each petri dish was randomly picked by gently touching the center of the colony with a pipette tip and dipped into the wells. The pipette tips were then carefully moved so that in the bacterial cells would be free in the dH_2O solution. Tips were then stroked on LB-agar containing antibiotics corresponding to the plasmid encoded resistance gene in carefully marked areas to obtain fresh cultures of the single colonies. PCR solution was added to the single colonies. 1 µl of positive control was added to one well in a addition to a negative control containing dH_2O.

PCR is used to amplify DNA *in vitro* during several cycles and is carried out by using a temperature stable DNA polymerase, nucleotide primers and nucleotide supply (dNTP).

PCR solution:

11 µl	H_2O
2.5 µl	PCR Buffer
2 µl	dNTP
0.5 µl	Primer forward / reverse
0.2 µl	AmpliTaq DNA Polymerase

16,2 µl	Total volume

Primers:

	Forward	Reverse
pTPC	pTPC-R	sgRNA-F1
pFGC	pFGC-F 3710	sgRNA-F1

PCR program:

94°C 5 min, (94°C 30 sec, 56°C 30 sec, 72°C 45 sec) x 40, 72°C 7 min and 4°C ∞.

The cultures were prepared by adding 5 ml of LB medium to a bottom flask with spectinomycin and kanamycin for pTPC and pFGC, respectively. Bacterial culture was added to the flask with a sterile plastic bacteriological loop and incubated at 37°C overnight at 180 rpm. 2 ml of the overnight culture was transferred to 2 ml tubes and centrifuged at 10000 rpm for 3 min to obtain a pellet and resuspended.

Plasmid DNA purification was done with QIAprep® Spin Miniprep Kit (Quiagen, Hilden, Germany) according to manufacturer's protocol. After purifying, DNA concentration was measured by NanoDrop™ 2000 to be between 30-100 ng/µl for all four samples. 20 µl of the DNA solution was prepared and sent for sequencing in GATC Biotech AG, Germany.

Primers used in sequencing:

	Forward	Reverse
pTPC	TPC_F	TPC_R
pFGC	pFGC-F 3710	pFGC_R

After sequencing, the plasmids were transformed into *A. tumefaciens* for transient and stable plant transformation. pTPC and pFGC without sgRNA expression cassettes were included as negative controls

Transformation of *A. tumefaciens*

The *MET1* CRISPR plasmid was introduced to two different strains of *A. tumefaciens*; EC58 and LBA4404. Both strains were premade with calcium chloride according to a protocol based on Holsters et al. (1978).

Freeze-thaw transformation of *A. tumefaciens* competent cells:

1. Tubes of approximately 50 µl frozen competent cells were thawed on ice for 30-50 min. 250 ng of plasmid DNA was added to the tubes, and tubes were incubated on ice for 5 min for the plasmid DNA to stick to the outer side of the cells. Empty vectors were also added to tubes to serve as control.
2. Tubes were frozen in liquid nitrogen for 5 min, and immediately transferred to a heat shock in 37°C water bath for 5 min, then returned to ice for 5 min. This temperature treatments makes the cells take up the plasmid DNA.
3. 1 ml of room tempered LB broth was added to each tube and incubated at 28°C with shaking at 200 rpm for 3-4 hours.
4. After incubation, 50 µl and 200 µl of each culture was plated out on LB agar plates containing rifampicin (50 µg/ml) selection agent combined with spectinomycin (50 µg/ml) and kanamycin (50 µg/ml) for pTPC and pFGC, respectively for culturing.

Plates were incubated for colony growth at 28°C for 2d for strain EC58 and 3d for the LBA4404 strain.

PCR screening

The PCR screening was done according to the previous protocol were 8 µl of H_2O was added to PCR plates, and six single colonies of each vector was picked from each agar plate to verify the transformation. Colonies were stroke on new agar plates to obtain fresh cultures. Colonies were grown overnight at 28°C with shaking at 200 rpm. Stocks of the overnight culture was prepared for freezing by adding 500 µl of the culture to 500 µl of glycerol in 1.8 ml tubes and stored at -80°C until use

4. Results

4.1. Detached leaf assay

4.1.1. Phenotypic effect of BABA treatment

A total of 16 detached leaves from each of the four treatments were incubated after infection to observe the phenotypic characteristics of the BABA treatment. Half of the leaves had been primed with BABA 8 days before drop-infecting the leaves with *B. cinerea*, and the aim was to observe whether the primed leaves were more or less resistant to infection by *B. cinerea* than the non-primed leaves. The leaves were observed during the 10-day incubation period, and phenotypic scoring was based on the development of brown, necrotic lesions around the drop-infected areas.

The observed disease development is illustrated in Figure 4.1, 4.2, and 4.3 where the NPI and PI leaves are presented after 5, 8 and 10 days, respectively. No symptoms were observed after 48 hours, and the control plants NPNI and PNI did not show any sign of disease development after the incubation period of 10 days and is therefore not presented.

After the experiment had been carried out, the previous grower of the strawberry plants informed that the plants used in the experiment had been treated for aphids, mites and powdery mildew prior to being given to this experiment. There were also concerns that the plants had been infected by the oomycete *Phytophthora cactorum*. Therefore, it's reason to assume that the insects and pathogens may have weakened the plants and interfered with the results. All plants were treated the exact same way after priming, and the detached leaves were all incubated in the same growth room under the same conditions.

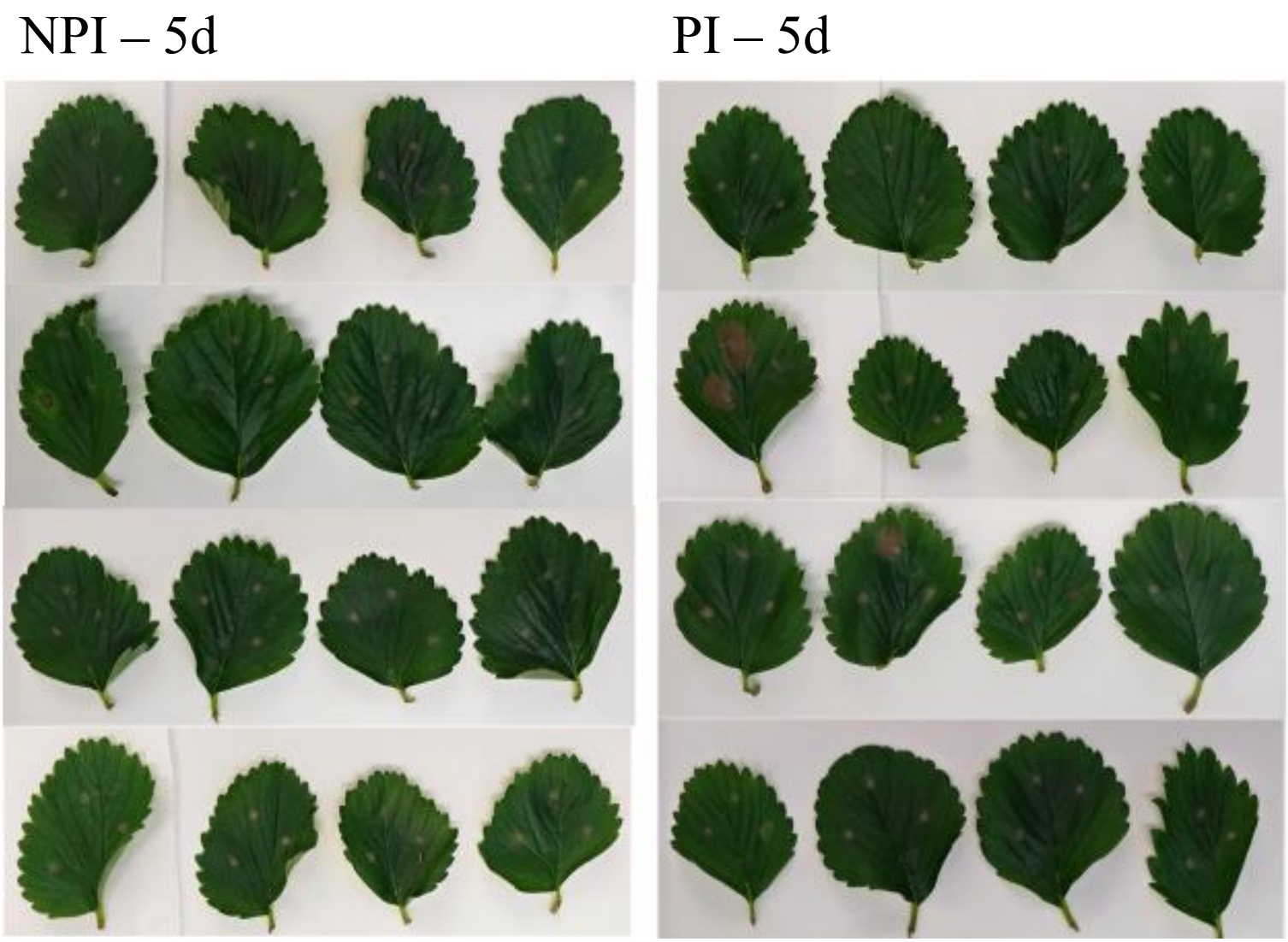


Figure 4.1. Disease development in NPI and PI leaves after 5 days. Disease development 5 days after infection. 1/16 leaves have developed necrotic lesions on the NPI leaves, and 3/16 leaves have lesion development on the PI treated leaves.

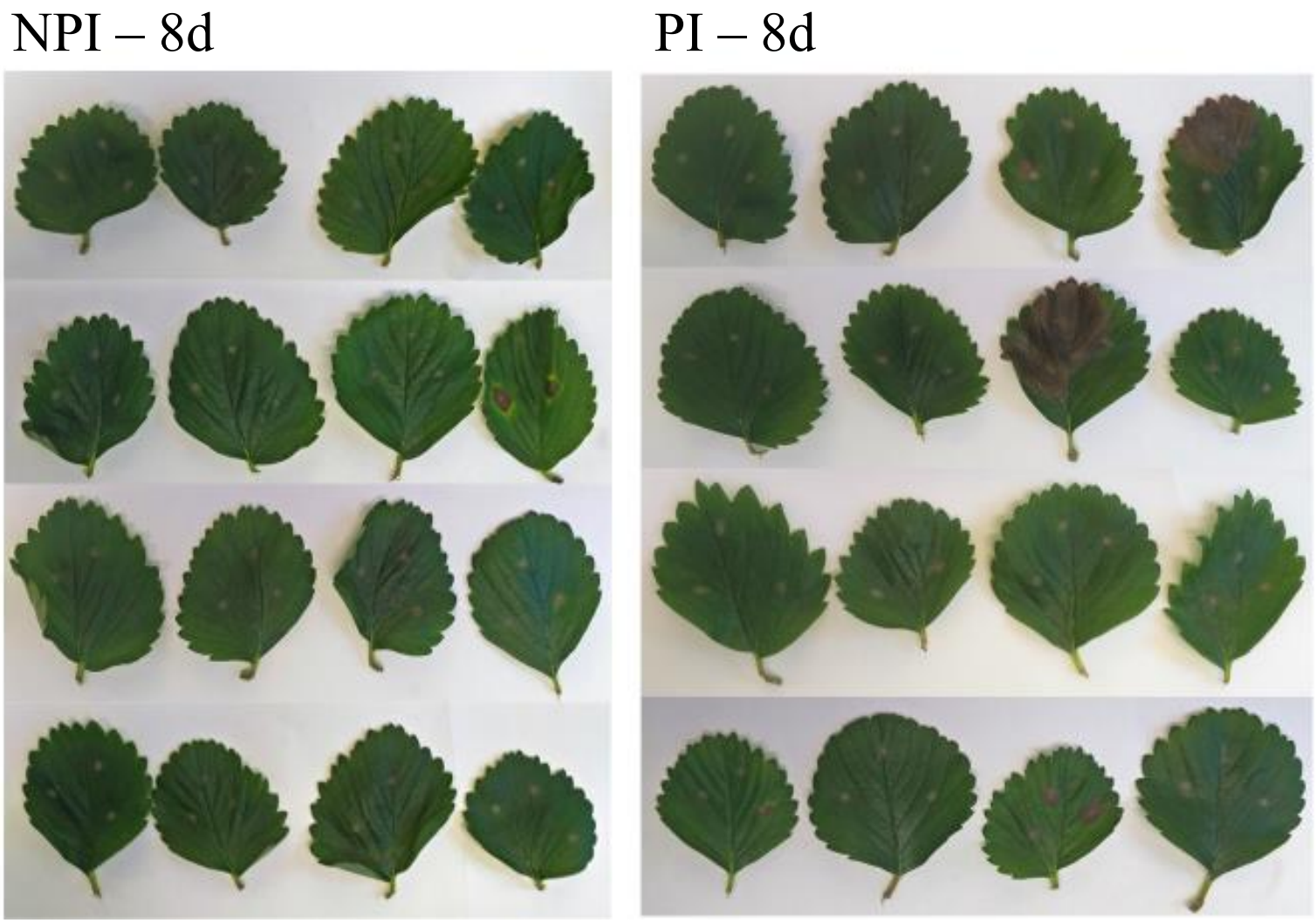


Figure 4.2. Disease development in NPI and PI leaves after 8 days. Disease development 8 days after incubation. 1/16 leaves have necrotic lesions on the NPI treated leaves, and 5/16 for the PI leaves.

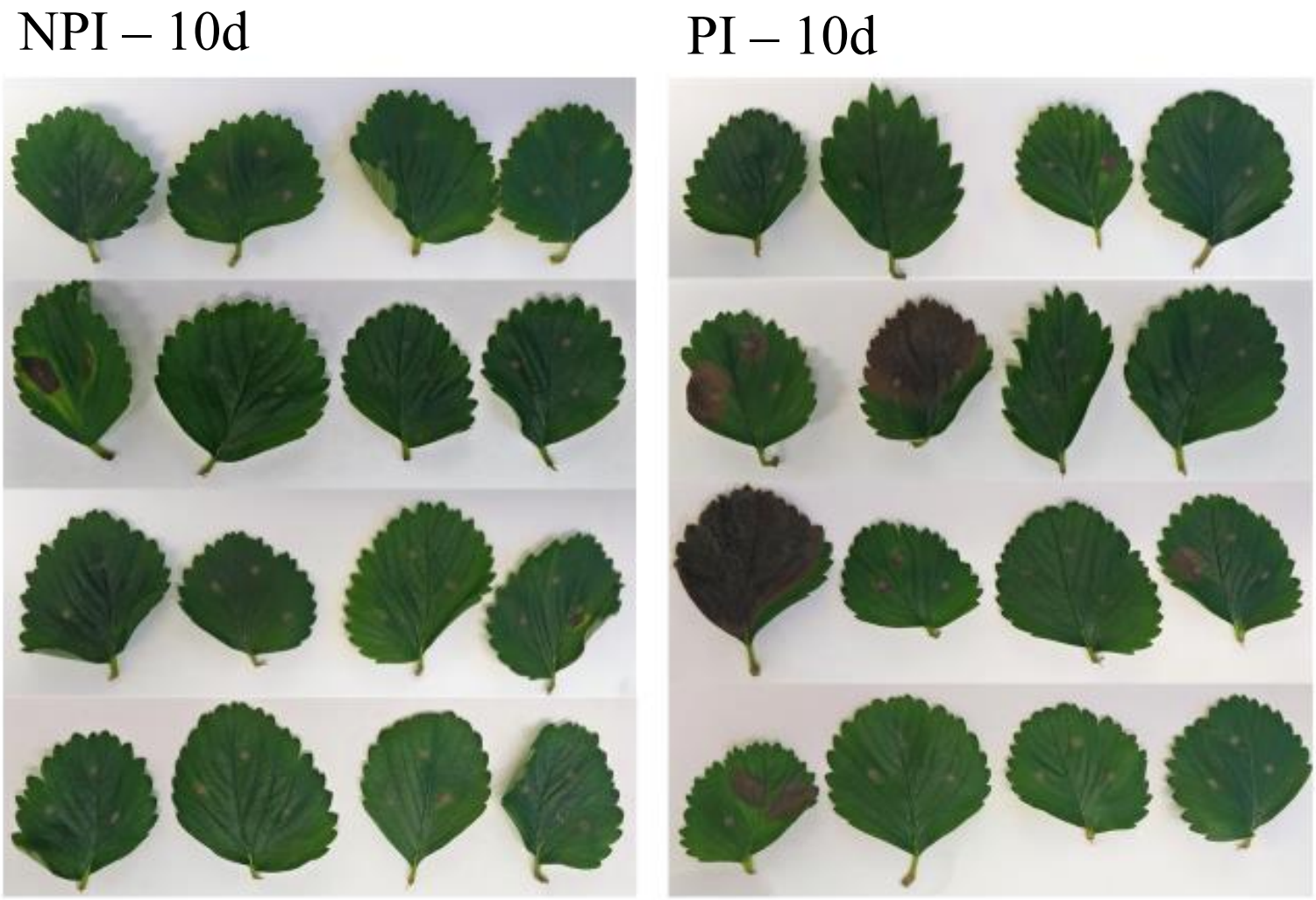


Figure 4.3. Disease development in NPI and PI leaves after 10 days. Disease development 10 days after infection. Of the NPI leaves 4/16 have developed necrotic lesions and 7/16 leaves from the PI treatment have necrotic lesion formation. The two leaves with the largest lesions on the PI treatment have sporulating fungi that can be seen as grey cotton-like structures on the lesions.

Ass seen from the results of Figure 4.1, 4.2 and 4.3, more leaves from the PI treatment had necrotic lesions compared to the NPI leaves. The fungus was also sporulating on two of most infected leaves on the PI treatment and there was no sign of sporulation on the NPI leaves.

To be able to compare the two treatments, the individual and total lesion size for each treatment at each timepoint was measured and calculated in the following figures and tables. The average infection development for NPI and PI treatment is also calculated.

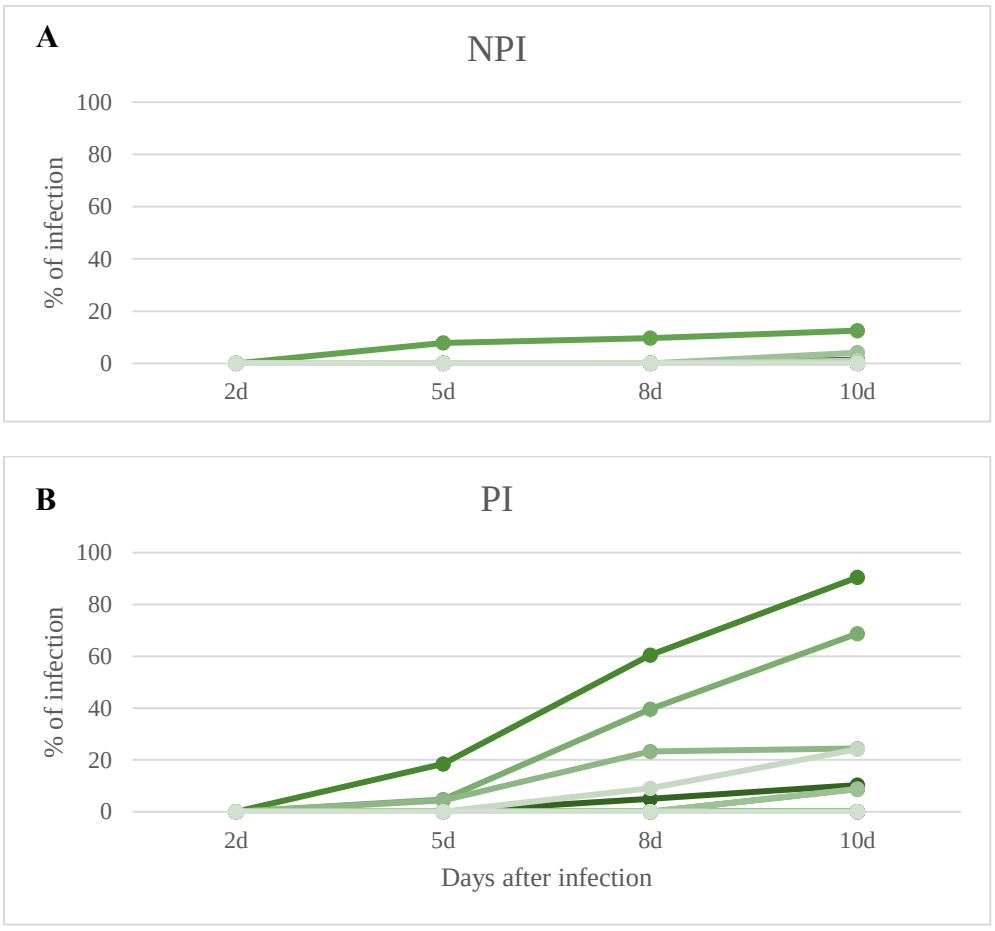


Figure 4.4. Infection development in NPI and PI leaves. Infection development on all 16 individual leaves of the (A) NPI and (B) PI treatment 2, 5, 8 and 10 days after infection. Infection was done at time point 0, and the development is presented as percentage of infected leaf area based on individual measurements of total leaf area and healthy leaf area. Each line represents individual leaves over the incubation period.

Table 4.1. Total lesion area and average infection development. The table presents the total lesion area and the average % of infection for all 16 leaves of NPI and PI treatment 2, 5, 8 and 10 days after infection. Lesion area measurements are given in cm^2 and the average percentage of infection is calculated from the individual measurements based on of total leaf area and healthy leaf area.

	2d		**5d**		**8d**		**10d**	
	Lesion area	% infection	Lesion area	% infection	Lesion area	% infection	Lesion area	% infection
NPI	0	0	0,20	0,41	0,22	0,49	0,47	1,12
PI	0	0	0,69	1,70	3,69	8,12	5,65	13,80

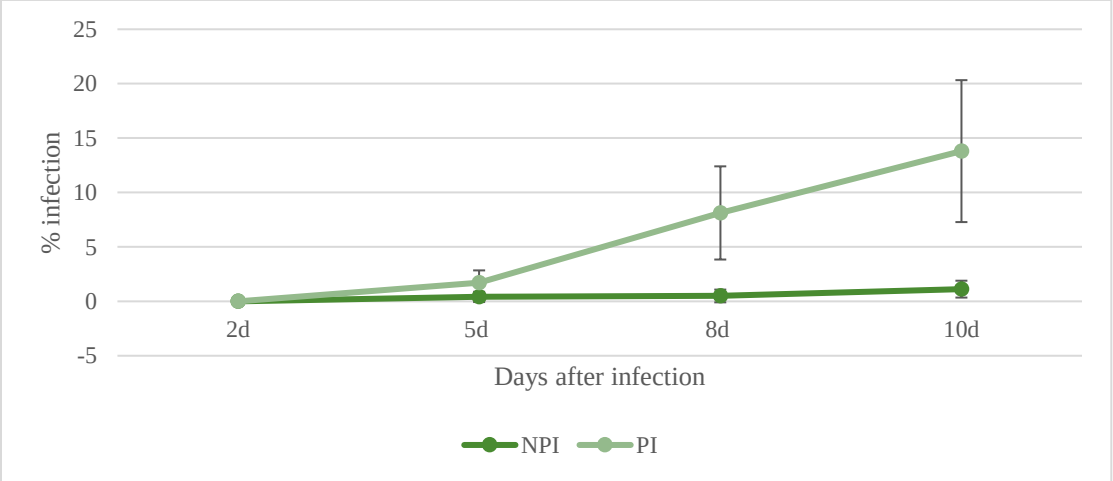


Figure 4.5. Average infection development for NPI and PI leaves. Comparison for the NPI and PI leaves 2, 5, 8 and 10 days after infection. Infection was done at time point 0, and the infection development is presented as percentage of infected leaf area. The calculations are based on individual measurements of total leaf area and healthy leaf area and is expressed as the average of all 16 leaf measurements with the calculated ±SE bars.

4.1.2. Optimization of RNA isolation method

Because of the importance of obtaining high quality RNA for further molecular studies, two different methods of RNA isolation were tested to establish which method is most efficient for isolation of strawberry tissue:

1. Spectrum method
2. CTAB/Spectrum method

RNA from both methods was tested and compared in Table 4.2.

Table 4.2. RNA measurements for comparison of isolation methods. RNA yield (ng / µl) and purity ($A_{260/280}$) measured by NanoDrop™ 200 and RNA yield measured by Qubit® 2.0.

		NanoDrop™ 2000		**Qubit® 2.0**
Method	**Sample**	**ng / µl**	**$A_{260/280}$**	**ng / µl**
Spectrum	NPNI-1	642.9	1.27	25.2
	PNI-1	514.7	1.16	37.2
	NPI-1	431.2	1.34	16.76
	PI-1	732.4	1.31	40.4
CTAB/Spectrum	NPNI-2	24.6	2.21	39.6
	PNI-2	136.7	2,08	85.0
	NPI-2	292.0	2,09	98.6
	PI-2	189.6	2,11	77.2

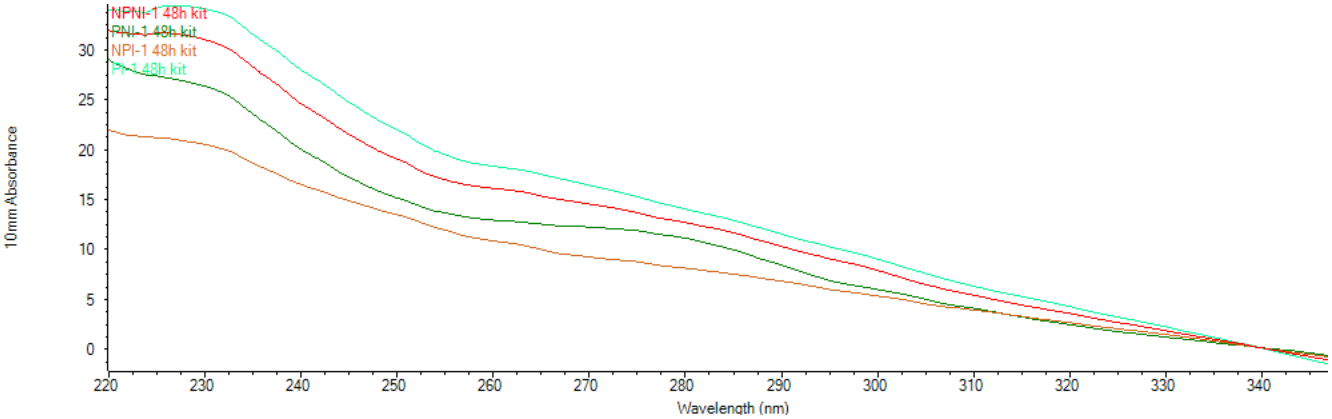


Figure 4.6. RNA measurements using Spectrum method. The different colors represent each sample from the Spectrum method; PI-1 = blue, NPNI 1 = red, PNI-1 = green, NPI-1 = brown. Measurements are done using NanoDrop™ 2000.

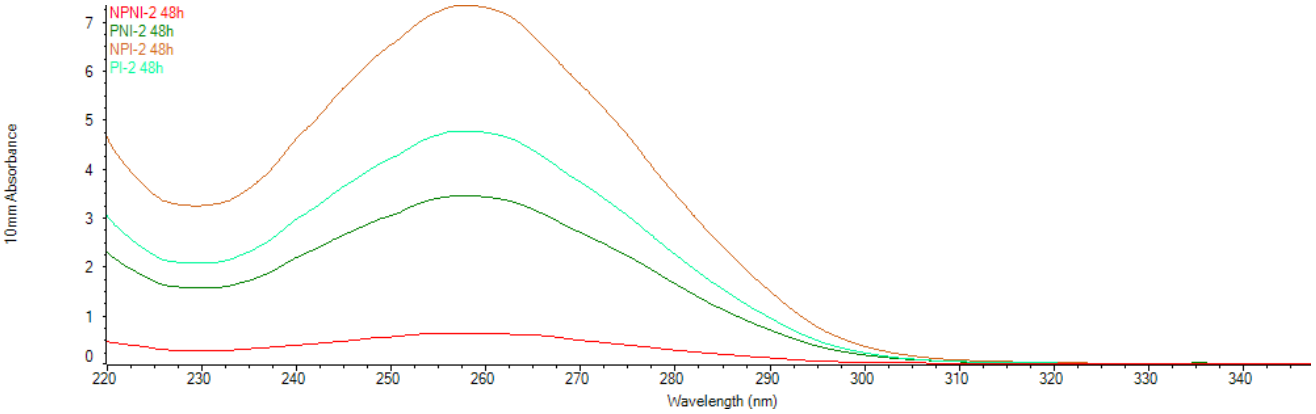


Figure 4.7. RNA measurements using CTAB/Spectrum method. The different colors represent each sample from the Spectrum method; NPI-1 = brown, PI-1 = blue, PNI-1 = green, NPNI 1 = red. Measurements are done using NanoDrop™ 2000.

To investigate whether an additional second round of DNase treatment would improve the quality of the RNA and remove contamination of DNA, a second DNase treatment was carried out for all the isolated RNA samples. RNA yield was measure before and after the second DNase treatment for comparison.

Table 4.3. Comparison of RNA yield. RNA yield was measured with Qubit® fluorometer before and after the second DNase treatment. The first DNase is on-column DNase-treatment according to the Spectrum™ Plant kit protocol and second DNase is treated with the additional DNase I Amplification grade after elution, and subsequent heat inactivation.

Time after infection	**Sample**	**First DNase (ng / µl)**	**Second DNase (ng / µl)**
48h	NPNI-1	168	134
	NPNI-2	196	120
	NPNI-3	142	95.6
	NPNI-4	200	178
	NPI-1	200	136
	NPI-2	200	156
	NPI-3	-	-
	NPI-4	200	120
	PNI-1	91.8	49.8
	PNI-2	132	91.4

	PNI-3	200	132
	PNI-4	200	134
	PI-1	200	132
	PI-2	200	118
	PI-3	200	112
	PI-4	200	118
5d	NPNI-1	200	164
	NPNI-2	200	88.8
	NPNI-3	200	152
	NPNI-4	200	98
	NPI-1	200	5.14
	NPI-2	200	4
	NPI-3	142	41.4
	NPI-4	200	180
	PNI-1	200	108
	PNI-2	200	122
	PNI-3	200	126
	PNI-4	200	122
	PI-1	200	55.6
	PI-2	196	12.66
	PI-3	200	9.68
	PI-4	200	9.64

Table 4.4. Mean Ct values for first DNase and second DNase treated samples after RT-qPCR.

ND = no detection of the cDNA template.

Time after infection	**Sample**	**First DNase EF1-α Ct**	**Second DNase EF1-α Ct**
48h	NPNI	20.89	22.84
48h	PNI	23.20	23.75
48h	NPI	20.62	23.64
48h	PI	20.90	23.64
5d	NPNI	21.16	24.95
5d	PNI	21.98	24.87
5d	NPI	21.43	27.61
5d	PI	22.27	30.03

48h	NPNI 48h*	36.31	38.26
48h	NPI 48h*	35.36	ND
	H_2O	ND	ND

*Control without reverse transcriptase enzyme

4.1.3. Effects of BABA treatment on gene expression analysis

To assess the mechanisms behind the defense responses involved in the BABA treated plants, gene expression analysis was performed to detect whether defense related genes were more expressed in the primed leaves than the non-primed leaves, and to investigate the duration of the activity. RNA from four leaves harvested 48 hours and 5 days after infection was isolated and prepared for cDNA synthesis and RT-qPCR. Four candidate genes related to defense responses were tested to quantify their transcript levels in the detached leaves of the different treatments; BG2-1, PR1, PR5.3 and PGIP.

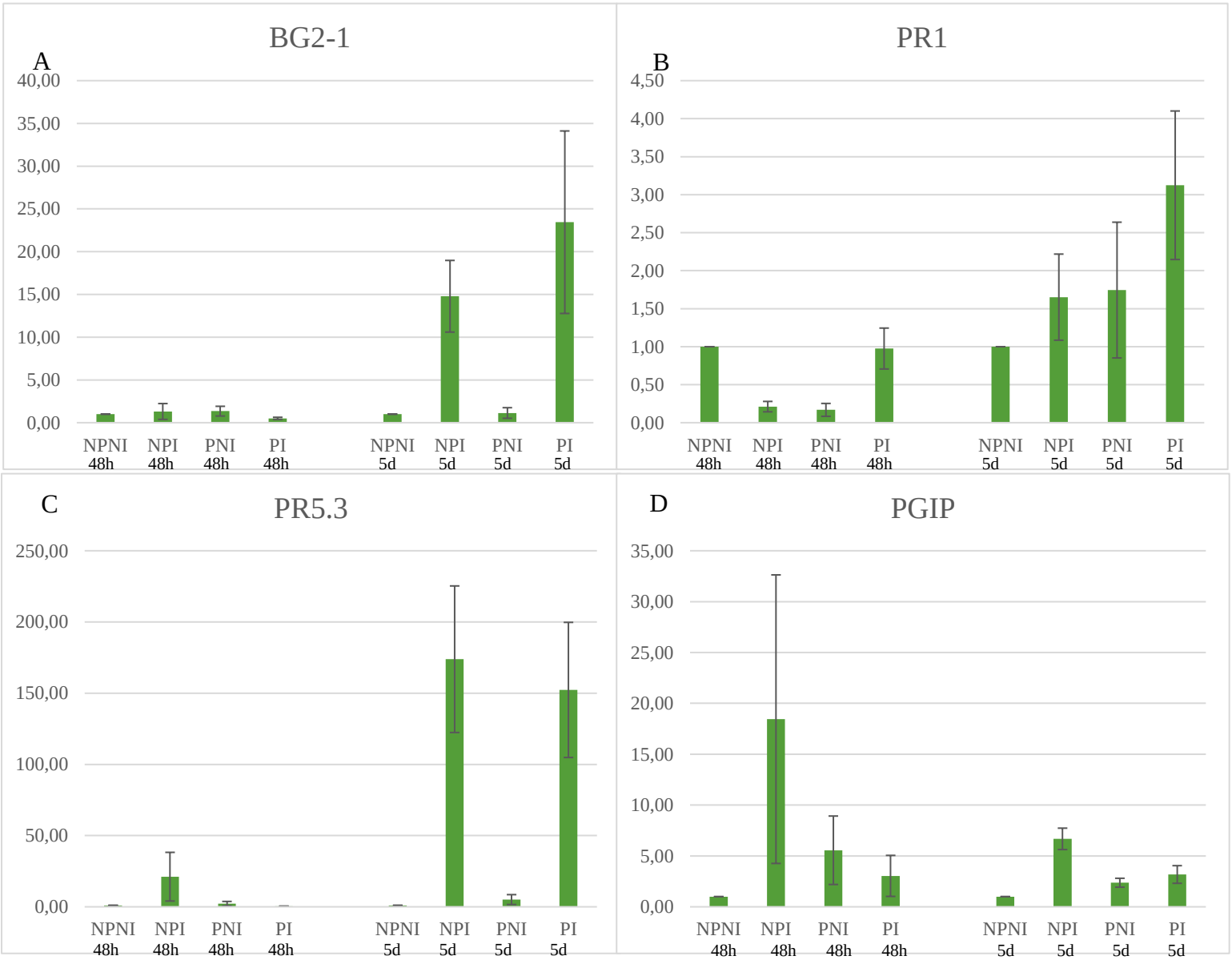


Figure 4.8. Expression of defense related genes. (A) BG2-1, (B) PR1, (C) PR5.3 and (D) PGIP. Leaves were infected with *B. cinerea* at time point 0, and gene expression was measured by RT-qPCR after 48 hours and 5 days. Values were normalized to EF1-α and calculated relative to the NPNI control at each time point, thereby set to 1. Data are expressed as the average of four samples from one experiment with the calculated ±SE bars.

The gene expression of BG2-1, PR1 and PR5.3 had increased from 48 hours to 5 days, relative to NPNI, although in varying degree. In contrast, PGIP activity was high for NPI after 48 hours and but decreased after 5 days.

4.2. Epigenetic regulation of defense

4.2.1. Quantification of *F. vesca* U6 promoters

Berries of *F. vesca* was agroinfiltrated with three CRISPR constructs carrying three different U6 promoters. The berries were harvested 2 days after the infiltration to be able to study the transient expression of the sgRNA under the different promoters U6-1, U6-2 and U6-8 using RT-qPCR. The aim was to determine which promoter gave highest expression of sgRNA for the genes of interest: *MET1* and *RPPL1*.

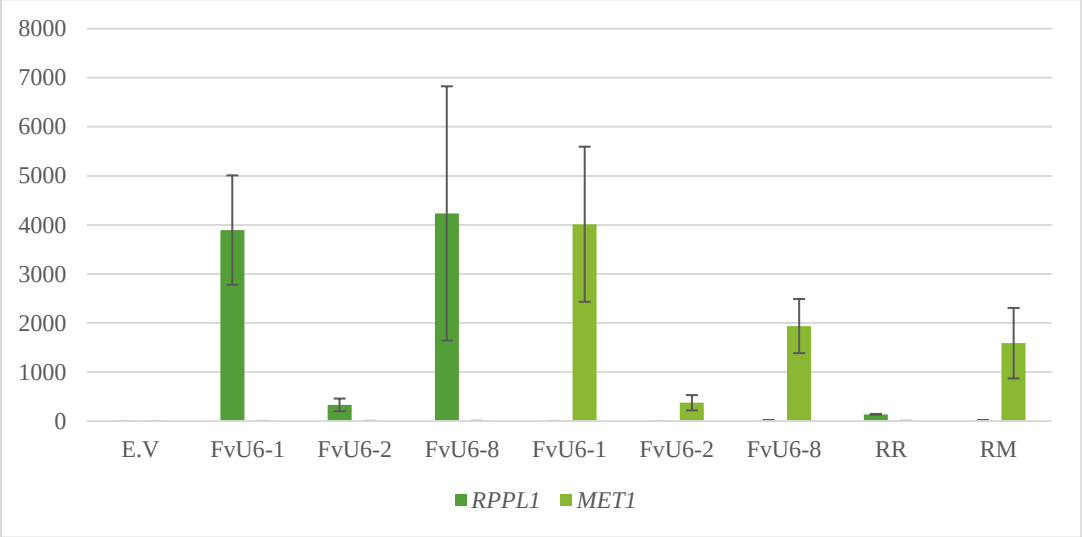


Figure 4.9. Expression of sgRNA under three different U6 promoters in *F. vesca*. Three different promoters of the sgRNA was tested for the *RPPL1* and *MET1* gene; U6-1, U6-2 and U6-8. RR and RM is RPPL1 and MET1 genes in *A. thaliana*, respectively. Gene expression analysis was measured by RT-qPCR 2 d after agroinfiltration using Bar1 as internal control. Values were normalized to isolated DNA and compared to the empty vector (E.V) control, thereby set to 1. Data are expressed as average of three assays from one experiment with the calculated ±SE bars.

4.2.2. Cloning of sgRNA into plant vectors

In order to make a site-specific mutagenesis in *F. vesca* using *Agrobacterium*-mediated transformation, the CRISPR sgRNA expression cassette in Figure 3.6. B in chapter 3.2.2. was cloned into the pTPC and pPFGC plasmids using PACI restriction enzyme. Followed by the cutting, a gel electrophorese was carried out in order to separate and cut the DNA fragments at the expected sizes in the gel. After 50min, sgRNA fragments from *MET1* were clearly

separated and cut out from the gel at the expected size of 1171 bp using a clean scalpel. The gel slice was weighted and put in 1.5 ml tubes and purified. The same procedure was done for the pTPC and pFGC vectors after 45 more minutes with gel electrophoresis at the expected size of 14 kb.

Before the final step of ligation, phosphorylation was carried out. In the ligation step, the insert *MET1* sgRNA-expression construct in Figure 3.6. B was ligated into the pTPC and pFGC binary vectors, respectively. For the ligase reaction a total of 8 tubes were set up, four tubes for plant vector combined with the insert, and the last four tubes contained plant vectors with and without CIP reaction with added dH_2O to serve as control. CRISPR vectors were transformed into *E. coli* for propagation of the construct.

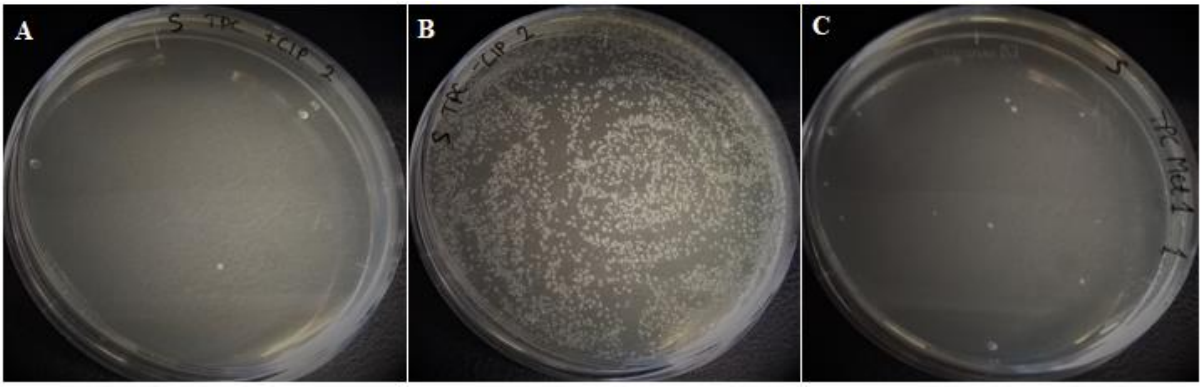


Figure 4.10. CIP verification after transformation in *E. coli*. (A) Empty pTPC vector with CIP treatment showed no bacterial colonies. (B) Empty pTPC vector without CIP treatment showed many bacterial colonies and (C) pTPC vector with insert *MET1* sgRNA expression construct showed some bacterial colonies. The same results were shown for the pFGC vector.

After running the PCR program, the samples were run on gel to visualize the binding of primers. 2 µl of dye was added to 18 wells, and 10 µl of the PCR solution was added and mixed before 10 µl of the solution was added to the gel. After 45 min at 90 V, and the product was visualized under UV-light. Positive bonds for pTPC were expected at size 328 bp and 912 bp and for pFGC at size 438 bp and 1022 bp, and was confirmed by positive controls.

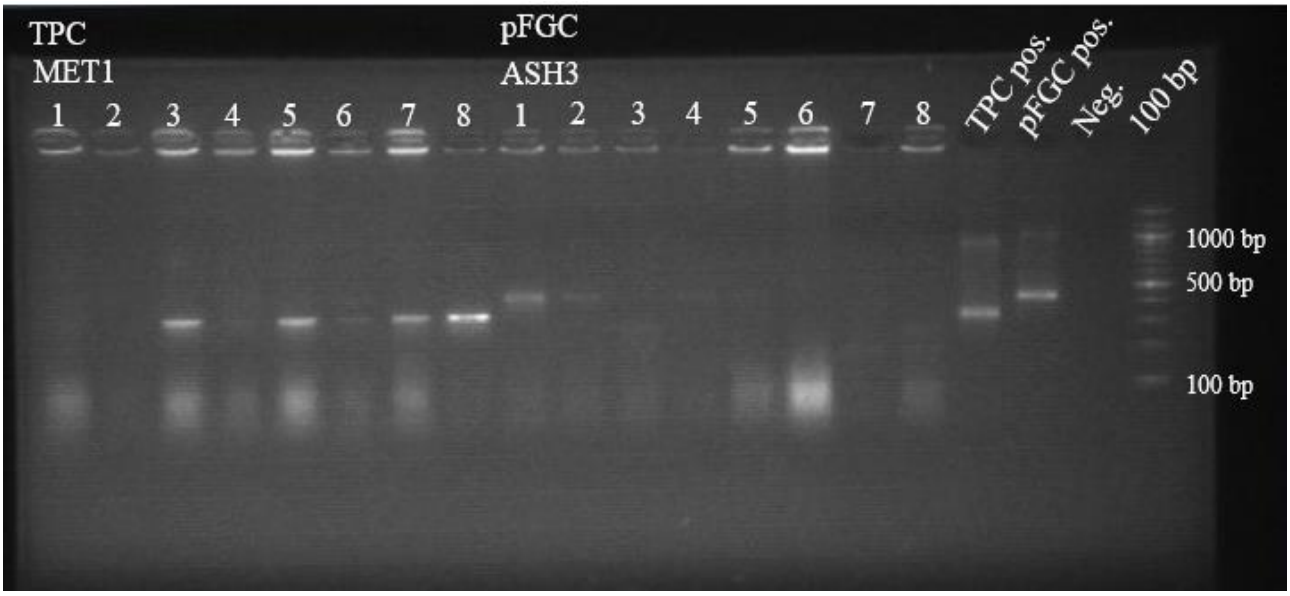


Figure 4.11. Visualization of correct primer binding and orientation. For pTPC MET1, sample 3,5,7 and 8 are positive for correct binding and orientation because of visualisation of bond at expected size. Sample 1 and 2 is negative, and sample 4 and 6 seems to have a positive bond, but less visible. The size of the positive bonds corresponds with the positive control at 328 bp. Visualization of approximately 900 bp is hardly visible at the positive pTPC samples, but can be seen in the positive control at 912 bp. The same visualization was done for pFGC MET1. ASH3 was run simultaneously but was not involved in the experiment.

After verifying the construct, *A. tumefaciens* was used in order to deliver the CRISPR/Cas9 components for transient expression. After verifying, two colonies from each construct were chosen and transferred to 5 ml of LB medium in 50 ml tubes with the appropriate antibiotics.

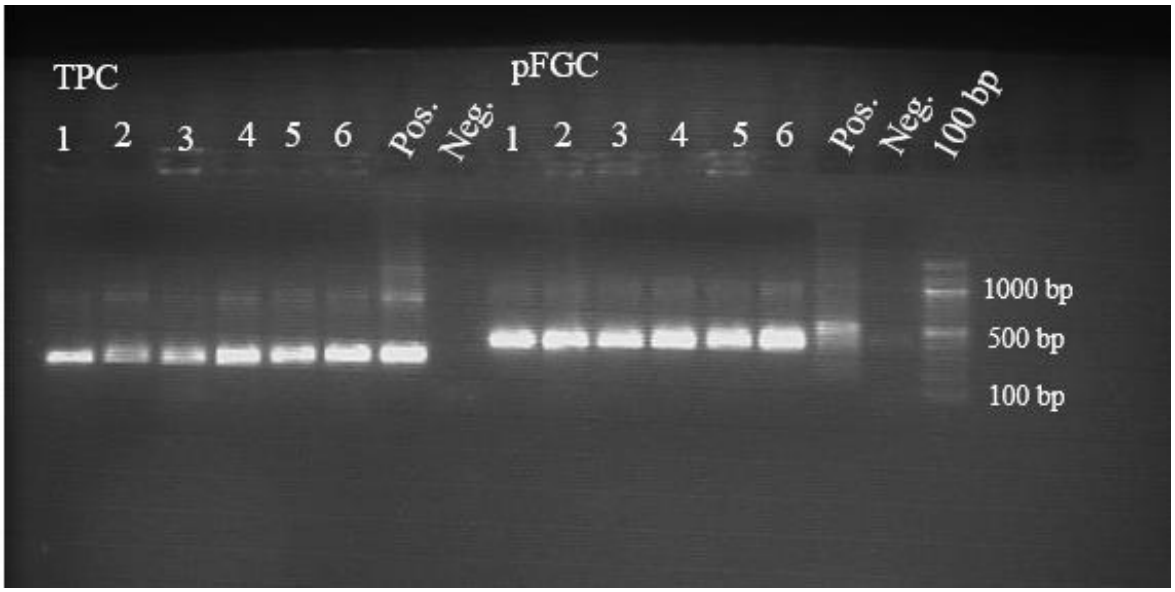


Figure 4.12. Visualization of transformation. Successful transformation in EC48 strain confirmed for all colonies tested. This is confirmed by the presence of expected bond size 328 bp and 912 bp for pTPC, and the same for pFGC with visible bonds at approximately 438 and 1022 bp. The positive controls confirm this. The same visualization was done for both binary vectors in the LBA4404 strain.

3.3.3.2. Agroinfiltration of *F. vesca*

A rapid and efficient *Agrobacterium*-mediated transient gene expression system in leaves of *F. vesca* was planned for the experiment, but because of time limitation, it was not possible to carry out the experiment.

5. Discussion

5.1. Detached leaf assay

Defense priming is as an important part of the innate immune system of plants. Common for induced resistance phenomena caused by priming is that they are associated with an enhanced capacity to express cellular defense responses, especially upon pathogen challenge. Different priming agents can be applied in order to increase the resistance in a susceptible plant, allowing the plant to combat possible future pathogens quicker than normal.

5.1.2. CTAB/Spectrum method for RNA isolation

It is essential for gene expression studies to have a reproducible and reliable RNA isolation method in order to detect the differences in expression between different treatments of plants. Because of the high content of polysaccharides, polyphenols and other secondary metabolites in strawberry tissues, we noticed large variations in quantity and quality between the samples from identically treated plants. This suggested that the RNA isolation protocol was not optimal. Thus, it was important to establish a good method to isolate and obtain RNA of best possible quality.

The two methods of Spectrum Kit and CTAB/Spectrum method was compared, and based on the results in chapter 4.1.2., the CTAB/Spectrum method was chosen as the most efficient method for RNA extraction of strawberry. Although Table 4.2. shows a higher RNA yield (ng / µl) by using the Spectrum method for RNA isolation compared to the CTAB/Spectrum method, higher absorption ratio for CTAB/Spectrum method are shown. 260/280 absorption ratio should be between 1.8 – 2.0 because lower ratios indicate contamination of secondary metabolites. This is also shown in Figure 4.6. and 4.7. where CTAB/Spectrum method gives purer RNA yield measurements.

After establishing the best method for isolating RNA, we noticed DNA contamination in the isolated RNA. Therefore, a second round of DNase treatment was carried out in order to investigate whether it could effectively remove the DNA contamination. When comparing the two methods, RNA yield was generally measured in lower concentrations after the second DNase treatment, and some of the sample measurements showed very low yield (<10 ng / µl)

as seen in Table 4.3. in chapter 4.1.2. The differences also seemed to be larger in the leaf tissue incubated for 5 days after infection compared to 48 hours of incubation, and may be because of a greater accumulation of secondary metabolites in the plant tissue over time due to stress (Jacobo-Velázquez et al., 2015), making it more difficult to extract RNA. The second DNase treatment may also have degraded some of the RNA, or the Qubit® fluorometer may not be 100% specific on RNA measurements because of the large differences before and after the second round of RNase treatment. Although the Ct values from the RT-qPCR in Table 4.4., show no DNA detection on the controls, the fist DNase treatment show lower and more stable values than the samples from the second DNase treatment. Therefore, it was decided to discard the second DNase treated results and go forward with the results from the first DNase treated RNA as described in the original protocol.

5.1.1. Phenotypic scoring and gene expression analysis

The overall results of the detached leaf assay in *F. x ananassa* did not show the expected BABA-induced defense against *B. cinerea* (Baccelli & Mauch-Mani, 2016). The phenotypic results in chapter 4.1.1. showed a significant enhanced susceptibility of the BABA treated leaves compared to the non-primed leaves. The phenotypic development in Figure 4.1. – 4.3. showed a higher disease incidence in the PI leaves compared to the NPI leaves. The phenotypic disease score was more severe both in terms of number of leaves infected, the percentage of infected individual leaves and the total lesion area. BABA did not seem to enhance the defense mechanisms as expected, but rather the opposite making the leaves more susceptible to infection by *B. cinerea*. The disease development was also more rapid in the PI leaves compared to the NPI leaves.

In general, the gene expression results in Figure 4.8. in chapter 4.1.3 were very dual and did not directly reflect upon the phenotypic results that were observed. If BABA would make the plants more susceptible to *B. cinerea*, it is expected to be reflected in the gene expression analysis and show significantly lower gene expression of the defense related genes in PI compared to the NPI. Although this is found in the gene expression of PGIP after 5 days, the remaining three candidate genes did not show similar effect. PGIP proteins are important in primary defense responses where it has been shown to reduce cell-wall degrading enzymes

secreted by necrotic fungi like *B. cinerea* (Kalunke et al., 2015), and could possibly explain the increased lesion size in the PI leaves.

If the priming had shown the expected effect of enhanced defense responses, the expression values of the primed leaves would be significantly higher than the non-primed leaves. Primed plants are expected to react in a more rapid and robust way (Conrath et al., 2015), and the increase in gene expression could therefore be expected to be seen already after 48 hours. The value of PI gene expression activity would also be expected to be significantly higher compared to the NPI expression after 5 days because of the long-lasting effect of BABA treatment that has previously been reported (Wilkinson et al., 2018). This long-lasting effect is not found in any of the candidate genes and may indicate that BABA did not have any effect at all. In BG2-1 gene expression, the results were significant between the infected and non-infected leaves, which indicates the BABA treatment did not have any effect. The results from BG2-1 were also applicable for the expression of PR5.3, which also showed a significant difference between infected and non-infected leaves, and underlines the assumption that BABA did not have any effect on inducing resistance. For the gene expression of PR1, the results could have indicated some effect of the priming because the expression of the primed leaves were enhanced after five days compared to the non-primed leaves, but the results were not significant.

It is important to note that several sources of error could have influenced the results, and it is therefore important to consider these possible errors when contemplating the data. One source of error that could have interfered is that although all leaves were drop inoculated with the same spore solution of *B. cinerea*, all leaves were not likely to be infected with an equal amount of spores. The syringe wounding of the leaves are also likely to be unequal, and may have facilitated for different establishment of *B. cinerea*. The leaves may also have been injured in other ways before the infection process, all of which could have affected the phenotypic results. Also, in the RNA isolation step, an unequal ratio of infected and uninfected plant material from individual samples could have had an impact on the final results.

BABA is well documented as a priming agent and have been shown to be efficient in inducing defense responses against a wide range of biotrophic and necrotrophic pathogens, in different plant species and with different application methods (Baccelli & Mauch-Mani, 2016; Conrath et al., 2015). A recent publication involving BABA showed that the compound is naturally synthesized in several plants as a response to stress (Thevenet et al., 2017), and could possibly underline the widespread efficacy of BABA in induced resistance. The amount of studies involving BABA in plant defense makes is difficult to explain how BABA could potentially increase susceptibility to *B. cinerea*, but the possibility that BABA has not had any effect is more likely.

Several previous studies have investigated the effect of priming in strawberry plants with different priming agents, with and without pathogen attack (Landi et al., 2014; Saavedra et al., 2017). A study made by Wang et al. (2016) reported that BABA effectively induced defense responses in detached berries of *F. x ananassa* when challenged with *B. cinerea*. These studies all investigate the effect on priming in berries and not in leaves. Different plant species and plant parts have different ability to defend themselves against pathogens because of different chemical compositions of the tissue. Detached leaf assays have in several previous studies been shown suitable for quantifying chemically induced resistance (Audenaert et al., 2002; Luna et al., 2016), and should therefore be a sufficient method for priming with BABA against *B. cinerea* in strawberry. Although studies involving this plant-pathosystem has, to my knowledge, not been published, a possible reason may be that the soil treatment with BABA and the subsequently expected priming effect is not showing in the whole plant.

van Hulten et al. (2006) reported that high doses of BABA could suppress plant growth when applied in too high dosages. This could raise the question whether the dosages of BABA were too high. In the experiment, a final concentration of 200 µM BABA was used in the soil drench and could potentially have been too high and thereby harmful for the plants making them more susceptible to *B. cinerea*. However, in previous publications using BABA soil drench in different plants before infection, the final concentrations are reported to be 100 µM (Martinez-Aguilar et al., 2016), 150 µM (Ton & Mauch-Mani, 2004), 200 µM (Po-Wen et al., 2013), 250 µM (Pastor et al., 2014) and 300 µM (Ton et al., 2005). These studies reported that

plants primed with BABA were less susceptible to pathogen challenges without being harmful to the plants. Based on these findings, the BABA concentration used in the detached leaf experiment should not be too high.

As mentioned in chapter 4.1.1., the plants used in the experiment had been treated with pesticides against powdery mildew, mites and aphids before given to the experiment, and this has most likely influenced the data. Stress caused by herbivores or pathogens prior to the experiment are likely to have induced defense mechanisms in the plants prior to the priming stimuli. There was also concern that the plants may have been infected with *P. cactorum*. Infection by *P. cactorum* could lead to less efficient uptake of BABA through the roots because of rotting roots (Toljamo et al., 2016), and could be another error of the experiment that needs to be taken in consideration when contemplating the results. Therefore, its reason to believe that the priming responses in the strawberry plants have been interfered by some of these factors, and underlines the importance of clean plant material in experimental work. Although the plant material was not efficient for a closed experiment like this, it is more applicable to natural conditions where plants are continuously exposed to different abiotic and biotic stress.

The overall result is that exogenous application of BABA did not enhance defense in *F. x ananassa*. It could be that the increased susceptibility in the phenotype that was observed were only due to deviations. Possible deviations and errors has been suggested earlier in this chapter, and could have limited the uptake of BABA through the roots or have facilitated for unequal establishment of *B. cinerea* in the detached leaves. Two of the most infected leaves are from the PI treatment, and if they are ruled out due to deviations, the incidence of disease could seem to be more similar between the two treatments PI and NPI. Thus, there is a great risk of biased results when removing deviating samples from the overall results, and more data are needed to conclude in this matter.

5.1.3. Further perspectives

Because of previous publications, there are reasons to believe that BABA could induce resistance against *B. cinerea* in strawberry plants, and further investigations are therefore

needed. The most important improvement would be to use clean plant material in the experiments to exclude variations due to stress and the possibility of a poor BABA uptake through the roots. An approach could be to test how different plant tissue such as berries or flowers respond to BABA treatment and *B. cinerea* infection, because these tissues are the primary target for the pathogen. Different inoculation methods could also be tested. The detached leaf method could also be applied in other species susceptible to *B. cinerea*. Because of the epigenetic mechanisms that might be involved in controlling the priming response, the next step after trying different methods would be to investigate chromatin alterations and DNA methylation responses after priming.

Priming in strawberry plants should still be investigated as it has great potential in agricultural practices. BABA has been shown to induce resistance in several plants and is shown to be synthesized in the plant in response to stress, showing that BABA is important in defense responses. BABA has also been shown to be effective at low concentrations without being harmful to plants, and low costs of application could therefore be achievable in commercial use in addition to its water-soluble properties which do not demand big investments for applications in the field. Because of the heritable and reversible epigenetic effects of the priming it is serves as a good strategy to deploy in perennial plants as strawberry that.

5.2. Epigenetic regulation of defense

Targeted genome editing using CRISPR/Cas9 provides an excellent method to introduce deletions, insertions or precise sequence changes in a broad spectrum of organisms and cell types with a high efficiency (Sander & Joung, 2014). The possibility to introduce targeted genomic changes into living plant cells is a powerful tool that can be used to improve disease resistance in plants. Transient transformation makes it possible to do rapid testing of gene functions.

Because the promoter used to drive sgRNA expression is dependent upon the plant species, it was important to determine which promoters to use in further CRISPR/Cas9 transient experiments in *F. vesca*. From the results in Figure 4.9. in chapter 4.3.1., the U6-1 promoter showed highest levels for sgRNA expression for MET1 and the U6-3 promoter had highest

expression for RPPL1. U6-2 showed lowest expression on both genes of interest, and the difference was significant. In terms of what promoter was highest expressed for both genes combined, U6-1 was found to be the most efficient. Based on this, the U6-1 promoter was chosen for further use in transient expression of CRISPR construct.

Also, in this experiment it is important to consider possible errors when contemplating the data. One source of error is that by agroinfiltrating the berries with a syringe, all tissue is not likely to be transformed. An unequal ratio of transformed and untransformed plant material from individual samples could have had an impact on the final results in isolating RNA. In addition, the syringe could have wounded the berries leading to a response in the plant that could have interfered with the results. The berries were infiltrated with the same bacterial suspension liquid solution, but all berries are not likely to be infiltrated with an equal amount of bacteria which could also have interfered with the final results.

The pFGC and pTPC plasmids containing expression cassettes for the 2 x sgRNA and the Cas9-endonuclease showed successful uptake in both strains of *A. tumefaciens*. The next step of the experiment was therefore to use the specific U6-1 promoter to express 2 x sgRNA in order to knock out the *MET1* gene. Because *MET1* is a major maintenance protein in DNA methyltransferase and has an impact on histone modification, knocking out this gene could potentially induce the plant to a primed state where the plant is able to respond faster and more robust to a pathogen challenge. *MET1* is an important part of the epigenetic map in *A. thaliana* (Espinas et al., 2016), and by indirectly editing the plant genome by modifications in the epigenetic machinery, it can be investigated whether this could potentially make strawberry plants more resistant not only to *B. cinerea*, but to a wide range of pathogens and abiotic stress. Once a mutation has been successfully initiated by the reparation system in the plant, the targeted sites are no longer recognized by the sgRNA and cannot be subject to further mutagenesis.

5.2.1. Further perspectives

Without time limitations, the transient expression experiment could have been carried out in *F. vesca*. During the time span of this thesis, a similar study in *F. vesca* was published that had investigated transient expression to study disease resistance proteins (Cui et al., 2017). In this publication vacuum infiltration was used in detached leaves of *F. vesca*, and serves as a good method for carrying out the next steps of this experiment. All of the plant material is infiltrated with this method and it does not involve damaging of the plant tissue.

The expected outcome of the transient expression experiment would be that the U6-1 promoter expressing the 2 x sgRNA would result in a two-sited cleavage by Cas9 between the two sgRNAs targeting the *MET1* gene. When the double stranded breaks are repaired after the cleavage by the plants own reparation systems, it is expected to result in a knock-out of function of the *MET1* gene. If the expression of either the 2 x sgRNA or Cas9 would fail, no deletion would be detected.

For further studies, stable transformation of the plants could be carried out, typically achieved through selection and callus culturing of transformed tissue. Stable transformation is more time consuming but can lead to persistent expression of transgenes in the plant.

6. Conclusions

6.1. Detached leaf assay

The overall conclusion of the experiment is that BABA did not induce the hypothesized resistance in *F. x ananassa* against *B. cinerea* in the detached leaf assay. Because the phenotypic results and the results of the gene expression analysis did not correspond, it is difficult to draw any clear conclusions on why the priming effect did not work as expected. What can be concluded is that the significant increase in gene expression that was found between the infected and the non-infected plants in BG2-1 and PR5.3 is that infection by *B. cinerea* alone is the reason for the elevated gene expression and that BABA did not have any effect in priming.

6.2. CRISPR transformation

The identification of U6-1 as an efficient promoter of sgRNA expression in *F. vesca* was an important step to be able to design an efficient CRISPR construct in order to knock out the putative *MET1* gene in *F. vesca*. This serves as an easy and rapid method to investigate defense related genes for enhancement of resistance in strawberry, and the method is also applicable to other plant species.

7. References

Agrios, G. N. (2005). *Plant Pathology, Fifth Edition*. Fifth edition ed.: Elsevier Academic Press.

Amil-Ruiz, F., Blanco-Portales, R., Muñoz-Blanco, J. & Caballero, J. L. (2011). The Strawberry Plant Defense Mechanism: A Molecular Review. *Plant and Cell Physiology*, 52 (11): 1873-1903.

Audenaert, K., Meyer, G. B. D. & Höfte, M. M. (2002). Abscisic Acid Determines Basal Susceptibility of Tomato to *Botrytis cinerea* and Suppresses Salicylic Acid-Dependent Signaling Mechanisms. *Plant Pathology*, 128 (2): 491-501.

Baccelli, I. & Mauch-Mani, B. (2016). Beta-aminobutyric acid priming of plant defense: the role of ABA and other hormones. *Plant Molecular Biology*, 91 (6): 703–711.

Balmer, A., Pastor, V., Gamir, J., Flors, V. & Mauch-Mani, B. (2015). The 'prime-ome': towards a holistic approach to priming. *Trends in Plant Science*, 20 (7): 443-452.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A. & Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science*, 315 (5819): 1709-12.

Belhaj, K., Chaparro-Garcia, A., Kamoun, S. & Nekrasov, V. (2013). Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. *Plant Methods*, 9 (1): 39.

Bruce, T. J. A., Matthes, M. C., Napier, J. A. & Pickett, J. A. (2007). Stressful "memories" of plants: Evidence and possible mechanisms. *Plant Science*, 173: 603-608.

Caarls, L., Pieterse, C. M. J. & Van Wees, S. C. M. (2015). How salicylic acid takes transcriptional control over jasmonic acid signaling. *Frontiers in Plant Science*, 6 (170).

Chan, S. W.-L., Henderson, I. R. & Jacobsen, S. E. (2005). Gardening The Genome: DNA Methylation in *Arabidopsis thaliana*. *Nature Reviews Genetics*, 6: 351–360.

Chang, S., Puryear, J. & Carnei, J. (1993). A simple and efficient method for isolating RNA from pine trees. *Plant molecular biology reporter*, 11 (2): 113-116.

Chisholm, S. T., Coaker, G., Day, B. & Staskawicz, B. J. (2006). Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response. *Cell*, 124 (4): 803-814.

Choquer, M., Fournier, E., Kunz, C., Levis, C., Pradier, J. M., Simon, A. & Viaud, M. (2007). *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphageous pathogen. *FEMS Microbiol Lett*, 277 (1): 1-10.

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., et al. (2013). Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* 339 (6121): 819-823.

Conrath, U., Beckers, G. J. M., Langenbach, C. J. G. & Jaskiewicz, M. R. (2015). Priming for Enhanced Defense. *Annual Review of Phytopathology*, 53 (1): 97-119.

Cui, M.-Y., Wei, W., Gao, K., Xie, Y.-G., Guo, Y. & Feng, J.-Y. (2017). A rapid and efficient *Agrobacterium*-mediated transient gene expression system for strawberry leaves and the study of disease resistance proteins. *Plant Cell, Tissue and Organ Culture*, 131 (2): 233-246.

Espinas, N. A., Saze, H. & Saijo, Y. (2016). Epigenetic Control of Defense Signaling and Priming in Plants. *Frontiers in Plant Science*, 7 (1201).

Esvelt, K. M., Mali, P., Braff, J. L., Moosburner, M., Yaung, S. J. & Church, G. M. (2013). Orthogonal Cas9 Proteins for RNA-Guided Gene Regulation and Editing. *Nature methods*, 10 (11): 1116-1121.

FAOSTAT. (2017). *Food & Agricultural Organization of the United Nations - FAOSTAT Database*. Available at: http://www.fao.org/faostat/en/#data/QC/visualize (accessed: 03/23-2018).

Fauser, F., Schiml, S. & Puchta, H. (2014). Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. *Plant J*, 79 (2): 348-59.

Fernández-Ortuño, D., Grabke, A., Bryson, P. K., Amiri, A., Peres, N. A. & Schnabel, G. (2014). Fungicide Resistance Profiles in *Botrytis cinerea* from Strawberry Fields of Seven Southern U.S. States. *Plant Disease*, 98 (6): 825-833.

Fernández-Ortuño, D., Torés, J. A., Chamorro, M., Pérez-García, A. & Vicente, A. d. (2016). Characterization of Resistance to Six Chemical Classes of Site-Specific Fungicides Registered for Gray Mold Control on Strawberry in Spain. *Plant Disease*, 100 (11): 2234-2239.

Folta, K. M. & Davis, T. M. (2006). Strawberry Genes and Genomics. *Critical Reviews in Plant Sciences*, 25 (5): 399-415.

Fu, Z. Q. & Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. *Annual Review of Plant Biology*, 64: 839-63.

Gasic, K., Hernandez, A. & Korban, S. S. (2004). RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. *Plant Molecular Biology Reporter*, 22 (4): 437-438.

Hahn, M. (2014). The rising threat of fungicide resistance in plant pathogenic fungi: *Botrytis* as a case study. *Journal of Chemical Biology*, 7 (4): 133–141.

Hahn, M., Viaud, M. & van Kan, J. (2014). *The Genome of Botrytis cinerea, a Ubiquitous Broad Host Range Necrotroph*: Springer.

Holsters, M., Waele, D. d., Depicker, A., Messens, E., Montagu, M. v. & Schell, J. (1978). Transfection and tranformation of *Agrobacterium tumefaciens*. *Molecular and General Genetics*, 163 (2): 181-187.

Iandolino, A. B., Silva, F. G. d., Lim, H., Choi, H., Williams, L. E. & Cook, D. R. (2004). High-quality RNA, cDNA, and derived EST libraries from grapevine (Vitis vinifera L.). *Plant Molecular Biology Reporter*, 22 (3): 269-278.

Jacobo-Velázquez, D. A., González-Agüero, M. & Cisneros-Zevallos, L. (2015). Cross-talk between signaling pathways: The link between plant secondary metabolite production and wounding stress response. *Scientific Reports*, 5: 8608.

Jaskiewicz, M., Conrath, U. & Peterhänsel, C. (2011). Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Reports*, 12 (1): 50-55.

Jones, J. D. & Dangl, J. L. (2006). The plant immune system. *Nature*, 444 (7117): 323-9.

Kalunke, R. M., Tundo, S., Benedetti, M., Cervone, F., De Lorenzo, G. & D'Ovidio, R. (2015). An update on polygalacturonase-inhibiting protein (PGIP), a leucine-rich repeat protein that protects crop plants against pathogens. *Frontiers in Plant Science*, 6: 146.

Kan, J. A. L. v., Shaw, M. W. & Grant-Downton, R. T. (2014). *Botrytis* species: relentless necrotrophic thugs or endophytes gone rogue? *Molecular Plant Pathology*, 15 (19): 957-961.

Kan, J. A. L. v., Stassen, J. H. M., Mosbach, A., Lee, T. A. J. V. D., Faino, L., Farmer, A. D., Papasotiriou, D. G., Zhou, S., Seidl, M. F., Cottam, E., et al. (2017). A gapless genome sequence of the fungus *Botrytis cinerea*. *Molecular Plant Pathology*, 18 (1): 75-89.

Krenek, P., Samajova, O., Luptovciak, I., Doskocilova, A., Komis, G. & Samaj, J. (2015). Transient plant transformation mediated by *Agrobacterium tumefaciens*: Principles, methods and applications. *Biotechnology Advances*, 33 (6): 1024-1042.

Kuc, J. (1987). Translocated Signals for Plant Immunizationa. *Annals of the New York Academy of Sciences*, 494 (1): 221-223.

Landi, L., Feliziani, E. & Romanazzi, G. (2014). Expression of Defense Genes in Strawberry Fruits Treated with Different Resistance Inducers. *Journal of Agricultural and Food Chemistry*, 62: 3047-3056.

Luna, E., Bruce, T. J. A., Roberts, M. R., Flors, V. & Ton, J. (2012). Next-Generation Systemic Acquired Resistance. *Plant Physiology*, 158 (2): 844-853.

Luna, E. & Ton, J. (2012). The epigenetic machinery controlling transgenerational systemic acquired resistance. *Plant Signaling & Behavior*, 7 (6): 615-618.

Luna, E., Beardon, E., Ravnskov, S., Scholes, J. & Ton, J. (2016). Optimizing Chemically Induced Resistance in Tomato Against *Botrytis cinerea*. *Plant Disease*, 100 (4): 704-710.

Martinez-Aguilar, K., Ramirez-Carrasco, G., Hernandez-Chavez, J. L., Barraza, A. & Alvarez-Venegas, R. (2016). Use of BABA and INA As Activators of a Primed State in the Common Bean (*Phaseolus vulgaris* L.). *Frontiers in Plant Science*, 7: 653.

Mauch-Mani, B., Baccelli, I., Luna, E. & Flors, V. (2017). Defense Priming: An Adaptive Part of Induced Resistance. *Annual Review of Plant Biology*, 68: 485-512.

Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J. & Almendros, C. (2009). Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology*, 155 (Pt 3): 733-40.

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D. & Kamoun, S. (2013). Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nature Biotechnology*, 31 (8): 691-693.

Niks, R. E., Parlevliet, J. E., Lindhout, P. & Bai, Y. (2011). *Breeding crops with resistance to diseases and pests*. Breeding crops with resistance to diseases and pests. The Netherlands: Wageningen Academic Publishers.

Oosumi, T., Gruszewski, H. A., Blischak, L. A., Baxter, A. J., Wadl, P. A., Shuman, J. L., Veilleux, R. E. & Shulaev, V. (2006). High-efficiency transformation of the diploid strawberry (*Fragaria vesca*) for functional genomics. *Planta*, 223 (6): 1219-1230.

Pandey, D., Rajendran, S. R. C. K., Gaur, M., Sajeesh, P. K. & Kumar, A. (2016). Plant Defense Signaling and Responses Against Necrotrophic Fungal Pathogens. *Journal of Plant Growth Regulation*, 35 (4): 1159-1174.

Pastor, V., Luna, E., Ton, J., Cerezo, M., García-Agustín, P. & Flors, V. (2013). Fine Tuning of Reactive Oxygen Species Homeostasis Regulates Primed Immune Responses in *Arabidopsis*. *Molecular Plant-Microbe Interactions*, 26 (11): 1334-1344.

Pastor, V., Balmer, A., Gamir, J., Flors, V. & Mauch-Mani, B. (2014). Preparing to fight back: generation and storage of priming compounds. *Frontiers in Plant Science*, 5: 295.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, 29 (9): e45.

Pfluger, J. & DorisWagner. (2007). Histone modifications and dynamic regulation of genome accessibility in plants. *Plant Biology*, 10 (6): 645-652.

Pieterse, C. M. & Van Loon, L. C. (2004). NPR1: the spider in the web of induced resistance signaling pathways. *Current Opinion in Plant Biology*, 7 (4): 456-64.

Pigliucci, M. (2005). Evolution of phenotypic plasticity: where are we going now? *Trends in Ecology & Evolution*, 20 (9): 481-486.

Po-Wen, C., Singh, P. & Zimmerli, L. (2013). Priming of the Arabidopsis pattern-triggered immunity response upon infection by necrotrophic *Pectobacterium carotovorum* bacteria. *Molecular Plant Pathology*, 14 (1): 58-70.

Reimer-Michalski, E.-M. & Conrath, U. (2016). Innate immune memory in plants. *Seminars in Immunology*, 28 (4): 319-327.

Saavedra, G. M., Sanfuentes, E., Figueroa, P. M. & Figueroa, C. R. (2017). Independent Preharvest Applications of Methyl Jasmonate and Chitosan Elicit Differential Upregulation of Defense-Related Genes with Reduced Incidence of Gray Mold Decay during Postharvest Storage of *Fragaria chiloensis* Fruit. *International Journal of Molecular Sciences*, 18 (7).

Sander, J. D. & Joung, J. K. (2014). CRISPR-Cas systems for genome editing, regulation and targeting. *Nature biotechnology*, 32 (4): 347-355.

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9: 671.

Sels, J., Mathys, J., De Coninck, B. M., Cammue, B. P. & De Bolle, M. F. (2008). Plant pathogenesis-related (PR) proteins: a focus on PR peptides. *Plant Physiology and Biochemistry*, 46 (11): 941-50.

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J. J., Qiu, J.-L., et al. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. *Nature Biotechnology*, 31: 686.

Shulaev, V., Sargent, D. J., Crowhurst, R. N., Mockler, T. C., Folkerts, O., Delcher, A. L., Jaiswal, P., Mockaitis, K., Liston, A., Mane, S. P., et al. (2010). The genome of woodland strawberry (*Fragaria vesca*). *Nature Genetics*, 43: 109.

Slaughter, A., Daniel, X., Flors, V., Luna, E., Hohn, B. & Mauch-Mani, B. (2012). Descendants of Primed *Arabidopsis* Plants Exhibit Resistance to Biotic Stress. *Plant Physiology*, 158 (2): 835-843.

Soppe, W. J. J., Jasencakova, Z., Houben, A., Kakutani, T., Meister, A., Huang, M. S., Jacobsen, S. E., Schubert, I. & Fransz, P. F. (2002). DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*. *The EMBO Journal*, 21 (23): 6549-6559.

Spoel, S. H., Koornneef, A., Claessens, S. M. C., Korzelius, J. P., Pelt, J. A. V., Mueller, M. J., Buchala, A. J., Métraux, J.-P., Brown, R., Kazan, K., et al. (2003). NPR1 Modulates Cross-Talk between Salicylate- and Jasmonate-Dependent Defense Pathways through a Novel Function in the Cytosol. *The Plant Cell*, 15 (3): 760-770.

Strømeng, G. M., Hjeljord, L. G. & Stensvand, A. (2009). Relative Contribution of Various Sources of *Botrytis cinerea* Inoculum in Strawberry Fields in Norway. *Plant Disease*, 39 (12): 1305-1310.

Strømeng, G. M. & Stensvand, A. (2017). Resistens mot soppmidler hos *Botrytis* – årsaken til gråskimmel i jordbær. Status for resistenssituasjonen i jordbærfelt i Agder. *NIBIO Rapport*, 3 (61).

Taiz, L., Zeiger, E., Møller, I. M. & Murphy, A. (2015). Biotic Interactions. In *Plant Physiology and Development*, pp. 693-730. Sunderland, MA U.S.A: Sinauer Associates, Inc. .

Tariq, M., Saze, H., Probst, A. V., Lichota, J., Habu, Y. & Paszkowski, J. (2003). Erasure of CpG methylation in *Arabidopsis* alters patterns of histone H3 methylation in heterochromatin. *Proceedings of the National Academy of Sciences of the United States of America*, 100 (15): 8823-8827.

Thevenet, D., Pastor, V., Baccelli, I., Balmer, A., Vallat, A., Neier, R., Glauser, G. & Mauch-Mani, B. (2017). The priming molecule beta-aminobutyric acid is naturally present in plants and is induced by stress. *New Phytol*, 213 (2): 552-559.

Toljamo, A., Blande, D., Karenlampi, S. & Kokko, H. (2016). Reprogramming of Strawberry (*Fragaria vesca*) Root Transcriptome in Response to *Phytophthora cactorum*. *PLoS One*, 11 (8): e0161078.

Ton, J. & Mauch-Mani, B. (2004). β-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. *The Plant Journal*, 38 (1): 119-130.

Ton, J., Jakab, G., Toquin, V., Flors, V., Iavicoli, A., Maeder, M. N., Métraux, J.-P. & Mauch-Mani, B. (2005). Dissecting the β-Aminobutyric Acid–Induced Priming Phenomenon in *Arabidopsis*. *The Plant Cell*, 17 (3): 987-999.

van Hulten, M., Pelser, M., van Loon, L. C., Pieterse, C. M. J. & Ton, J. (2006). Costs and benefits of priming for defense in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, 103 (14): 5602-5607.

van Kan, J. A. (2006). Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends in Plant Science*, 11 (5): 247-53.

Wang, K., Liao, Y., Xiong, Q., Kan, J., Cao, S. & Zheng, Y. (2016). Induction of Direct or Priming Resistance against Botrytis cinerea in Strawberries by beta-Aminobutyric Acid and Their Effects on Sucrose Metabolism. *Journal of Agriculutal and Food Chemistry*, 64 (29): 5855-65.

Wilkinson, S. W., Pastor, V., Paplauskas, S., Pétriacq, P. & Luna, E. (2018). Long-lasting β-aminobutyric acid-induced resistance protects tomato fruit against *Botrytis cinerea*. *Plant Pathology*, 67 (1): 30-41.

Williamson, B., Tudzynski, B., Tudzynski, P. & van Kan, J. A. (2007). *Botrytis cinerea*: the cause of grey mould disease. *Molecular Plant Pathology*, 8 (5): 561-80.

Woodhead, M., Taylor, M. A., Davies, H. V., Brennan, R. M. & McNicol, R. J. (1997). Isolation of RNA from blackcurrant (Ribes nigrum L.) fruit. *Molecular Biotechnology*, 7 (1): 1-4.

Zhou, J., Wang, X., He, K., Charron, J. B., Elling, A. A. & Deng, X. W. (2010). Genome-wide profiling of histone H3 lysine 9 acetylation and dimethylation in *Arabidopsis* reveals correlation between multiple histone marks and gene expression. *Plant Molecular Biology*, 72 (6): 585-95.

Zipfel, C. (2009). Early molecular events in PAMP-triggered immunity. *Plant Biology*, 12 (4): 414-420.

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