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# Functional characterization of putative disease resistance genes of strawberry

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# Functional characterization of putative disease resistance genes of strawberry

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## Abstract

The octoploid strawberry (*Fragaria × ananassa*) is highly valued horticultural crop. The fruit crop is often challenged by several phytopathogens including a hemibiotrophic oomycete, *Phytophthora cactorum*. The pathogen has been known to cause two distinct diseases in strawberry: crown rot and leather rot. Both diseases are reported to cause significant economic losses in strawberry production globally. The high heterozygosity, absence of qualitative/natural resistance and progressive phaseout of chemical means of disease control has made it obvious for identification and characterization of putative resistance genes from their wild accessions.

In this study, two *Fragaria vesca* genes, *WAK* (*Wall Associated Kinases)* and *FvCDIP1 (Fragaria vesca Cell Death Inducing Protein 1)*, were selected for their functional characterization. *Agrobacterium tumefaciens* mediated transient expression of *WAK* gene in *Nicotiana benthamiana* was performed followed by an inoculation of *P. cactorum.* Diseased area in terms of percentage was calculated to analyse the effect of *WAK* gene expression on defence. A reduced disease severity in comparison to the mock controls was observed for the *WAK* gene expressed plants.

Whereas for the functional study of *FvCDIP1* gene two different approaches were used. First approach included generation of transgenic *N. benthamiana* lines which stably expresses *FvCDIP1* gene. A gel-based PCR analysis confirmed the successful integration and expression of *FvCDIP1* gene in *N. benthamiana* genome. To study the influence of *FvCDIP1* on defence signalling pathways in the transgenic plants, the relative expression of four marker genes, *PR-1a, PR-5, PDF1.2* and *EDS1* were analysed using a qRT-PCR. An upregulation of *PDF1.2* defensin gene associated with Jasmonic Acid/Ethylene signalling pathway was observed.

The second approach was *Agrobacterium* mediated delivery of a *FvCDIP1* gene knockout construct pCas9-TPC::FvCDIP1_2XgRNA into strawberry tissue using a recombinant plant expression vector pCas9-TPC. The knockout construct consisted of two guide RNAs targeting two different sites close to the start codon of the *FvCDIP1* gene. Through gel-based RT-PCR analysis the presence and expression of the knockout construct was confirmed. In future, a generation of mutant strawberry impaired in *FvCDIP1* gene function could elaborate the functional characteristic of the gene.

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**Abbreviations**

| | |
|---|---|
| AFLP | Amplified Fragment Length Polymorphism |
| ATP | Adenosine triphosphate |
| BAP | 6-Benzylaminopurine |
| bp | Base pair(s) |
| CBEL | Cellulose binding elicitor lectins |
| cDNA | Complementary DNA |
| CDPK | Calcium dependent protein kinases |
| CNL | Coiled coil NLR |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| CRN | Crinkling and necrosis |
| crRNA | CRISPR RNA |
| CTAB | Cetyltrimethyl ammonium bromide |
| DAMP | Damage associated molecular pattern |
| dH2O | Distilled water of Milli-Q quality |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| dpi | Days post infection |
| DSB | Double stranded break |
| ET | Ethylene |
| ECM | Extra cellular matrix |
| ETI | Effector triggered immunity |
| gDNA | Genomic DNA |
| HDR | Homology-dependent repair |
| hpi | Hours post infection |
| HR | Hypersensitive response |
| IAA | Indole-3-acetic acid |
| JA | Jasmonic acid |
| kb | Kilo base pair(s) |
| LRR | Leucine-rich repeat |
| MAMP | Microbe-associated molecular pattern |
| MAPK | Mitogen associated protein kinases |
| MMLV | Moloney murine leukaemia virus |
| NB-LRR/NLR | Nucleotide-binding leucine-rich repeat |
| NBS | Nucleotide binding sites |
| NHEJ | Non-homologous end joining |
| NIBIO | Norwegian Institute of Bioeconomic Research |
| NRC | NLR- required for cell death |
| PAM | Protospacer-adjacent motif |
| PAMP | Pathogen associated molecular pattern |
| PcF | Phytophthora cactorum factor |
| PCR | Polymerase chain reaction |
| PR | Pathogenesis related |
| PRR | Pattern recognition receptors |
| PTI | Pattern triggered immunity |
| QTL | Quantitative trait loci |
| RAMS | Random Amplified Microsatellite |
| RenSeq | Resistance gene enrichment sequencing |
| RLK | Receptor like kinase |
| RNA | Ribonucleic acid |

| | |
|---|---|
| ROS | Reactive oxygen species |
| RxLR | Arginine-any amino acid-Leucine-Arginine |
| SA | Salicylic acid |
| SAR | Stramenopiles–alveolata–rhizaria |
| T-DNA | Transfer DNA |
| TALEN | Transcription activator like effector nuclease |
| TDZ | Thidiazuron |
| TNL | Toll/interleukin-1 receptor NLR |
| tracrRNA | Trans activating crRNA |
| V/V | Volume/volume |
| WAK | Wall associated kinases |
| ZFN | Zinc finger nuclease |

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# 1. Introduction

## 1.1. Strawberry

Strawberry is a widely cultivated soft fruit crop belonging to family Rosaceae, desired for its appealing aroma, colour and organoleptic characters. The cultivated strawberry *Fragaria × ananassa* Duchesne is an unpremeditated hybrid between two different wild ecotypes *Fragaria virginiana* and *Fragaria chiloensis* from North America and South America, respectively (Darrow, 1966). The hybridization supposedly happened approximately 250-300 years ago in southern France (Darrow, 1966; Hancock, 1999), which makes cultivated strawberry *Fragaria × ananassa* one of the youngest crop species. *Fragaria × ananassa* (2n=8x=56) is an allo-octoploid species and has eight sets of chromosomes which is derived from four different diploid ancestors (Hummer et al., 2011). Two of these four diploid progenitors have been identified (Tennessen et al., 2014) while the other two still remain unknown (Edger et al., 2019). *Fragaria × ananassa* shares a high degree of genetic sequence resemblance with the diploid species *F. vesca* also known as woodland strawberry (2n=2x=14). Unlike *F. × ananassa* with a large genome (813.4 Mb), the small genome size (~265 mb) of *F. vesca* along with features like short regeneration period, readily transformable and small herbaceous nature makes it a model plant for use in genetic research and studies within the Rosaceae family (Edger et al., 2018; Shulaev et al., 2011; Wilson et al., 2019). The cultivated species *F. × ananassa* is widely acknowledged for its health benefits. Phytochemical and epidemiological studies have evinced anti-inflammatory, antioxidant, and antihypertensive properties with strawberry consumption (Basu et al., 2014; Giampieri et al., 2014; Hannum, 2004). Today, multiple breeding programs throughout Europe and the United States has resulted in hundreds of cultivars with wide variability (Mezzetti et al., 2018). The global production of strawberry as of 2019 is 8.88 million tonnes produced in 3.96 thousand hectares land area (FAOSTAT, 2021). Strawberry is also widely cultivated in northern European countries like Sweden, Finland, and Norway. The ever-expanding market and demand of fresh and processed strawberry products has led to even more possibilities to breed and exploit the present quality products.

### 1.1.1. **Production of strawberry in Norway**

Strawberry is one of the most important horticultural fruit crops in Norway. The first introduction of strawberry into the country is predicted to have happened by monks in the 19$^{th}$ century, which at that time had its production only in the monasteries (Nes, 1998). Today, it is produced in all the counties of Norway, however, the majority of the production is in the mid-Eastern region (Haslestad, 2016). Predominant cultivars in Norway includes Florence, Korona, Polka and Sonata which are popular for their unique traits including higher yield and suitability for processing (Sønsteby et al., 2017). The strawberry production in Norway starts from the beginning of June and lasts to the middle of September (Døving et al., 2017). The annual production of strawberry in 2020 was 7027 tonnes produced on 1250.08 hectares of area (SSB Norway, 2021). Although strawberry accounts for 70% of the total berry production (Øverby, 2021) in Norway, the domestic production holds for only 33.8% of total strawberry consumption, while the rest is imported (OFG, 2021). The per capita consumption of strawberry in 2020 was 2.0 kg per year (OFG, 2021). The limitations in commercial production of strawberry in Norway include marginal climatic condition, seasonality in productions, high production costs, a need to import planting material and most importantly occurrence of diseases (Engelseth et al., 2011; Sønsteby et al., 2004; Sønsteby et al., 2013; Sønsteby & Heide, 2017). Important disease causing pathogens of strawberry in Norway include the biotrophic fungus *Podosphaera aphanis* (Suthaparan et al., 2013), the necrotrophic fungus *Botrytis cinerea* (Strømeng et al., 2009) and the hemibiotrophic oomycete *Phytophthora cactorum* (Pettersson et al., 2020).

## 1.2. **Phytophthora cactorum**

*Phytophthora cactorum* (Lebert and Cohn) Schröter, which belongs to the class Oomycetes, is a homothallic pathogen that persists in soil and plant debris. The occurrence of *P. cactorum* has caused huge economic losses as the pathogen can cause latent infection and remain unnoticed in planting material (Nellist, 2018; Shaw et al., 2006). Because of its morphological characters, filamentous growth, mode of nutritient uptake and ecological niches, oomycetes resemble fungi (Leonard et al., 2018; Richards et al., 2006). Despite these similarities with fungi, oomycetes belong to stramenopiles; SAR (stramenopiles–alveolata–rhizaria) eukaryotic supergroup,

which means they are closer to diatoms and brown algae (Burki et al., 2020). They differ from fungi primarily by having diploid-aseptate hyphae and cellulosic cell wall rather than chitin (Erwin & Ribeiro, 1996). The class Oomycetes includes highly diverse orders and has some notorious pathogens which infect hosts ranging from vertebrates, arthropods, algae, protists, fungi and plants (Beakes et al., 2012; Phillips et al., 2008). The hemibiotrophic genus *Phytophthora* (plant destroyers; Greek) comprises several devastating plant pathogens which have the history of causing serious ecological and economical damage (Judelson & Blanco, 2005). *Phytophthora infestans, P. ramorum* and *P. cinnamomi* are examples of *Phytophthora* spp. with noticeable impediment to agriculture and forest ecosystem management. The hemibiotrophic lifestyle of *Phytophthora* requires a living cell in its early infection stage which subsequently undergo a physiological transition from an asymptomatic biotrophic phase to a necrotrophic phase characterized by tissue degradation and disease symptoms (Chepsergon et al., 2020).

*Phytophthora cactorum* was first identified in cactus in 1870 and was first known by the name *Peronospora cactorum* (Lebert & Cohn, 1870). It has a wide host range and infects over 200 plant species from 160 genera that includes several ornamentals, woody species and fruit crops including strawberry, peach and apple (Deutschmann, 1954; Erwin & Ribeiro, 1996; Hantula et al., 2000; Jones & Benson, 2001; Nienhaus, 1960). On strawberry, it causes two distinct diseases, rotting of rhizome (crown rot) and rotting of fruit (leather rot) (Parikka, 1991). *Phytophthora cactorum* was first identified in strawberry from infected fruit samples in the USA (Rose, 1924), whereas, crown rot was first reported in Germany in 1952 (Deutschmann, 1954). In Norway, *P. cactorum* was first detected as crown rot in strawberry in 1992 (Stensvand et al., 1999).

The pathogen has been described as a generalist pathogen (Grenville-Briggs et al., 2017; Yang et al., 2018), however, studies also support the evidence of host specialisation. Isolates from different hosts appear to be genetically different and does not seem to infect all host species. Pathogenicity trials of *P. cactorum* have shown that crown- and leather rot of strawberry are caused by two specialised pathotypes; the crown rot pathotype and the leather rot pathotype. Isolates from different hosts such as apple, pear, rhododendron, silver birch cannot cause crown rot in strawberry and vice versa (Hantula et al., 2000; Nellist et al., 2021). Similarly, Eikemo et al. (2004)

found that isolates from hosts other than strawberry along with four isolates from strawberry fruits/leaves did not develop crown rot symptoms in the susceptible strawberry cultivar 'Inga'. These isolates were designated as the leather rot pathotype. Leather rot pathotypes are not specialized to their original host and are capable of developing leather rot symptoms in strawberry fruit. In contrast, crown rot of strawberry is caused by a host specialised pathogen (Nellist et al., 2021; Seemüller & Schmidle, 1979). These isolates are designated crown rot pathotypes. The crown rot pathotype can also be found on fruit as the cause of leather rot (Eikemo et al., 2004; Seemüller & Schmidle, 1979). Genetic variation of crown rot and leather rot pathotypes of *P. cactorum* have been assessed using Random Amplified Microsatellite (RAMS) (Hantula et al., 1997; Hantula et al., 2000) and Amplified Fragment Length Polymorphism (AFLP) (Bhat et al., 2006; Eikemo et al., 2004; Huang et al., 2004). These assessment techniques have been proved useful in detecting the source of primary inoculum, characterizing individual isolates, disease forecasting and moreover in formulating efficient disease management strategies.

### 1.2.1. **Disease cycle and epidemiology**

*Phytophthora cactorum* has both sexual and asexual modes of reproduction. The homothallic property of the pathogen allows the isolates to produce gametangia; antheridia (male reproductive cell) and oogonia (female reproductive cell) in the absence of another mating type. A proper synchrony in the meiosis of individual gametangia will lead to a successful formation of a diploid nucleus which later forms the hardy oospore. The oospores (resting spore) are the primary source of inoculum and can survive in harsh edaphic and climatic conditions. Due to their thick-walled nature, they remain viable in plant residues and the soil for longer period of time (Agrios, 2005). The resilient character of the oospores allow them to germinate even after several years, and under natural conditions this happens after overwintering or when the soil temperature exceeds 7.5°C. Ultimately, oospores germinate by producing a sporangium or hypha (Erwin & Ribeiro, 1996) under saturated soil conditions.

In asexual reproduction, sporangia can either germinate directly or release numerous zoospores, leading to quick and rapid dispersal of the pathogen. Released zoospores are mononucleated cells devoid of cell wall but have two lateral flagella which allows them to propel in thin water film (Erwin & Ribeiro, 1996). These flagella are the

indispensable feature of the *Phytophthora* spp. which use electrical, chemical and molecular stimuli to reach, aggregate and colonize their potential host (Donaldson & Deacon, 1993; Galiana et al., 2019; Walker & van West, 2007). When zoospores arrive to a suitable site they discard their flagella from the cell wall and undergo synchronized encystment (Galiana et al., 2008; Harris, 1991). At this moment, adhesive mucin-like protein substances and polysaccharides are secreted, and ultimately a germ tube is produced which penetrates and colonizes the host tissue (Walker & van West, 2007).

### 1.2.2. **Disease symptoms**

As mentioned earlier, *P. cactorum* causes two distinct diseases in strawberry plants: crown rot and leather rot. Although the *Phytophthora* species is the same, AFLP analysis showed that two different genotypes are responsible for causing these two different diseases (Eikemo et al., 2004). Strawberry plants infected with the crown rot pathotype develop symptoms when warm weather conditions prevail or during early to mid-summer. The diseased plant develops necrotic tissue along the leaf margin and in between the veins. The youngest leaves are the first to show the wilt signs and they gradually turn bluish. The wilt eventually expands throughout the plant resulting in complete collapse of the plant and ultimately death. When the crown is split longitudinally, reddish-brown discoloration can be observed, which in most cases protrudes downwards (Maas, 1998). This symptom in crown rot infected strawberry plants can resemble the symptoms caused by *Colletotrichum acutatum*, especially during the later stage of disease development. Crown rot symptoms also include the rotting of the root, fewer secondary roots and runner lesions in young plants. Often the symptoms are first noticed in the lower areas of the field that are prone to water accumulation.

Leather rot isolates can infect strawberry fruit at any stage of their development. When immature green berries are infected, evidence of brown margin lining the diseased area is visible along with a leathery-rough texture which spreads throughout the entire fruit. Whereas for mature red fruit, the discoloration is comparatively harder to notice, as it shows only slight change in colour from dull pink to lavender or purple. The diseased fruit also develop white mould on the surface, an unpleasant putrid odour which alters the taste of the fruit. When affected fruit are cut cross-sectionally, a darkening of the xylem tissue leading to each seed can be observed. At the later stage of disease development, the affected fruit dries, stiffens and mummifies. Leather rot

has a serious impact in the post-harvest activities such as jam and jelly production. Due to a little or unnoticeable colour change, the damaged fruits appear normal and when processed with healthy fruits it results in off flavoured jam and jelly (Ellis & Grove, 1983).

## 1.3. Plant defence

Under natural conditions plants are closely associated with a variety of microbes, and like other living creatures' plants often get attacked by a multitude of microbes and pathogens. They are continuously exposed to many potential pathogenic organisms likely to invade but have the ability to defend themselves against these invaders. It is a well-known fact that most plant species are resistant to most pathogens, and disease is an exception, a condition where plants fail to defend themselves. In other words, some organisms are pathogenic to some plants while not to others. So, it would be wrong to say that plants lack resistance against pathogen attack, because they do. In fact, plants have a cascade of defence systems which is complex but effective against diverse pests and pathogens (Slater et al., 2008).

Oomycetes invaginate plant cells, using their specialised hyphae called haustoria into the plasma membrane where they form a tight membrane interface with the haustoriated cells (Bozkurt & Kamoun, 2020). The defence strategy used by the plant depends on how successful the pathogen is in overcoming the subsequent defence barrier. The first line of defence includes anatomical structures where the plant protects itself from external threats by means of morphological and structural barriers. Strawberry also has some impressive anatomical structure such as the glandular trichomes with oxidative enzymes which ward off spider mites (Steinite & Ievinsh, 2003). The bacterial pathogen *Xanthomonas fragariae*; causal organism of angular leaf spot, was reported to be restricted by the leaf vein by Kennedy and King (1962). The importance of cell wall as a physical barrier against invading pathogen is well known. Osorio et al. (2008) observed that the transgenic strawberry plants altered in pectin methyl esterase 1 gene (*FaPE1*) provided higher resistance against *Botrytis cinerea* due to reduced degree of demethylation of cell wall oligogalacturonides.

The second line of defence deployed by plants is the use of pre-formed chemical barriers or antimicrobial proteins. This includes several secondary metabolites such as terpenes, phenolics, alkaloids, saponins which are derived from phenylpropanoid,

isoprenoid, alkaloid or fatty acid pathways, respectively (Kliebenstein, 2004). Products like proanthocyanidins from the flavonoid pathway are synthesized and accumulated in the early stage of fruit ripening. Cultivars with high concentration of proanthocyanidins were found to be resistant against *Botrytis cinerea* (Di Venere et al., 1998; Hébert, 2001; Jersch et al., 1989). Strawberry achenes along with their well protected pericarp have pre-formed compounds like cyanogenic glucosides (Aharoni & O'Connell, 2002), propanoids and ellagitannins (Fait et al., 2008) which are toxic to pathogens and pest. Volatile alcohols and aldehydes are also reported to pose defence properties. An aromatic volatile product (E)-hex-2-enal showed antifungal property against the causal agent of black spot disease; *Colletotrichum acutatum* in strawberry fruit (Arroyo et al., 2007). Besides fruit, leaves of the strawberry plant are also laden with antimicrobial metabolites. The compound fragarin was reported to alter cell membrane permeability and disrupt membrane function in *Clavibacter michiganensis* (Filippone et al., 2001) and restrict the growth of *Colletotrichum acutatum, C. fragariae,* and *C. gloeosporioides* (Filippone et al., 1999). Likewise, lower flavonol concentration in the leaves of strawberry plants was correlated with increased susceptibility to grey mold (Hanhineva et al., 2009). There are also some phenolic compounds which are present in the root region which ward off root pathogens like *Alternaria alternata, Pythium irregulare and Rhizoctonia solani* (Nemec, 1976). Characterization and enhancement of such compounds through genetic manipulation and priming treatments is considered to further strengthen the plant defence against a broad range of pathogens. Though the abovementioned preformed defence plays a vital role to create a shield, it is often insufficient to arrest pathogenesis. Therefore, induced immunity is also required which provides greater protection.

The next level of defence relies on protein synthesis as a response triggered by several extra/intracellular immune receptors towards invading pathogens. These receptors are responsible for detection of various immunogenic signals that include pathogen or host derived molecules as well as several proteins/non-protein compounds released by the pathogen during the process of pathogenesis. This defence relies broadly on (1) surface-localized pattern recognition receptors (PRRs) and (2) intracellular nucleotide-binding site–leucine-rich repeat (LRR) receptors (NLRs), which recognize apoplastic elicitors and cytoplasmic effectors, respectively (Jones & Dangl, 2006).

PRRs detect microbe-, pathogen-, or damage associated molecular patterns (MAMPs/PAMPs/DAMPs (Boller & Felix, 2009). DAMPs are the host derived endogenous molecular signals which include cytosolic proteins, plant peptides and cell wall fragments (Nürnberger & Kemmerling, 2018). DAMPs are released upon mechanical injuries from damaged cells or secreted by cells during pathogen invasion (Hou et al., 2019). Whereas, MAMPs/PAMPs are the signature molecules or structures that are conserved across a large class of microorganisms (Boutrot & Zipfel, 2017). DAMPs as well as PAMPs display distinct roles and sometimes compensate for one another when one of the signals is compromised (Hou et al., 2019). Plants employ PRRs that recognize non-self-signals (PAMPs) directly and modified-self-signals (DAMPs) indirectly, ultimately inducing both local and systemic resistance. The membrane bound PRRs are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) which are located in the plant plasma membrane. They are also known as modular transmembrane proteins (Monaghan & Zipfel, 2012). RLKs are composed of an extracellular ligand-binding ectodomain, a transmembrane-spanning domain and a cytoplasmic kinase domain whereas RLPs comprise similar structural organization except for the intracellular kinase domain (Zipfel, 2014). Both RLP and RLK ectodomains contain leucine-rich repeats (LRRs), lysin-motifs (LysMs) or lectin motifs in order to chemically bind diverse ligands (Macho & Zipfel, 2014; Zipfel, 2014). For example, PRRs with LRR-type ectodomain bind proteins/peptides such as bacterial flagellin, endogenous plant elicitor peptides or bacterial elongation factor Tu (EF-Tu) (Nürnberger et al., 2004; Yamaguchi et al., 2006; Zipfel & Felix, 2005). Whereas other type of PRR ectodomains are responsible for recognition of extracellular ATP and molecules containing carbohydrates like bacterial peptidoglycans, fungal chitin or cell wall derived oligogalacturonides (Brutus et al., 2010; Choi et al., 2014; Kaku et al., 2006; Willmann et al., 2011).

A unique subfamily of the RLK includes transmembrane Wall-associated Kinases (WAKs) proteins that are known to serve as sensors monitoring cell wall integrity depending upon the state of the pectin fraction of the plant cell wall. WAK proteins are characterised by the presence of N-terminal extracellular epidermal growth factors (EGF-) like domain and C-terminal intracellular kinase domain (Kohorn, 2015). The positioning and role played by WAKs is of prime significance as WAKs physically link cell wall to the plasma membrane and promote communication between extra cellular

matrix (ECM) and the cytoplasm (Kohorn, 2015). WAKs bind to pathogen/damage–induced pectin fragments or oligogalacturonides and communicate the these damage signals, thereby modulating defence response (Kohorn & Kohorn, 2012). Recently, from a network based analysis module, Li et al. (2020) found that majority of WAK gene are found in octoploid strawberry fruits compared to the diploid strawberry.

Both RLKs and RLPs are responsible for activating a defence response known as pattern-triggered immunity (PTI) (Chisholm et al., 2006). Because of the conserved nature of PAMPs (e.g., bacterial flagellin, fungal chitin), PTI provides resistance against multiple pathogenic as well as non-pathogenic microbes (Zipfel, 2014). PTI is also known as basal/horizontal resistance or surface immunity and is responsible for activation of an early defence signalling cascade through mitogen associated protein kinases (MAPKs) and calcium dependent protein kinases (CDPKs). These downstream immune signals are transduced into multiple defence responses, including production of reactive oxygen species (ROS), biosynthesis of phytohormones and ethylene, callose deposition, stomatal closure, transcriptional and metabolic reprogramming including induction of defence genes (Boller & Felix, 2009; Macho & Zipfel, 2014; Sun et al., 2013). Although PTI prevents plants from a wide array of microbes and diseases, successful pathogens deploy several effector proteins (virulence factors) to suppress/inhibit PTI (Espinosa & Alfano, 2004). As the plant immunity is thwarted it results in effector-triggered susceptibility as outlined by Jones and Dangl (2006) in their zig-zag model of plant-microbe interaction. Nevertheless, some plants are able to recognize effectors by employing a varied class of intracellular immunoreceptors called nucleotide-binding/leucine-rich repeat (NB-LRR or NLRs) receptors as an outcome of host-pathogen co-evolution cycle (Chisholm et al., 2006; Dangl & Jones, 2001). This newly described arms race between plants and pathogens intercepted by NLRs is known as effector-triggered immunity (ETI).

NLR proteins are encoded by specific disease resistance genes known as “R genes”, which recognize potential immunosuppressing effector molecules. NLRs are multidomain molecules with variable N-terminal domains, central nucleotide-binding site (NBS) domains followed by C-terminal LRR domains (Lukasik & Takken, 2009; Takken & Goverse, 2012). NLR proteins are broadly classified into two subclasses based on the N-terminal domains, one with a Toll/interleukin-1 receptor (TIR) domain called TIR-NBS-LRR or TNL and one with a coiled-coil (CC) domain called CC-NBS-

LRR or CNL (Griebel et al., 2014). CNLs are present in both monocot and dicot plants whereas TNLs are present only in dicot plants (Jacob et al., 2013). Most often the NLRs detect non-conserved polymorphic pathogenic effectors (Dangl & Jones, 2001) and undergo a confirmation change from a condensed ADP-bound “off” state to an open ATP-bound “on” state at their central NBS domain (Cui et al., 2015; Griebel et al., 2014; Hu et al., 2013). The recognition of pathogen effectors by NLRs is similar to PRRs but unlike PRRs, NLRs are usually located in the cytoplasm. Some NLR functions as a single unit for both detection and immune signalling, these are referred as ‘singleton NLRs’ (Adachi et al., 2019). These NLRs can detect effectors either via direct recognition or via indirect recognition (Barragan & Weigel, 2020). In direct recognition, the effectors are detected by direct physical interaction with NLRs (Dodds et al., 2006). Whereas in indirect recognition, the NLRs detect the modifications in the host proteins or decoy proteins (Dodds & Rathjen, 2010) where the host/decoy protein is a guardee, guarded by NLRs (Cesari, 2018). Studies have revealed that singleton NLRs have evolved and functionally specialised as ‘sensor’ and ‘helper’ NLRs (Adachi et al., 2019). This indicates that certain NLRs require other NLR proteins to function (Gabriëls et al., 2007). Some of these work in pair and are known as ‘pair NLRs’, while other operate in a complex network fashion (Adachi et al., 2019; Feehan et al., 2020). In pair NLRs, one member is responsible for sensing the pathogen while another member is responsible for defence initiation and executing immune signals (van Wersch et al., 2020). Likewise, intricate clustered-networks of NLRs entail a clade of helper NLRs, known as NLR- Required for Cell Death (NRC), which are required for functioning of a larger number of sensor NLRs (Wu et al., 2017). These NLRs ultimately provide strong resistance against pathogens by rebooting and amplifying the PTI basal transcriptional programs (Cui et al., 2015; Eulgem et al., 2004) and most importantly initiates the programmed cell death response also called hypersensitive response (HR), marked by apoptotic cell death and localized necrosis (Dodds & Rathjen, 2010; Jones & Dangl, 2006).

Both PTI and ETI are responsible for mediating several immune response signalling pathways. However, both tiers of defence function on common signalling components and are interwind (Cook et al., 2015; Lu & Tsuda, 2021; Thomma et al., 2011). This common down-stream signalling includes the influx of $Ca^{2+}$ ions from extracellular space into the cytosol, ROS burst, activation of MAPK cascades, induction of defence

genes and biosynthesis of several phytohormones (Lu & Tsuda, 2021; Tsuda & Katagiri, 2010). Apart from common signalling pathways, much recent studies have shown that ETI potentiate PTI response to induce a robust defence response in plants against pathogen attack (Ngou et al., 2021) and PTI components are required for inducing cell death mediated by ETI (Yuan et al., 2021). Thus, the immune systems overlap extensively with each other where the defence response associated with the plant receptors and recognized molecules does not necessarily justify the PTI-ETI dichotomy (Cook et al., 2015; Hatsugai et al., 2017; Thomma et al., 2011; Wang, Y. et al., 2018; Yuan et al., 2020). Such blurred distinction between the two justifies the need for an alternative molecular module for a clearer nomenclature which better describes the signalling pathways.

## 1.4. **Oomycete pathogenicity**

Phytopathogenic oomycetes have evolved to escape enzymatic activity of the host pathogenesis related (PR) proteins and have several counter-defence strategies to bypass the host's defence barrier (Kamoun, 2006), one of which is their effector repertoires. Effectors are enzymatic or non-enzymatic proteins, small RNAs or secondary metabolites (Wang et al., 2019). Oomycetes are laden with arsenals of effector proteins which disintegrate host cell components and modulate the host immune response (Asai & Shirasu, 2015; Wawra et al., 2012). Based on their localization, effectors are broadly classified into two classes: apoplastic and cytoplasmic. Recent biochemical and live cell imaging data of *P. infestans* has revealed that diverse classes of effector proteins are secreted at haustorial interfaces and interact with the host plasma membrane before entering into the host cytoplasm, primarily via host mediated vesicle trafficking (Petre et al., 2021; Wang et al., 2017; Wang, S. et al., 2018).

### 1.4.1. **Apoplastic effectors**

Apoplastic effectors are released by haustoria at the plant-derived extrahaustorial matrix (Bozkurt & Kamoun, 2020) and are active at the host-pathogen interface (Giraldo & Valent, 2013). These effector proteins assist the pathogen to elude the host's hydrolytic enzymes which target the mycelial wall, thereby facilitating the pathogen to breach the host cell wall structure (Wang & Wang, 2017). One category of apoplastic effectors released by oomycetes include hydrolytic enzymes such as

glycoside hydrolases, pectinases and proteases that play a role in degradation of the host's cell wall components (McGowan & Fitzpatrick, 2017). A second class of apoplastic effectors comprised of protease-inhibitors and glucanase-inhibitors whose primary function is inhibition of PR proteins and prevention of the pathogen's cell wall degradation (Wawra et al., 2012). For example, host proteases like tomato apoplastic cysteine proteases (Rcr3), Phytophthora inhibited proteases (PIP1) and papain-like cysteine proteases (C14) are targeted for inhibition by the apoplastic effector EPIC1 (Kaschani et al., 2010; Song et al., 2009; Tian et al., 2007).

Oomycetes like *Phytophthora* also encode extracellular toxins such as necrosis-and-ethylene-inducing proteins (Nep1-like protein; NLPs) and phytotoxic proteins (*Phytophthora cactorum* factor; PcF) to enhance their invasion success (Jiang & Tyler, 2012; Kamoun, 2006; McGowan & Fitzpatrick, 2020). The genes encoding NLPs are present in high copy numbers in *Phytophthora* spp. (McCarthy & Fitzpatrick, 2017). NLPs are responsible for inducing ethylene accumulation leading to tissue necrosis (Qutob et al., 2006), thereby contributing to the transition from biotrophy to necrotrophy during the infection (Qutob et al., 2002). Moreover, NLPs' similarity with the pore forming toxins of sea anemones and mutant analyses suggests that NLPs are involved in cytolysis through plasma membrane disruption (Ottmann et al., 2009). Several NLPs including their nontoxic variants are recognized as MAMPs which trigger immunity in *Arabidopsis* (Böhm et al., 2014; Oome et al., 2014). In a recent attempt to identify and characterize candidate effectors of *P. cactorum*, Armitage et al. (2018) identified 24 NLP encoding genes. Unlike NLPs, genes encoding PcF proteins are present in low copy numbers (Haas et al., 2009). PcF was originally identified from a culture of *P. cactorum* involved in induction of cell necrosis in strawberry and tomato plants (Orsomando et al., 2001). PcFs are small cysteine-rich (SCR) proteins with three disulfide bridges. Due to their intramolecular S-S bonding (Orsomando et al., 2001), PcF proteins are predicted to withstand proteolysis in the protease rich apoplast of the host (Chen et al., 2016b). A transient expression of PcF effector SCR96 from *P. cactorum* triggered plant cell death in *Nicotiana benthamiana* and its silencing resulted in loss of pathogenicity conferring to its role in virulence of the pathogen (Chen et al., 2016b).

Apart from the apoplastic effectors, oomycetes also release elicitins, cellulose-binding elicitor lectins (CBELs) and transglutaminases. These molecules are recognized by

the host as MAMPs (Oome et al., 2014). Elicitins are extracellular sterol binding proteins secreted mainly by *Phytophthora* and *Pythium* species (Panabières et al., 1997; Yu, 1995). Elicitins bind to sterol and facilitate the assimilation of these into *Phytophthora*, consequently overcoming the inability of *Phytophthora* spp. to synthesize their own sterols (Kamoun, 2006). Altogether, 47 such sterol binding proteins were identified in *P. cactorum* (Armitage et al., 2018). Elicitins like INF1 trigger cell death and induce defence response in *Nicotiana* spp. and *Capsicum annuum* (Kamoun et al., 1993; Liu et al., 2015; Ricci et al., 1989). In addition to this, the presence of fatty acid and sterols stimulate sexual reproduction (Judelson, 2007). Elicitins are speculated in contributing to interspecies variation in *Phytophthora* giving rise to more virulent strains through genetic recombination (Chepsergon et al., 2020). CBEL proteins are cell wall localized proteins which are perceived as a potential elicitor and activate expression of host defence genes (Larroque et al., 2011; Raaymakers & Van den Ackerveken, 2016). CBEL proteins possess two carbohydrate binding modules that allow binding to cellulose and to plant cell walls (Mateos et al., 1997). Binding to the plant cell wall is essential for the CBEL-induced defence reaction (Dumas et al., 2008; Gaulin et al., 2006). In addition to this, CBEL plays a major role in the cell wall integrity of *Phytophthora* spp. by interacting with the cellulose components of the cell wall (Gaulin et al., 2002). Transglutaminases are enzymes which belong to a protease class that enables cross linking of glutamine and lysine residues in proteins (Li, H. et al., 2013). This leads to irreversible conformational changes forming high molecular weight polymers (Martins et al., 2014). A fragment, Pep-13, derived from the $Ca^{2+}$ dependent cell wall associated transglutaminase glycoprotein GP42 present in *P. sojae* was found to act as an elicitor of host defence (Brunner et al., 2002). Pep-13 elicits defence response in plants like potato, parsley, grapevine and *N. benthamiana* (Halim et al., 2004; Nürnberger & Kemmerling, 2009). Due to the formation of covalent bonds between the amino acid residues (Reiss et al., 2011) transglutaminases in oomycetes are presumed to protect the cell wall from hydrolytic host enzymes and thereby conferring resistance to proteolysis (Raaymakers & Van den Ackerveken, 2016).

### 1.4.2. **Cytoplasmic effectors**

Cytoplasmic effectors are secreted and translocated inside the host cytoplasm or intracellular compartments (Armitage et al., 2018). Three major groups of cytoplasmic

effectors are known, all of which consist of conserved motifs. RxLR (Arginine-any amino acid-Leucine-Arginine) proteins are considered to be a rapidly evolving group of effectors that contain an N-terminal signal peptide which facilitate the effector delivery into the host, followed by an RxLR-EER motif (Whisson et al., 2007), and a highly divergent C terminal domain carrying W, Y and L motifs (Win et al., 2012). These RxLR effectors manipulate host defence by suppressing host cell death and PTI, ultimately enhancing susceptibility (Anderson et al., 2015). For example, the RxLR effector AVR3a of *Phytophthora infestans* was able to suppress the cell death in *N. benthamiana* induced by the apoplastic effector INF1 from the same pathogen (Bos et al., 2006). RxLRs are also found to interfere in the signalling pathways of hormones like salicylic acid (Asai et al., 2014), jasmonic acid (Gimenez-Ibanez et al., 2014) and auxin (Evangelisti et al., 2013). The RxLRs are perceived most often by NB-LRR containing R- proteins (Armstrong et al., 2005; Kourelis & Van Der Hoorn, 2018). RxLR effectors are abundant in *Phytophthora* spp., e.g., *P. cactorum* is predicted to encode 200 RxLRs (Armitage et al., 2018). It has been shown that genes encoding RxLR effectors reside in fast-evolving regions of the pathogen genome which contains high frequency of transposons, and that housekeeping genes are located in more stable and slow-evolving regions of the genome (Haas et al., 2009; Raffaele et al., 2010). This allows pathogens to discard deleterious RxLR genes (Anderson et al., 2015) and provide space for diversifying selection and gene conversion (Jiang & Tyler, 2012).

CRN (Crinkling and necrosis) proteins are named after a protein family which produce a leaf crinkling and necrosis phenotype when overexpressed in transient expression assays in *N. benthamiana* and tomato plants (Torto et al., 2003). Like RxLRs, CRNs also contain conserved motifs: an N-terminal LxLFLAK-motif following a signal peptide sequence (Schornack et al., 2010), a DWL domain followed by the conserved C-terminal HVLVVVP-motif (Haas et al., 2009). The LxLFLAK motif mediates translocation of the effector proteins into the host cell (Kamoun, 2007). Unlike RxLRs which are localized in different cell organelles including the nucleus, almost all *Phytophthora* CRNs are reported to localize and target the host nucleus (Hicks & Galán, 2013; Schornack et al., 2010; Stam et al., 2013). Such nuclear localization is considered important for promoting cell death to enhance the PTI, especially in the necrotrophic stage of oomycete hemibiotrophy (Stam et al., 2013). However, the

mechanism behind the trafficking of the CRNs into the nucleus still remains unknown (Amaro et al., 2017). An exception, however, violates this general feature where a *Phytophthora sojae* CRN effector, CRN78, was found to localize on the host plasma membrane arresting the ROS accumulation (Ai et al., 2021). The CRNs are expressed during cyst germination as well as during early and late infection stages (Haas et al., 2009; Ye et al., 2011). While some CRNs in *Phytophthora,* like PsCRN115 in *P. sojae,* have been shown to suppress the host cell death process, other CRNs, like PsCRN63, promote host cell death. Likewise, two closely related CRN proteins showed antagonistic function upon expression in plant (Liu et al., 2011) and only one of two CRNs was found to be associated with cell death (Stam et al., 2013). Thus, CRNs might have more diverse function in pathogenesis than previously anticipated.

CHXC-effectors is a more recently identified class of cytoplasmic effectors from the obligate biotrophic oomycete *Albugo laibachii* (Kemen et al., 2011). Similar to other cytoplasmic effectors, CHXC effectors consist of a conserved CHxC motif within 50 amino acids of the signal peptide cleavage site which facilitate effector translocation into the host cells. This motif has functional similarities to the RXLR in terms of their delivery into the host cell (Kemen et al., 2011). Although the CHXC motif was found to be present in some *Pythium* and *Phytophthora* species, their functionality has yet to be determined (Jiang & Tyler, 2012).

### 1.5. **Disease resistance in strawberry**

Due to its cultivation style and its perennial nature, sensitive-soft-nutrient-rich tissue and proneness of its contact with the soil (Farzaneh et al., 2015), the cultivated strawberry is a vulnerable host to several insects and pests, including necrotrophic, biotrophic and hemibiotrophic phytopathogens. Climate change and erratic pattern of weather phenomenon have made it challenging to manage disease in this already vulnerable fruit crop (Parikka & Tuovinen, 2014). In addition, reliance on chemical means of disease management is now progressively being phased out as legal provisions like Council Directives 91/414/EEC in the European Union (EFSA, 2013), and the Montreal Protocol (Velders et al., 2007) restricts the majority of previously effective fungicides and fumigants, including methyl bromide 1,3-dichloropropane and chloropicrin. Rapid elimination of chemicals for disease management evokes an urgency to seek for alternative disease management strategies which ought to be both

economically and environmentally justified (Maas, 2014). For these reasons, development of disease resistant strawberry cultivars is a promising strategy in the long run.

Disease resistance in allo-octoploid strawberry can be maneuvered first, by characterizing and exploiting the gene pool, especially R-genes, followed by pyramiding of resistance genes in a new genotype (Amil-Ruiz et al., 2011; Poland & Rutkoski, 2016). Identification of candidate genes in strawberry has made use of DNA markers, assisted genetic mapping of quantitative trait loci (QTL), mapping of NB-LRR-type R genes using resistance gene enrichment sequencing (RenSeq), transcriptomic analyses, genotyping-by-sequencing (GBS) and genomic selection, including RNAi-mediated gene silencing and recombinant technologies for functional validation (Barbey et al., 2019; Davik et al., 2015b; Gezan et al., 2017; Härtl et al., 2017; Jia et al., 2021; Lee et al., 2021; Pincot et al., 2018; Toljamo et al., 2016). High quality genome of octoploid strawberry by Edger et al. (2019) and high-resolution genotyping in octoploid strawberry have facilitated in revealing disease resistance loci against a wide array of strawberry pathogens, but specific genes effectuating resistance in these loci is still unresolved (Barbey et al., 2019).

QTL-mapping links phenotypic data with genotypic data and allows identification of genes governing the polygenic trait in the genome, through statistical analysis (Nellist et al., 2019; Young, 1996). The *P. cactorum* resistance trait in cultivated strawberry is a polygenic trait with multiple QTLs being involved (Denoyes-Rothan et al., 2004; Shaw et al., 2006). However, there is a lack of strong QTLs that provide quantitative resistance. In addition, physiological status of the plant can make significant differences in resistance. For example, Eikemo et al. (2000) found that young, cold-stored and/or wounded plants are more prone to disease than old non-wounded plants. Davik et al. (2015a) identified potential resistance genes within a locus named *RPc-1* (*Resistance* to *Phytophthora cactorum 1)* in an $F_2$ population (Bukammen × Haugastøl) of *F. vesca*. They observed a 3.3 Mb QTL region on the proximal end of linkage group (LG) 6 with 801 genes, 69 of which putatively conferred resistance against *P. cactorum*. Toljamo et al. (2016) further identified L-type-lectin-RLKs and NBS-LRR resistance genes in the *RPc-1* locus, which were highly upregulated in *F. vesca* Hawaii 4 roots inoculated with *P. cactorum*. Similarly, a pedigree-based QTL analysis of multi-parental populations revealed a QTL in LG 7D named *FaRPc2*

(*Fragaria* Resistance to *P. cactorum* locus/gene2) conferring resistance to *P. cactorum* (Mangandi et al., 2017). Another QTL analysis in a bi-parental cross of octoploid strawberry showed three QTLs; *FaRPc6C*, *FaRPc6D* and *FaRPc7D* segregated for resistance against *P. cactorum* (Nellist et al., 2019). A PCR-based NBS profiling followed by transcriptional observation showed 15 out of 17 identified resistance gene analogues (RGAs) in resistance genotypes that were quickly expressed after inoculation with *P. cactorum* compared in the susceptible one (Chen et al., 2016a). Furthermore, QTLs conferring resistance to strawberry pathogens other than *P. cactorum* have been identified over the past years, e.g., resistance locus *FaRCa1* on LG 6B conferred resistance against the fungal pathogen *Colletotrichum acutatum* in *F. × ananassa* (Salinas et al., 2019). In the same way, Anciro et al. (2018) observed *FaRCg1* as a resistance locus on LG 6B of *F. × ananassa* linked to resistance against *Colletotrichum gloeosporioides.* Resistance to the bacterial pathogen *Xanthomonas fragariae* in two wild *F. vesca* accessions was highly controlled by a major locus in LG 6D designated *FaRXf1* (Roach et al., 2016). Recently, expression-QTL analysis of octoploid strawberry revealed 76 putative R-genes of which the majority were NLR genes expressed in the roots and leaves (Barbey et al., 2019).

WRKY transcription factors (WRKYTF), which regulate gene expression during defence, was studied in *F. × ananassa* by Chen and Liu (2019). They observed a total of 47 different WRKY genes among which *FaWRKY32* and *FaWRKY45* were upregulated during continuous cropping. These genes supposedly play a role in expression of defence related genes by activating the expression of defence-related proteins such as PR1 protein and peroxidase and assist in hormonal signalling. Similarly, *FaWRKY11* was found to be upregulated against *B. cinerea* (Wang et al., 2021), whereas *FaWRKY1* was found to negatively regulate resistance against *C. acutatum* (Higuera et al., 2019).

Genome wide association studies integrated with phenotypic and transcriptome data allow us to predict candidate genes involved in disease resistance. After the identification of candidate resistance genes, the next step is to functionally validate the putative resistance genes before integrating the desirable trait into the improved cultivar (Gaston et al., 2020). Several approaches can be applied to analyse the effect of candidate genes on disease resistance, one of which includes the study of a mutant

phenotype where the gene is altered. Molecular analysis uses forward genetics and reverse genetic approaches to create mutants. Forward genetics find the genetic basis of a phenotype/trait by the use of a pre-existing mutant phenotype of interest followed by progeny analysis and genetic mapping/sequencing (Zuryn & Jarriault, 2013). Conversely, reverse genetics uses a known sequence of the gene in first-hand and proceeds backwards to create a mutant phenotype gene followed by its analysis. Tools used in reverse genetics include gene silencing by RNA interference, homologous recombination and insertional mutagenesis via T-DNA or use of plant transposons (Aklilu, 2021; Sinha et al., 2018). These reverse genetics tools are being increasingly adopted for molecular and physiological-biochemical analysis of a chosen gene (Guidarelli & Baraldi, 2015; Tyurin et al., 2020). Amongst these, transient and stable genetic transformation through insertional mutagenesis with T-DNA from *Agrobacterium tumefaciens* is widely used. Transient gene expression allows a more rapid assay than stable transformation to infer gene function, characterise gene and protein localisation. Whereas a stable plant transformation allows integration of the gene of interest into the plant genome for inheritance and allows the long term expression of the transgene (Krenek et al., 2015). The details of these approaches are discussed in the following sections.

## 1.6. *Agrobacterium*-mediated gene transformation

The soil bacterium *Agrobacterium tumefaciens* causes crown gall disease in a range of plant species by transferring its DNA segment called transfer-DNA (T-DNA) into the plant cell and ultimately integrates into the host genome (Chilton et al., 1977). Recombinant *Agrobacterium* strains were created in the 1980s by successfully disarming the virulent strains and replacing the native T-DNA with a gene of interest (Fraley et al., 1983). Later, T-DNA binary vector plasmids for efficient transfer to plants were made (Hoekema et al., 1983).

*Agrobacterium* strains used for transformation contains a small binary vector plasmid with cloning sites and a separate helper plasmid which constitutes *vir* genes that is devoid of the native tumour inducing region. Expression of the helper plasmid's *vir* genes assists in transferring the T-DNA region of the binary vector to the plant. Expression of *vir* genes is enhanced by various factors, including pH, temperature, acetosyringone, phenolic compounds and sugar (Shimoda et al., 1990; Turk et al.,

1991). Binary vectors are capable of being multiplied in both *Escherichia coli* and *Agrobacterium* and can be modified to have different antibiotic selection markers, regulatory elements to drive expression of genes of interest as well as translational enhancers (Hellens et al., 2000). Specially, selectable markers provide a convenient way to differentiate between transformed and non-transformed plant tissue. Naturally, plants do not have any sort of antibiotic resistance genes. The transformed tissue with selectable markers gains the ability to survive on selective media containing antibiotics while the non-transformed tissue fails. There are several commercially available non-oncogenic *Agrobacterium* strains, including LBA4404 (Hoekema et al., 1983), AGL0 and AGL-1 (Lazo et al., 1991), EHA105 (Hood et al., 1993) and numerous other derivatives with distinct selectable markers and reporter genes.

*Agrobacterium* mediated transformation is preferred over other plant transformation methods such as protoplast or biolistic transformations, due to the ease in obtaining single copy integration of the DNA into the host genome (Bartlett et al., 2008). Ever since the first successful transformation of tobacco, *Agrobacterium* mediated transformation of plants has been extensively used to study the function of plant genes in molecular biology (De Block et al., 1987), including strawberry. This includes agroinfiltration mediated transient/stable expression (Cui et al., 2017; Guidarelli & Baraldi, 2015; Zhang et al., 2016) and agroinoculation-mediated virus-induced gene silencing (Jia & Shen, 2013; Li, C. et al., 2019). Several plant segments are used as explant material in transformation, the most widely used are leaf disks (Yau et al., 2017). The present study used *N. benthamiana* explants for plant transformation.

*Agrobacterium*-mediated transient expression (agroinfiltration) is simply the infiltration of *Agrobacterium* cells with plant expression vector into the intact plant tissues (Ma, Lisong et al., 2012). The T-DNA from the vector is translocated from the cytoplasm into the host nucleus where it integrates into the chromosome (Krenek et al., 2015). However, during this integration process not all plant cells are necessarily transformed. The non-integrated T-DNA copies remain transcriptionally competent for several days, and this allows for transient (short-term) expression of the gene construct in a plant cell (Hellens et al., 2005).

Agroinfiltration is a simple and inexpensive technology during which the stress impact on plant cells is minimised (Vaghchhipawala et al., 2011). As the transgenic-bacteria

are actively delivered into the intercellular space, higher expression and inheritance efficiency is achieved (Chen & Lai, 2015). Delivery of the bacteria can be mediated either by using a needleless syringe or by vacuum infiltration of the plant material. Syringe agroinfiltration offers an advantage to infiltrate either a single gene alone or a combination of genes within a single leaf. This allows a more homogeneous interpretation for gene expression of a single gene or multiple genes along with the controls at the same time. Whereas vacuum infiltration can be applied on entire plant or whole leaves at once without injuring plant material.

Agroinfiltration is a rapid and efficient method for studying disease resistance genes functions in plants (Cui et al., 2017). The majority of heterologous gene expression and functional validation assay of plant's *R*-genes and pathogens' *Avr* (avirulance) genes (Ma, L. et al., 2012) has been done in the solanaceous model plant *N. benthamiana* (Zhang et al., 2020). Features like simple propagation, short life cycle, large infiltratable leaves, and absence of necrosis upon infiltration makes *N. benthamiana* an ideal model plant in plant-pathogen interaction assays (Goodin et al., 2008; Tyurin et al., 2020). An *Agrobacterium*-mediated transient expression assay of two *F. vesca* genes *FvCaM* and *FvCML*, responsible for calcium and salicylic acid signal transduction in *N. benthamiana* leaves, showed increased resistance to *A. tumefaciens* (Zhang et al., 2016).

Besides *N. benthamiana*, strawberry can also be used for transient expression of candidate genes. The majority of agroinfiltration performed in strawberry fruits so far relates to horticultural traits like firmness, flavours, ripening and aspects related to fruit biology (Carvalho et al., 2016). However recently, Lu et al. (2020) transiently overexpressed the *FabZIP46* gene associated with stress response and defence into 200 strawberry fruit which substantially delayed and lowered the incidence of damage associated with *B. cinerea*. Similarly, Li, Q. et al. (2013) inoculated strawberry fruits with *Agrobacterium* carrying the tobacco rattle virus-gene silencing machinery targeting β-glucosidases associated gene *FaBG3*, which resulted in higher level of resistance to *B. cinerea* compared to the control fruits. Recently, overexpression of the WRKY associated gene *FaWRKY25* using *Agrobacterium* in strawberry fruits resulted in reduced resistance to *B. cinerea* while its silencing significantly increased resistance (Jia et al., 2021). Gene validation by agroinfiltration of strawberry leaves is also possible. For example, Cui et al. (2017) have demonstrated a versatile

*Agrobacterium*-mediated transient gene expression system in strawberry where they used vacuum infiltration to heterologously express the *Arabidopsis thaliana* broad spectrum disease resistance gene (*AtRPW8.2*) in strawberry leaves. The present study entailed *Agrobacterium* mediated transient and stable expression of candidate resistant genes.

### 1.7. **CRISPR/Cas9 mediated gene knockout**

Genome editing has allowed single and multiple mutations by both insertion and deletion of governing traits in plants for functional genomic studies. Recent advancement in use of programmable sequence specific nucleases has allowed in introducing desired modification of genomes (Langner et al., 2018). An important usage of this technology has been made in monitoring and regulating gene expression related studies. To date there are three major genome editing tools with programmable nucleases, namely, **z**inc **f**inger **n**ucleases (ZFNs) (Klug, 2010), **t**ranscription **a**ctivator-**l**ike **e**ffector **n**ucleases (TALENs) (Christian et al., 2010) and RNA-guided nucleases in CRISPR (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats) (Langner et al., 2018). CRISPR-associated Cas protein (CRISPR-Cas) has been able to outstand itself as it is user-friendly, cheaper and faster than other genome editing technologies (Ding et al., 2016; Wada et al., 2020).

CRIPSR-Cas9 is an adaptive immune system of bacteria that protects them from invading foreign DNA, for example from bacteriophages and viruses (Jinek et al., 2012). The most common RNA guided nuclease used in genome editing is the Cas9 nuclease derived from the type II CRISPR/Cas9 system of *Streptococcus pyogenes* (Jinek et al., 2012). The CRISPR-Cas9 genome editing tool comprises two major components - (1) the monomeric DNA endonuclease Cas9 and (2) crRNA (CRISPR RNA) along with tracrRNA (trans activating crRNA) fused into a chimeric synthetic RNA called single guide RNA (sgRNA). Together they form a sgRNA/Cas9 complex which scans for a conserved three-nucleotide protospacer-adjacent motif (PAM) sequence for cleavage in the target DNA (Langner et al., 2018). The PAM sequence varies in length and nucleotide composition depending on the Cas nuclease. The PAM sequence recognised by the Cas9 nuclease is 5'-NGG-3' where "N" stands for any of the four nucleotide bases (Jinek et al., 2012). The sgRNA directs the complex 20 nucleotides upstream of the PAM using base complementarity whereas Cas9 creates

a blunt end double-strand break at a position three base pairs upstream of the PAM sequence (Jinek et al., 2012). The double-strand break is later repaired by either of the two different host-cell repair mechanisms (1) non-homologous end joining (NHEJ) or (2) homology dependent repair (HDR). The NHEJ repair mechanism is an error prone pathway which results in either short insertion and/or deletions (indels) leading to a frame shift and/or early stop codon, ultimately generating a knockout in the target gene.

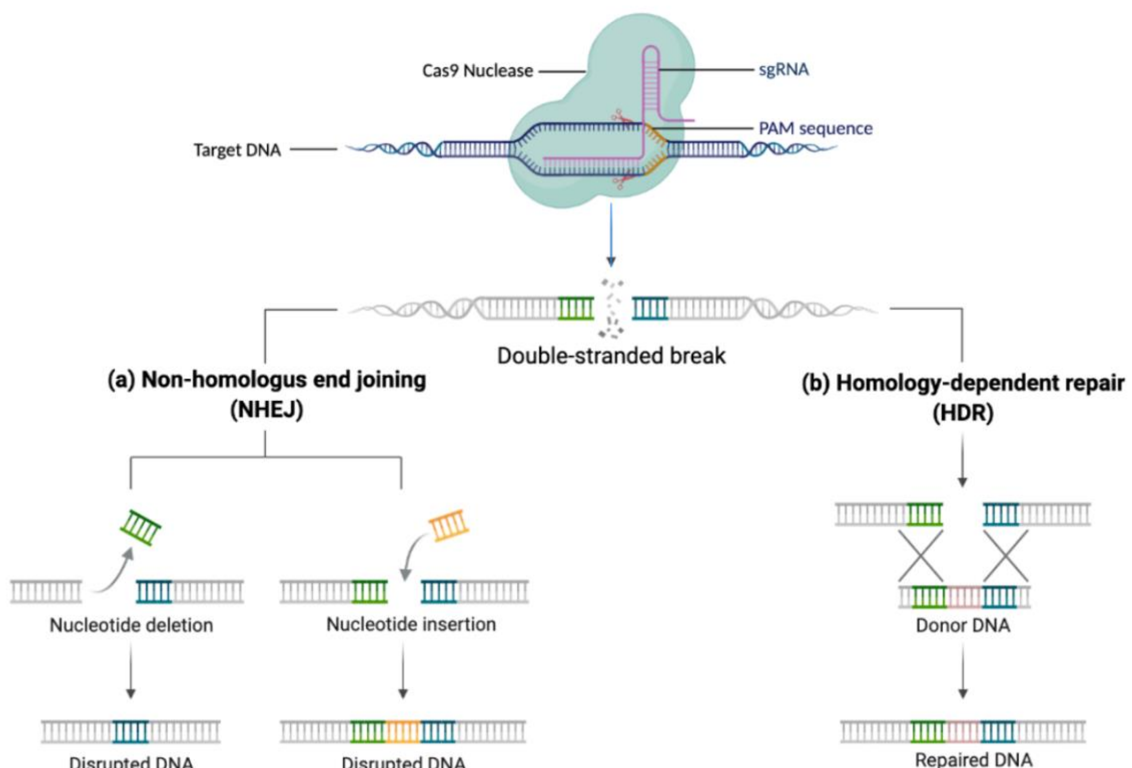


Figure 1: Schematic representation of repair mechanisms of double stranded break (DSB) in DNA. The Cas9 nuclease is directed to the target DNA region by base pairing. The Cas9/sgRNA complex recognizes the PAM motif downstream of the gRNA and creates a (DSB). This DSB is subsequently repaired by (a) error-prone Non-Homologous End Joining (NHEJ) or (b) Homology-Dependent Repair (HDR) pathways. In NHEJ, the cellular machinery incorporates small insertions or deletions of nucleotides at the cut points leading to loss of function of the gene. DSB in knock-out experiments is repaired by NHEJ. In the HDR pathway, a donor DNA acts as a template which is copied into the target site resulting in precise repair. DSB in knock-in experiments is repaired by HDR. (Created in BioRender)

CRISPR-Cas9 offers unprecedented opportunity to plant pathologists in introduction of precise mutations in the targeted genes and has aided in disease management applications in both hosts and the pathogens via two distinct strategies: pathogen-gene and plant-gene approach (Dort et al., 2020). The pathogen-gene approach entails engineering of viral DNA derived sgRNA and its transformation into the plant genome which subsequently targets the virus DNA/RNA during evasion and thereby

restrain pathogenesis (Zhang et al., 2018). The pathogen-gene approach is beyond the scope of this study so this literature review will further focus on plant-gene approach. The plant-gene approach relates to the modification of host genes involved in pathogen interaction to either boost the host immunity or to interfere with the pathogen-host recognition pathway (Makarova et al., 2018).

The reverse genetics approach through plant gene knockout provides a direct insight into functional characterization of a chosen gene in situ. Most of the plant-gene approach focuses on manipulation or knocking out of plant disease susceptibility genes (S-genes) to verify its role. However, mutants generated by knockout of R-genes that are overexpressed during pathogenesis can also provide an insight into its role in plant defence. Such loss of function causes changes in phenotype of the plant. This allows to unravel the role of a gene in plant defence by phenotypic comparison of the knockout mutant lines with the wildtypes. The molecular construct for CRISPR/Cas9-knockout (Cas9 nuclease and sgRNA) can be delivered 1) directly via protoplast transformation or particle bombardment mediated transformation, 2) *Agrobacterium* mediated transformation, 3) virus-derived vector transformation.

This study aimed to create a DSB in the *FvCDIP1* (*F. vesca Cell Death Inducing Protein 1*) gene that has been previously found to be uniquely expressed in two resistant *F. vesca* genotypes against *P. cactorum* (Gogoi et al. NMBU, unpublished). The gene has shown to enhance resistance against *P. cactorum* in *N. benthamiana,* by inducing cell death (A. Gogoi, NMBU Unpublished Phd. thesis). Likewise, this study also aimed to generate stable *N. benthamiana* transformants mediated by recombinant *Agrobacterium* with *FvCDIP1* gene. The third objective was to transiently express another *F. vesca* gene; *WAK* (*Wall Associated Kinase*) gene, in *N. benthamiana* and study the effect of the gene in response to *P. cactorum* inoculation. Thus, the hypothesis is that the *N. benthamiana* plants expressing the *F. vesca* genes would show enhanced resistivity against *P. cactorum.* Therefore, in this study, an *Agrobacterium* mediated transformation approach was used to 1) transiently express a candidate gene in *N. benthamiana* leaves; 2) introduce a CRISPR-Cas9 gene knockout construct in strawberry cells; 3) stably transform *N. benthamiana* for heterologous expression of a *F. vesca* gene.

# 2. Materials and Methods

All experiments were conducted at Norwegian Institute of Bioeconomic Research (NIBIO) Ås, Norway.

## 2.1. *Agrobacterium* mediated transient expression of WAK gene

**Selection of the candidate gene:**

Based on a previous transcriptome study (Gogoi et al.), a gene encoding wall-associated receptor like kinase (WAK) protein was selected as a candidate gene for the transient expression assay. The *WAK* gene encodes a wall-associated receptor like kinase protein and is located in the resistance gene locus *RPc-1* previously identified by Davik et al. (2015a). The gene was selected based on a transcriptome study by A. Gogoi et al.( NMBU, Unpublished). RNA was isolated from *P. cactorum* inoculated resistant strawberry genotypes NCGR1603 and Bukammen 48 hpi (hours post infection) and cDNA was synthesized. The synthesized cDNA from Bukammen was used as a template in Gateway recombination cloning.

### 2.1.1. Gateway cloning of the WAK gene

Gateway recombination cloning allows the cloning of the flanked gene of interest with the vector carrying compatible Gateway attachment sites. To generate attachment sites (*attB1* and *attB2*) on the gene, Gateway primer sets were used. These attachment sites are the 5' extensions at both ends of the gene. The primers were designed in such a way that they had 29 base pair *attB* sites with four guanine residues at their 5' end followed by 20 bp of the gene-specific sequence.

To start the Gateway cloning, the amplification of the *WAK* gene was carried out on cDNA as a template which was previously synthesised (provided by Anupam Gogoi, NMBU).

Table 1: Components added to the PCR mastermix for the Gateway cloning of *WAK* gene.

| Reagents | Volume | Program | |
|---|---|---|---|
| 5X Phusion HF Buffer | 10 µL | | |
| dNTPs | 3.2 µL | 95°C | 5 minutes |
| Forward Primer (Gw_WAK_F) | 2.5 µL | 95°C | 30 seconds |
| Reverse Primer (Gw_WAK_R) | 2.5 µL | 58°C | 30 seconds |
| $MgCl_2$ | 2 µL | 72°C | 2 minutes |
| DMSO | 1.5µL | 72°C | 7 minutes |

| Phusion Polymerase | 0.5µL | 4°C | ∞ |
|---|---|---|---|
| dH2O | 21.6µL | | |

Total: 43.6 µL + 6.4 µL template DNA

Fifty ng of eluted DNA was added to the PCR mastermix. To further purify the WAK gene, the amplified PCR product was loaded on agarose gel (0.7%) and run at 100V for 50 minutes. This allowed the identification of the desired DNA fragment based on the size. The visualised band with expected size was cut with clean sterilized scalpel and with minimal exposure to ultraviolet light (UV). The sliced gel was placed in a 2 mL microcentrifuge tube and weighed. Purification of the cut sample was done as per the instructions for the DNA extraction protocol from NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG) kit to remove the DNA fragments obtained from gel elution with the flanked *attB* sites that were cloned using Gateway® Cloning (Invitrogen).

#### 2.1.1.1. **BP recombination reaction**

The BP recombination reaction was carried out between the a*ttB*-flanked WAK-gene; and the *attP*-containing donor vector pDONR™/Zeo, a 4291-bp circular DNA. The vector contained a zeocin™ resistance gene which allowed selection of *E. coli*.

Table 2: Components added for performing the BP recombination reaction.

| Components | Volume |
|---|---|
| Gel eluted PCR product: WAK_attB (200 ng) | 2.4µL |
| pDONR™/Zeo (200 ng) | 1 µL |
| 5X BP Clonase™ reaction buffer | 1 µL |
| $dH_2O$ | 5.6 µL |
| | Total: 10 µL |

The above components (Table 2) were mixed briefly in a 1.5 mL microcentrifuge tube and incubated at 25°C overnight. On the next day, to stop the reaction and to get rid of any protein or nucleases 1 µL of Proteinase K solution was added to the reaction mixture. The reaction mixture was incubated at 37°C for 10 minutes. The BP reaction was followed by *E. coli* transformation

#### 2.1.1.2. **Transformation of *Escherichia coli***

The transformation of Library Efficiency® DH5α™ (Invitrogen) chemically competent cells was performed using heat-shock, as per the instruction from the Invitrogen protocol with slight modifications. Fifty µL Library Efficiency® DH5α™ (Invitrogen)

chemically competent *E. coli* was thawed on ice. Approximately 5 ng (4 µL) of the BP recombination reaction mixture was chilled in a 1.5 mL microcentrifuge tube. Fifty µL of the thawed competent cells was added to the BP recombination reaction mixture and was mixed without pipetting or vortexing. The mixture was incubated on ice for 30 minutes. A heat shock in a 42°C water bath for 90 seconds was applied, and the mixture was immediately transferred into the ice for 2 minutes. To obtain maximum transformation efficiency, 500 µL of room temperature nutrient rich S.O.C (Super Optimal Catabolite) media was added to the tube. The tube was then vigorously shaken at 200 rpm at 37 °C for 1.5 hours in an incubator. Later, 250 µL of the culture was spread on two prewarmed zeocin (50 µg/mL) LB selection plates with the help of a spreader. Finally, the plates were incubated at 37°C overnight.

#### 2.1.1.3. **Analysis of transformants**

To confirm that the transformants contained the gene of interest, PCR was performed on randomly picked colonies using gene specific primers. Before PCR, colonies were transferred onto new plates containing antibiotics and incubated at 37°C overnight as a stock. From the original plate, the remains of the colony were placed in a 1.5 mL microcentrifuge tube containing 30 µL $dH_2O$. To osmotically lyse and release the plasmid DNA from the bacterial cell, the bacterial solution was then incubated at 99°C for 15 minutes, and the tube was centrifuged at 10000 g for 1 minute. Two µL of the supernatant was used as a template for the PCR reaction mix.

Table 3: The PCR requirements for amplification of the WAK gene for the analysis of E. coli transformants.

| Primer name | Amplicon size (bp) | Annealing temperature | Elongation time |
|---|---|---|---|
| GW_WAK_F | 2476-bp | 58°C | 1.5 minutes |
| GW_WAK_R | | | |

The PCR products were then visually analysed in 1.5% agarose gel electrophoresis made on 10X TBE (Tris-borate EDTA) buffer. Transformed positive colonies were cultured overnight in 20 mL of LB broth containing zeocin (50 µg/mL) at 37°C for plasmid isolation.

#### 2.1.1.4. **Plasmid Isolation from *E. coli* transformants**

10 mL of saturated *E. coli* LB culture was taken from the overnight culture for plasmid isolation. The plasmid isolation was performed as defined in the protocol of the NucleoSpin® plasmid DNA purification kit from Macherey-Nagel GmbH & Co. KG.

First, the pelleted bacterial cells were resuspended. A buffer containing sodium dodecyl sulphate and sodium hydroxide was used to liberate plasmid DNA from the bacteria. The resulting lysate was neutralised by a buffer which created an appropriate condition for binding of plasmid DNA to the silica membrane column. Centrifugation was performed to remove the undesired proteins, genomic DNA, and cell debris. The supernatant was loaded onto a column and ethanolic buffer was used to remove salts and soluble macromolecular components. Finally, the pure plasmid DNA was eluted in a slightly alkaline (pH 8.5) buffer.

#### 2.1.1.5. **DNA sequencing**

For the confirmation of the *WAK* gene insertion into the entry clone, the obtained plasmids were diluted to a final concentration of 50 ng/ µL, and submitted for Sanger sequencing at Eurofins Genomics, Germany. Five primers were chosen for the sequencing, which covered the entire target region (Fig. 10).

#### 2.1.1.6. **LR recombination reaction**

After successfully generating the entry construct, an LR recombination reaction was performed between the entry construct (pDONR_WAK) and the destination vector (pGWB454).

Table 4: Components added for performing the LR recombination reaction.

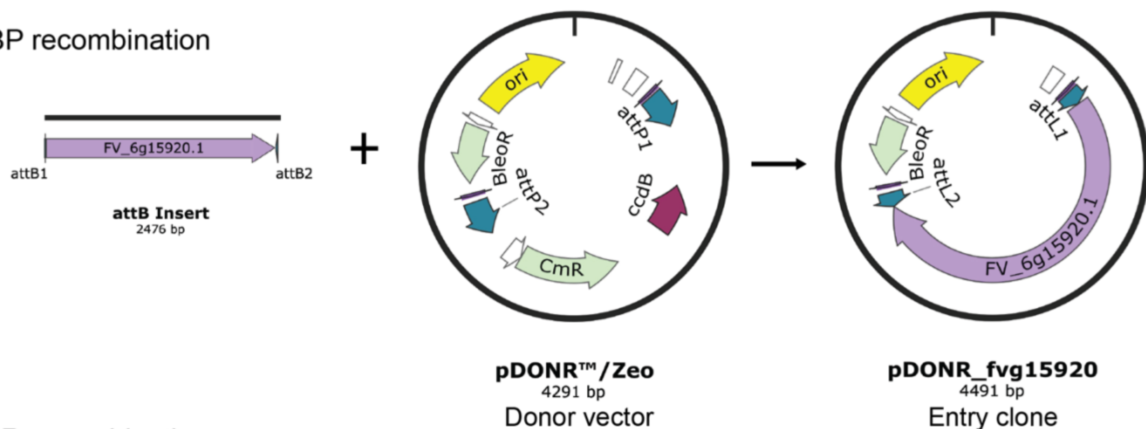
| Components | Volume |
|---|---|
| pDONR_WAK (300ng) | 2.2 µL |
| Destination vector: pGWB454 (300ng) | 1.5 µL |
| 5X LR Clonase™ reaction buffer | 1 µL |
| d$H_2O$ | 5.3 µL |
| | Total: 10 µL |

The LR reaction mixture (Table 4) was incubated overnight at 25°C. On the next day, LR reaction was stopped by addition of 1 µL of Proteinase K to the reaction mixture, and it was subsequently incubated at 37°C for 10 minutes. The LR reaction mix was then transformed into the *E. coli*.

#### 2.1.1.7. **Transformation of *E. coli* and verification of transformants**

Chemically competent “Library Efficiency® DH5α™ (Invitrogen)” cells were transformed by using 4 µL LR recombination reaction mixture in a similar manner as before. Following the transformation, bacteria were cultured in LB-media selection plates containing spectinomycin (75 µg/mL) for the selection of a positive expression vector.

Afterwards, the confirmation of the bacterial transformation was done through colony PCR following a similar procedure as previously described above. PCR was carried



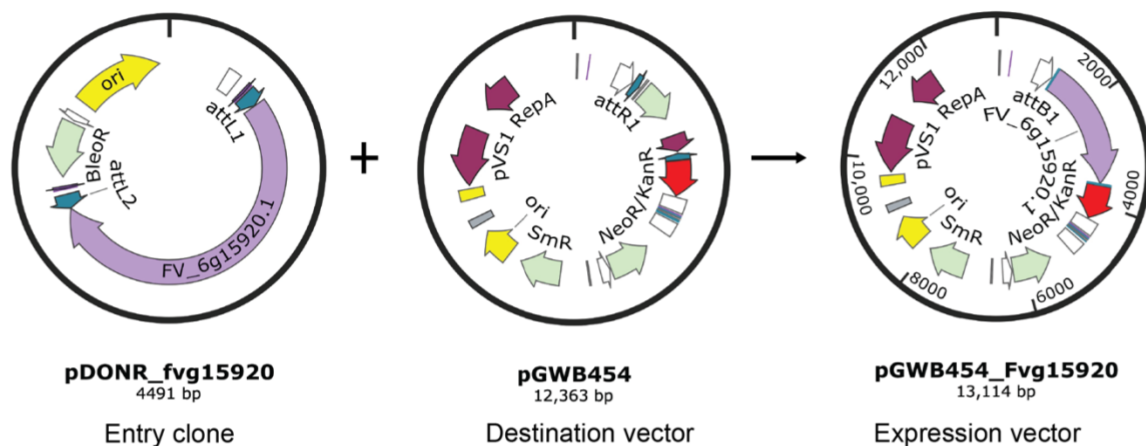




Figure 2: Schematic illustration of Gateway cloning of *Fragaria vesca WAK* gene. (a) A BP recombination reaction performed between the *attB*-flanked PCR fragment and the donor vector: "pDONR/Zeo". In BP recombination reaction, the region between *attP* sites of the donor vector is replaced with the *aatB*-flanked gene of interest, resulting in an entry construct. (b) An LR recombination reaction performed between the entry construct and destination vector. In LR recombination reaction the gene of interest is transferred from the entry construct to the destination vector: "pGWB454" resulting in the final expression construct. The expression construct carries the backbone of the destination vector along with the insert from the entry construct. During LR recombination everything between the *attR* sites is replaced with the insert.

out using gene specific forward and reverse primers (WAK_P2F and WAK_P2R). PCR analysis of the transformants included the following components (Table 5).

Table 5: Components for colony PCR of the *E. coli* transformants.

| Reagents | Volume | Program | |
|---|---|---|---|
| 10X Taq AB Buffer | 2 µL | 95°C | 5 minutes |
| dNTPs | 1.6 µL | 95°C | 1 minute |
| Forward Primer (WAK_P2F) | 0.5 µL | 55°C | 30 seconds |
| Reverse Primer (WAK_P2R) | 0.5 µL | 72°C | 1 minute |
| Amp. Taq Pol (5U/µL) | 0.1 µL | 72°C | 7 minutes |
| dH2O | 13.3 µL | 4°C | ∞ |

Total: 18µL + 2 µL Template

#### 2.1.1.8. **Plasmid Isolation from *E. coli***

After successful generation of the expression construct, plasmid was isolated from the transformed *E. coli* cells grown overnight in 20 mL LB liquid media with spectinomycin (75 µg/mL) at 37°C. The plasmid was isolated following the manufacturer's guidelines from the NucleoSpin® plasmid DNA purification kit (Macherey-Nagel GmbH & Co. KG, Germany).

#### 2.1.1.9. **Sanger DNA sequencing**

For further confirmation, the obtained plasmid (expression clones) was diluted to a final concentration of 50 ng/µL and was verified using sanger sequencing at Eurofins Genomics, Germany. The sequencing was targeted for observation of correct positioning of the constitutive 35S-promoter in the expression vector.

### 2.1.2. **Transformation of *Agrobacterium tumefaciens***

Before starting the transformation, calcium chloride ($CaCl_2$) competent *Agrobacterium* cells were prepared. *Agrobacterium* stock (150 µL) was added in 50 mL of LB liquid-media in a 125 mL flask and incubated at 28°C overnight at 200 rpm in a rotating shaker. The culture was grown until the $OD_{600}$ reached 0.8. The culture was chilled on ice for 30 minutes. Forty-five mL of the chilled culture was transferred into a prechilled 50 mL tube. The tube was centrifuged for 10 minutes at 2000g at 4°C. The supernatant was discarded, and the pellet was gently resuspended in 5 mL ice cold $CaCl_2$ (20 mM). The resuspended cell was centrifuged again for 5 minutes at 2000g at 4°C. The supernatant was discarded followed by a resuspension of pelleted cells in 1 mL chilled $CaCl_2$ (20 mM). The resuspended bacterial cell was transferred into a prechilled 1.5 mL microcentrifuge tube in an aliquot of 100 µL. The aliquot was immediately frozen in liquid nitrogen and stored at -80°C for subsequent use.

Chemically competent *Agrobacterium* cells (LBA4404) stored at -80°C was thawed on ice for 30 minutes. Plasmid DNA (500 ng) was added to the vial of the chemically competent cell and kept on ice for 1 hour. The mixture was kept in liquid nitrogen for 5 minutes. Afterwards, heat shock was applied at 37°C in a water bath for 5 minutes and returned immediately to ice and incubated for 5 minutes. One mL of LB broth was added to the tube, and it was incubated at 28°C for 3 to 4 hours on a rotating shaker.

Later, 250 µL of the culture was plated on LB media plates containing spectinomycin (75 µg/mL). The plates were incubated for 2 days at 28°C.

### 2.1.3. **Analysis of transformants**

The confirmation of transformation of the *Agrobacterium* competent cell with pGWB454::WAK was done through colony PCR using gene specific forward and reverse primers. Ten random colonies were chosen for the analysis. The procedure for obtaining template plasmid DNA for colony PCR was described previously. PCR analysis of the transformants was performed with the following specification (Table 6). Amplified PCR products were then visualized on a 1.5% agarose gel.

Table 6: The PCR requirements for amplification of the *WAK* gene segment for the analysis of *Agrobacterium* transformants

| Primer name | Amplicon size (bp) | Annealing temperature | Elongation time |
|---|---|---|---|
| WAK_P2F | 818-bp | 55°C | 1 minutes |
| WAK_P2R | | | |

### 2.1.4. **Transient gene expression in *Nicotiana benthamiana***

After successful transformation of *Agrobacterium* competent cells, a single colony from the transformed *Agrobacterium* was cultured in a 100 mL flask with 45 mL LB broth containing 75 µg/mL spectinomycin and 25 µg/mL rifampicin at 28°C overnight in a rotating shaker at 220 rpm. $OD_{600}$ of the culture was measured in a spectrophotometer to determine the bacterial cell concentration. The bacterial culture was centrifuged at 2000g for 10 minutes at 4°C. As much as possible of the supernatant was discarded, and the pelleted cells were resuspended in a resuspension buffer (Table 7) adjusting the $OD_{600}$ to 0.2.

Table 7: Components required for preparation of resuspension buffer.

| Components | Volume |
|---|---|
| MES hydrate (10 mM) | 2 mL |
| $MgCl_2$ (10 mM) | 2 mL |
| Acetosyringone (200 µM) | 200 µL |
| | Total: 200 mL |

The bacterial cell suspension was incubated at room temperature for 2.5 to 3 hours to induce the *vir*-genes before infiltration. For *Agro*-infiltration, 6- to 8-weeks-old, four plants were used. Each plant was tagged on two leaves for infiltration. The leaves were infiltrated with *Agrobacterium* on the abaxial side using a needleless 1- mL

syringe by applying pressure. An empty vector construct without the candidate gene was also infiltrated as a control.

### 2.1.5. **Isolation of total RNA**

Leaves from *Agro*-inoculated *N. benthamiana* plants were harvested at different time points; 0 hpi (hours post inoculation), 48 hpi, 72 hpi and 96 hpi. Inoculated leaves were flash frozen in liquid nitrogen and stored at -80°C before proceeding to RNA isolation. The stored leaves were disrupted and finely ground in liquid nitrogen using mortar and pestle. Approximately 100 mg (wet weight) of ground leaves per sample was used for RNA isolation. Total RNA was isolated from the plant material using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich®, USA) with slight modification, where the initial step included CTAB (cetyltrimethyl ammonium bromide) extraction buffer. To start the lysis step, 2 mL of CTAB buffer was added to a 2 mL tube containing 40 mg of PVPP (polyvinylpyrrolidone). The mixture was incubated on a heat block at 60°C for 30 minutes with continuous vortexing. Pre-heated CTAB buffer mixture (900 µL) was added to 100 mg of ground leaves. A reducing agent, β-Mercaptoethanol (10 µL), was added to the tube before incubating at 60°C for 15 minutes to avoid degradation by RNases. Incubation was followed by a centrifugation step at 13000 rpm for 5 minutes. The supernatant was pipetted carefully to a new 2 mL tube without disturbing the pellets. An equal volume of freshly prepared chloroform:isoamylalcohol (24:1) was added to the supernatant and mixed well by inverting the tube several times. The mixture was centrifuged at 13000 rpm for 10 minutes at 4°C before transferring into the filtration column provided by Sigma-Aldrich®. From this step onward the manufacturer's protocol was followed. During isolation, on-column DNase digestion was carried out to remove DNA from the RNA sample using "On-column DNase I Digestion Set" from Sigma-Aldrich®. The isolated RNA samples were tested for their concentration using NanoDrop™ 2000 spectrophotometer.

As the isolated total RNA was to be used for reverse transcription reaction, it was vital to have RNA with no DNA contamination. For this reason, the obtained RNA was further taken through an additional DNase digestion step using a DNase I, RNase-free (Thermo Scientific™) kit. The final obtained product was used directly as a template for reverse transcription.

### 2.1.6. cDNA synthesis and RT-PCR

cDNA was synthesized from the isolated RNA using the iScript$^{TM}$ Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) (Table 8). The cDNA synthesis included reverse transcription reaction facilitated by a modified form of reverse transcriptase from Moloney murine leukaemia virus (MMLV). During this cDNA synthesis process, RNA-dependent DNA polymerase incorporates dNTPs in the pre-existing single stranded RNA, synthesizing a cDNA strand. RNase-H present within reverse transcriptase with endoribonuclease activity then specifically degrades the existing RNA from the RNA-DNA complex. The obtained cDNA represents the genes that are actively expressed at the time of harvesting the plant material.

Table 8: Reaction setup for the synthesis of cDNA.

| Components | Volume | Program | |
|---|---|---|---|
| 5X iScript reaction mix | 4 µL | 25°C | 5 minutes |
| iScript Reverse transcriptase | 1 µL | 46°C | 20 minutes |
| RNA template (100 ng) | 2 µL | 95°C | 1 minute |
| Nuclease free H2O | 13 µL | 4°C | ∞ |
| Total | 20 µL | | |

A PCR was carried out to check the quality of the obtained cDNA (Table 9), by targeting a housekeeping gene *EF1α* (elongation factor gene-1α). Housekeeping genes are stably expressed and are used for normalization of the target gene under specific experimental conditions (Amil-Ruiz et al., 2013).

Table 9: The PCR requirements for amplification of the housekeeping gene (*EF1α).*

| Primer name | Amplicon size (bp) | Annealing temperature | Elongation time |
|---|---|---|---|
| EF1α_NB_F | 116-bp | 55°C | 30 seconds |
| EF1α_NB_R | | | |

### 2.1.7. *WAK* gene expression analysis

After validating the presence of the housekeeping gene in the isolated cDNA, RT-PCR was conducted (Table 10) using cDNA as a template for the expression analysis of the *WAK* gene in agroinfiltrated *N. benthamiana* leaves*.* An equal concentration of RNA was used as a control.

Table 10: PCR reaction setup for the expression analysis of *WAK* gene.

| Components | Volume | Program | |
|---|---|---|---|
| 10X Taq AB Buffer | 2 µL | 95°C | 5 minutes |
| dNTPs | 1.6 µL | 95°C | 1 minute |

| | | | |
|---|---|---|---|
| Forward Primer (WAK_RTF) | 0.5 µL | 58°C | 30 seconds |
| Reverse Primer (WAK_RTR) | 0.5 µL | 72°C | 30 seconds |
| Amp. Taq Pol (5U/µL) | 0.1 µL | 72°C | 7 minutes |
| dH2O | 13.8 µL | 4°C | ∞ |

Total 18 µL + 2 µL template

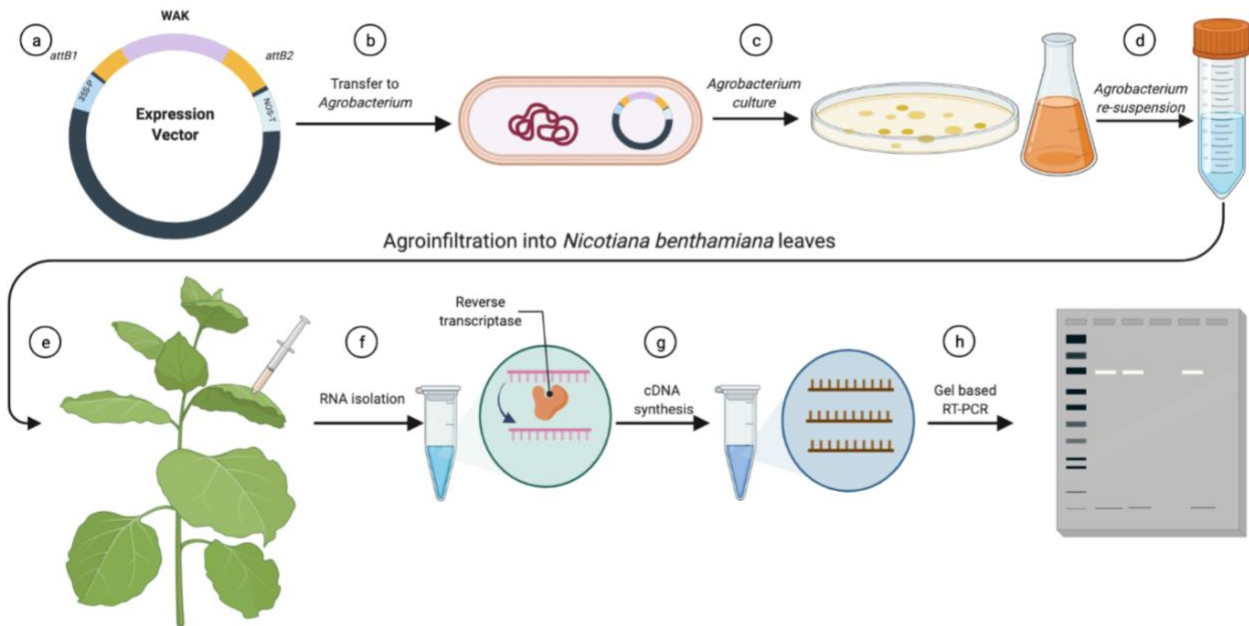



Figure 3: Schematic representation of the steps involved in transient expression of the WAK gene. (a) The plant expression vector harbouring the WAK gene, which is guided by the CaMV-35 S promoter is obtained after Gateway cloning; (b) the plant expression vector is transferred into *Agrobacterium*; (c) the obtained bacterial transformants are cultured in appropriate antibiotics and (d) suspended to $OD_{600}$ 0.2; (e) agroinfiltration of resuspended bacterial culture in the abaxial surface of *N. benthamiana* with a needleless syringe; (f) total RNA isolation from agroinfiltrated *N. benthamiana* leaves at different time points (0 hpi, 48 hpi, 72 hpi and 96 hpi); (g) cDNA synthesis from the obtained total RNA; (h) gel based RT-PCR for WAK gene expression analysis.

### 2.1.8. **Phytophthora cactorum infection assay**

*Phytophthora cactorum* isolate 10300 (Armitage et al., 2018), was grown on 10% (v/v) V8 juice agar plates at room temperature in the dark for a week. To release the zoospores the culture was dipped into 25 mL sterilized pond water and placed at 4°C for 30 minutes followed by incubation at room temperature under a light source for about 30 minutes. To calculate the concentration, 10 µL of zoospore suspension was adjusted by haemocytometer counts. Zoospore inoculation was performed two days post infiltration. Five plants were chosen, and two leaves from each plant were inoculated at two different spots with 30 µL of zoospore suspension with a concentration of 17 zoospores per microliter on the abaxial surface of the leaf.

To evaluate the extent of disease symptoms, the leaf samples were photographed at 5 dpi (days post infection). To calculate the leaf area "ImageJ" software was used. First, the total leaf area for individual leaves was calculated, afterwards the area covered by the lesion was calculated (Fig. 4). Finally, an average percentage of lesion coverage for each treatment group was computed.

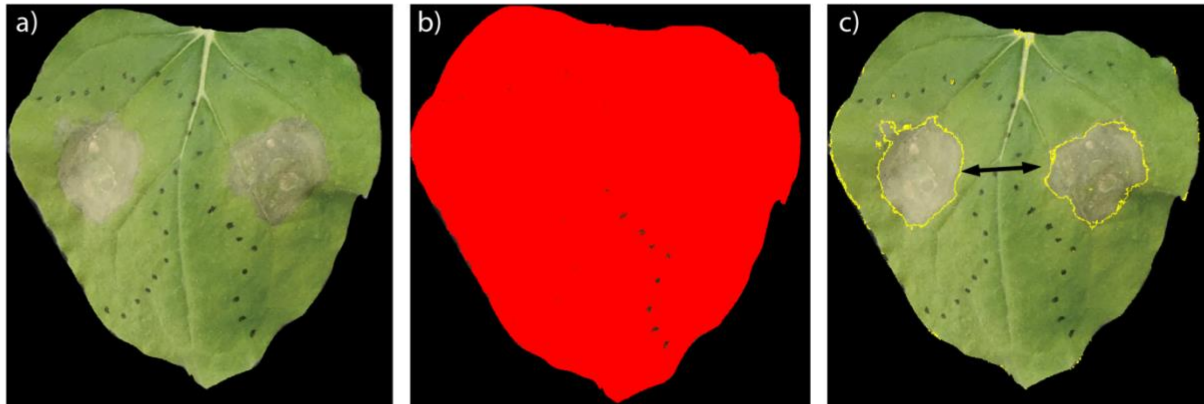



Figure 4: The lesion coverage calculation method: (a) A cropped leaf sample; (b) red-colour highlighted region represents the total leaf area after colour threshold correction in ImageJ; (c) yellow circled region indicated by arrowheads represents the calculated lesion area.

## 2.2. Transient expression of CRISPR/Cas9 gene-knockout construct

### 2.2.1. Designing CRISPR/Cas9 construct

The putative CRISPR/Cas9 target site for *FvCDIP1* corresponding to two sgRNAs (single guide RNAs) were designed using the online tool CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/). CRISPR-P 2.0 is an online platform for designing sgRNA with minimal off-target potentials for 49 plant species. This tool provides GC content, microhomology score and the secondary structure of the sgRNA for better accessing the sgRNA efficiency (Liu et al., 2017). The microhomology score estimates the frequency of out-of-frame indels at nuclease target sites (Bae et al., 2014). sgRNA with higher microhomology scores were chosen to obtain higher frequency of out of frame-indel mutations. Potential off-target sites with off-score ≤ 0.30 and targeting a smaller number of protein coding sequence were considered. Two 20 bp target regions adjacent to the PAM (5'- NGG), G-$N_{20}$-NGG, were chosen from Exon-1 (Table 11). Both of these target sites were close to the 5' of the coding sequences of the gene and at 91-bp distance apart from each other.

Table 11: List of guide RNA designed using online tool CRISPR-P 2.0. The GC% represents the guanine-cytosine abundance in the sgRNA. Microhomology score represents the frequency of out-of-frame indels at nuclease target sites for each sgRNA.

| Name | Sequence | GC% | Microhomology score |
|---|---|---|---|
| sgRNA1 | AGAGAACTACATCGTGTACG | 45 | 52.4 |
| sgRNA2 | CCATTATTAATATACCTCAC | 30 | 92.2 |

#### 2.2.1.1. Transformation of *E. coli*

A Met-2xgRNA backbone construct for the sgRNA cassette was provided by Tage Thorstensen (NIBIO, Ås). This Met-2XgRNA has previously been tested in transient expression of gene CRISPR-knockout construct of the *F. vesca* gene methyltransferase *MET1* (Haugland, 2018). This backbone construct along with two sgRNA was obtained in a chimeric vector (pMA-RQ::FvCDIP1_2XgRNA) from Invitrogen. Library Efficiency® DH5α™ (Invitrogen) chemically competent *E. coli* was transformed with pMA-RQ_FvCDIP1_2XgRNA plasmid to produce sufficient clones for subsequent cloning. The transformation procedure was similar to the technique previously described above. The transformed bacteria were plated and incubated on spectinomycin (75 µg/mL) selection LB plates overnight at 37°C.

#### 2.2.1.2. Analysis of *E. coli* transformants

Five random colonies were chosen for PCR analysis of the transformants. M13 primer sets (forward and reverse) that bind outside the insert were used to amplify the target DNA for PCR based analysis (Table 12).

Table 12: Setup for colony PCR of the *E. coli* transformants.

| Reagents | Volume | Program | |
|---|---|---|---|
| 10X Taq AB Buffer | 2 µL | 95°C | 5 minutes |
| dNTPs | 1.6 µL | 95°C | 1 minute |
| Forward Primer (M13_F) | 0.5 µL | 55°C | 30 seconds |
| Reverse Primer (M13_R) | 0.5 µL | 72°C | 1.5 minute |
| Amp. Taq Pol (5U/µL) | 0.1 µL | 72°C | 7 minutes |
| dH2O | 13.3 µL | 4°C | ∞ |
| Total:18 µL + 2 µL template DNA | | | |

PCR amplified products were loaded in 1.5 % agarose gel for visualisation. Based on the results, two colonies were chosen for culture to generate plasmids for restriction digestion. Bacteria were cultured in 20 mL LB broth containing spectinomycin (75 µg/mL) overnight at 37°C at 200 rpm in two 50 mL tubes.

### 2.2.1.3. **Plasmid isolation from *E. coli* transformants**

On the following day, 10 mL bacterial culture was precipitated for plasmid isolation. Plasmid isolation was performed using the QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany) as defined in the manufacturer's protocol.

### 2.2.1.4. **Restriction digestion and purification of plasmid**

Both the plasmid with guide RNA (pMA-RQ_FvCDIP1_2XgRNA) and the circular vector (pCas9-TPC) were cut with FastDigest *Pac*I (Thermo Scientific™) restriction enzyme to linearize the plasmid structure before the ligation (Table 13).

Table 13: Reaction setup for restriction digestion.

| Components | | PMA-RQ_sgRNA | pCas9_TPC |
|---|---|---|---|
| 10X Buffer *Pac*I | | 5 µL | 5 µL |
| Plasmid DNA (5 µg) | | 32 µL | 29.10 µL |
| Restriction enzyme (*Pac*I) | | 2.5 µL | 2.5 µL |
| $dH_2O$ | | 10.5 µL | 13.4 µL |
| | Total | 50 µL | 50 µL |

A total volume of 100 µL for each plasmid were prepared (i.e. two tubes each). The reaction mixtures were briefly mixed and incubated at 37°C for 4 hours. The reaction was heat inactivated at 65°C for 20 minutes. Restriction digested products with sticky ends were loaded in 0.7% agarose gel made in 10X TBE buffer and ran for 45 minutes at 100 V. One drop of Ethidium bromide (0.5 µg/mL) was added for visualization under a UV light source. Visual DNA bands were then cut, and plasmid DNA was cleaned using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG, Germany) kit according to the manufacturer's instructions.

### 2.2.1.5. **Dephosphorylation and sticky-end ligation**

The pCas9_TPC vector was treated with alkaline phosphatase (Thermo Scientific FastAP™ Thermosensitive Alkaline Phosphatase) to prevent the vector from re-circularisation during the ligation reaction. The alkaline phosphatase catalysed the release of 5'- and 3'- phosphate from DNA/RNA. The following components were mixed thoroughly and incubated for 10 minutes at 37° (Table 14). To inactivate the enzyme activity, the tube with reaction mixture was heated for 5 minutes at 75°C.

Table 14: Reaction condition for dephosphorylation of pCas9-TPC.

| Components | | Volume |
|---|---|---|
| Nuclease free $H_2O$ | | 4 µL |
| 10X Thermo Scientific$^{TM}$ FastDigest$^{TM}$ Buffer | | 2 µL |
| Plasmid DNA (1.8 µg) | | 12.5 µL |
| FastAP$^{TM}$ Thermosensitive Alkaline Phosphatase | | 1.5 µL |
| | Total | 20 µL |

After the dephosphorylation step, the linear vector was further cleaned using the NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG) kit, according to the manufacturer's instructions. To obtain the final CRISPR-construct (pCas9-TPC::FvCDIP1_2XgRNA) (Fig. 5-b), the insert DNA with the guide RNA scaffold cassette (FvCDIP1_2XgRNA) was ligated with the dephosphorylated linear vector plasmid (pCas9_TPC) facilitated by T4 DNA ligase from Thermo Fisher Scientific. Two different molar ratios (5:1 and 7:1) were used and the volume was calculated using the NEBioCalculator$^{TM}$ (https://nebiocalculator.neb.com/#!/ligation). Two molar ratios for ligation reaction were used to see the difference in the ligation efficiency and if one fails to yield proper ligation another could be used as a backup. The following components were added to complete the ligation and was kept at 16°C for 12 hours (Table 15).

Table 15: Reaction condition for ligation of pCas9-TPC and FvCDIP1_2XgRNA with two molar ratios.

| Components | | 5:1 | 7:1 |
|---|---|---|---|
| Insert DNA (FvCDIP1_2XgRNA) | | 1.25 µL | 1.76 µL |
| Linear Vector DNA (pCas9_TPC) | | 3.09 µL | 3.09 µL |
| 10X T4 DNA Ligase buffer | | 2 µL | 2 µL |
| Thermo Scientific T4 DNA Ligase | | 1 µL | 1 µL |
| $dH_2O$ | | 12.66 µL | 12.15 µL |
| | Total | 20 µL | 20 µL |

The reaction was inactivated at 70°C for 5 minutes. The ligation was confirmed by loading 8 µL of the ligation mixture on 1% agarose gel and visualised in UV-light. This confirmation was based on the expected difference in bands of the ligated product relative to the undigested empty pCas9_TPC vector.

#### 2.2.1.6. **Transformation of *E. coli* and analysis of transformants**

After visualization of successful ligation, heat shock transformation of Library Efficiency® DH5α™ (Invitrogen) chemically competent *E. coli* (50 µL) was carried out

using 50 ng of ligated plasmid (pCas9-TPC::FvCDIP1_2XgRNA). The procedure of transformation is described above. The culture (250 µL) was spread on two pre-warmed LB agar plates containing spectinomycin (50 µg/mL) at 37°C overnight. Seven random colonies from two different molar ratio plates were chosen for colony PCR. The PCR was carried out using two sets of primers: sgRNA1_F/sgRNA_R and TPC_F/ TPC_R targeting two different sites (Table 16).

Table 16: Setup for colony PCR of the *E. coli* transformants targeting sgRNA1 DNA segment.

| Reagents | | Volume | Program | |
|---|---|---|---|---|
| 10X Taq AB Buffer | | 2 µL | 95°C | 5 minutes |
| dNTPs | | 1.6 µL | 95°C | 30 seconds |
| Forward Primer (sgRNA1_F) | | 0.5 µL | 56°C | 30 seconds |
| Reverse Primer (sgRNA_R) | | 0.5 µL | 72°C | 30 seconds |
| Amp. Taq Pol (5U/µL) | | 0.1 µL | 72°C | 7 minutes |
| dH2O | | 13.3 µL | 4°C | ∞ |
| | Total | 18 µL + 2 µL | | |

Table 17: Setup for colony PCR of the *E. coli* transformants.

| Reagents | | Volume | Program | |
|---|---|---|---|---|
| 10X Taq AB Buffer | | 2 µL | 95°C | 5 minutes |
| dNTPs | | 1.6 µL | 95°C | 1 minute |
| Forward Primer (TPC_F) | | 0.5 µL | 56°C | 30 seconds |
| Reverse Primer (TPC_R) | | 0.5 µL | 72°C | 1.5 minutes |
| Amp. Taq Pol (5U/µL) | | 0.1 µL | 72°C | 7 minutes |
| dH2O | | 13.3 µL | 4°C | ∞ |
| | Total | 18 µL + 2 µL | | |

#### 2.2.1.7. **Plasmid isolation from *E. coli* transformants**

Two positive colonies from 7:1 molar ratio ligated plasmid were cultured in 20 mL LB broth containing spectinomycin (50 µg/mL) at 37°C overnight at 200 rpm. On the next day, 10 mL of the culture was taken for plasmid isolation using the QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. The concentration of the isolated plasmid was measured in NanoDrop™ 2000 spectrophotometer.

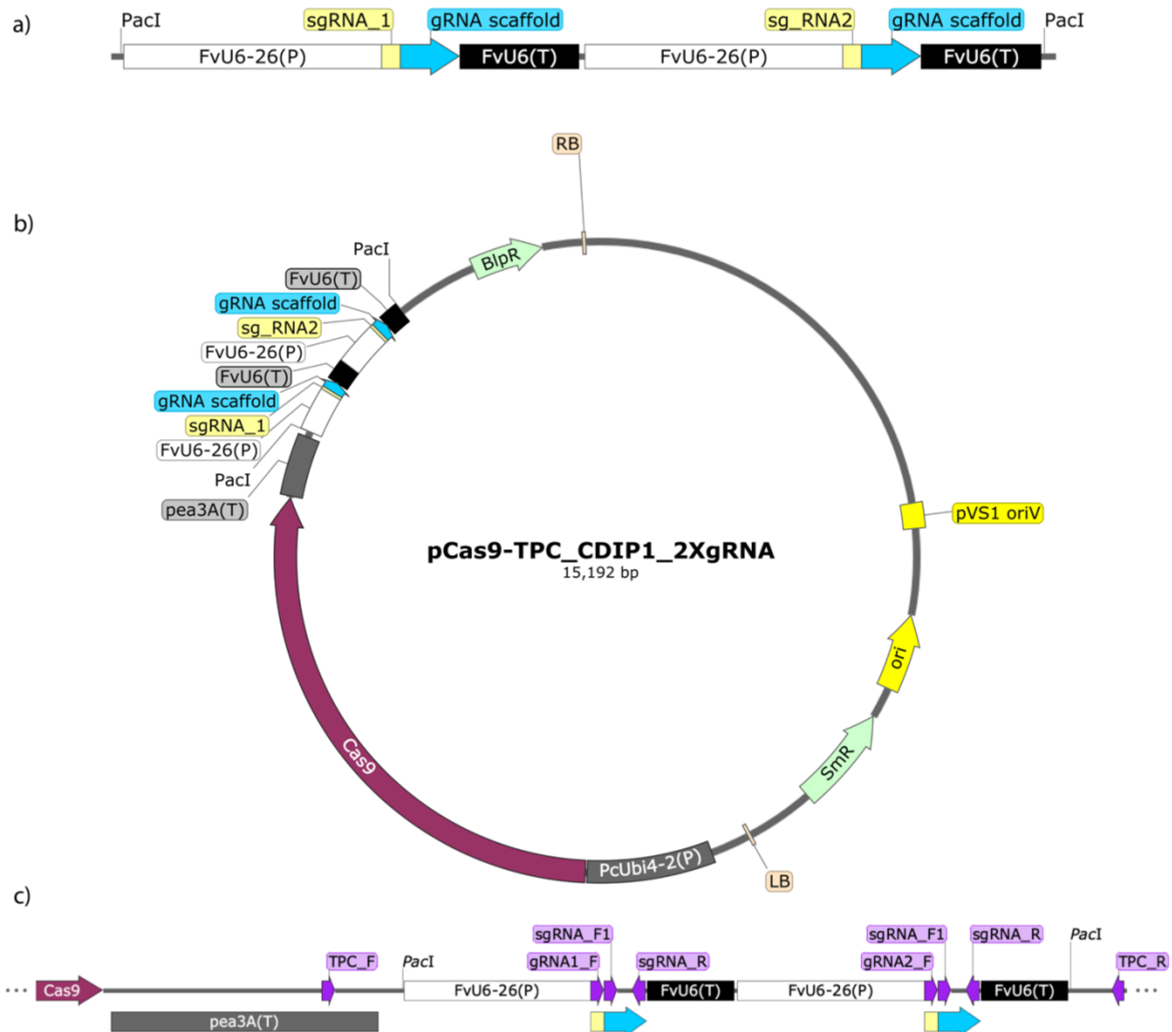



Figure 5: Schematic representation of the construct designed for disruption of the *FvCDIP1* gene. (a) Expression cassette with two guide RNAs (yellow); sgRNA_1 and sgRNA_2 followed by corresponding gRNA scaffold (blue) under the control of the FvU6-26 promoter. The cassette consisted of *Pac*I restriction sites on both ends to facilitate ligation; (b) The final CRISPR/Cas9 expression plasmid with ligated expression cassette. The *Cas9* gene (magenta) was controlled by the PcUbi4-2 promoter. The spectinomycin resistance gene (*SmR*) confered bacterial selection; (c) Eight different primer binding sites (purple) along the expression cassette.

### 2.2.2. ***Agrobacterium* transformation using CRISPR-construct**

Two different strains of *Agrobacterium* were taken for transformation: LBA4404 and AGL-1. Freeze-thaw transformation of chemically competent cells were carried out in the previously explained manner. In this transformation step, 50 µL of competent cells were used along with 1 µg of CRISPR-expression-plasmid (pCas9-TPC::FvCDIP1_2XgRNA). Transformed bacteria (250 µL) were plated on two different antibiotic selection LB-agar plates for individual *Agrobacterium* strains. Carbenicillin (50 µg/mL), chloramphenicol (25 µg/mL) and spectinomycin (100 µg/mL) were used

as selective agents for the AGL-1 strain, whereas rifampicin (20 µg/mL) and spectinomycin (70 µg/mL) were used for the LBA4404 strain. Analysis of transformants was performed by running a colony-PCR test on the obtained colonies. Ten random colonies for each of the bacterial strains were selected and amplified using specific primers. The same sets of primers were selected for PCR amplification which were used to screen positive *E. coli* colonies in the previous step (Table 18).

Table 18: PCR requirements for amplification of target DNA for the analysis of *Agrobacterium* transformants.

| Primer name | Amplicon size (bp) | Annealing temperature | Elongation time |
|---|---|---|---|
| sgRNA1_F | 95-bp<br>682-bp | 56°C | 30 seconds |
| sgRNA_R | | | |
| TPC_F | 1410-bp | 56°C | 1.5 minutes |
| TPC_R | | | |

### 2.2.3. *Agrobacterium*-mediated transient transformation of strawberry

Preparation of *Agrobacterium* resuspension media was done as previously described from an overnight culture. Healthy strawberry fruit (*Fragaria* × *ananassa* cv. Polka) grown under controlled environmental conditions were chosen for agroinfiltration. Fruits at two different stages: ripe (red) and unripe (white) were used. These fruits were rinsed four times by dipping in distilled water, and surface dried on a sterilised filter paper. The *Agrobacterium* solution was injected into the fruits with a 1 mL hypodermic needle in a manner that ensured an even distribution of the bacteria (Fig. 6-a). Most of the solution was injected from the stalk region into the hollow pith in a single attempt, while the remaining volume was injected from the other side, with minimum damage to the fruit tissue. The fruits were placed in the dark for 72 hours before examining them.

Two months old Bukammen leaves, grown *in*-*vitro* in MS-agar media without growth regulators, were used for vacuum infiltration. Leaves were submerged in 30 mL of *Agrobacterium* suspension inside a glass beaker. The air from the glass chamber was sucked out, and this condition was maintained for 25 minutes (Fig. 6-c). The pressure valve was slowly released, allowing the air to enter into the chamber. Soaked leaves were pat dried with sterilised filter paper and placed on MS media at a 14 hours light/10 hours dark cycle at 22°C.

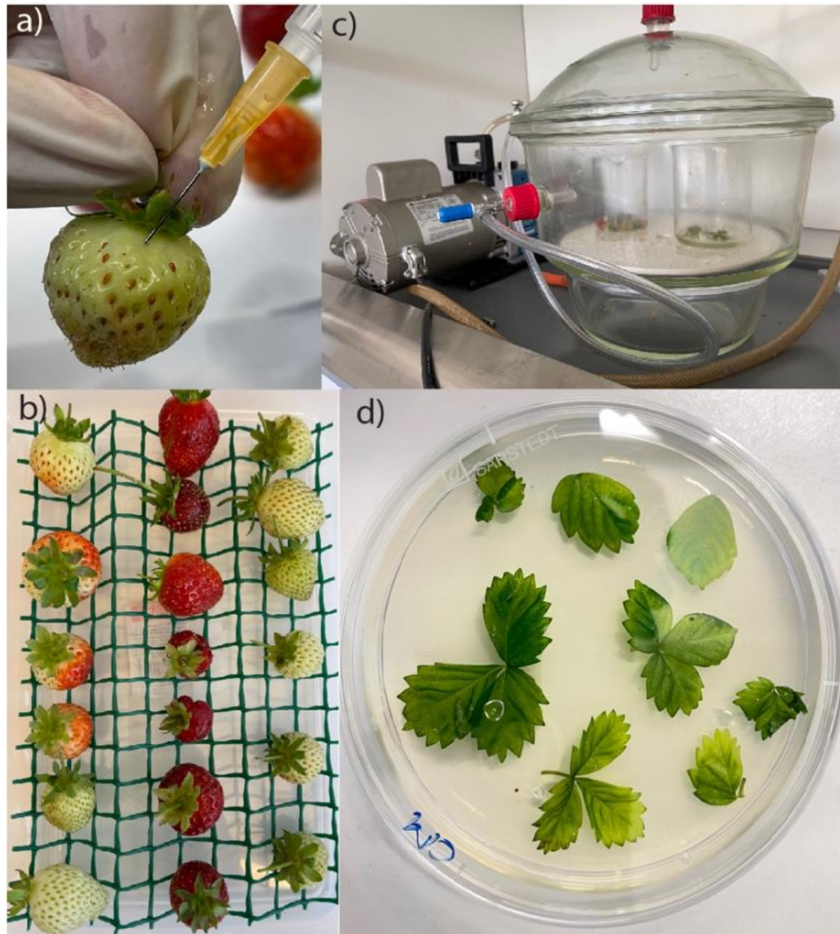



Figure 6: *Agrobacterium* mediated transient expression of the CRISPR/Cas9 knockout cassette in strawberry fruits and leaves. (a) Agroinfiltration of the strawberry fruit (cv. Polka) with a hypodermic needle near the stalk region; (b) Arrangement of inoculated fruit samples on a malleable plastic mesh before incubation in the dark (left and right columns; unripe fruits, middle column; ripe fruits); (c) Vacuum infiltration of *F. vesca* (genotype Bukammen) leaves inside a suction chamber; (d) Vacuum infiltrated leaves of *F. vesca* (genotype Bukammen) plated on MS medium.

### 2.2.4. **Total RNA isolation, cDNA synthesis and RT-PCR**

Agroinfiltrated strawberry fruit samples along with the vacuum infiltrated leaves were crushed and made into fine powder in liquid Nitrogen using mortar and pestle. Fruit and leaf samples were kept at -80 °C before RNA isolation. One hundred mg of the fine powder was placed in a 2 mL microcentrifuge tube, and RNA isolation was performed using a similar protocol as described before. The RNA obtained with on-column DNA digestion was further purified to eliminate any trace of DNA using a DNase I, RNase-free (Thermo Scientific$^{TM}$) kit, according to the manufacturer's protocol. The RNA obtained after purification was used to synthesize the cDNA using RT-PCR. Gene expression analysis was performed using the cDNA as a template for target DNA fragments viz; 1) Cas9 2) sgRNA_1 and 3) sgRNA_2. Gene specific primer

were used for the PCR amplification of the target region (Table 19). PCR amplified products were visualised in 1.5% agarose gel.

Table 19: PCR requirements for amplification of target DNA for expression analysis of the CRISPR cassette after transient expression.

| Primer name | Target | Amplicon size (bp) | Annealing temperature (°C) | Elongation time |
|---|---|---|---|---|
| Cas9_RTF | Cas9 | 259 bp | 60°C | 30 seconds |
| Cas9_RTR | | | | |
| sgRNA1_F | sgRNA1 | 95 bp | 56°C | 30 seconds |
| sgRNA_R | | | | |
| sgRNA2_F | sgRNA2 | 95 bp | 56°C | 30 seconds |
| sgRNA_R | | | | |

## 2.3. Plant transformation using *Fragaria vesca* gene (*FvCDIP1*)

Plant transformation of *N. benthamiana* was according to the transformation protocol described by Pathi et al. (2013) with a few modifications. The gene *FvCDIP1*, was previously cloned into the *Agrobacterium* strain LBA4404 before the start of this thesis and was provided for this experiment by Anupam Gogoi (NMBU). For this purpose, a complementary DNA reverse transcript generated from total RNA of *F. vesca* genotypes resistant to *P. cactorum* was used as an insert into the plant expression vector.

### 2.3.1. Preparation of explants

*Nicotiana benthamiana*, susceptible *Fragaria vesca* genotype NCGR1218 and susceptible *Fragaria × ananassa* cv. Polka were used for *Agrobacterium* mediated stable plant transformation (Fig. 7).

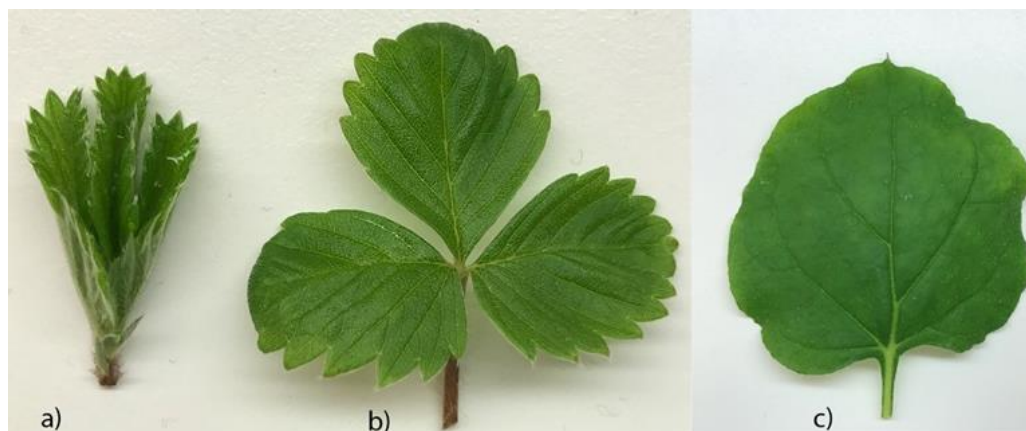



Figure 7: Selection of 1-2 month old leaves for plant transformation. (a*) Fragaria vesca* (b) *Fragaria* x *ananassa* (c) *Nicotiana benthamiana*

Healthy leaves were taken from plants grown under controlled environmental conditions. Leaves were washed in running distilled water for few minutes. The leaves were then washed with commercial bleach (NaOCl, 0.6%) supplemented with 1 drop of Tween 20 per 50 mL water in a beaker. The leaves were allowed to soak in the washing solution for approximately 5 minutes. The leaves were then rinsed four times with distilled water and dried in a sterilized filter paper. Later, circular cuts of 1 cm diameter were made in the leaves, using a leaf puncher, avoiding petiole, midrib, and borders.

### 2.3.2. **Inoculation of explants with *Agrobacterium***

Before the start of this research, the *FvCDIP1* gene had been cloned into the plant expression vector *pGWB454.* A glycerol stock of *Agrobacterium* cells carrying the gene of interest was cultured overnight at 28°C in 220 rpm in a flask with 50 mL LB media containing spectinomycin (75 µg/mL) and rifampicin (25 µg/mL). The preparation of bacterial suspension in a resuspension buffer was carried in a similar manner as previously described. The resuspension buffer of 500 mL consisted of; 2.2 g MS-including vitamins, 15 g sucrose and 200 µL acetosyringone (200 µM). After induction of the *Vir*-genes, the circular leaf disks were placed in a Petridish containing the *Agrobacterium* construct with the gene of interest and suspended in MES resuspension medium. Explants were thoroughly dipped, and an even soaking was assured by continuous shaking of the inoculated leaves in the Petridish. The explants of *Fragaria* were slightly injured with a scalpel to provide infection sites for the bacteria. Non-inoculated leaf disks with no selective agent were used as positive control to check the efficiency of the regeneration medium. Similarly, non-inoculated leaf disks with selective agents were used as negative controls to see the efficiency of the antibiotics.

### 2.3.3. **Plating of inoculated explants**

The explants were dried one last time on sterilized filter paper and placed in specific co-cultured MS-media (Table 20) for two days, with the adaxial side up in dark conditions at room temperature. Six to nine explants were placed in each plate, and the plates were sealed with parafilm. After two days, explants were transferred to the selection media with appropriate antibiotics and hormones (Table 20).

Table 20: Composition of two different selection media used for plant transformation.

| Components | Selection Media | Co-culture Media | Components | Selection Media | Co-culture Media |
|---|---|---|---|---|---|
| | (Strawberry) | | | (Tobacco) | |
| MS including Vitamins | 4.4 g/L | 4.4 g/L | MS including Vitamins | 4.4 g/L | 4.4 g/L |
| Sucrose | 30 g/L | 30 g/L | Sucrose | 30 g/L | 30 g/L |
| Agar | 8 g/L | 8 g/L | Agar | 8 g/L | 8 g/L |
| BAP | 3 mg/L | 3 mg/L | BAP | 1 mg/L | 1 mg/L |
| IBA | 0.2 mg/L | 0.2 mg/L | NAA | 1 mg/L | 1 mg/L |
| Carbenicillin | 250 mg/L | - | Carbenicillin | 250 mg/L | - |
| Kanamycin | 75 mg/L | - | Kanamycin | 75 mg/L | - |
| Timentin | 50 mg/L | - | Timentin | 50 mg/L | - |

The explants were subsequently moved to newly prepared selection media with appropriate antibiotics every two weeks. However, the failure in optimisation of transformation protocol for strawberry led to a decision to discard the strawberry explants. Therefore, only *N. benthamiana* was continued in the plant transformation experiment.

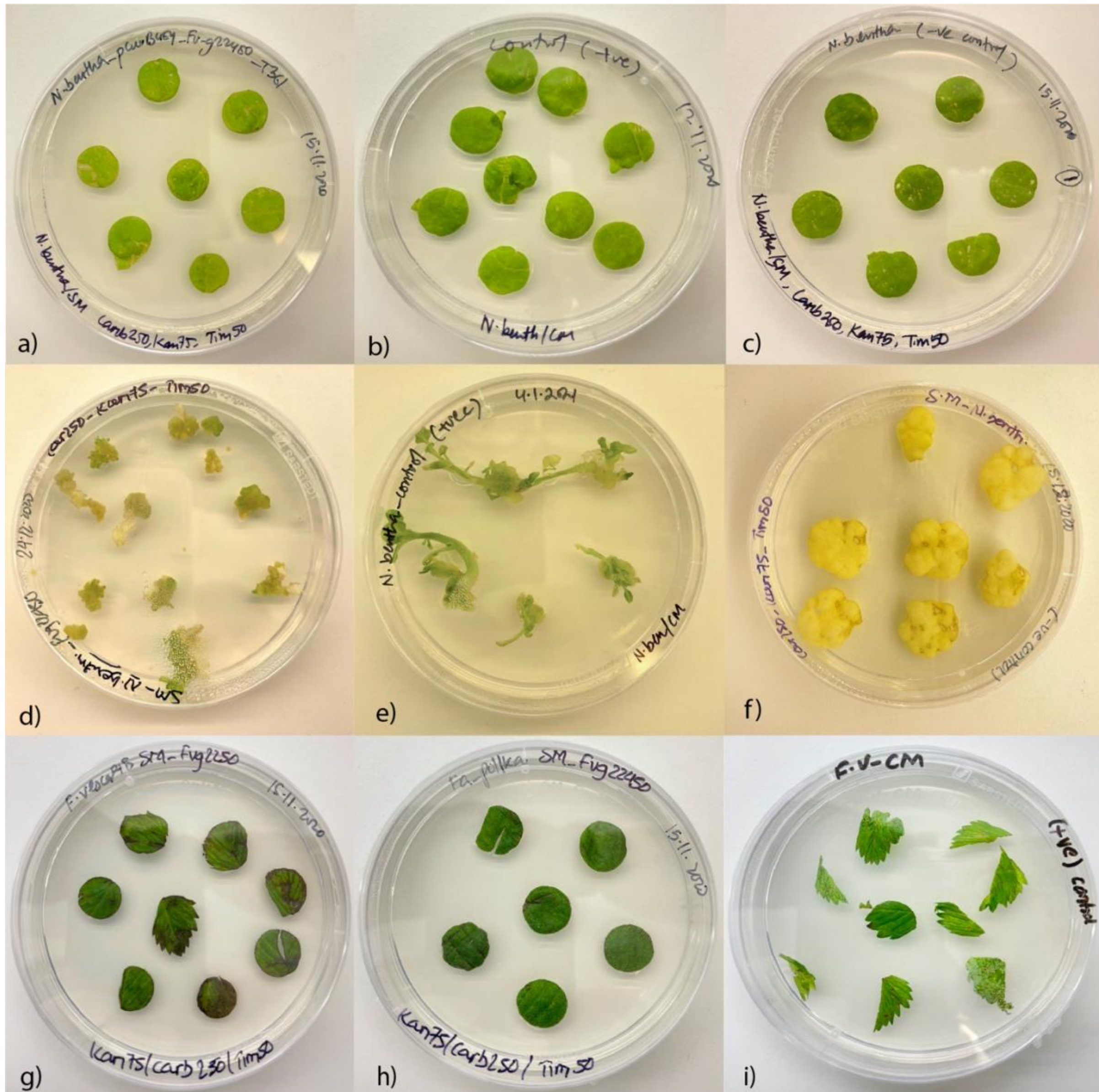



Figure 8: The treatments for different plant materials used in plant transformation. Explants of *Nicotiana benthamiana* (top two rows) plated on different media after co-cultivation. *N. benthamiana* explants transformed; (a) with *Agrobacterium* carrying pGWB454_FvCDIP1 on selection media with appropriate antibiotics, (b) without *Agrobacterium* inoculation and antibiotics on co-culture media; positive control, (c) without bacteria inoculation on selection media with antibiotics; negative control. (d) Callus formed in *N. benthamiana* explants after one month in selection media. (e) Shoot emergence on positive control explants after 1.5 months of transformation (f) Explants showing cell death due to antibiotics for plant selection. Explants of *F. vesca* genotype 1218 (g) and *F. × ananassa* cv. Polka (h) transformed with *Agrobacterium* carrying pGWB454_FvCDIP1 and (i) *F. vesca* explants without *Agrobacterium* inoculation and antibiotics on co-culture media; positive control.

### 2.3.4. **Transfer to Rooting media**

Regenerated shoots were placed in 70 mL MS rooting media (RM) in Magenta boxes after carefully removing excess callus tissue while leaving sufficient bulbous parts in the rooting region to allow root proliferation. The rooting media included MS with

vitamins (4.4 g/L), sucrose (30 g/L), agar (5 g/L), cefotaxime (250 mg/L) and kanamycin (75 mg/L).

### 2.3.5. **Hardening for *ex vitro* adaptation**

Plants with fine hair-like roots were carefully removed from the MS-growth media and rinsed thoroughly using tap water to get rid of growth media. The plants were placed in wet substrate into a pot with complete burial of the newly emerged roots. Plants were covered with transparent plastic film to ensure a humid condition around its canopy.

### 2.3.6. **Analysis of transformants**

100 mg of ground plant materials from each plant were used for genomic DNA isolation. DNA was isolated following the DNeasy® Plant Mini Kit (QIAGEN®) defined protocol. Genomic DNA was used as a template to carry out PCR to test for presence of the heterologous gene (*FvCDIP1*) (Table 21). Verification of the quality of the gDNA with a PCR on the housekeeping gene *EF1α* (elongation factor gene-1α) was performed as an internal control (Table 21).

Table 21: PCR requirements for amplification of target DNA for the analysis of transgene in FvCDIP1-N. benthamiana.

| Primer name | Target | Amplicon size (bp) | Annealing temperature (°C) | Elongation time |
| --- | --- | --- | --- | --- |
| EF1α_NB_F | EF1α | 116 bp | 56 | 30 seconds |
| EF1α_NB_R | | | | |
| GW_FvCDIP1_F | FvCDIP1 | 1363 bp | 60 | 1.5 minutes |
| GW_ FvCDIP1_R | | | | |

After confirming successful integration of the transgene, further analysis for the verification of the gene expression was conducted. For this, total RNA was isolated using a similar protocol as mentioned above and 1000 ng of RNA was used to synthesize cDNA via RT-PCR. To check the expression of FvCDIP*1* gene*,* a PCR on the obtained cDNA was performed using gene specific primers (Table 22).

Table 22: RT-PCR requirements for amplification of the target DNA for expression analysis of FvCDIP1-*N. benthamiana*.

| Primer name | Target | Amplicon size (bp) | Annealing temperature (°C) | Elongation time |
| --- | --- | --- | --- | --- |
| FvCDIP1_RT_F | FvCDIP1 | 177 bp | 55 | 30 seconds |
| FvCDIP1_RT_R | | | | |

### 2.3.7. Determination of transcript level of PR-genes

To investigate the relative expression of defence genes associated with signal transduction, a qRT-PCR (quantitative RT-PCR) was performed on the *FvCDIP1* overexpressing transgenic and wild type *N. benthamiana* plants. For this, three transgenic plants were chosen. Relative expression of four different defence associated genes: *Pathogenesis related-1a* (*PR-1a)*, *Pathogenesis related-5 (PR-5)*, *Plant defensin1.2* (*PDF 1.2*) and *Enhanced Disease Susceptibility 1* gene (*EDS1)* were observed. An equal concentration of total RNA and cDNA was maintained. Target gene specific primers were used (Supplementary Table 1). A constitutively expressed reference gene, *EF1α,* was used as an indigenous control to calculate relative transcriptional level. qRT-PCR was performed in a BIO-RAD qPCR machine in a reaction volume of 10 µL using SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) under the following program: initialisation at 95°C for 30 seconds followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 30 seconds. A melting curve from 65°C to 95°C was established. Three biological replicates were used for each of the treatments and control. The $2^{-\Delta\Delta CT}$ method was used for the quantification of the relative expression of the PR-genes.

# 3. Results:

## 3.1. Transient gene expression of the *Wall Associated Kinase* gene

Expression of *WAK* gene in *N. benthamiana* was mediated through *Agrobacterium*. The *WAK* gene was inserted into the plant expression vector pGWB454, using Gateway cloning which resulted in pGWB454::WAK construct.

### 3.1.1. Generation of the entry construct

To begin the Gateway cloning, *attB* attachment sites were generated by PCR amplification with Gateway primers (Gw_WAK_F and Gw_WAK_R) using a cDNA template obtained from Anupam Gogoi (NMBU). A PCR product with the expected band size of 2476-bp was obtained (Supplementary Fig. 1). The band was excised, and purified, followed by a BP recombination reaction. During the BP recombination reaction, the region between *attP* sites of the donor vector is replaced by the *attB* flanked PCR product (Fig. 2-a) yielding entry constructs. These obtained entry constructs were transformed into chemically competent *E. coli* cells. The transformed

*E. coli* were selected on LB agar plates containing appropriate antibiotic. The obtained transformants were verified by performing a colony PCR from randomly picked colonies. Bands of the expected size (2476 bp) were observed in all the selected colonies (Fig. 9), thus confirming the *E. coli* transformation. Plasmids were isolated from the verified transformants and sent for sequencing along with five different primers that covered the entire length gene of the *WAK* gene. The sequencing data from the isolated plasmid verified the correct orientation of the *WAK* gene in the recombinant plasmid (Fig. 10).

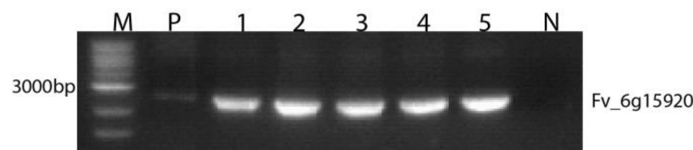



Figure 9: Colony PCR result of *E. coli* cells carrying entry construct. PCR products obtained after amplification of WAK fragment from plasmid DNA of five random *E. coli* colonies using GW_WAK_F and GW_WAK_R*.* The expected band size if 2476-*bp.* (P) Positive plasmid control. The positive control used is the attB-flanked PCR amplified gene product*,* (N) Negative control without template DNA. (M) 100-bp standard molecular weight marker

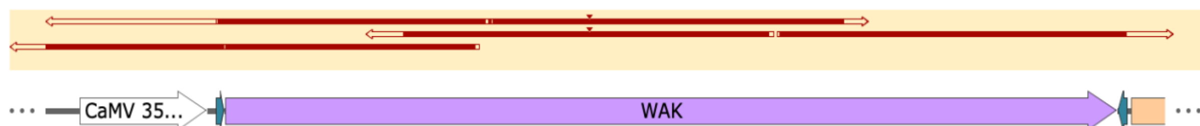



Figure 10: Aligned sequence reads using five different primers to cover the target WAK gene**.** The red coloured lines with arrowhead represent the sequencing data for individual primer. The inconsistencies in the sequence are represented as gaps in the red coloured primers' coverage bar*.* The regions with discrepancies are compensated by a correct read of one of the other sequencing results. The WAK gene (purple) is cloned downstream of CaMV 35S promoter (white).

### 3.1.2. **Generation of the expression construct**

Transformation of the sequence-verified entry construct pDONR::WAK was recombined into the destination vector (pGWB454), generating the expression construct pGWB454::WAK. During the LR recombination reaction, the region between the *attR* sites of the destination vector including the toxic *ccdB* gene is replaced by the *attL* sites of the entry construct, yielding an expression construct (Fig. 2-b). The expression construct was then used for transforming *E. coli* cells. The transformed *E. coli* cells were grown on selection media. Colony PCR was performed on randomly selected colonies using WAK_P2F and WAK_P2R primers targeting *WAK* fragment. The PCR results confirmed that seven out of eight colonies had a band (818-bp) corresponding in size to the band of the positive plasmid control (Fig. 11).

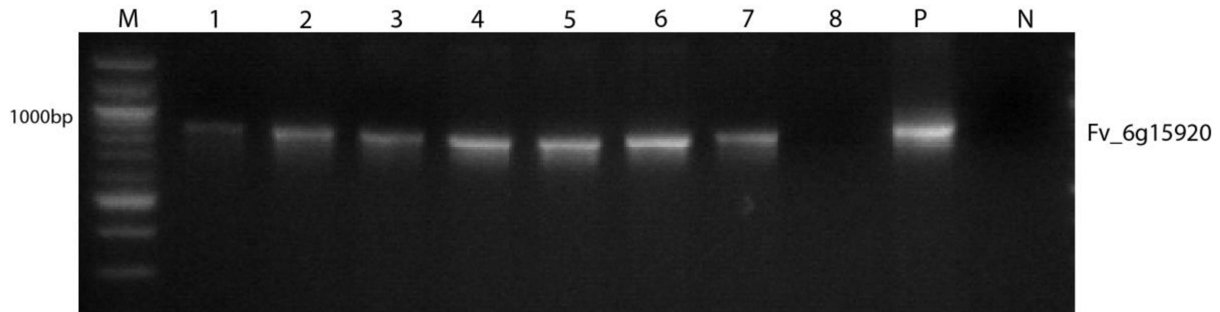


Figure 11: Agarose gel electrophoresis of colony PCR products from *E. coli* transformed with the expression construct (pGWB454::WAK)**.** The expected amplicon size is 818-bp. (P) Positive plasmid control. (N) Negative control without template DNA, (M) 100 bp standard molecular weight marker.

The verification of positive clones from the colony PCR was followed by the isolation of plasmid DNA from positive *E. coli* transformants and sequencing of these. The sequencing results confirmed that the WAK gene was successfully cloned downstream of the CaMV 35S promoter region.

### 3.1.3. **Transformation of *Agrobacterium* cells**

*Agrobacterium* (LBA4404) was transformed with the sequence verified expression plasmid pGWB454:: WAK. The transformation mixture was spread onto LB agar plates with appropriate antibiotics for selection of transformed cells. Numerous colonies were obtained, and colony PCR was performed on ten randomly selected colonies, using WAK_P2F and WAK_P2R primers. The PCR product of the transformed *Agrobacterium* carrying plant expression vector was analysed on agarose gel (Fig. 12), and all the colonies (1 to 10) yielded a band of the expected size (818 bp), indicating a successful transformation of expression plasmid.

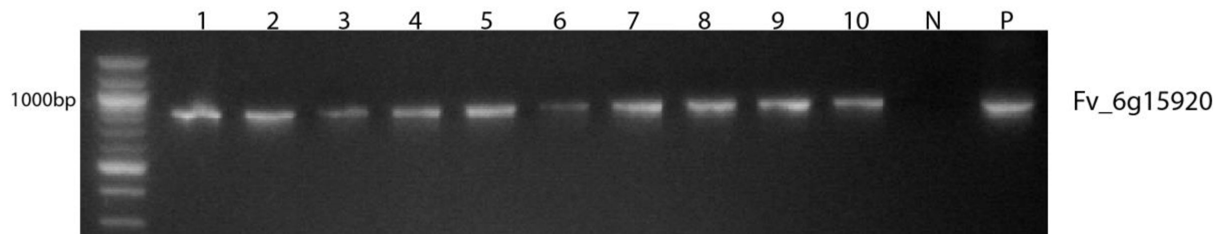


Figure 12: Result of gel electrophoresis of colony PCR of *Agrobacterium* (LBA4404*)* with expression construct (pGWB454::WAK). PCR amplified products obtained from the amplification of WAK gene region. PCR was performed on 10 plasmid DNA samples of randomly chosen bacterial colonies using WAK_P2F and WAK_P2R primers with an amplicon size of 818-bp. A presence of similar band on all the tested colonies corresponds with the positive plasmid control (P). The positive control used is the PCR amplified plasmid verified with Sanger sequencing. (N) Negative control without template DNA, (M) 100 bp standard molecular weight marker.

### 3.1.4. **Transient gene expression in *N. benthamiana***

To explore the role of the *WAK* gene, *Agrobacterium* with the plant expression plasmid pGWB454::WAK was infiltrated in *N. benthamiana* leaves. To verify that the *WAK* gene was transiently expressed, total RNA was isolated and tested using gel-based RT-PCR. The RNA was isolated from the infiltrated *N. benthamiana* leaves at four different time points (0, 48, 72 and 96 hours post infiltration). Gene specific primers WAK_RTF and WAK_RTR targeting the *WAK* gene with an expected amplicon size of 160-bp, were used. The RT-PCR showed that the WAK gene is transiently expressed in agroinfiltrated *N. benthamiana* plants. The gene was expressed only in the 48 hpi and 72 hpi *Agro*-infiltrated plant samples (Fig. 13). An absence of band for RNA samples further verified the results.

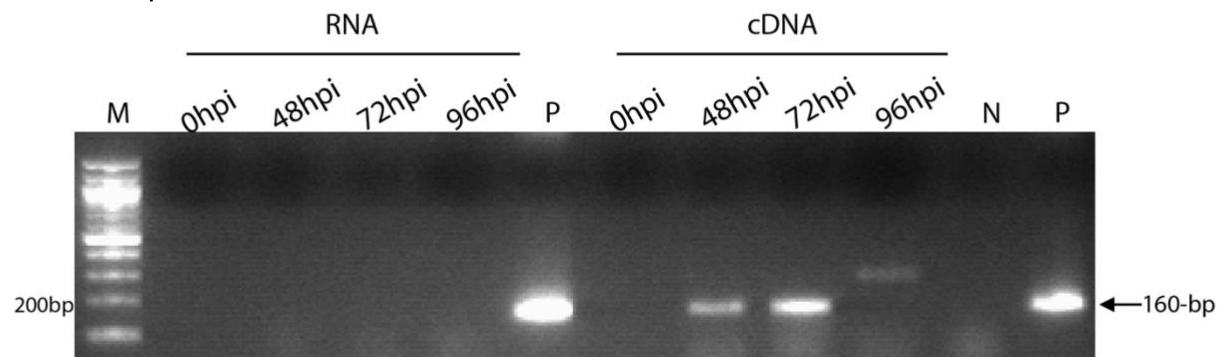



Figure 13: Results from transient expression analysis of the *WAK* gene in *Nicotiana benthamiana* leaves. RT-PCR products obtained after infiltration of *Agrobacterium tumefaciens* harbouring WAK gene construct in *N. benthamiana.* Samples were harvested at four different time points (0hpi, 48hpi, 72hpi and 96hpi). Gene specific short primers (WAK_RTF and WAK_RTF) targeting a fragment of the *WAK* was used. The expected band size is 160-bp. (P) Positive plasmid control (M) 100 bp standard molecular weight marker, (N) Negative control without template DNA.

### 3.1.5. **Expression of *WAK* reduce susceptibility of *Nicotiana benthamiana* to *P. cactorum***

To investigate the effect of the transiently expressed *WAK* gene on defence in *N. benthamiana*, the leaves were inoculated with *P. cactorum* zoospores and disease severity was assayed at 5 days post inoculation A visual assessment of the inoculated leaves showed that the disease severity in leaves expressing WAK were lower than in the control leaves (Fig. 14). The average lesion coverage for the WAK-expressing leaves was 16 % whereas for mock controls it was 32.6% (Fig. 15). However, there was no significant difference between the two treatments (student t-test *P*-value = 0.72).

Figure 14: Effect of transient expression of *WAK* gene in *Nicotiana benthamiana* on infection with *P. cactorum*. The photographs were taken five days after zoospore inoculation. (a) *N. benthamiana* leaves with transient expression of *WAK* gene; (b) mock control.

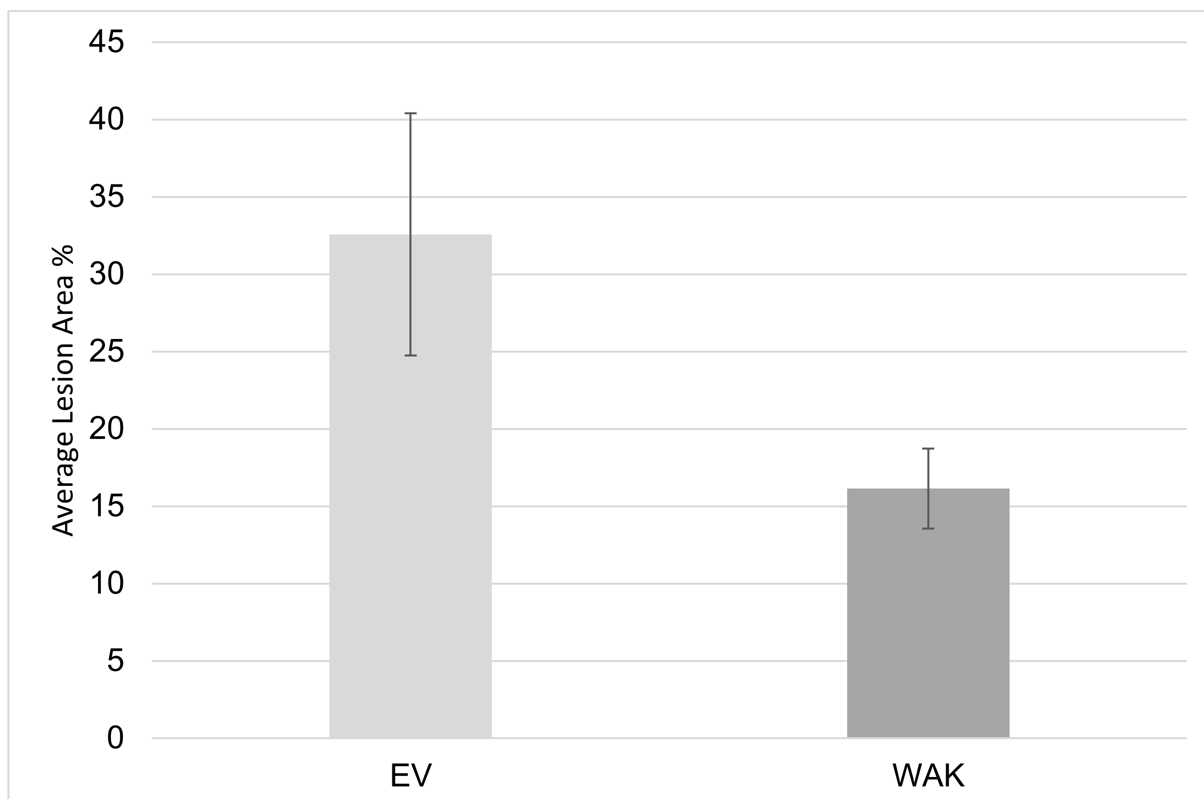



Figure 15: The average lesion area of *Nicotiana benthamiana* leaves transiently expressing *WAK* gene, after inoculation with *Phytophthora cactorum*. Leaves of *N. benthamiana* were agroinfiltrated with either empty vector (EV) or with an expression vector carrying the WAK gene. Pathogen inoculated leaves were imaged under visible light 5 days post inoculation. Each bar represents the average lesion area in % of total leaf area after inoculation with *P. cactorum*. The error bars represent the standard error of the mean. Statistical analysis was performed by Student *t*-test to determine whether the observed difference was statistically significant. (P=0.072).

## 3.2. CRISPR/Cas9 mediated knockout of *FvCDIP1*

### 3.2.1. Generation of the CRISPR-construct

The chimeric plasmid (pMA-RQ::FvCDIP1_2XgRNA) was transformed into *E. coli*. The transformed *E. coli* cells were confirmed by colony PCR. Amplified PCR products were detected from all five randomly picked bacterial colonies (Supplementary Fig. 2). Plasmids from subsequently generated clones of transformed *E. coli* cells and the desired plant expression vector pCas9-TPC, were digested using the restriction enzyme *Pac*I. The digested products were visualised after agarose gel electrophoresis, excised, and purified to perform a subsequent ligation. The ligated product obtained after the ligation of *Pac*I digested plant expression vector (pCas9-TPC) and *Pac*I digested expression cassette (FvCDIP1_2XgRNA) was visualised to confirm ligation. This confirmation was based on the comparison of visible bands from

the ligated product with the undigested pCas9-TPC vector without the insert. The final construct, pCas9-TPC::FvCDIP1_2XgRNA (Fig. 5-b), containing the PcUbi (*Petroselinum crispum* Ubiquitin) promoter for expression of Cas9 and the AtU6-26 (*Arabidopsis thaliana* U6-26) promoter for FvCDIP1_2XgRNA was obtained. Chemically competent *E. coli* cells were transformed with the ligated product (pCas9-TPC::FvCDIP1_2XgRNA). Ligation mixtures with both molar ratios were used for the transformation. The confirmation of *E. coli* transformation was later done by a colony PCR from the randomly picked colonies. Two different primers (TPC_F/TPC_R and sgRNA1_F/sgRNA_R) targeting two different DNA segments were used for the PCR-screening of the putative positive colonies. Expected bands of 1410-bp were observed from PCR products amplified with TPC_F/TPC_R (Fig. 16 upper panel). Along with this, an unspecified band at ca. 1000-bp was also observed. Empty plasmids lacking the expression cassette are seen with the band size of 235-bp (Fig. 16 upper panel).

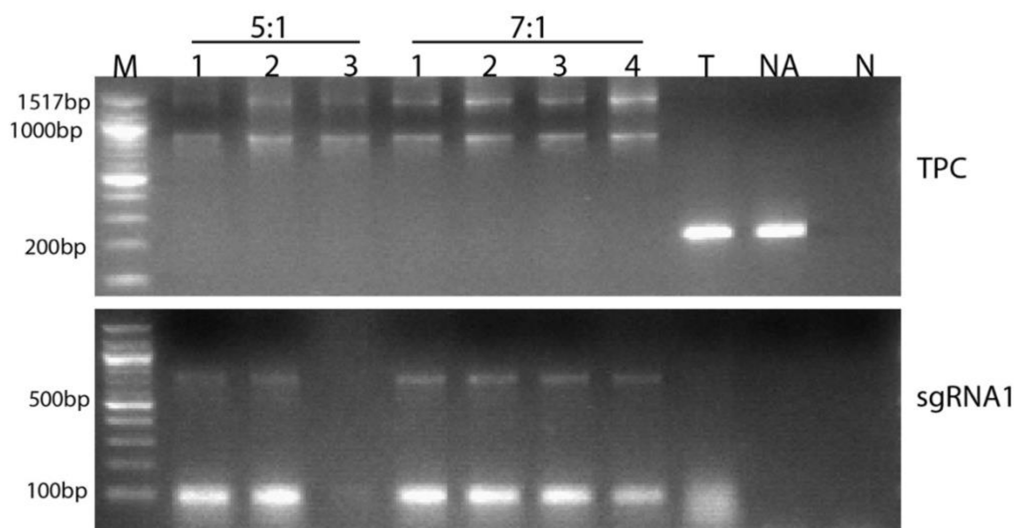



Figure 16: Agarose gel electrophoresis of colony PCR products from *E. coli* transformants. PCR analysis for the confirmation of transformation of *E. coli* by amplification of plasmid DNA using two different primer sets TPC_F/TPC_R (upper panel) and sgRNA1_F/sgRNA_R (lower panel). Lanes 1-3 are PCR products from ligated plasmid (pCas9-TPC::FvCDIP1_2XgRNA) with 5:1 molar ratio while lanes 4-7 represent ligated plasmids with 7:1 molar ratio. The expected band size is 1410-bp (upper panel. Lower panel; amplified products of sgRNA1 DNA segment. The expected band sizes are 95-bp and 682-bp. T (empty plasmid; pCas9_TPC), NA (digested empty plasmid without alkaline phosphatase treatment) (N) Negative control without template DNA, (M) 100-bp standard molecular weight marker.

The PCR products obtained from the amplification DNA segment of sgRNA1 with sgRNA1_F/sgRNA_R primers gave two different bands at 95-bp and 682-bp (Fig. 16 lower panel) because of two binding sites for the reverse primer sgRNA_R (Fig. 5-C). Faint band was observed for lane 3 of the 5:1 molar ratio ligated plasmid due to a pipetting error. The detection of bands with expected sizes (Fig. 16) from the gel image

suggested the presence of the finalised CRISPR/Cas9 expression cassette pCas9-TPC::FvCDIP1_2XgRNA in the selected colonies of *E. coli*.

### 3.2.2. **Verification of *Agrobacterium* transformation with pCas9-TPC::FvCDIP1_2XgRNA**

After the confirmation of *E. coli* transformation, plasmids were isolated from cultured *E. coli* cells and transformed into chemically competent *Agrobacterium* cells (strains LBA4404 and AGL-1). After transformation, *Agrobacterium* cells were plated and grown overnight with appropriate antibiotics and the colonies were screened using

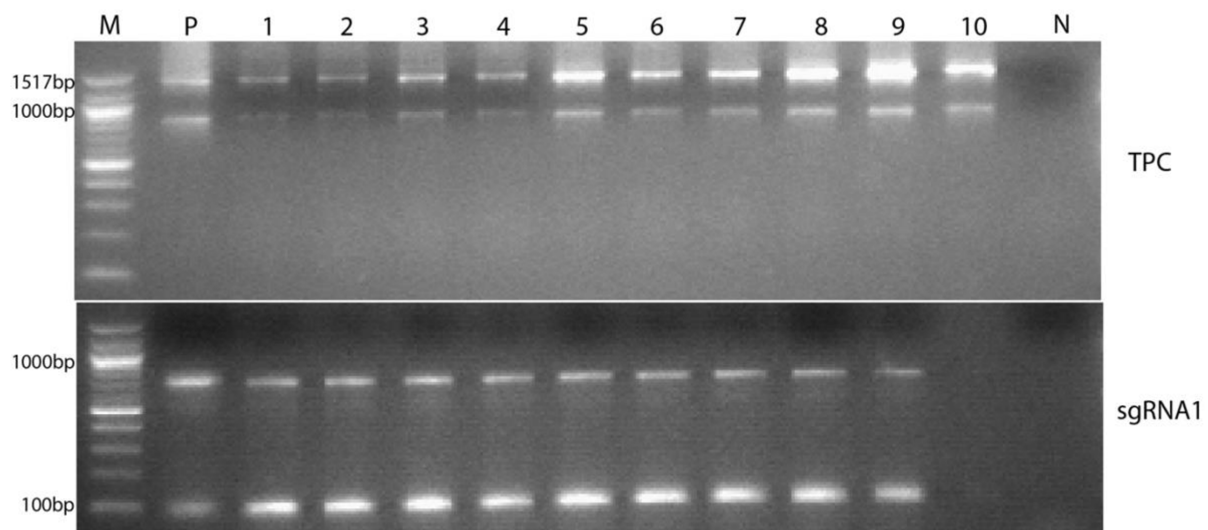



Figure 17: Agarose gel electrophoresis of colony PCR products from of *Agrobacterium* transformants. PCR analysis for the confirmation of transformation of *Agrobacterium (LBA4404)* by amplification of plasmid DNA using two different primer sets TPC_F/TPC_R (upper panel) and sgRNA1_F/sgRNA_R (lower panel). The expected band size is 1410-bp. Lower panel; the expected bands sizes are 95-bp and 682-bp. No PCR product was obtained in lane 10 due to pipetting error. (M) 100-bp standard molecular weight marker, (P) positive plasmid control, (N) Negative control without template DNA.

PCR with the primer sets TPC_F/TPC_R and sgRNA1_F/sgRNA_R. Expected bands of 1410-bp were observed in all the selected colonies which corresponded with the bands from the positive plasmid control (Fig. 17 upper panel). As with the *E. coli* transformants, an unspecified band of ca. 1000-bp was observed in the colony PCR of *Agrobacterium* transformants. As the reverse primer (sgRNA_R) had two binding sites, the PCR products obtained with sgRNA1_F/sgRNA_R primers gave two distinct bands at 95-bp and 682-bp (Fig. 17 lower panel). In lane 10 (Fig. 17 lower panel) no PCR amplification was observed due to a possible pipetting error. Bands of expected sizes were detected for both the primer pairs, proving the presence of the construct in all the selected colonies (Fig. 17). A similar confirmation was made for AGL-1 (Supplementary Fig. 3).

### 3.2.3. Confirmation of expression of CRISPR/Cas9 and FvCDIP1_2XsgRNA.

In order to study the performance of the pCas9-TPC::FvCDIP1_2XgRNA, transient transformation of strawberry fruits cultivar Polka and leaves of *F. vesca* genotype Bukammen was performed using of *Agrobacterium* (LBA4404) harbouring the construct. The vacuum infiltrated leaves of Bukammen, plated on MS-medium were green and viable at 48-hours post inoculation (hpi) (Fig 6-d). Whereas for the fruits, a slight change in pigmentation was observed. Greenish/white fruits developed red pigmentation at 48-hpi (Fig. 18).

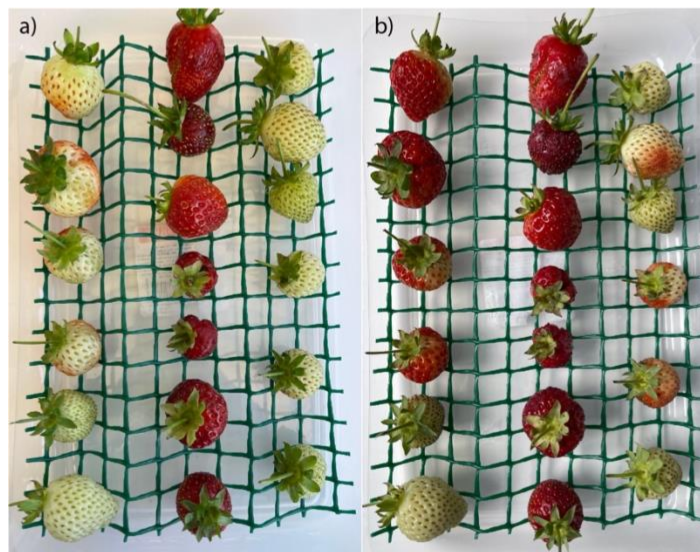



Figure 18: **Visual observation of *Agrobacterium* inoculated strawberry fruits with CRISPR/Cas9 expression cassette.** Twelve unripe fruits including seven ripe fruits were inoculated. A slight change in pigmentation is visible between the inoculated strawberries at 0 hpi (a) and 48 hpi (b).

For evaluating the expression of the CRISPR construct, gel-based RT-PCR was performed on the cDNA synthesized from the isolated RNA. The PCR products from the cDNA samples included a 95-bp fragment corresponding to the target region sgRNA1 and sgRNA2 as well as a 259-bp from Cas9 (Fig. 19). Two differential bands at 95-bp and 682-bp positions were observed for the positive control (fig. 19-a) due to two binding sites for the reverse primer sgRNA_R (Fig. 5-c). No specific amplification was observed from the corresponding RNA samples signifying the purity of the isolated RNA. This result indicated that the CRISPR/Cas9 knockout cassette was successfully expressed in the strawberry samples.

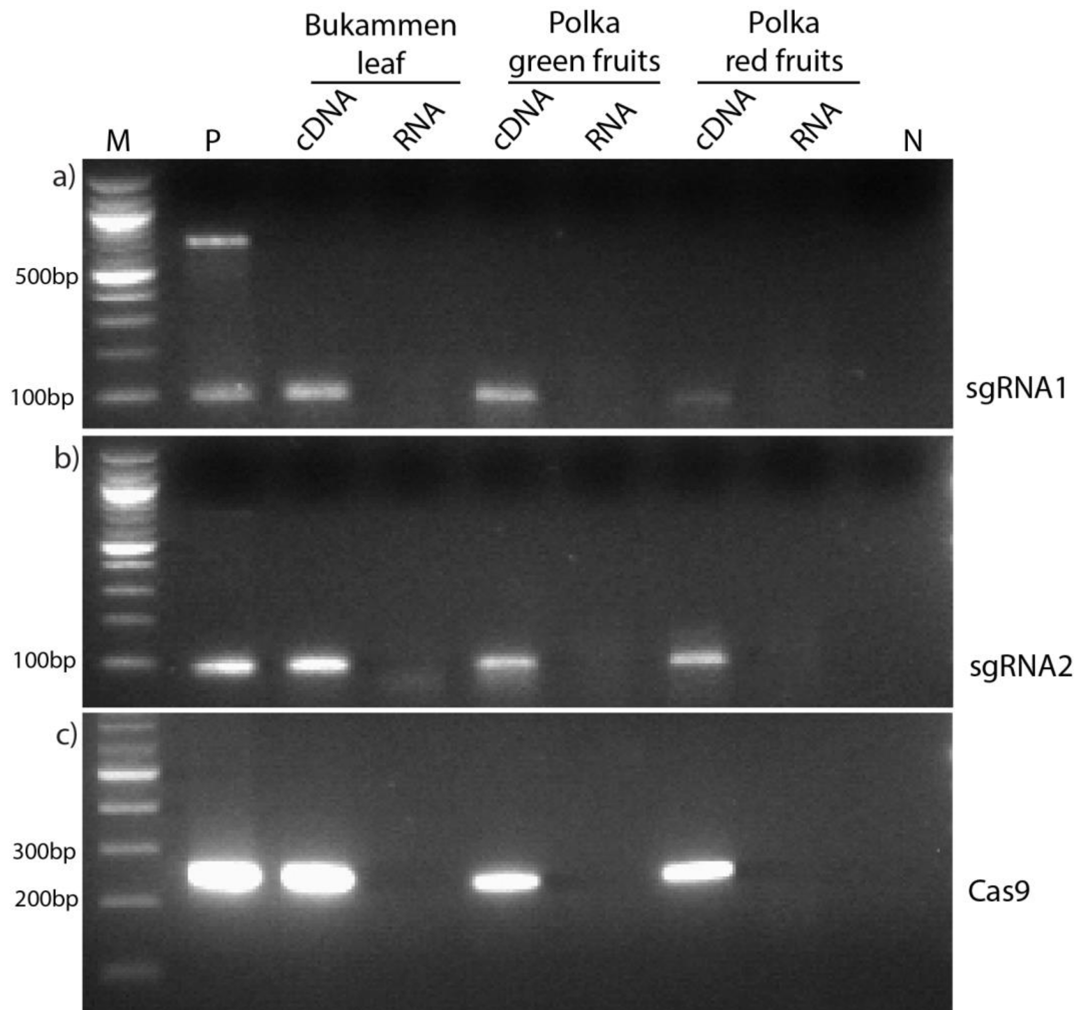



Figure 19: **PCR analysis for expression of sgRNA1, sgRNA2 and Cas9 *nuclease* after transient transformation *in strawberry.*** Each horizontal panel represents PCR products amplified with primers specific to sgRNA1, sgRNA2 and Cas9 as indicated on the right side. (a) PCR amplification of sgRNA1 with the expected band size of 95-bp. (b) PCR amplification of sgRNA2 with the expected band size of 95-bp. (c) PCR amplification of Cas9 nuclease with the expected band size of 259-bp. (N) Negative control without template DNA. (M) 100-bp standard molecular weight marker. (P) Positive plasmid control; (pCas9_TPC::FvCDIP1_2XgRNA).

## 3.3. Generation of transgenic *Nicotiana benthamiana*

Three different plant species were selected for *Agrobacterium* mediated stable transformation: 1) *Nicotiana benthamiana*; 2) *Fragaria vesca*; 3) *Fragaria* x *ananassa*.

### 3.3.1. **Callus induction and regeneration**

After infecting with *Agrobacterium,* both tobacco and strawberry explants were provided with similar growth conditions, except for the compositions of the growth hormones in the co-culture media (Table 20). Callus development was visible in the explants of *N. benthamiana* after two weeks. There was no sign of regeneration for the negative controls, due to the presence of selection antibiotics (Fig. 8-f). Unfortunately, the strawberry explants, failed to revive, and showed the signs of necrosis (Fig. 20). Several repetitive transfers of the explants into newly prepared growth media with increasing growth regulator concentration were made, without signs of growth. So, only results of the experiments with *N. benthamiana* are reported below.

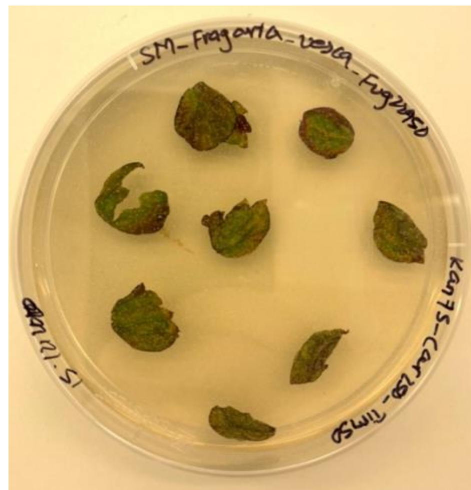



Figure 20: *Fragaria vesca* explants showing signs of necrosis. A one-month-old explant from *F. vesca* failed to produce callose in the selected growth medium and had necrotic symptoms on the leaf blades.

### 3.3.2. **Shoot induction, root regeneration and hardening**

Shoot initiation from the *N. benthamiana* explants was observed after 25 days of *Agrobacterium* inoculation. The number of shoots developed per leaf disk ranged from one to six. There were also some calluses which grew in size but did not aid in the shoot development process. The larger calluses with several shoots were excised and sub-cultured in freshly prepared MS-selection media. Subsequent sub-culturing in selection media was done at two-week intervals.

Elongated shoots with adequate length (approximately 3-4 cm) were transferred to rooting media in a Magenta box. Fine roots were visible three weeks after the transfer (Fig 21-b). After complete root development plants were transferred into a soil substrate (4-5 weeks after transfer to rooting media). Plants with no visible roots were

sub-cultured in freshly prepared rooting media every two weeks. At the final stage in the rooting media, plants were fully stretched throughout the length of the magenta box. A total of 21 plants with sufficient root growth were transferred to the soil substrate for hardening (Fig. 21-c). However, for further analysis only ten plants which were transferred at a same time were selected.

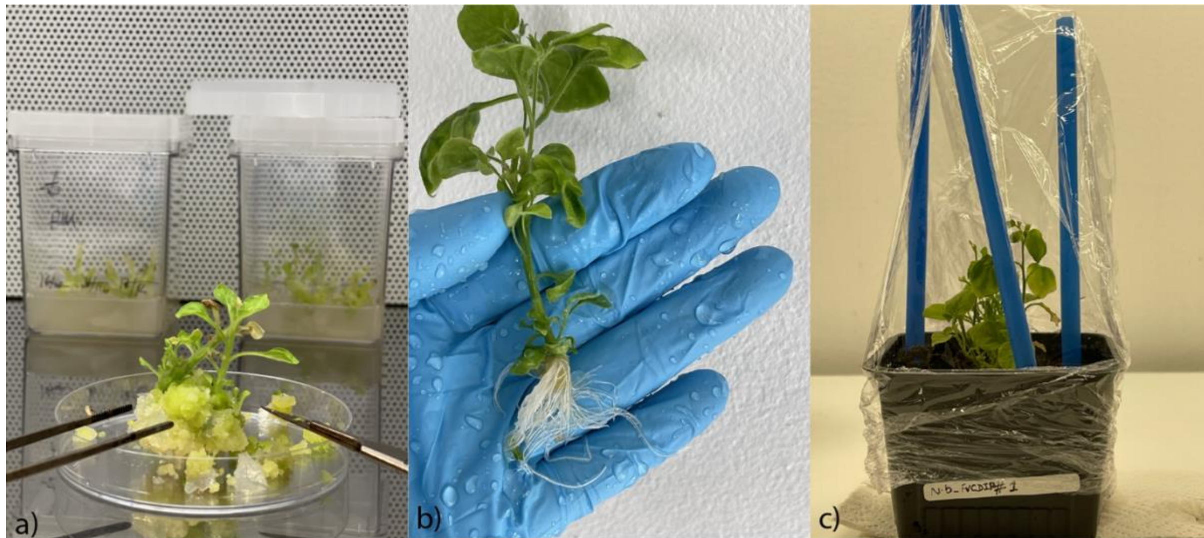



Figure 21: Different stages of plant transformation of *Nicotiana benthamiana* after shoot emergence. (a) A 3-month-old *N. benthamiana* ready for transfer into rooting media. The excess callus was removed before transferring the plants into the rooting media; (b) A *N. benthamiana* transgenic plant with proliferated roots ready for the transfer into soil substrate; (c) A transgenic plant planted in soil substrate and covered with a transparent plastic film to maintain high relative humidity around the plant's canopy.

### 3.3.3. **Analysis of putative transgenic *N. benthamiana* plants**

Once all the ten plants were properly established in the soil substrate, they were further analysed. Phenotypic aberrations in the shoots of the transgenic plants, especially in the leaves, were observed. The transgenic plants showed unrestricted cell death like necrotic lesions along with chlorosis symptoms (Fig. 22). Genomic DNA (gDNA) was isolated from ten putatively transformed *N. benthamiana* plants and tested for the presence of the target gene *FvCDIP1* using PCR with gene-specific primers (GW_FvCDIP1_F/ GW_FvCDIP1_R). A 1363-bp DNA was amplified from the putative transgenic lines, along with a 116-bp fragment of the housekeeping gene *EF1α*, which was used as an internal control (Fig. 23).

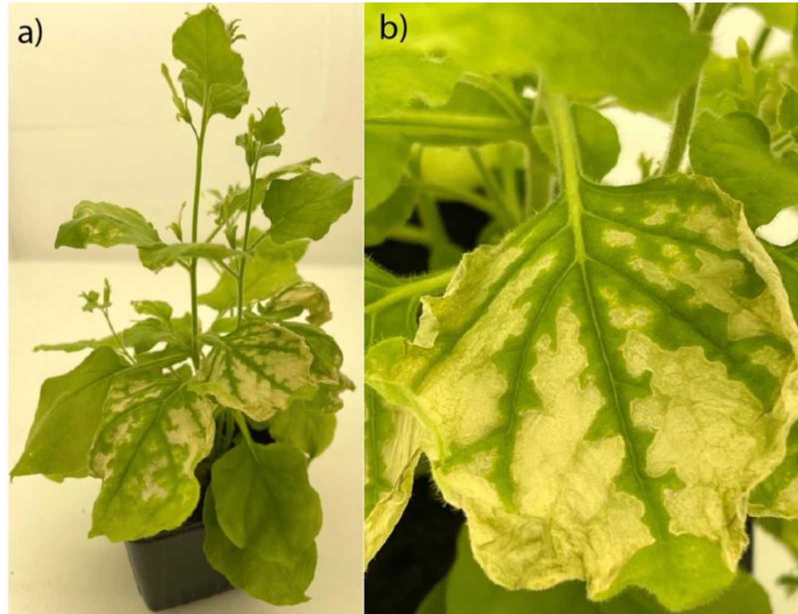



Figure 22: A typical phenotype of *FvCDIP1*transgenic *Nicotiana benthamiana*. (a) Unrestricted cell death like necrotic lesions in $T_0$ generation; (b) An enlarged view of a typical lesion plant.

The detection of amplified PCR product for *EF1α* gene in all the samples suggested that the template gDNA used for verification of the transgenic plants was of good quality (Fig. 23-lower panel). All the transgenics plants showed the expected band size (1363-bp) corresponding to the positive control which suggested that the *FvCDIP1* gene was successfully integrated into the genome of all the tested *N. benthamiana* plants (Fig. 23).

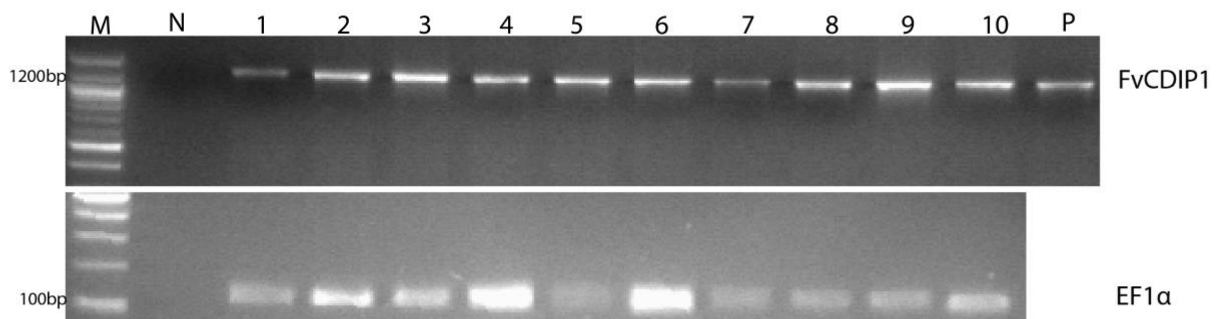



Figure 23: PCR analysis of the transgenic *FvCDIP1- Nicotiana benthamiana* plants. Upper panel: PCR products obtained after amplification of genomic DNA from ten plants (lanes 1-10) using the primers GW_FvCDIP1_F/ GW_FvCDIP1_R. The expected band size is 1363-bp; Lower panel: PCR product obtained after amplification of a fragment of the housekeeping gene *EF1α,* using primers EF1α_NB_F/EF1α_NB_F. The expected band size is 116-bp. (M) 100-bp standard molecular weight marker, (N) Negative control without template DNA, (P) Positive plasmid control.

Afterwards, the *FvCDIP1* transgenic *N. benthamiana* plants were checked for the expression of *FvCDIP1* using gel-based RT-PCR. For this purpose, total RNA was isolated, and cDNA was synthesized by reverse-transcription. PCR amplification of *FvCDIP1* was carried out using primers; FvCDIP1_RT_F/ FvCDIP1_RT_R. Amplified PCR products of 177-bp corresponding to the positive plasmid control were obtained from the cDNA samples from all positive transgenic *N. benthamiana* plants (Fig. 24), suggesting that the *FvCDIP1* gene is constitutively expressed under the 35S promoter. No amplification was detected in the RNA samples, which implied that the isolated RNA was free from DNA contamination.

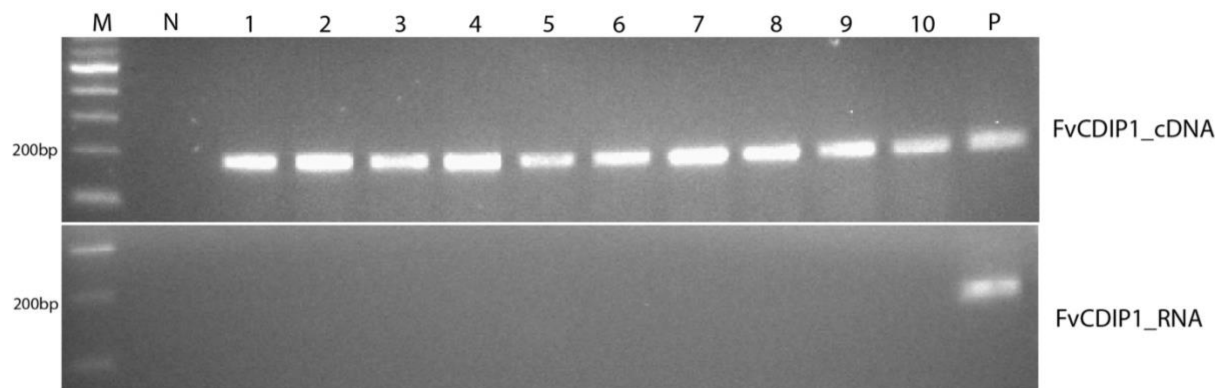



Figure 24: PCR analysis of *FVCDIP1* gene expression in $T_0$-*Nicotiana benthamiana*. Upper panel: PCR products obtained after amplification of cDNA samples from ten transgenic *N. benthamiana* plants (lanes 1-10). The RT-PCR was performed with the primer pair FvCDIP1_RT_F/ FvCDIP1_RT_R. Expected band size is 177-bp. Lower panel: PCR analysis using RNA as a template (M) 100-bp standard molecular weight marker (NEB, USA), (N) Negative control without template, (P) Positive plasmid control (pGWB454::FvCDIP1 construct).

To study the influence of *FvCDIP1* on defence signalling pathways in the transgenic plants, the relative expression of signalling related marker genes, *PR-1a, PR-5, PDF1.2* and *EDS1* were analysed using a qRT-PCR. A single defence related gene, *PDF1.1*, associated with the jasmonic acid signalling pathway was upregulated in the *FvCDIP1* transgenic *N. benthamiana* relative to the untransformed wild type (Fig. 25). Contrarily, a decrease in expression for the other defence related genes (EDS1, PR5 and PR1a) was observed. This pattern of relative expression was witnessed in another replicate of the experiment.

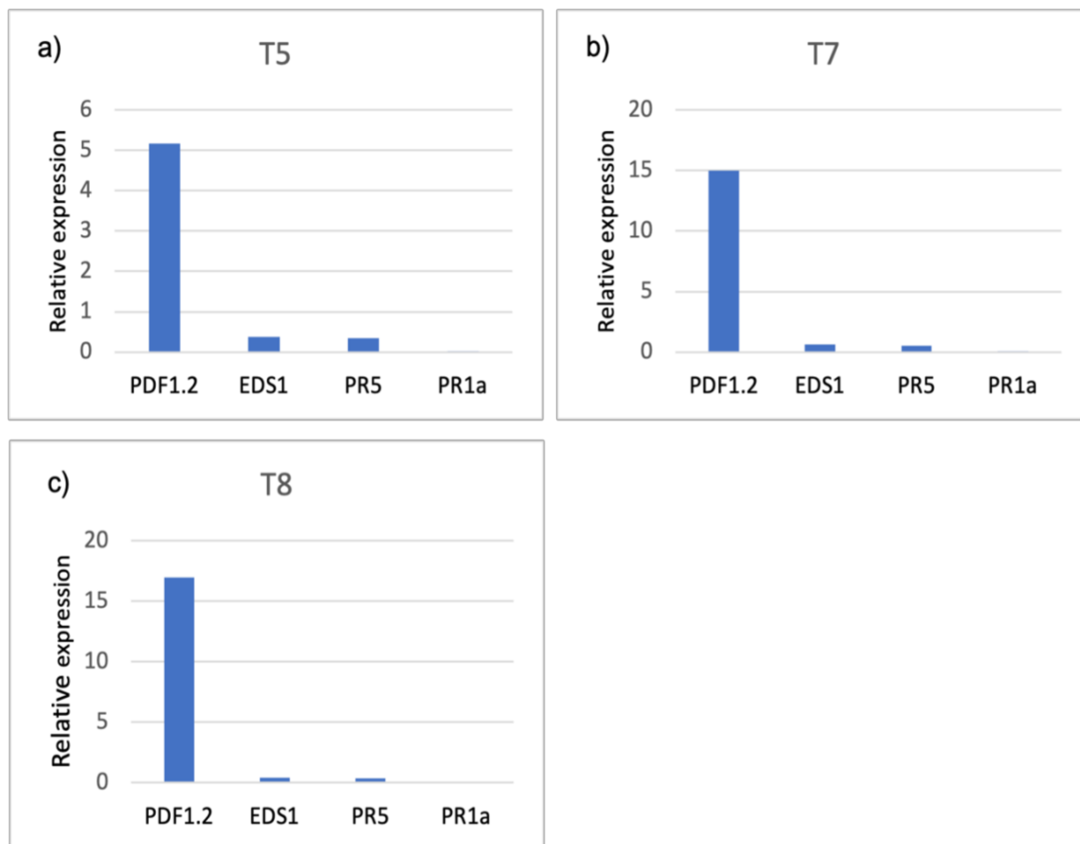



Figure 25: Relative expression of four PR-genes in three transgenic-*Nicotiana benthamiana* lines.

## 4. Discussion

To understand the role played by a particular gene, identification of its sequence and its expression pattern are the starting steps. In this thesis, the *F. vesca* genes *FvCDIP1* (*Fragaria vesca Cell Death Inducing Protein 1*) and a *WAK* (*Wall-associated kinases*) gene*;* associated with disease resistance response against the hemibiotrophic oomycete *P. cactorum* were studied. These genes were previously found to be uniquely expressed in two *F. vesca* resistance genotype (NCGR1603 and Bukammen) inoculated with *P. cactorum* and not in the susceptible genotypes (Gogoi et al.).

In this study 1) the *WAK* gene expression in *N. benthamiana* leaves conferred moderate resistance to *P. cactorum,* 2) transgenic *N. benthamiana* plants expressing the heterologous gene *FvCDIP1* showed upregulation of JA/ET associated *PDF1.2* defensin gene*,* 3) the CRISPR/Cas9 knockout cassette showed transient expression of its knockout machinery targeting the *FvCDIP1* gene.

## 4.1. Transgenic *FvCDIP1-Nicotiana benthamiana*

In this study, transgenic *Nicotiana benthamiana* lines harbouring the *FvCDIP1* gene were obtained. The original plan was to infect the transgenic *N. benthamiana* with *P. cactorum* zoospores and observe the difference in disease progression in comparison to the wildtype plants. However, COVID-19 regulations and restricted permission to the laboratory interrupted in the planed laboratory work. This hinderance directly impacted on drawing a convincing conclusion about the gene's function. Nonetheless, the $T_0$ *FvCDIP1- N. benthamiana* plants developed pathogen independent cell-death-like response. The unrestricted cell death like necrotic lesions followed by chlorosis at the later stage of plant maturity. This, somewhat expected, response in the transgenic plants could be either a response to non-self-recognition by the plant or it could be due to the constitutive expression of the *F. vesca* gene controlled by the 35S promoter. Similar phenotypic response was observed in the $T_0$ generation of transgenic *N. benthamiana* plants harbouring *Pinellia ternate* genes (Abbas et al., 2018). The authors concluded that it was a non-self-recognition, based on a similar HR symptom in another plant species (*Lycopersicon esculentum*), while no cell death was noticed when the genes were overexpressed in the parent plant *P. ternate*. Interestingly, the authors observed that the transgenic plants obtained from $T_1$ generation onwards showed normal morphology with no phenotypic variation. In this study, due to time limitations, no such assessment was performed to explicate the phenotypic response as a non-self-recognition phenomenon. Also, it was not possible to assess it beyond the $T_0$ transgenics as the $T_0$ plants were still at their flowering phase.

The *FvCDIP1* gene has been reported to induce cell death in *N. benthamiana* in a transient expression analysis (Gogoi, Unpublished Phd. thesis). This provides a speculation that the *FvCDIP1* gene expression could attribute towards the aberrated cell death response in the transgenic lines. Studies have shown that a constitutive expression of defence related transgenes is often associated with uncontrolled defence reactions which includes spontaneous necrosis and/or runaway cell death of the plant tissue (Gurr & Rushton, 2005). Therefore, the constitutive expression of the *F. vesca*'s *FcCDIP1* gene, which was under control of the constitutive promoter CaMV 35S might be an explanation for the phenotypic response in the transgenic *FvCDIP1-N. benthamiana*. Nonetheless, it needs to be investigated if the response continues in the subsequent generations of *FvCDIP1* transgenic *N. benthamiana*.

### 4.1.1. **Relative expression of PR-genes**

Expression analysis using quantitative RT-PCR for these defence associated-genes in $T_0$-*FvCDIP1-N. benthamiana* plant showed an increased expression of JA-associated marker gene *PDF1.2* whereas a reduced expression was observed for SA-responsive genes (*PR1-a, PR-5 and EDS1*) relative to the wild-type plants. This result provided a speculation that *FvCDIP1* works in association with jasmonic acid pathway towards the resistance mechanism. Interestingly, in line with this result, it has been observed that JA and SA usually manifest reciprocal antagonism (Beckers & Spoel, 2006; Li, N. et al., 2019; Thaler et al., 2012). For example, transgenic *N. benthamiana* plants showed an increased expression of *PDF1.2* by 12-fold, meanwhile the expression of a SA-associated gene *PR1* was reduced by 38% (Xu et al., 2018). In another study, *Arabidopsis* plants impaired with SA accumulation showed 25-fold higher level of JA along with enhanced expression of JA-responsive gene including *PDF1.2* (Spoel et al., 2003). Contrarily, majority of the studies have shown a downregulation of JA-responsive gene when SA-associated gene is upregulated. For instance, Cui et al. (2019) found an upregulation of SA-responsive gene (*PR1* and *BGL2*) while the transcript level of *PDF1.2* was significantly repressed. Such crosstalk between the two signalling pathways has become an indispensable mechanism for plants to control their induced defence responses thereby reducing the cost of defence linked adaptations (Chen et al., 2021).

Phytohormones JA and SA are the major plant hormones which regulate and fine tune the plant's induced defence mechanisms by a network of cross-communicating pathways. Several studies have reported the change in gene expression level of biochemical pathway associated signalling hormones: *PDF1.2* associated with JA/ET (Abbas et al., 2018; Leon-Reyes et al., 2010), *PR1-a* and *PR-5* associated with SA (Leonetti et al., 2017) and *EDS1* an essential SA transcriptional inducer (Lapin et al., 2020) in plants.

Although phytohormones are important part of defence signalling pathway, JA itself has not been implicated as a major regulator of the cell death response. However, Yoon et al. (2009) collected evidences regarding pathogen-induced cell death in connection to JA-mediated signalling. But, in this case, only the relative expression of defence associated genes were analysed, and the obtained findings are not sufficient to claim its role in observed phenotypic aberrations.

### 4.1.2. **Transformation of strawberry explants**

In this study, the explants for both the species of strawberry (*Fragaria × ananassa* and *Fragaria vesca*) failed to survive in the selection media. The strawberry explants started to develop necrotic patches on the deliberately injured sites which was intended for increasing the transformation efficiency. The necrotic patches were also noticeable on the peripheral region of the explants. On the other hand, *N. benthamiana* explants receiving similar treatment and handling showed appropriate signs of regenerations.

As *Agrobacterium* mediated *in vitro* regeneration is a complex process it is influenced by several environmental and genetic factors. A right combination and optimisation of factors including choice of plant species and explants, bacterial strain and cell density, growth regulators, antibiotics and the physical conditions determine the success of plant transformation (Husaini et al., 2011). Moreover, in case of strawberry, the variation in agro-morphological characteristic of the accessions is reflected in the variation of its transformation protocol. Several studies have shown a varied range of regeneration efficiency among the selected cultivar/genotype (Husaini, 2010). As majority of strawberry transformation has been achieved using leaf disk as explant material, this study also made use of leaf disk as explant material. Passey et al. (2003) compared the adventitious regeneration of seven different commercial strawberry cultivars using stipules, root, petioles, and leaf disks. The highest regeneration was achieved for leaf disk and found a strong genetic component amongst the tested cultivars which influenced their regeneration capacity.

Besides this, choices of plant growth regulators and their amount can make a considerable difference in the regeneration of explants. A different combination of 6-Benzylaminopurine (BAP) and indole-3-acetic acid (IAA), BAP and indole-3-butyric acid (IBA), BAP and 2,4-dichlorophenoxyacetic acid (2,4-D), IBA and Thidiazuron (TDZ) has been recorded in successful shoot/root regeneration of strawberry cultivars in different scientific observations (Husaini et al., 2011). In this study, a combination of BAP (3 mg $L^{-1}$) and IBA (0.2 mg $L^{-1}$) were used in the selection media along with appropriate selection agents. Later, the concentrations of BAP and IBA were slightly increased to 3.5 mg $L^{-1}$ and 0.5 mg $L^{-1}$ respectively with the expectations of possible improvements in regeneration. However, due to time limitations the protocol could not

be optimized further as necessitated. This failure in optimization of protocol led to a discontinuance in transplant of strawberry explants.

### 4.1.3. Further perspective of *FvCDIP1-Nicotiana benthamiana*

The higher relative expression of the plant defensin gene *PDF1.2,* but not genes associated with other signalling pathways, suggests that *FvCDIP1*-mediated defence regulation is mostly specific to defensins. However, to mark an unbiased interpretation, analysis of detail mechanism and confirmation of resistance level in subsequent generations is necessary. In addition to this, challenging the transgenic *FvCDIP1-N. benthamiana* plants with pathogen could further widen the current understanding of the gene function in association to pathogen defence. Lastly, alternative use of endogenous/native promoter or the use of inducible promoters through promoter engineering that express the transgene only when and where it is needed (Gurr & Rushton, 2005) could help avoid uncontrolled defence reactions in uninfected plant.

## 4.2. Transient expression of CRISPR/Cas9 *FvCDIP1* knockout construct

Targeted gene knockout in plants and the generation of inheritable mutant alleles offers an unprecedented opportunity for functional study of genes. This part of the study was intended to generate targeted mutants of strawberry using *Agrobacterium*-mediated delivery of a CRISPR/Cas9 expression cassette. The expression cassette was designed to knock out the *FvCDIP1*. The hypothesis was that a frameshift knockout mutation in the target gene of in a disease resistant *F. vesca* genotype would lead to a disease susceptibility relative to its wild-type accessions.  However, the planned task was too optimistic for generating knockout mutant lines. So in this study, only the transient expression of the *FvCDIP1*-CRISPR/Cas9 knockout construct was checked using gel-based RT-PCR, where it was found to successfully express its machinery inside the host tissue.

### 4.2.1. Construction of CRISPR/Cas9 *FvCDIP1* knockout construct

In this study, taking advantage of the *PacI* restriction site, the expression cassette with two sgRNAs (FvCDIP1_2XgRNA) was ligated into pCas9-TPC vector producing pCas9-TPC::FvCDIP1_2XgRNA.

Intriguingly, in addition to an expected 1410-bp product, another unspecific band of ca. 1000-bp was obtained from the PCR amplified products of transformed *E. coli* and *Agrobacterium* (Fig. 16 and Fig. 17). The binding sites for the primers (TPC_F and TPC_R) were outside of the expression cassette (Fig. 5-c), were supposed to yield a 1410-bp product. An identical observation was made by Blanc (2018) where the author observed a similar unexpected band throughout the colony-PCR results of bacterial transformants harbouring a similar two sgRNAs cassette in the pCas9-TPC recombinant plasmid. Where did this unspecified band come from? - cloning of the unexpected band and its sequencing could, in a way, provide an answer to the question. Another explanation for this could be an intraplasmid rearrangement, where a Rec-A independent mechanism could contribute to the formation of deletion and amplification of the product in both *E. coli* and *Agrobacterium* (Bočkor et al., 2013). The tendency of such replication misalignment is dependent on the homology and proximity of the repeat sequence (Bočkor et al., 2013; Bzymek & Lovett, 2001). Since, majority of the sgRNA expression cassette had an identical sequence, including the homology for the two-FvU6-26 promoter, two-sgRNA scaffold and two-FvU6 terminator, the unexpected band could be present because of intraplasmid rearrangement. As, the pCas9-TPC vector was originally tested to work with a single sgRNA (Fauser et al., 2014), the intraplasmid rearrangement could have happened yielding an unspecific band. Nevertheless, the construct development was successful as both the sgRNAs and Cas9 nuclease were expressed after a transient expression assessment in strawberry plant materials.

#### 4.2.2. **Transient expression of pCas9-TPC::FvCDIP1_2XgRNA construct**

Gel image from PCR amplification of the cDNA template obtained from transiently expressed tissues showed visible bands for the targeted fragments of sgRNA1, sgRNA2, and Cas9 (Fig. 18). This proves the expression of the knockout machinery present in the recombinant plant expression vector pCas9-TPC::FvCDIP1_2XgRNA. As no bands were detected for the RNA templates, it further validates the obtained results. Since different tissue has different expression efficiencies (Hwang et al., 2017), in this case, two different strawberry tissues; leaf and fruit were taken. For leaves of *F. vesca* genotype Bukammen, a vacuum infiltration approach was adopted. Whereas the fruits of *F. × ananassa* cultivar Polka were *Agro*-infiltrated with a 1mL hypodermic needle.

The majority of the transient expression in strawberry has been performed by infiltration of *Agrobacterium* on strawberry fruits (Carvalho et al., 2016). Besides this, agroinfiltration in strawberry leaves via particle bombardment (Li, C. et al., 2019) and vacuum infiltration (Cui et al., 2017) is also common. However, the choice of *Agrobacterium* strain, the extent of cuticular penetration and the abundance of mesophyll cells in the leaves can, to some extent, determine the transformation efficiency (Hwang et al., 2017). There are other aspects that are reported to play roles in a efficient transformation of vacuum infiltrated leaves, which include seedling age, leaf position and time of vacuum exposure (Cui et al., 2017). In our case, we used fully opened first position leaves from a 2 months old *in-vitro* grown strawberry plantlets, which, as suggested by reports (Cui et al., 2017), have a higher expression efficiency.

### 4.3. Expression of WAK gene and reduced disease progression

In this part of the study, we attempted to study the function of a selected candidate *WAK* gene*,* by transiently expressing it into *N. benthamiana* plant. The transient expression was followed by *P. cactorum* inoculation assay which showed a reduced disease progression in comparison to the mock plants. The average lesion area in the *WAK*-gene expressed *N. benthamiana* plants was half of the lesion area compared the mock control. This suggests that the reduced lesion in transiently expressed plants was indeed attributed to *WAK* associated response. However, from the statistical observation, the P value showed a insignificant result (P= 0.72). Since the experiment was performed a single time, multiple replications with larger sample size can be conducted to lower the insignificant observations.

The *WAK* gene was chosen based on the transcriptome study from *P. cactorum* inoculated strawberry genotypes (NCGR1603 and Bukammen) (Gogoi), where it was found to be uniquely expressed. A similar observation for wall associated receptor like kinases was made by (Toljamo et al., 2016) where they found 12 out of 18 WAK associated *F. vesca* genes were upregulated after *P. cactorum* inoculation. This signifies a potential role of *WAK*-gene to enhance disease resistance against *P. cactorum.*

# 5. Conclusion and future perspective

The cultivated strawberry is a highly vulnerable horticultural crop from a disease susceptibility perspective. The projection of global climate change and the progressive phase-out of pesticides has made it imperative to seek for alternative techniques to make the fruit crop more resilient. The diploid strawberry (*F. vesca*), a model plant, could be used as a natural genetic source of resistance against a multitude of pathogen, including *Phytophthora cactorum*.

In this study, two candidate genes from diploid strawberry were chosen from a previously conducted transcriptomic study of *P. cactorum* infected strawberry plants. Successful transient expression of *WAK* gene and stable expression for *FvCDIP1* was achieved in another model plant *N. benthamiana*. The expression of the *WAK* gene showed a reduced disease severity upon *P. cactorum* inoculation. At the same time, the heterologous expression of *FvCDIP1* in transgenic *N. benthamiana* showed increased relative expression of JA-associated gene *PDF1.2*, suggesting defensin associated role of the uncharacterized gene *FvCDIP1.* However, analysis in the subsequent generation of transgenics with pathogen infection assays could provide sufficient evidence to reach an unbiased conclusion. Besides, we were able to transiently express the CRISPR/Cas9 knockout construct designed to bring a frameshift mutation in *F. vesca FvCDIP1* gene. Further testing by generating functionally impaired stable mutant strawberry lines would allow in understanding its defensive role against *P. cactorum*.

As the new regulations emphasise more on the alternative disease management approaches including the use of disease resistant plant material, in particular, the identification and deep-molecular characterization of the resistance gene followed by its integration in the existing elite genotypes can offer a durable resistance in the long run.

## 6. Supplementary materials:

Supplementary Table 1: List of primer-pairs used in the PCR amplification

| Primer name | Sequence ( 5' to 3') | Amplicon size (bp) |
|---|---|---|
| Gw_WAK_F | GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCAA CTTTTCTTTTTCAA | 2476 |
| Gw_WAK_R | GGGGACCACTTTGTACAAGAAAGCTGGGTTCAACA TGTCTTCTCAAACA | |
| pDONR-F | CTGAACGAGAAACGTAAAATGA | 856 |
| WAK_P1-R | TGCATAGTCCTTGCAATCCA | |
| WAK_P2F | TGAAACAAGAAGAAATGCGATG | 818 |
| WAK_P2R | CTGCCACAATTCTTCCATCC | |
| WAK_P3-F | GCATGTTGATGGATGGAAGA | 1015 |
| pDONR-R | TGTAAAACACAACATATCCAGTCA | |
| WAK_RTF | ACCAAACTCACCTGACCACA | 160 |
| WAK_RTR | TCGTCTGACCTTGTTACCGG | |
| NbPR1a-RT-F | CCTCGTACATTCTCATGGTCAAT | 219 |
| NbPR1a-RT-R | CCATTGTTACACTGAACCCTAGC | |
| NbPR-5-RT-F | CCGAGGTAATTGTGAGACTGGAG | 107 |
| NbPR-5-RT-R | CCTGATTGGGTTGATTAAGTGCA | |
| NbEDS1-RT-F | AGGCCGAAGCGTTATAGGTT | 203 |
| NbEDS1-RT-R | AAAACATCATCGCCCAGAAG | |
| NbPDF1.2-RT-F | GGAAATGGCAAACTCCATGCG | 182 |
| NbPDF1.2-RT-R | ATCCTTCGGTCAGACAAACG | |
| EF1α_NB_F | AGCTTTACCTCCCAAGTCATC | 116 |
| EF1α_NB_R | AGAACGCCTGTCAATCTTGG | |
| GW_FvCDIP1_F | GGGGACAAGTTTGTACAAAAAAGCAGGCTN20* | 1255 |
| GW_FvCDIP1_R | GGGGACCACTTTGTACAAGAAAGCTGGGTN20* | |
| FvCDIP1_RT_F | N21* | 177 |
| FvCDIP1_RT_R | N20* | |
| Cas9_RTF | TGGTTTCGATTCTCCTACCG | 259 |
| Cas9_RTR | GCGAGCATCCTCTTTCTACCG | |
| TPC_F | TCTTGAATTGGTTTGTTTCTTCAC | 235 without insert / 1410 with insert |
| TPC_R | TAGACAAGCGTGTCGTGCTC | |
| sgRNA1_F | N23* | 95 / 682 |
| sgRNA_R | N23* | |
| sgRNA2_F | N22* | 95 |
| sgRNA_R | N23* | |
| sgRNA_F1 | N24* | 327[1] |

[1] * Primers designed at NIBIO – manuscript in progress.
N- Number of nucleotides.

| TPC_R | TAGACAAGCGTGTCGTGCTC | |
|---|---|---|

Figure 1: PCR product obtained after amplification of WAK gene from cDNA sample of *Fragaria vesca* genotype Bukammen inoculated with *Phytophthora cactorum*. Gateway primers (GW_WAK_F and GW_WAK_R) were used to amplify the full segment of the WAK gene. The amplicon size for the target gene is 2476 bp. 1-kb standard molecular weight marker was used.

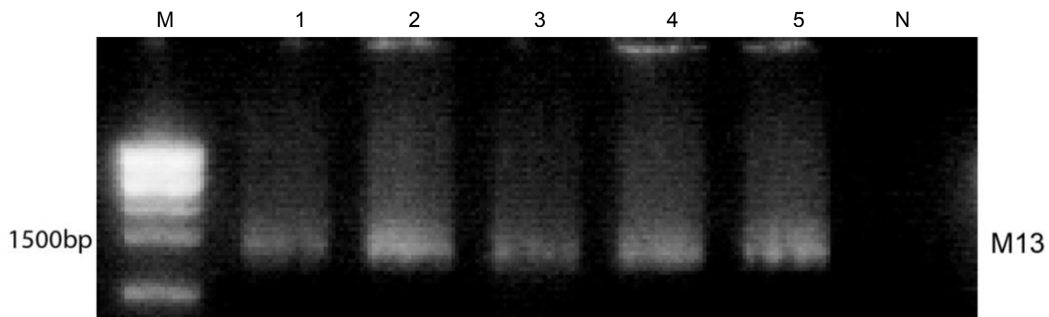



Figure 2: Gel electrophoresis result from colony PCR of *E. coli* transformants. PCR amplified products obtained after amplification of target DNA from plasmid DNA of five randomly picked *E. coli* colonies. PCR was carried using M13_F/M13_R primer pair. The expected size is 1500-bp. (M)100 bp standard molecular weight marker. (N) Negative control without template.

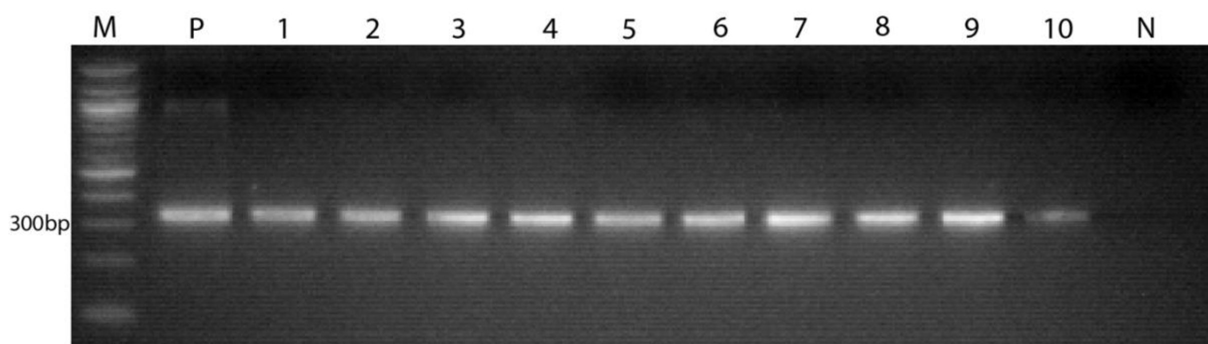



Figure 3: PCR analysis of *Agrobacterium* transformants (AGL-1). PCR amplified products obtained after the amplification of sgRNA1 region from plasmid DNA of ten random colonies of Agrobacterium *(AGL-1)* transformants. Primers: sgRNA_F1 and TPC_R. All the tested colonies (Lane 1 to 10) were confirmed as positive transformants with band size of 327-bp which corresponds to the positive plasmid control (P). (N) Negative control without template, (M) 100-bp standard molecular weight marker.

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