



**STUDENT RESEARCH CONFERENCE**

**November 20, 2024**

**Studies for the development of *R. solanacearum* resistance in potatoes using antisense gene silencing and overexpressing techniques**

**A *R. solanacearum* rezisztencia kifejlesztését célzó tanulmányok burgonyában az antiszensz géncsendesítés és túlexpresszáltatás módszereinek felhasználásával**

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**Gödöllő  
2025**

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## 1. INTRODUCTION

Potato (*Solanum tuberosum*), is a vital food crop within the *Solanaceae* family, playing a crucial role in global agriculture. It is rich in bioactive compounds such as flavonoids and carotenoids, which significantly influence oxidative processes in biological systems. The various potato varieties display a wide diversity in flavor, texture, and color, reflecting their genetic adaptability and ability to thrive in different environmental conditions (FAO, 2022; Potato Grower Magazine, 2023). Native to the Andes in South America, potatoes have been cultivated in a wide range of regions around the world, adapting to various climatic conditions. Today, the top producers are China, with approximately 95 million tons annually, and India, with 56 million tons. Other major producers include Ukraine, Russia, and the United States, each producing around 20 million tons until 2021 (Potato Grower Magazine, 2024; Word Population Review, 2021).

Despite its agricultural importance, the potato crop faces significant biological threats, such as pathogens that cause severe losses in yield and quality. Among these, *Ralstonia solanacearum*, which causes bacterial wilt, is particularly concerning as it leads to rapid wilting and eventual plant death, severely affecting potato productivity (Hanika et al., 2021). The infection mechanism of *Ralstonia solanacearum* relies on detecting vulnerable plant roots and entering them. Once inside, the pathogen colonizes internal structures and blocks water flow through the production of exopolysaccharides (EPS). In response, the plant activates a two-phase defense mechanism, first recognizing the pathogen and then producing secondary metabolites to limit the infection (Isah, 2019; Miedes et al., 2014).

Biotechnology has offered solutions to enhance potato varieties' resistance to pathogens. Key tools include gene editing, gene silencing and overexpression, allowing for controlled gene expression in the plant to reduce susceptibility to bacterial infections and improve overall crop resistance (Kilikevicius et al., 2022). This study utilized gene silencing and protein overexpression techniques to enhance the potato plant resistance. Gene silencing inhibits the expression of specific genes, while overexpression introduces a vector to increase the production of a key protein, often achieved through *Agrobacterium*-mediated genetic transformation (El-Sappah et al., 2021).

## **2. OBJECTIVES**

### **2.1. General objective**

- Develop potato varieties with improved resistance to *Ralstonia solanacearum* using gene silencing and overexpression techniques.

### **2.2. Specific objectives**

- Determine the candidate genes by literature analysis.
- Designing constructs (sense and antisense) using bioinformatics tools.
- Transform plants mediated by *Agrobacterium tumefaciens*.
- Micropropagate transformant potato lines by *in vitro* culture techniques.
- Quantify the expression level of candidate genes into *in vitro* transformed plants by RT-qPCR.

## **3. LITERATURE REVIEW**

### **3.1. Potato (*Solanum tuberosum*)**

Genetically, potato plants are of heterozygous genotype, but vary in their ploidy levels, as they can be diploid, but there is triploid, tetraploid, pentaploid and hexaploid varieties (Slater et al., 2014). It is a plant domesticated for its tuber approximately 8 000 years ago in the central Andes region between Bolivia and Peru (Vincent et al., 2013). It is basically a temperate climate crop and has grown in about 100 countries in temperate, subtropical and tropical conditions. Asia and Europe are major potato producing regions of the world. China is the largest potato-producing country, followed by India, but the country with the largest area planted with potato is Bangladesh (Kennedy et al., 2019).

Potato is the fourth most important food crop in the world behind only wheat, maize and rice, with more than 314 million tons of fresh weight produced in 2006 where half come from Asia, Africa and Latin America. Potato is a staple food that contributes to the United Nations Millennium Development Goals of providing food security and eradicating poverty (Bradshaw & Ramsay, 2009). Likewise, potato is a high carbohydrate crop, and wild species can be found that are adapted to environments with low temperatures and semi-desert soils and its varieties are subdivided into commercial and non-commercial (Slater et al., 2014).

### **3.1.1. *Solanum tuberosum* cv. *Desirée***

This tetraploid potato cultivar is recognized for its reddish skin, oval shape, and high yield. *Desirée* is a widely used commercial cultivar originating from the Netherlands. *Desirée* is commonly employed in transgenesis studies, genetic editing (such as CRISPR/Cas9), and crop improvement efforts due to its efficient transformation via *Agrobacterium tumefaciens* (Kumari et al., 2024). While it shows some resistance to diseases like late blight (*Phytophthora infestans*), it remains vulnerable to other pathogens (e.g. *R. solanacearum*), positioning it as a valuable model for studying disease resistance through genetic manipulation (Amundson et al., 2023).

## **3.2. Bacterial resistance in plants**

There are multiple genera and species of pathogenic bacteria that affect plants. However, plants deploy a complex set of defensive responses to prevent infections. These defense mechanisms can perceive bacterial elicitors capable of disrupting cellular, biochemical, and physiological functions (Lanna-Filho, 2023). Bactericides, fungicides, and other pesticides play a significant role in managing plant diseases, though their use may lead to the accumulation of residues in both plants and the environment, potentially causing harmful effects (Miller et al., 2022).

Plants respond differently to pathogens depending on the type of tissue affected, such as leaves, roots, or flowers. Plant defense mechanisms can be preformed (primary) or induced (secondary). The first step in the defense process is the recognition of the microorganism through elicitor molecules, which function as signal compounds in low concentrations. These molecules trigger the plant's defense by distinguishing them from toxins, which can affect the plant at higher concentrations (Singh & Singh, 2018).

Pathogens produce effectors, these are molecules that help them evade plant immune defenses. These effectors, encoded by *Avr* genes, trigger the synthesis of proteins recognized as threats by the plant. The plant detects pathogen molecules, known as elicitors, through specialized cell surface receptors, activating defense-related genes. These genes produce proteins and compounds, such as kinases, chitinases, and phytoalexins, which inhibit pathogen growth and strengthen defenses. In localized infections, the plant may initiate a hypersensitive response (HR), causing programmed cell death in affected areas to contain the pathogen (see Figure 1) (Thakur & Sohal, 2013).

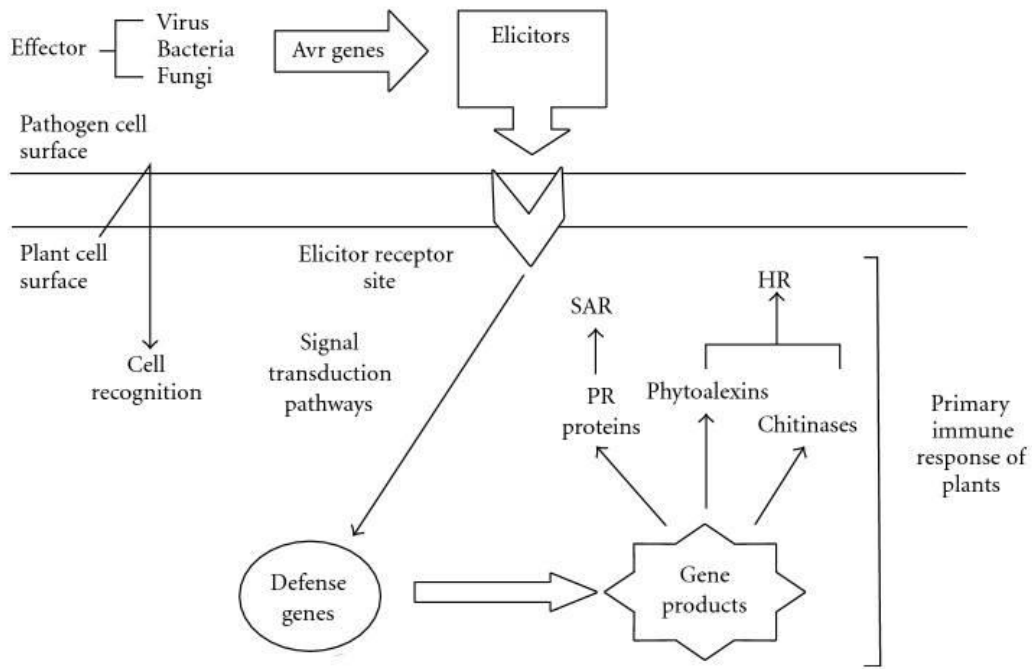


Figure 1. Process flow diagram of primary plant defense adapted from (Thakur & Sohal, 2013).

Once the signaling pathways are activated, the plant induces the expression of genes with various defense functions. It also detects pathogen-associated molecular patterns (PAMPs) using specific receptors on plant cells, initiating immune responses known as PAMP-triggered immunity (PTI) (Pieterse et al., 2009; Thomma et al., 2011).

These defense responses include the transcription of specific genes that produce antimicrobial compounds, the accumulation of reactive oxygen species (ROS), and the activation of cell wall defense mechanisms along with defense hormones like salicylic acid (SA), ethylene, and jasmonic acid (JA) (Jones & Dangl, 2006).

### 3.2.1. Characteristics of *Ralstonia solanacearum* and its life cycle and modes of transmission

Five key stages have been characterized in the life cycle of *Ralstonia solanacearum*. The above-mentioned transmission stages are detailed in Figure 2.

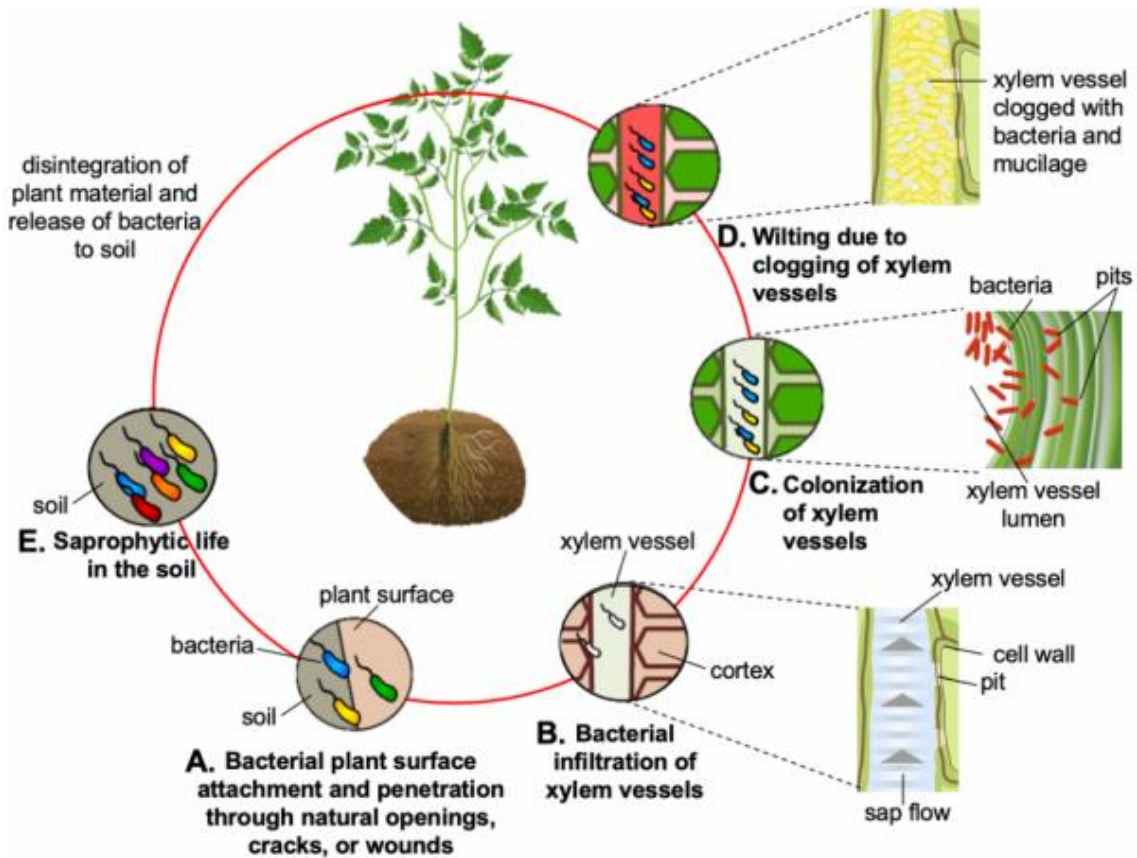


Figure 2. Distinct phases of the *Ralstonia solanacearum* life cycle, obtained from (Tariq & Sachin, 2020).

- *Environmental Survival:* *R. solanacearum* thrives in soil and water, where it can persist for extended periods even in the absence of a host. The bacterium can remain viable in plant debris or within the natural environment, awaiting an opportunity to infect susceptible hosts like potatoes (Wang et al., 2023; Xu et al., 2022).
- *Host Entry:* The bacterium typically enters the potato plant through root wounds, cracks, or natural openings. Root tips or secondary root emergence zones are common entry points. *R. solanacearum* is attracted to root exudates and initiates infection upon contact (Balamurugan et al., 2020).

- *Xylem Colonization*: After penetrating the roots, *R. solanacearum* migrates to the xylem vessels (responsible for water transport), where it multiplies rapidly. The bacterium produces exopolysaccharides (EPS), which block the xylem vessels, leading to wilting by preventing water transport throughout the plant (Balamurugan et al., 2020).
- *Systemic Spread*: Once established in the xylem, the bacterium spreads throughout the plant, causing systemic infection that leads to characteristic wilting symptoms and, ultimately, plant death (Xu et al., 2022).
- *Transmission and Spread*: *R. solanacearum* is primarily transmitted through contaminated soil and water. Infected plant material, such as potato tubers, can function as a reservoir for the pathogen. It can also spread via contaminated tools, equipment, or asymptomatic host plants (Wang et al., 2023).

*Ralstonia solanacearum* detects its host plant roots through root exudates and enters the plant via vulnerable areas such as the root elongation zone, wounds, or points where secondary roots emerge. In this early phase, it colonizes the intercellular spaces of the root (apoplast), which forms the first line of plant defense. To establish a successful infection, *R. solanacearum* must penetrate the vascular cylinder and colonize the xylem, eventually blocking water flow due to the production of exopolysaccharides (EPS) and bacterial multiplication, leading to wilting symptoms in the plant (Jose et al., 2023).

The infection process involves various virulence factors, with the Type III Secretion System (T3SS) being the most significant. This system introduces effector proteins into plant cells, manipulating their functions for the benefit of the bacterium. Additionally, EPS plays a crucial role by obstructing the xylem vessels and protecting the bacteria from plant defenses. The Type II Secretion System is also vital, as it secretes cell wall-degrading enzymes in the apoplast, facilitating colonization. These enzymes, including cellulases and pectinases, are essential for virulence, as their removal significantly reduces pathogenicity (Jose et al., 2023; Planas-Marquès et al., 2020).

Bacterial motility is equally important, especially in the initial stages of infection, as shown by mutants deficient in flagellar components, which exhibit reduced virulence. Furthermore, *R. solanacearum* can utilize nitrate as an energy source in the xylem environment, which, although hypoxic, is rich in nitrates, allowing the pathogen to sustain rapid growth under these conditions (Dong & Ronald, 2019; Jose et al., 2023).

### 3.2.2. Economic and agronomic impact of bacterial wilt

The pathogenic species of *Ralstonia spp.* have an extensive geographical distribution and a broad host range. In potatoes alone, it is estimated that the bacterium causes approximately \$1 billion annual losses, impacting around 3 million farming families across 1.5 million hectares in nearly 80 countries (Campos & Ortiz, 2019). Yield reductions in potato crops vary significantly, ranging from 20 % to 100 %, depending on the region and the management practices in place. In heavily infected areas, such as parts of Asia, Africa, and Latin America, the disease can render land unsuitable for potato cultivation for several years (Wei et al., 2023).

### 3.3. Defense genes involved in bacterial resistance of *Solanum tuberosum*

In *Solanum tuberosum*, several defense-related genes have been identified that contribute to bacterial resistance, particularly against *Ralstonia solanacearum*. These genes are often involved in the production of proteins that enhance plant immune responses, inhibit pathogen growth, and strengthen the structural integrity of plant tissues. Key defense genes include:

- *Pathogenesis-related (PR) genes*: These genes encode proteins involved in plant defense mechanisms, such as PR-1, PR-2 ( $\beta$ -1,3-glucanases), and PR-5 (thaumatin-like proteins). These proteins help break down bacterial cell walls and play a role in immune signaling in plants (Jose et al., 2023).
- *Phenylpropanoid pathway genes*: This pathway is crucial for the synthesis of lignin and other secondary metabolites, such as flavonoids, which strengthen cell walls and function as antimicrobial agents. Genes involved in this pathway, such as phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), are upregulated during bacterial infection (de Pedro-Jové et al., 2021).
- *Glutathione S-transferase (GST) genes*: These genes participate in detoxifying reactive oxygen species (ROS) that accumulate during the defense response. ROS plays a critical role in signaling and direct antimicrobial activity, but they need to be regulated to prevent damage to plant tissues (Das & Roychoudhury, 2014).
- *Cell wall-modifying genes*: Genes related to cell wall synthesis and modification, such as those encoding expansins and pectin methylesterases (PMEs), are often induced in response to bacterial attacks. These proteins modify the plant cell wall to reinforce it or facilitate its degradation for defense signaling (de Pedro-Jové et al., 2021).

- *Nucleotide-binding leucine-rich repeat (NLR)* proteins: These resistance genes play a key role in recognizing bacterial effectors and activating effector-triggered immunity (ETI), a strong immune response against pathogens like *Ralstonia solanacearum* (Dong et al., 2022).

### **3.3.1. Function and regulation of WAT-1 gene in plants**

Walls Are Thin 1 (WAT-1) is a conserved gene in higher plants, primarily located in the cell membranes of xylem cells, where it plays a significant role in vascular development. WAT-1 is essential for lignin deposition in xylem fibers, contributing to the formation of thick, lignified cell walls. Lignin is crucial for the mechanical strength and rigidity of plants (Ranocha et al., 2011).

The WAT-1 gene plays a key role in cell wall formation and the production of secondary metabolites, both of which are essential for plant defense mechanisms. In *Arabidopsis*, mutations (knock-out mutations) in the WAT-1 gene lead to a weakening of cell wall integrity, underlining its importance in maintaining plant structure and supporting immune responses against vascular pathogens (Hanika et al., 2021).

### **3.3.2. Function and regulation of OSML15 genes in plants**

The Osmotin-like protein 15 (OSML15 gene) in *Arabidopsis thaliana* is part of the osmotin/thaumatin family of proteins, which are classified under the broader group of pathogenesis-related proteins (PR-5). These proteins are primarily involved in plant defense mechanisms and environmental stress responses (Bashir et al., 2020).

OSML15 plays a crucial role in helping plants cope with osmotic stress. It aids in water retention and ionic balance regulation, which are essential during drought or high salinity conditions. Like other PR-5 proteins, OSML15 exhibits antifungal properties, making it a key player in the plant's immune response. By interacting with the fungal cell wall, it inhibits fungal growth, thereby enhancing the plant's resistance to pathogens (Kim & Park, 2021).

The OSML15 gene plays a critical role in several plant functions, particularly in strengthening cell walls and producing secondary metabolites, which are vital for plant defense. OSML15 enhances plant resistance to pathogens by reinforcing cell walls, thereby forming a physical barrier that helps limit pathogen invasion. Additionally, secondary metabolites generated in response to stress or pathogen exposure function as chemical defenses, either by preventing pathogen growth or by aiding in tissue repair (Liu et al., 1994).

### **3.4. Antisense gene silencing technique**

Gene silencing is a highly effective molecular procedure that inhibits the expression of specific genes, functioning as a regulatory mechanism for gene expression. In nature, gene silencing plays a crucial role in determining cell fate and controlling metabolic activities throughout the eukaryotic organism's life. In plants, gene silencing is vital for defending against abnormal RNA, which can be lethal if it accumulates in the cell's RNA pool. This protection is facilitated through nonsense-mediated mRNA decay (NMD), which may also serve antisense therapeutic purposes (Horser et al., 2002).

The antisense gene silencing technique purposefully prevents gene expression by generating double-stranded RNA (dsRNA) in the cytoplasm (see Figure 3). Antisense mediated gene silencing technology involves sequence-specific antisense oligonucleotide molecules to create an antisense copy of the target messenger RNA (mRNA). This complementary strand, known as antisense RNA, binds to the specific mRNA, regulating or inhibiting the expression of the corresponding protein, by either suppressing the translation or triggering the degradation of the target mRNA (Saurabh et al., 2014).

As shown in Figure 3. The antisense RNA transcribed from the sense strand of DNA binds to the sense mRNA to block translation. This Figure illustrates how antisense RNA, transcribed from the sense strand, pairs with sense mRNA to prevent it from being translated. Sense mRNA, which carries genetic information from DNA to the ribosome for protein synthesis, is blocked when antisense RNA, transcribed from the antisense strand of DNA, binds to it. Base pairing between the complementary sequences prevents the ribosome from translating the mRNA into protein, effectively stopping the expression of the protein encoded by the sense mRNA (El-Sappah et al., 2021).

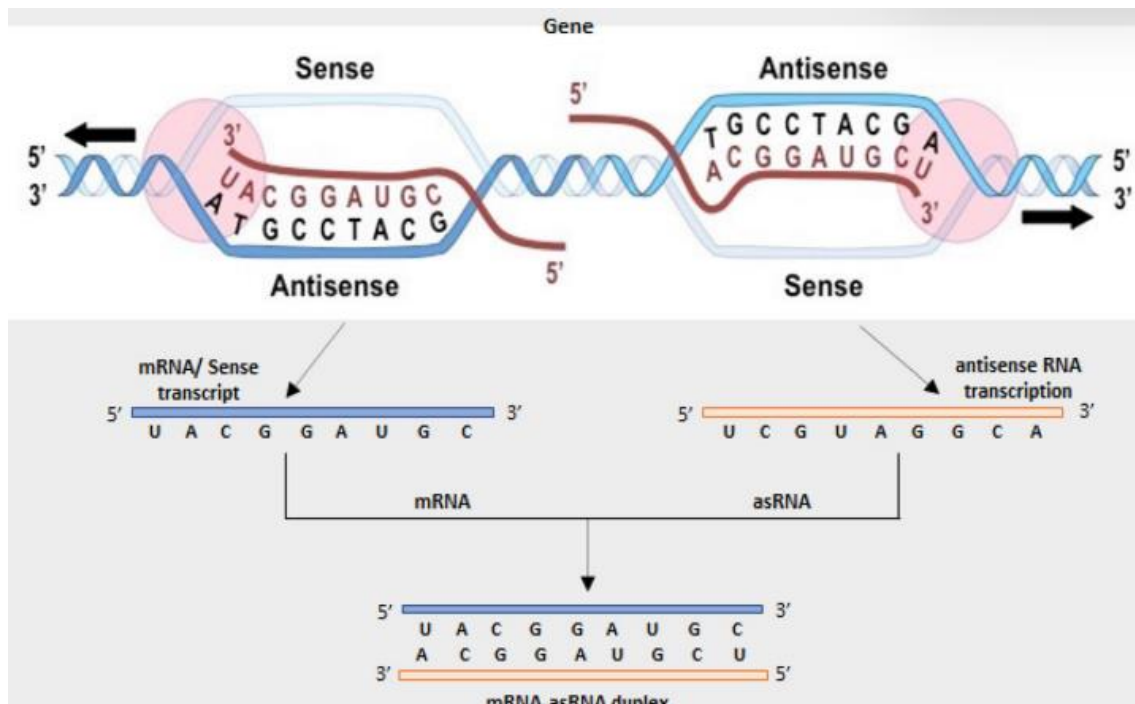


Figure 3. The mechanism by which antisense RNA, transcribed from the sense strand, binds to sense mRNA to inhibit translation (Animasaun & Lawrence, 2023).

Bacterial diseases present significant challenges to crops such as tomatoes, soybeans, bananas, and potatoes due to their rapid spread and difficulty in control. As a result, it is crucial to develop bacterial-resistant varieties of food crops to minimize infections, reduce yield losses, and lower production costs. One promising approach to enhancing bacterial resistance is the use of antisense RNA (asRNA) (Kilanowska & Studzińska, 2020).

In a study conducted by (Animasaun & Lawrence, 2023), the silencing of two bacterial genes (*iaaM* and *ipt*) successfully reduced the formation of crown gall tumors caused by *Agrobacterium tumefaciens* in *Arabidopsis* to nearly zero. This finding suggests the potential to engineer resistance to crown gall disease in trees, ornamental plants, and even food crops.

### 3.5. Overexpression technique

The mechanism of protein overexpression in plants begins with the construction of an expression vector. This vector carries the gene encoding the protein of interest, regulated by a strong promoter that ensures a high transcription rate. In plants, constitutive promoters, such as the 35S promoter from the cauliflower mosaic virus, are commonly used to maintain continuous protein expression throughout various plant tissues (Valencia-Lozano et al., 2023).

Using methods like *Agrobacterium tumefaciens*-mediated transformation we can introduce the DNA of interest into the plant genome and its expression is maintained by 35S. Once integrated, the transcription machinery of the plant produces excess copies of mRNA from the gene. The mRNA produced by the overexpressed gene is then translated by ribosomes to generate the desired protein in higher amounts comparing to the wild type (O. X. Dong & Ronald, 2019). Pathogenesis-related (PR) proteins, such as chitinases or  $\beta$ -1,3-glucanases play a key role in breaking down the cell walls of pathogens like fungi and bacteria, thereby enhancing the plant's resistance to infections. For instance, in potatoes, the overexpression of thaumatin-like protein (CsTLP) has been shown to improve resistance to infections caused by *Phytophthora infestans*, a major cause of late blight, which is highly detrimental to potato crops (Acharya et al., 2013).

## **4. MATERIALS AND METHODS**

### **4.1. Experimental plants**

- Taxon: *Solanaceae*
- Genus: *Solanum L.*
- Species: *Solanum tuberosum L.*
- Cultivar: *Desirée*

See: Section 3.1.1.

### **4.2. Designing of construct of transformed potato lines**

#### **4.2.1. Selection of specific genes for modification**

Based on the research outlined by (Hanika et al., 2021), the homolog of the WAT-1 gene from *Arabidopsis* was identified in tomato as *WAT1 Solyc04g080940* (SIWAT1). Using techniques such as virus-induced gene silencing, RNAi, and CRISPR/Cas9, researchers transiently silenced SIWAT1 in tomato plants. The findings revealed that inactivating this gene conferred broad-spectrum resistance against multiple vascular pathogens in tomatoes. Notably, many of these pathogens also affect potato plants, suggesting a potential shared defense mechanism between these related crops.

In the study conducted by (Liu et al., 1994), constructs containing the osmotin gene (OSML) were developed, utilizing the 35S promoter from the cauliflower mosaic virus to achieve overexpression of the osmotin protein. The transformation of both potato and tobacco plants was mediated by *Agrobacterium tumefaciens*. The results revealed that osmotin expression constituted approximately 2 % of the total cellular protein. Furthermore, transgenic potato plants displayed a delayed onset of disease symptoms when inoculated with *Phytophthora infestans*, the pathogen responsible for potato late blight, demonstrating the potential of osmotin overexpression to enhance disease resistance. Considering the potential as genes involved in resistance against pathogens, the WAT-1 gene was selected for antisense silencing and OSML15 for gene overexpression.

#### 4.2.2. Characteristics of the vector

In this study, the pCP60 vector was used. pCP60 is a derivative of pBIN19 (Bevan, 1984) constructed and was generously provided by P. Ratet, CNRS, Paris, France. This vector is binary and is used for *Agrobacterium tumefaciens* and *Escherichia coli* mediated transformation of plant cells. The vector size is about 12 kilobases and *Bam*HI (5'- GGATCC; 3'- CCTAGG) , and *Eco*RI (5'- GAATTC; 3'-CTTAAG), restriction enzymes (produced by *THERMO FISHER* ®) were used for the insertion of our fragment as shown in Figure 4.

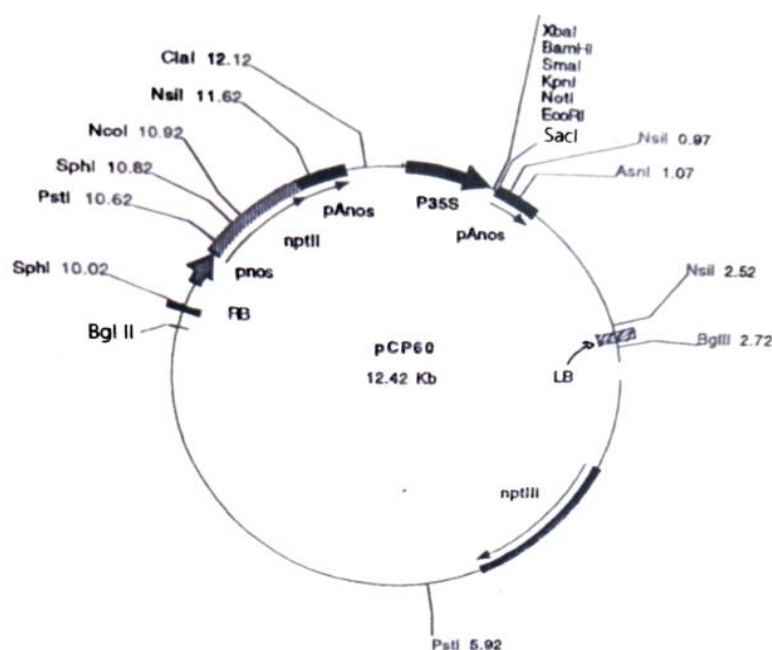


Figure 4. Structure of pCP60 (Bevan, 1984).

### 4.3. Gene silencing technique

For the gene silencing technique, WAT-1 was used as the gene of interest. Table 1 shows the pair of primers (oligo manufacturer: *IDT* ®) used considering the genome of the *S. Phureja* variety presented in the SPUD database (<https://spudb.uga.edu/>), gene identifier: *Soltu.DM.04G036030*.

Table 1. Primer design for WAT-1 fragment.

| Code           | Type    | Sequence (5'-3')                      |
|----------------|---------|---------------------------------------|
| WAT1 406bp aFw | Forward | T <b>GGATCC</b> GCACCAGAGTGAAGTAGCCA  |
| WAT1 406bp aR  | Reverse | C <b>GAATTC</b> ATGTGTAGCTGGAGCATCAGT |

The expected length of the PCR product was 321 bp. In the case of the WAT-1 fragment insertion, the direction is antisense 3'-5'. The direction of the insert was checked by PCR.

### 4.4. Overexpression technique

For the overexpression technique, OSML15 was used as the gene of interest. Table 2 shows the pair of primers (oligo manufacturer: *IDT* ®) used considering the same reference genomic database, gene accession number: *Soltu.DM.08G027290*.

Table 2. Primer design for OSML15 fragment.

| Code             | Type    | Sequence (5'-3')                        |
|------------------|---------|-----------------------------------------|
| OSM_new FwBamHI  | Forward | T <b>GGATCC</b> ATTGCAAAAAGCCGCCACAC    |
| OSM_new RevEcoRI | Reverse | C <b>GAATTC</b> TCAGATCTTAGCCACTTCAAGCA |

The expected length of the PCR product was 968 bp. In the case of the OSML15 fragment insertion, the direction is in sense 5'-3'. The direction of the insert was checked by PCR as well. Primers were designed using Primer-Blast software from the National Center for Biotechnology Information (NCBI).

### 4.5. Amplification of the fragments from genomic DNA

The plant material used was potato plants of the Desirée cultivar. The conditions and reagents used for PCR are described in Table 3 and 4. Forward and reverse primers are presented above.

Table 3. PCR protocol used for both fragments.

| Compounds                                      | Concentration | X [ $\mu$ L] |
|------------------------------------------------|---------------|--------------|
| Water                                          | -             | 19.85        |
| Dream Taq Buffer<br><i>THERMO FISHER</i> ®     | 10 X          | 2.50         |
| DNTPs                                          | 10 mM         | 0.50         |
| Primer (Forward)                               | 10 $\mu$ M    | 0.50         |
| Primer (Reverse)                               | 10 $\mu$ M    | 0.50         |
| Dream Taq Polymerase<br><i>THERMO FISHER</i> ® | 5 U/ $\mu$ L  | 0.15         |
| Template                                       | -             | 1.00         |
| Total (volume)                                 | -             | 25.00        |

Table 4. PCR settings in thermocycler for each fragment.

| Gene   | Temperature (°C)              | Time (min) | Cycles |
|--------|-------------------------------|------------|--------|
| WAT-1  | 95                            | 5:00       | 30     |
|        | 95                            | 00:30      |        |
|        | 59 (Temperature of annealing) | 00:30      |        |
|        | 72                            | 1:00       |        |
|        | 72                            | 10:00      |        |
|        | 4                             | $\infty$   |        |
|        | Temperature (°C)              | Time (min) | Cycles |
| OSML15 | 95                            | 5:00       | 24     |
|        | 95                            | 00:30      |        |
|        | 60 (Temperature of annealing) | 00:30      |        |
|        | 72                            | 1:00       |        |
|        | 72                            | 10:00      |        |
|        | 4                             | $\infty$   |        |

After the amplification of each fragment, an electrophoresis was performed for the observation of the amplified fragments of each gene. The fragments were observed in a 1 % agarose gel.

#### 4.6. Restriction enzyme digestion

After precipitation of the PCR products with 3x absolute ethanol and 1/20 4M sodium acetate, with both fragments (WAT-1 and OSML15) the same protocol was followed using 10  $\mu$ L per PCR product, 1  $\mu$ L of buffer, 1  $\mu$ L of BamHI and 1  $\mu$ L of EcoRI. Samples were incubated at 37 °C for 30 minutes in a thermoblock. Then, they were precipitated with 30  $\mu$ L absolute ethanol and 0.5  $\mu$ L 4M sodium acetate pH 5.2 at -20 °C overnight. The samples were then centrifuged at 13 000 RPM per 25 min, and the supernatant was discarded, the pellet was washed twice with 1 mL of 70 % (v/v) ethanol. Finally, the samples were dried in a laminar flow cabinet and the samples were suspended in 20  $\mu$ L of ultra-sterile water.

For the vector, 30  $\mu$ L of pCP60, 3  $\mu$ L of BamHI, 3  $\mu$ L of EcoRI and 3  $\mu$ L of buffer were transferred to an Eppendorf tube. The sample was incubated at 37 °C for 1 hr. The fragment was checked by 1 % agarose gel and precipitated with 108  $\mu$ L absolute ethanol and 1.4  $\mu$ L 4M sodium acetate pH 5.2 at -20 °C overnight. The samples were then centrifuged at 13 000 RPM per 25 min, then the supernatant was discarded, and the pellet was washed twice with 1 mL of 70 % (v/v) ethanol. Finally, the samples were dried in a laminar flow cabinet and resuspended in 20  $\mu$ L of ultra-sterile water.

#### **4.7. Ligation of the fragments into the vector (Cloning)**

For ligation of each PCR product (WAT-1 and OSML15 respectively), 5  $\mu$ L of ligation buffer *THERMO FISHER*®, 1  $\mu$ L of T4 DNA ligase *THERMO FISHER*®, 2  $\mu$ L of the digested pCP60 vector and 2  $\mu$ L of the digested PCR product of each were used. Likewise, 2 negative controls were established, which were a ligation reaction without T4 DNA ligase and another ligation reaction without any DNA fragment of interest (WAT-1 or OSML15), in this case, 2  $\mu$ L of ultra sterile water was used.

#### **4.8. Transformation of *E. coli* cells**

Competent *E. coli DH5  $\alpha$*  cells were thawed on ice. Then 100  $\mu$ L of the competent cells were added to the ligation mix, which was incubated on ice for 20 minutes. Subsequently, a heat shock was applied at 42 °C for 50 seconds and immediately placed on ice for 2 minutes. 1 mL of SOC medium and 20  $\mu$ L of glucose solution were added to the mixture and incubated for 1.5 hours at 37 °C with 200 RPM shaking. After 1.5 hours, the cell suspension was plated in dishes with LB medium supplemented with kanamycin (25 mg/L) and incubated at 37 °C for 24 hours.

The bacteria finally containing the pCP60 vector were expected to survive kanamycin treatment. Single colonies of supposedly transformed bacteria were picked with sterile swabs, and 48 streaks were made in a dish with LB medium supplemented with kanamycin (25 mg/L). Finally, each inoculated bacterial strain was diluted in liquid LB medium and incubated at 37 °C with 200 RPM shaking for 3 hours. Composition of the SOC and LB medium are listed in Table 5.

Table 5. Composition of LB and SOC medium.

| Medium | Reagent              | Quantity per liter |
|--------|----------------------|--------------------|
| LB     | Tryptone             | 10 g               |
|        | Yeast extract        | 5 g                |
|        | NaCl                 | 10 g               |
|        | Agar                 | 15 g               |
| SOC    | Yeast extract        | 5 g                |
|        | Tryptone             | 20 g               |
|        | 1M NaCl              | 10 mL              |
|        | 1M KCl               | 2.5 mL             |
|        | 1M MgCl <sub>2</sub> | 10 mL              |
|        | 1M MgSO <sub>4</sub> | 10 mL              |

#### 4.9. Extraction of plasmid DNA from *E. coli*

For plasmid DNA isolation, the following protocol: Transfected cells were collected by centrifugation at 13 000 RPM for 5 min from 1 mL of cell suspension and the supernatant was discarded. The cells were then resuspended in 100 µL of SOL I and each sample was vortexed. After five minutes, 200 µL of fresh SOL II was added and promptly mixed by inverting and left for 5 minutes at room temperature. Finally, 150 µL of SOL III was added and each sample was homogenized.

The samples were placed on ice for 5 minutes and then the tubes were centrifuged at 4 °C for 5 minutes at 13 000 RPM. The supernatant, approximately 2x 165 µL, was collected. To each sample, 1 mL of absolute ethanol was added, gently mixed and left for 2 minutes in the room. After an additional centrifugation step at 4 °C for 5 minutes at 13 000 RPM, the supernatant was discarded, and each sample was washed two times with 1 mL of 70 % (v/v) ethanol and centrifuged for 10 minutes under the above conditions. Finally, the supernatant was discarded, and the Eppendorf tubes were air dried.

The pellet obtained was resuspended in 50 µL of ultra-sterile water and 2 µL of RNase for each sample. The Eppendorf tubes were then incubated at 37 °C for 30 min in the thermoblock. Finally, 2.5 µL of 4M sodium acetate and 150 µL of absolute ethanol were added to each sample. Samples were stored at -20 °C overnight. The Eppendorf tubes were centrifuged at 13 000 RPM for 25 minutes, then the supernatant was removed, and 1 mL of 70 % ethanol was added and again, centrifuged at 13 000 RPM for 10 minutes (this process was performed twice).

Finally, each Eppendorf tube was dried and resuspended in 30  $\mu$ L of sterile water. SOL I, II and III mixtures are described in Table 6.

Table 6. Composition of each reagent for extraction DNA plasmid.

| Reagent | Compounds             | Concentration or Volume |
|---------|-----------------------|-------------------------|
| SOL I   | Glucose               | 50 $\mu$ M              |
|         | Tris pH 8             | 25                      |
|         | EDTA                  | 10                      |
|         | Lysozyme              | 5-10 mg/mL              |
| SOL III | NaOH                  | 0.2 N                   |
|         | SDS                   | 1% (w/v)                |
| SOL III | CH <sub>3</sub> COONa | 5 M                     |
|         | CH <sub>3</sub> COOH  | 11.5 mL                 |
|         | Water                 | 28.5mL                  |

#### 4.10. DNA quantification

For the determination of DNA concentration, a NanoDrop ND 1000 UV-VIS Spectrophotometer (NanoDrop Instruments, USA) was used, and the results were reported in ng/ $\mu$ L.

#### 4.11. Checking the presence and direction of WAT-1 and OSML15 fragments in the plasmid constructions

For amplification of the fragments used in the constructions from plasmid DNA, the PCR protocol and settings described in Tables 3 and 4 and the primer pairs described in Tables 1 and 2 were used. To verify the orientation of the fragments, a 35S promoter primer (*oligo manufacturer: IDT®*) functioned as a forward primer, 5'-CTATCCTTCGCAAGACCCTTC-3', together with the formerly used reverse primers. The fragments were observed on a 1% agarose gel supplemented with 0.07  $\mu$ l/ml ethidium-bromide using a BIO-RAD Geldoc EZ Imager instrument.

#### 4.12. Sequencing

The amplified DNA fragments were Sanger sequenced by Biomi, and the results were analyzed through the NCBI webpage and MEGA X software.

#### 4.13. Triparental mating

Triparental mating was performed using donor-, recipient- and helper strains. The transformed *E. coli* plasmid donor strains were grown in solid LB medium, which was supplemented with 25 mg/L Kanamycin at 37 °C for 24 hours. The *E. coli* SI18 helper strain was grown at 37 °C in solid LB medium supplemented with 10 mg/L kanamycin for 24 h. The electrocompetent *Agrobacterium tumefaciens* LBA4404 INVITROGEN® recipient strain was grown in YEB medium supplemented with magnesium sulfate and 100 mg/L rifampicin at 28 °C for 48 hours. A loopful of each bacterial colony was taken using heat sterilized inoculating loops and suspended in 100 µL of sterile water. The composition of YEB media and the bacterial strains used are shown in Table 7 and 8 respectively, and the design of the process of triparental mating is detailed in Table 9 and 10.

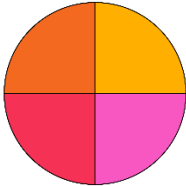
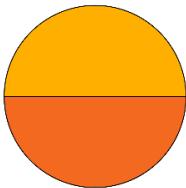
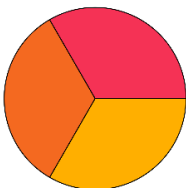
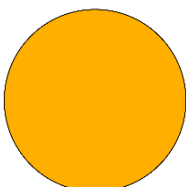
Table 7. Composition of YEB media.

| Media | Reagent                     | Quantity per liter |
|-------|-----------------------------|--------------------|
| YEB   | Yeast extract               | 1 g                |
|       | Beef extract                | 5 g                |
|       | Casein hydrolysate          | 5 g                |
|       | Sucrose                     | 5 g                |
|       | Agar (only for solid media) | 15 g               |

Table 8. Bacterial strains used in triparental mating.

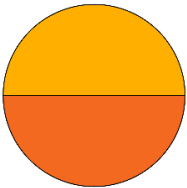
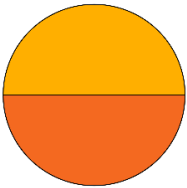
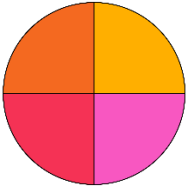
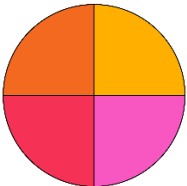
| Bacteria                         | Strains | Culture growth temperature (°C) |
|----------------------------------|---------|---------------------------------|
| <i>Escherichia coli</i>          | DH5α    | 37                              |
| <i>Escherichia coli</i> (helper) | SI18    | 37                              |
| <i>Agrobacterium tumefaciens</i> | LBA4404 | 28                              |

Table 9. Scheme of petri dishes used in triparental mating 1<sup>st</sup> part.

| N                                         | Treatment                                                                                                                                                                            | Growing media                 | Distribution of the petri dish                                                             |
|-------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|--------------------------------------------------------------------------------------------|
| Positive control of the different strains | <b>AG:</b> <i>Agrobacterium tumefaciens</i><br><b>HS:</b> Helper strain<br><b>TS:</b> Transformed strain<br><b>NC:</b> Negative control ( <i>E.coli</i> containing the empty vector) | YEB media + No antibiotics    | <br>A   |
| Crossings                                 | <b>AG + HS + TS</b><br><b>AG + HS + NC</b>                                                                                                                                           | YEB media + No antibiotics    | <br>B   |
| Negative control of <i>coli</i> strains   | <b>HS:</b> Helper strain<br><b>TS:</b> Transformed strain<br><b>NC:</b> Negative control (no transformed strain <i>E. Coli</i> strain containing the empty vector)                   | LB media + Rifampicin 100mg/L | <br>C  |
| Negative control of <i>Agrobacterium</i>  | <b>AG:</b> <i>Agrobacterium tumefaciens</i>                                                                                                                                          | YEB media + Kanamycin 25 mg/L | <br>D |

After inoculation of the bacterial dilutions mentioned in Table 9, the petri dishes were incubated at 28 °C for 48 hours. Then, serial dilutions up to 10<sup>-6</sup> of the crossings were performed, the distribution of the experiment shows in Table 10.

Table 10. Shame of petri dishes used in triparental mating 2<sup>nd</sup> part.

| N                                                       | Treatment    | Concentration of dilution | Growing media                                       | Distribution of the petri dish                                                        |
|---------------------------------------------------------|--------------|---------------------------|-----------------------------------------------------|---------------------------------------------------------------------------------------|
| Positive control of crossings                           | AG + HS + TS | 10 <sup>0</sup>           | YEB media + No antibiotics                          |    |
|                                                         | AG + HS + NC | 10 <sup>0</sup>           |                                                     |                                                                                       |
| Negative control of crossings                           | AG + HS + TS | 10 <sup>0</sup>           | YEB media + Tetracycline                            |    |
|                                                         | AG + HS + NC | 10 <sup>0</sup>           |                                                     |                                                                                       |
| Selection of transformed Agrobacteria                   | AG + HS + TS | 10 <sup>0</sup>           | YEB media + Kanamycin 25 mg/L + Rifampicin 100 mg/L |   |
|                                                         |              | 10 <sup>-2</sup>          |                                                     |                                                                                       |
|                                                         |              | 10 <sup>-4</sup>          |                                                     |                                                                                       |
|                                                         |              | 10 <sup>-6</sup>          |                                                     |                                                                                       |
| Selection of Agrobacteria transformed with empty vector | AG + HS + NC | 10 <sup>0</sup>           | YEB media + Kanamycin 25 mg/L + Rifampicin 100 mg/L |  |
|                                                         |              | 10 <sup>-2</sup>          |                                                     |                                                                                       |
|                                                         |              | 10 <sup>-4</sup>          |                                                     |                                                                                       |
|                                                         |              | 10 <sup>-6</sup>          |                                                     |                                                                                       |

The petri dishes shown on Table 10 were incubated at 28 °C for 48 hours.

Three independent colonies of each positive (WAT-1 and OSML15) and one colony of the negative (containing the empty vector) transformant Agrobacterial strains were inoculated over a new petri dish filled with solid YEB supplemented with 25 mg/L kanamycin and 100 mg/L rifampicin.

Finally, plasmid DNA was extracted from the samples by adding an extra step in the extraction protocol mentioned in section 4.9 where the cell dilution was resuspended with 1 mL of 0.1 M sodium chloride. The presence of the studied gene fragments of WAT-1 and OSML15 was checked by PCR following the protocol described in section 4.5.

#### **4.14. Transformation of potato plants**

For the purification of the colonies transformed into *Agrobacteria*, they were recultured in YEB liquid medium supplemented with magnesium sulfate: (a) bacterial solution with the WAT-1 gene, (b) bacterial solution with the OSML15 gene, (c) bacterial solution containing the empty vector, negative control. 10 mL of bacterial cultures were incubated at 28 °C with overnight shaking. Subsequently, 1 mL of each treatment was transferred to Eppendorf tubes.

The Eppendorf tubes were centrifuged at 8000 RPM per 10 minutes, the supernatant was discarded, and 1 mL of Murashige-Skoog liquid medium (MS) was added and centrifuged under the same conditions. Finally, the supernatant was discarded, and 1 mL of MS liquid medium was added, and the bacterial contents were dissolved. Twelve petri dishes were prepared with liquid MS medium (each petri dish with 25 mL volume). The model was represented with the following structure: 4 petri dishes for the WAT-1 gene, 4 petri dishes for the OSML15 gene and 4 petri dishes as negative control (empty vector containing strains). For the transformation of potato plants (of each study gene separately), leaves of plants obtained *in vitro* were used.

The plant material used were potato plants of the Desirée cultivar, which were previously cultivated *in vitro*. The leaves were cut at the height of the center of the stem and the leaves most distant from the root were used. The leaves selected for *agrobacterium*-mediated transformation were cut three times in their internal structure with a scalpel and the shoot tips were recultured in fresh culture medium for propagation.

Leaves were then placed in petri dishes and 100 µL of bacterial culture was added to each petri dish containing leaves with liquid MS medium. The samples were kept in the petri dishes in darkness for 48 hours. After 48 hours, the leaves were transferred to a filter paper to remove the excess liquid. Additionally, the dried leaves were placed with a sterile forceps in Callus Induction medium (CIM) supplemented with kanamycin 50 mg/L and 250 mg/L Claforan. The petri dishes were incubated in the lightroom for 7 days at 24 °C (16 h light/8 h dark).

After 7 days, potato leaves were transferred to Shoot Induction medium (SIM) selective medium supplemented with 50 mg/L Kanamycin and 250 mg/L Claforan for 6-8 weeks in the lightroom. Leaves were transferred to fresh SIM medium supplemented with antibiotics every week. Finally, regenerated seedlings were separated and placed on selective medium in test tubes.

The composition of the MS, CIM and SIM media are described in the Tables 11, 12, 13.

Table 11. Composition of MS medium.

| Reagent | Compounds                                          | Quantity (g/L) | Total volume (per liter) |
|---------|----------------------------------------------------|----------------|--------------------------|
| RM I    | NH <sub>4</sub> O <sub>3</sub>                     | 33 g/L         | 50 mL                    |
|         | KNO <sub>3</sub>                                   | 38 g/L         |                          |
|         | CaCl <sub>2</sub> . 2H <sub>2</sub> O              | 8.8 g/L        |                          |
|         | MgSO <sub>4</sub> .7H <sub>2</sub> O               | 7.4 g/L        |                          |
|         | KH <sub>2</sub> PO <sub>4</sub>                    | 3.4 g/L        |                          |
| RM II   | FeSO <sub>4</sub> .7H <sub>2</sub> O               | 5.35 g/L       | 1 mL                     |
|         | Na <sub>2</sub> EDTA.2H <sub>2</sub> O             | 7.46 g/L       |                          |
| RM III  | KI                                                 | 0.17 g/L       | 5 mL                     |
|         | H <sub>3</sub> BO <sub>3</sub>                     | 1.24 g/L       |                          |
|         | MnSO <sub>4</sub> .4H <sub>2</sub> O               | 4.46 g/L       |                          |
|         | ZnSO <sub>4</sub> .7H <sub>2</sub> O               | 1.72 g/L       |                          |
|         | N <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O | 50 g/L         |                          |
|         | CuSO <sub>4</sub> .5H <sub>2</sub> O               | 5 g/L          |                          |
|         | CoCl <sub>2</sub> .6H <sub>2</sub> O               | 5 g/L          |                          |
| RM IV   | Myo-inositol                                       | 0.02g/mL       | 5 mL                     |
|         | Nicotinic acid                                     | 0.2 mg/mL      |                          |
|         | Pyridoxine                                         | 0.1mg/mL       |                          |
|         | Thiamine                                           | 0.1 mg/mL      |                          |
|         | Glycine                                            | 0.4 mg/mL      |                          |
| Sucrose | -                                                  | -              | 20 g                     |
| Agar    | -                                                  | -              | 8 g                      |

Table 12. Composition of CIM medium.

| Reagent | Compounds                                          | Quantity (g/L) | Total volume (per liter) |
|---------|----------------------------------------------------|----------------|--------------------------|
| RM I    | NH <sub>4</sub> O <sub>3</sub>                     | 33 g/L         | 50 mL                    |
|         | KNO <sub>3</sub>                                   | 38 g/L         |                          |
|         | CaCl <sub>2</sub> . 2H <sub>2</sub> O              | 8.8 g/L        |                          |
|         | MgSO <sub>4</sub> .7H <sub>2</sub> O               | 7.4 g/L        |                          |
|         | KH <sub>2</sub> PO <sub>4</sub>                    | 3.4 g/L        |                          |
| RM II   | FeSO <sub>4</sub> .7H <sub>2</sub> O               | 5.35 g/L       | 1 mL                     |
|         | Na <sub>2</sub> EDTA.2H <sub>2</sub> O             | 7.46 g/L       |                          |
| RM III  | KI                                                 | 0.17 g/L       | 5 mL                     |
|         | H <sub>3</sub> BO <sub>3</sub>                     | 1.24 g/L       |                          |
|         | MnSO <sub>4</sub> .4H <sub>2</sub> O               | 4.46 g/L       |                          |
|         | ZnSO <sub>4</sub> .7H <sub>2</sub> O               | 1.72 g/L       |                          |
|         | N <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O | 50 g/L         |                          |
|         | CuSO <sub>4</sub> .5H <sub>2</sub> O               | 5 g/L          |                          |
|         | CoCl <sub>2</sub> .6H <sub>2</sub> O               | 5 g/L          |                          |
| RM IV   | Myo-inositol                                       | 0.02g/mL       | 5 mL                     |
|         | Nicotinic acid                                     | 0.2 mg/mL      |                          |
|         | Pyridoxine                                         | 0.1mg/mL       |                          |
|         | Thiamine                                           | 0.1 mg/mL      |                          |

|                         |         |           |       |
|-------------------------|---------|-----------|-------|
|                         | Glycine | 0.4 mg/mL |       |
| Naphthalene acetic acid | -       | 5 mg/mL   | 5 mL  |
| 6-Benzylaminopurine     | -       | 2.5 mg/mL | 40 µL |
| Glucose                 | -       | -         | 16 g  |
| Agar                    | -       | -         | 6 g   |

Table 13. Composition of SIM medium.

| Reagent                 | Compounds                                          | Quantity  | Total volume (per liter) |
|-------------------------|----------------------------------------------------|-----------|--------------------------|
| RM I                    | NH <sub>4</sub> O <sub>3</sub>                     | 33 g/L    | 50 mL                    |
|                         | KNO <sub>3</sub>                                   | 38 g/L    |                          |
|                         | CaCl <sub>2</sub> . 2H <sub>2</sub> O              | 8.8 g/L   |                          |
|                         | MgSO <sub>4</sub> .7H <sub>2</sub> O               | 7.4 g/L   |                          |
|                         | KH <sub>2</sub> PO <sub>4</sub>                    | 3.4 g/L   |                          |
| RM II                   | FeSO <sub>4</sub> .7H <sub>2</sub> O               | 5.35 g/L  | 1 mL                     |
|                         | Na <sub>2</sub> EDTA.2H <sub>2</sub> O             | 7.46 g/L  |                          |
| RM III                  | KI                                                 | 0.17 g/L  | 5 mL                     |
|                         | H <sub>3</sub> BO <sub>3</sub>                     | 1.24 g/L  |                          |
|                         | MnSO <sub>4</sub> .4H <sub>2</sub> O               | 4.46 g/L  |                          |
|                         | ZnSO <sub>4</sub> .7H <sub>2</sub> O               | 1.72 g/L  |                          |
|                         | N <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O | 50 g/L    |                          |
|                         | CuSO <sub>4</sub> .5H <sub>2</sub> O               | 5 g/L     |                          |
|                         | CoCl <sub>2</sub> .6H <sub>2</sub> O               | 5 g/L     |                          |
| RM IV                   | Myo-inositol                                       | 0.02g/mL  | 5 mL                     |
|                         | Nicotinic acid                                     | 0.2 mg/mL |                          |
|                         | Pyridoxine                                         | 0.1mg/mL  |                          |
|                         | Thiamine                                           | 0.1 mg/mL |                          |
|                         | Glycine                                            | 0.4 mg/mL |                          |
| Naphthalene acetic acid | -                                                  | 5 mg/mL   | 40 µL                    |
| 6-Benzylaminopurine     | -                                                  | 2.5 mg/mL | 100 µL                   |
| Zeatin riboside         | -                                                  | 2 mg/mL   | 1 mL                     |
| Sucrose                 | -                                                  | -         | 16 g                     |
| Agar                    | -                                                  | -         | 6 g                      |

#### 4.15. Checking the construct in the *in vitro* regenerated plants

##### 4.15.1. Isolation of RNA

The plants selected were exclusively those transformed with the WAT-1 gene, since the plants regenerated with the OSML15 gene are still in the growth stage. A 100 mg ground sample was collected from the *in vitro* regenerated plants, grinding was carried out with liquid nitrogen. There were added 600 µL of phenol and 600 µL of isolation buffer (see Table 14).

The samples were then vortexed for 60 seconds and left at room temperature for 10 minutes. The samples were then centrifuged at 13000 RPM at 4 °C for 10 minutes. The supernatant was transferred to new Eppendorf tubes containing 600 µL of 1:1 Phenol: Chloroform solution, vortexed and the samples were centrifuged at the above-mentioned conditions, this process was performed twice.

Table 14. Compounds of isolating solution.

| Reagent             | Concentration | Amount per sample (µL) |
|---------------------|---------------|------------------------|
| Sodium Acetate      | 3 M pH 5.2    | 39.6                   |
| EDTA                | 0.5 M         | 12                     |
| Ultra sterile water | -             | 488                    |
| SDS                 | 10 %          | 60                     |

Then 600 µL supernatant was transferred to a new Eppendorf tube and mixed with 200 µL of 10 M LiCl and the samples were incubated overnight at -20 °C. The next day, the samples were centrifuged at 13 000 RPM at 4 °C per 10 min, the supernatant was discarded. 1 mL of 2.5M LiCl was transferred and diluted. Likewise, the samples were centrifuged under the above conditions and the supernatant was discarded. 1 mL of 70 % ethanol was placed, and the supernatant was discarded, this process was performed twice. Finally, the samples were dried in a laminar flow chamber and then diluted in 30 µL of ultra sterile water. The RNA concentration was determined in ng/uL considering the method described in the section. Samples were diluted to 100 ng/uL with ultra sterile water.

#### 4.15.2. cDNA synthesis and RT-PCR

For cDNA synthesis from RNA, 1 µL of dsDNAase buffer *THERMO FISHER*®, 1 µL of dsDNAase enzyme *THERMO FISHER*® was used, and 1 µL of RNA sample (100 ng/µL) was transferred with ultra-sterile water to 10 µL. Samples were incubated at 37 °C for 2 min. Then 1 µL of random primers *THERMO FISHER*®, 1 µL of dNTP , 4 µL of RT enzyme *THERMO FISHER*® and 1 µL of enzyme mix *THERMO FISHER*® were placed. The mixture was made up to 10 µL using sterile water (total volume: 20 µL). The samples were incubated at 25 °C for 10 minutes, then 50 °C at 30 minutes and 85 °C for 5 minutes. Fragment amplification was carried out as mentioned in the PCR protocol described in section 4.5 and the primers used are described in Table 15. Primers were designed using Primer-Blast software from the National Center for Biotechnology Information (NCBI). The cDNA fragments were observed on a 4% agarose gel.

Table 15. Primer design for RT-PCR.

| Gen    | Code      | Sequence (5'-3')      |
|--------|-----------|-----------------------|
| WAT-1  | WAT-1 qFw | ATGGTGCATTGACAGAGGGG  |
|        | WAT-1 qR  | CGAAGGAAGCCATCAGAGCA  |
| OSML15 | OSM qFw   | GCCGGGAAAATGTCATCCAAT |
|        | OSM qR    | TGGACCTTGGGTGCAACAAT  |

## 5. RESULTS AND DISCUSSION

### 5.1. PCR products of WAT-1

In Figure 5A, the amplification of two WAT-1 fragments in PCR made with two different primer pairs designed on two different exons (485 bp long and 406 bp long) was observed on agarose gel. The exon under study here was the 406 bp long exon. Figure 5B shows gel electrophoresis of restriction enzyme digestion of the same fragments. In Figure 5C, after *E. coli* mediated transformation, it was obtained that 4/24 studied bacterial colonies (sample W2, W5, W16, W24) showed the designed construct within their genome. In Figure 5E, the direction of the fragment inside the vector was checked, which determined that the samples that amplified were inserted in the correct direction.

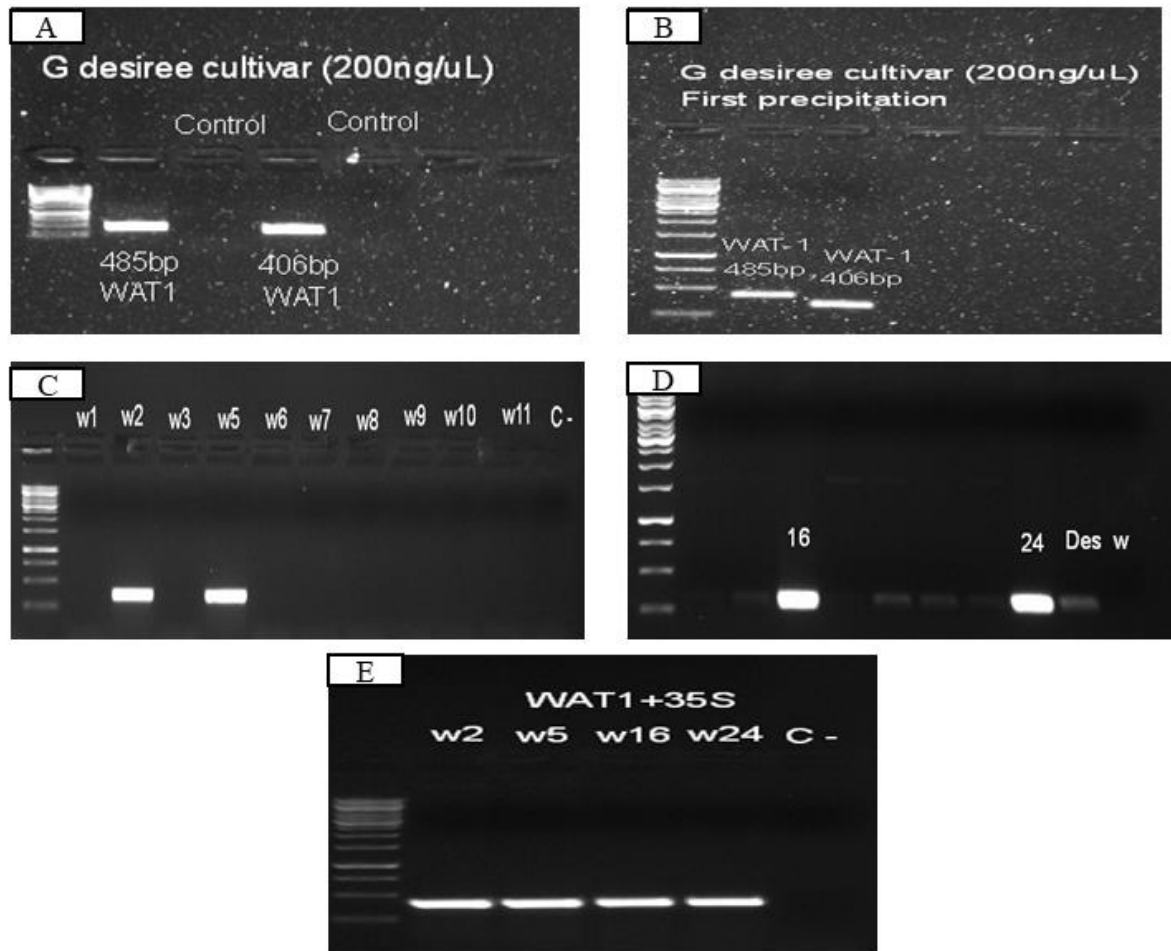


Figure 5. Fragments observed on agarose gel from WAT-1 gene (PCR products). A) Amplification of fragments of two exons of WAT-1 from genomic DNA of the Desirée cultivar. B) Amplification of the fragments digested with BamHI and EcoRI. C) Amplification of plasmid DNA fragments from transformed *E. coli* samples, part 1. D) Amplification of plasmid DNA fragments from transformed *E. coli* samples, part 2. Genomic DNA of the Desirée cultivar was used as positive control. E) Checking the direction of insertion of the fragment in the plasmid DNA by PCR.

## 5.2. PCR products of OSML15

Figure 6A shows the amplification of the OSML15 fragment amplified with the primers described in section 4.4, the length of the fragment was 968 bp. Figure 6B shows the fragment digested by the restriction enzymes mentioned in materials and methods. In Figure 6C, the direction of the inserts in the constructs was determined, by using 35S forward and OSM\_new RevEcoRI reverse primers in the PCR reaction resulting in the amplification of the fragment having the proper length. In Figure 6D, after the plasmid DNA extraction of 12 transformed *E. coli* colonies only two contained the desired fragment (samples O1 and O5).

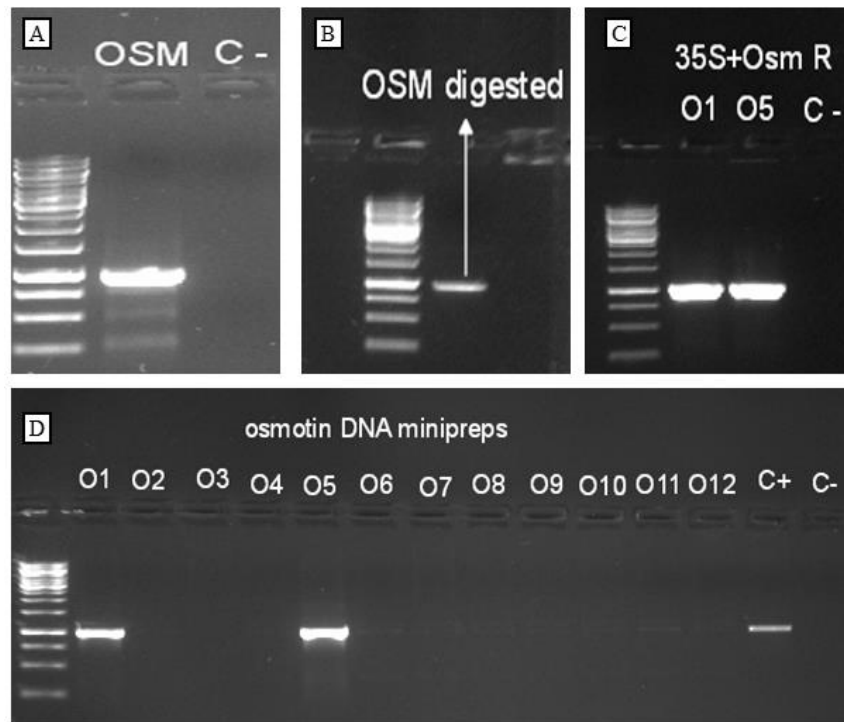


Figure 6. Fragments observed on agarose gel from OSML 15 gene (PCR products). A) Amplification of fragments of two exons of WAT-1 from genomic DNA corresponding to the Desirée cultivar. B) Amplification of the fragments digested with *Bam*HI and *Eco*RI. C) Checking the direction of the fragment by PCR in the plasmid DNA. D) Amplification of fragments from plasmid DNA of transformed *E. coli* samples, genomic DNA of Desirée was used as a positive control.

### 5.3. Triparental mating

As mentioned in materials and methods, in *Agrobacterium*-mediated transformation, colonies with the WAT-1 and OSML15 genes, respectively, were identified. The colonies consisted of the following construct: *E. coli helper::Agrobacterium tumefaciens::E.coli donor strain (DH5α)* containing the cloning vectors with fragments of the study genes (WAT-1 or OSML15).

In Figure 7A, 3 DNA fragments belonging to 3 colonies of the bacterial construct with the OSML15 gene were observed, in the fourth column the same fragment amplified from Desirée genomic DNA can be seen, while in the last three columns, colonies containing empty plasmids were used as negative controls. In Figure 7B, 2 DNA fragments corresponding to the plasmid DNA isolated from colonies of the bacterial construct with the WAT-1 gene were observed; likewise, the same reaction with strains containing empty plasmids was used as a negative control. In the last two columns, fragments amplified from Desirée's genomic DNA and reaction mix without DNA are shown.

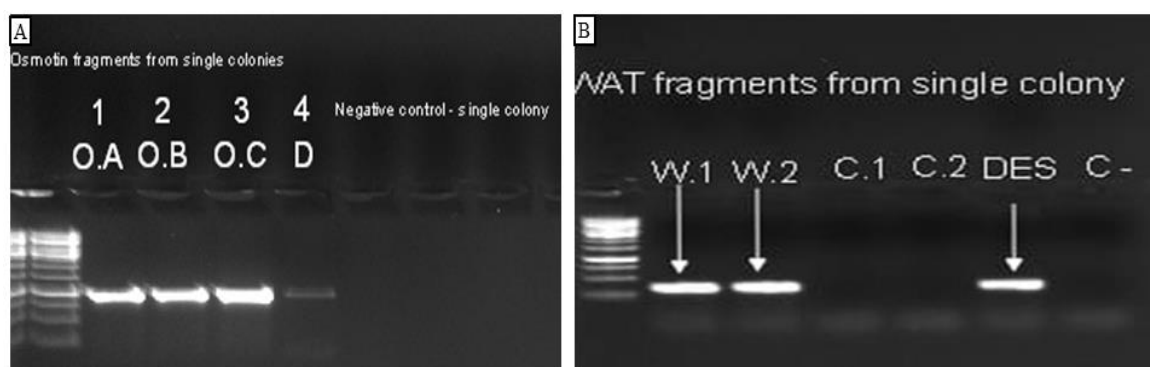


Figure 7. Fragments observed on agarose gel from plasmid DNA purified from transformed *Agrobacterium* colonies. A) Osmotin fragments (968 bp) amplified from plasmid DNA purified from three *Agrobacterium* independent colonies. B) WAT1 fragments (321 bp) amplified from plasmid DNA purified from two independent *Agrobacterium* colonies.

### 5.4. Development of transgenic plants

In the process of infection of potato *in vitro* plants with the bacterial constructs, in Figure 8A, the leaves were placed in MS medium containing the suspension of transformed bacteria (see section 4.14), no callus sprouts or external contamination were observed. Figure 8B shows the first callus sprouts after three weeks on SIM medium. Figure 8C shows the first small shoots developed *in vitro* from callus cells, these were observed from week 6 in SIM medium. Figure 8D shows the *in vitro* regenerated transgenic plants.

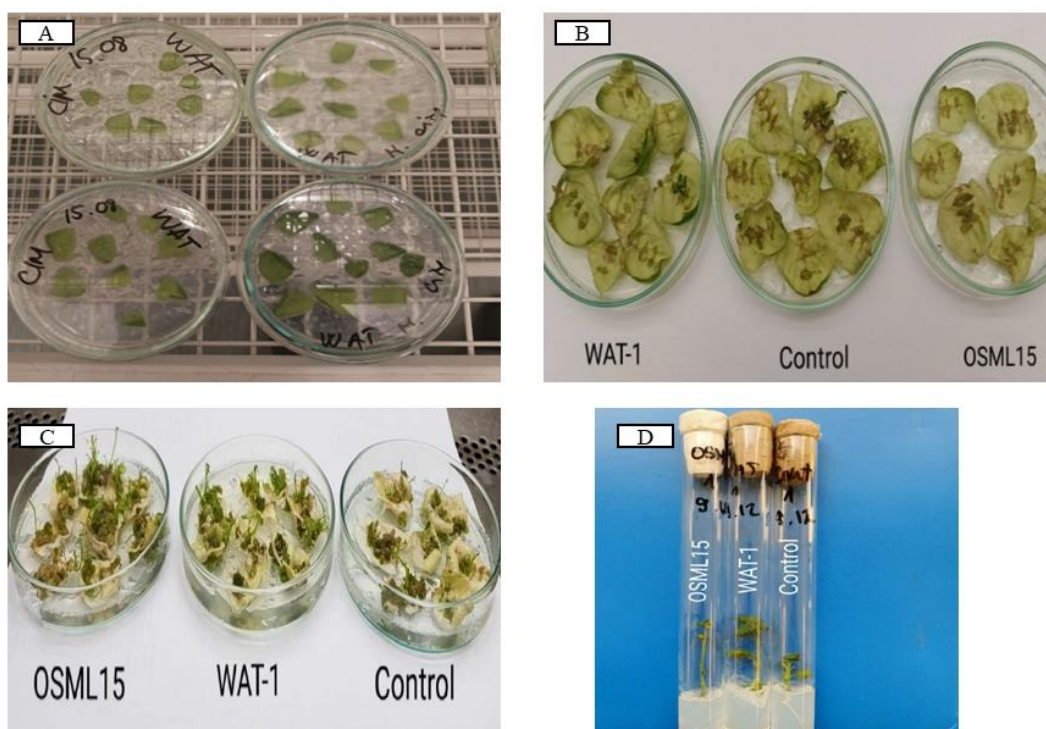


Figure 8. *In vitro* regeneration of transformed plants in selective medium. A) *Agrobacterium* infection of leaves in MS medium. B) First appearance of callus induction on SIM medium. C) First shoots of regenerated seedlings post-infection process. D) First lines of transformed plants growing in selective medium.

### 5.5. PCR of *in vitro* regenerated transgenic plants (WAT-1 lines)

As detailed in the materials and methods section, RNA was obtained from *in vitro* regenerated potato plants. After cDNA synthesis from the RNA, PCR was implemented using WAT-1 qFw and WAT-1 qR primers (see Table 15). Figure 9 demonstrates the lower expression level of WAT-1 gene of the *in vitro* regenerated plants compared to the wild type. Primers described in Table 15 were used in RT-PCR, where two cDNA samples from *in vitro* regenerated transgenic plants (W7 and W10) showed lower amplification levels beside the wild type of Désirée cDNA (GD1 and GD2), the length of the fragment shown was 86 bp.



Figure 9. Results of RT-PCR showing gene expression levels of the first regenerated WAT1 silenced plants. C4 and C7: negative control plants, however because of technical problems during sample uploading, C4 and C7 bands are not informative. W7 and W10: WAT1 silenced plants. GD1 and GD2: control Desirée plants.

## 6. CONCLUSIONS AND FUTURE AIMS

In this study, we demonstrated the present state of our research and detailed the steps we have taken so far towards reaching the final aims. These steps include the successful cloning of fragments from each gene under study (WAT-1 and OSML15), both of which were digested with restriction enzymes and ligated into the pCP60 vector using molecular biology techniques. These constructs, designed in antisense and sense orientations, respectively, were transformed into *E. coli* DH5  $\alpha$  strains, and bacterial colonies containing each gene were obtained, plasmid DNA were isolated, and the presence of the desired fragments was proved by gel electrophoresis after PCR. Additionally, at this stage of the research, the first *in vitro* regenerated potato seedlings were obtained from WAT-1 transformed lines, and the difference in the expression level of the target gene was confirmed by RT-PCR. After checking the gene expression of several transgenic lines by RT-qPCR, the final goal is to obtain stable low WAT-1 expression level and high OSML15 expression level potato lines and evaluate their phenotype and resistance against *Ralstonia solanacearum*.

## 7. ACKNOWLEDGEMENTS

I am grateful to Kiss Monika Tekla for her assistance and help with all the experiments performed in this research. Special thanks to Dr. Bánfalvi Zsófia and Dr. Villányi Vanda for their advice and imparting their knowledge as it was crucial for the implementation of this research.

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