FACULTY OF BIOSCIENCE ENGINEERING

THE SALICYLIC ACID BIOSYNTHESIS IN RICE: ELUCIDATING THE ROLE OF THE PAL PATHWAY

Thibault Cloet
Student number: 01505334

Promotors: Prof. dr. Godelieve Gheysen
Dr. Lander Bauters

Tutor: Hannes Lefevere

Master's Dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Science in Bioscience Engineering: Cell and Gene Biotechnology

Academic year: 2019 - 2020

GHENT UNIVERSITY

Prologue

At first, I would like to thank my promotor Prof. Gheysen to make it possible to write this masters dissertation. Together with Lander you also promoted my bachelor thesis. This project waked my interest to study plant biotechnology. I learned a lot in your laboratory and when there was a small problem, it was always possible to enter your office for questions or remarks. After the restrictions of the corona crisis, your involvement and new insights in my thesis by our weekly Microsoft Teams meetings were really encouraging and this is something many master students could only dream of. Also another special thank you for Lander, to read over my project and also to help me when I had questions. Our road trip to ILVO was very inspiring with a lot of good talks.

Next, I want to thank my supervisor and friend Hannes. During this year, you were always there to help me when I needed it. There were a lot of difficulties to overcome, but after a brainstorm, we came up with an idea to handle the situation. Thank you for advising and helping me and learning me everything I needed this year. During the writing of this project, you were also writing a very interesting review about the biosynthesis of salicylic acid. It would be an honour if you were the first source used in my project [1].

Further, I would like to thank all lab members. If I had an urgent question I could always rely on you to help me where needed. Also when the CRICK PCR machine gave again an error message you were always there to let me know. Thank you all!

At last, I would like to thank Melissa, my family and friends for keeping supporting me during this year. You were all a very great help, making me able to succeed this last year. Also thank you to let me try my thesis presentation although most of you thought I was talking another language.

The last five years of bio-engineering were a fantastic preparation for what is coming next. I made a lot of friendships, I learned a lot of very interesting material and I was formed to the man I am today. I thank everyone that was, in one way or another, involved in this period. This project is the final piece of this education program and I hope you enjoy reading it.

Impact of the corona virus

In this masters dissertation, the role of the *PAL* genes and the *AIM1* gene in the biosynthesis of salicylic acid (SA) is investigated in rice. For this, CRISPR mutants and overexpression lines of all genes separately are generated. In the end, for each gene; the CRISPR mutant, the overexpression line and a wild type plant will be tested for their concentration of SA and their susceptibility for the root pathogen *Meloidogyne graminicola* and the leaf pathogen *Fusarium*. With the collection of all this data, most likely, a conclusion can be made for each gene of their involvement in the biosynthesis of SA and the following resistance towards different kinds of pathogens. To further prove the involvement of the genes of interest in resistance towards pathogens, an infection experiment will be performed in which half of the plants are infected and the up- or downregulation of the *PAL* genes will be examined and compared between both groups. Furthermore, another infection experiment will be performed to infer the importance of the PAL enzyme by treating half of the plants with a PAL-inhibitor. All plants are then infected and a symptom analysis will be performed.

Due to the corona crisis and the early termination of all laboratory works, some steps of this masters dissertation could not be carried out. For the CRISPR mutants, it is shown that the used gRNA for *AIM1* does not cleave *in vitro* but another one capable of doing this was found. However, a new rice transformation series with this new gRNA could not be started. For the overexpression lines, *Agrobacterium* containing the *AIM1* gene is present, but a rice transformation series could also not be started here. Due to the large time range to grow transformed rice plants and the death of all transformed plants, the metabolome analysis and susceptibility experiments could not be performed. Rice plants for both infection experiments with *M. graminicola* were grown before the start of the corona crisis. However, the supervisor of this project did all subsequent steps and measurements. The analysis of the results was done by the author and the tutor of this project via online meetings. Also, the qPCR infection experiment was performed by the supervisor. However, because multiple samples did have too large error bars, no conclusion could be made and due to the shortened time range, the qPCR experiment could not be redone. At last, the infection experiments using *Fusarium* could also not be started.

This project is finished, based on the material that was already available before the start of the corona crisis with the addition of the infection experiments that were started before the crisis and were finished by the supervisor.

'This preamble has been prepared in consultation with the student and the supervisor and approved by both'.

Table of content

List of abbreviations

AIM1	Abnormal inflorescence meristem 1
AOPP	α-aminooxy-β-phenylpropionic acid
Cas	CRISPR-associated proteins
cDNA	Complementary DNA
CDS	Coding sequence
CM	Chorismate mutase
CRISPR	Clustered regulatory interspaced palindromic repeats
DHBA	Dihydroxybenzoates
DNA	Deoxyribonucleic acid
EDS5	Enhanced disease susceptibility 5
EPS1	Enhanced *Pseudomonas* susceptibility 1
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
gRNA	Guide RNA
HR	Hypersensitive response
ICS	Isochorismate synthase
IPL	Isochorismate pyruvate lyase
Kan	Kanamycin
MAPK	Mitogen-activated protein kinase
MeSA	Methyl salicylate
MeSAG	Methyl salicylate glucoside
MFP	Multifunctional protein
NHP	N-hydroxypipecolic acid
NPR1/3/4	Non-expressor of pathogenesis-related genes 1/3/4
PAL	Phenylalanine ammonia-lyase
PAM	Protospacer adjacent motif
PAMP	Pathogen-associated molecular pattern
PBS3	avrPphB susceptible 3
PCR	Polymerase chain reaction
PR-gene	Pathogenesis-related gene
PTI	Pattern triggered immunity
Rif	Rifampecillin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SAG	Salicylic acid glucoside
SAR	Systemic acquired resistance
SGE	Salicylic acid glucose ester
Spec	Spectinomycin
TAL	Tyrosine ammonia-lyase
tCA	trans-cinnamic acid

Abstract

In evolution, plants have developed a very complex and efficient immune system. This is necessary because plants are very often exposed to all kinds of pathogens and pests. A plant immune system is composed of a complicated signaling network which includes many receptors, hormones and other factors that can initiate several immune responses.

In *Arabidopsis thaliana*, a model plant, it has been discovered that salicylic acid is an important plant hormone to initiate these immune responses. Further research on this compound showed that the biosynthetic pathway of salicylic acid consists of two branches: the ICS and the PAL pathway. The ICS pathway was found to be the most important pathway for the formation of salicylic acid.

However, in rice, one of the most important nutritional food crops, not much is known about the importance of both pathways in the biosynthesis of salicylic acid.

The objective of this master thesis project is to look into the role of different genes involved in the PAL pathway for the biosynthesis of salicylic acid in rice. This will be accomplished by making CRISPR rice mutants with a knock-out in each *PAL* gene or *AIM1* and, on the other hand, making transgenic rice plants with overexpression in a *PAL* gene assumed in literature to be important. When these mutants are obtained, a comparison can be made between the CRISPR mutants, wild type plants and the transgenic overexpression plants based on levels of salicylic acid and resistance to certain pathogen attacks. In this project, the first steps in achieving this data have been improved to make future experiments less complicated.

Also, the role of the PAL enzyme was tested in nematode infection and here, a first indication is given that inhibition of the PAL enzyme will enhance susceptibility of the rice plants to *M. graminicola.*

Samenvatting

Planten hebben in de evolutie een complex en efficiënt immuunsysteem ontwikkeld. Dit is noodzakelijk doordat planten zeer vaak blootgesteld worden aan verschillende pathogenen en plagen. Het plantimmuunsysteem bestaat uit een ingewikkeld signaalnetwerk met vele receptoren en hormonen die verschillende immuunresponsen kunnen initiëren.

In de modelplant *Arabidopsis thaliana* is ontdekt dat salicylzuur een belangrijk planthormoon is dat deze immuunresponsen kan initiëren. Verder onderzoek heeft uitgewezen dat de pathway die salicylzuur vormt bestaat uit twee takken: de ICS- en de PAL-pathway. Er werd ook aangetoond dat de ICS-pathway de belangrijkste pathway is in de productie van salicylzuur.

In rijst, een van de meest belangrijke voedselgewassen ter wereld, is echter nog niet veel geweten over het belang van beide pathways in de productie van salicylzuur.

Het doel van deze masterscriptie is het onderzoeken van de rol van verschillende belangrijke genen betrokken in de PAL-pathway voor de biosynthese van salicylzuur in rijst. Hiervoor zullen CRISPR-rijstmutanten gemaakt worden met een knock-out in elk *PAL*-gen of *AIM1*. Ook zullen er transgene rijstplanten gemaakt worden die overexpressie van enkele, in de literatuur voorgesteld als belangrijke, *PAL*-genen tot gevolg hebben. Wanneer deze mutanten verkregen zijn, zal een vergelijking gemaakt worden tussen de CRISPR-mutant, een wild type plant en de transgene overexpressieplant gebaseerd op salicylzuurconcentratie en vatbaarheid voor bepaalde pathogenen. In dit project werden de eerste stappen van dit experiment geoptimaliseerd om verder onderzoek te vergemakkelijken.

Als laatste werd ook de rol van het PAL-enzyme tijdens nematodeninfectie onderzocht en werd een eerste indicatie gevonden dat inhibitie van het PAL-enzyme de vatbaarheid van rijstplanten voor *M. graminicola* verhoogt.

A

Literature survey

1 Introduction

Plants are, due to their sessile lifestyle, confronted with changing environmental stimuli like temperature, the incidence of light, pathogens and many more. For the adaptation of the plant to these events, the appropriate responses are required and for this reason plant hormones, also called phytohormones, are produced. These hormones are small, simple molecules with different chemical compositions, derived from the secondary metabolism of the plant. At very low concentrations these compounds can already influence a lot of biological activities such as growth, development, biotic or abiotic stress responses and even cell death. The adaptation of a plant to a new stimulus starts with a very complex signaling network where multiple hormones will initiate several cell responses. When a hormone is produced in one part of the plant, it can be transported and affect many other parts of the plant as well [2], [3].

The five major types of plant hormones are auxins, cytokinins, ethylene, gibberellins and abscisic acid. For a long time, these hormones are known to intervene in some very important plant processes. Many physiological experiments have been performed on these compounds, which led to a huge amount of available data revealing their role in plant processes. However, in the last decades, it was found that next to these five phytohormones a wide range of other hormones, like salicylic acid (SA), also play major roles in plant processes. Therefore, these compounds are called hormones of the new generation [4], [5].

Because of the importance of phytohormones, these molecules have been the focus of extensive research. Researchers have succeeded in finding new compounds with a similar chemical structure that affect plants in a similar way as phytohormones do. These chemical analogues are often easily producible and can have much more activity at the same concentration compared to their natural analogues. By adding naturally occurring hormones or synthetic variants to plants, a lot of plant processes can be controlled by people. In a world where the human population keeps increasing and the amount of farmland decreases, adding phytohormones to plants to have more yield and less pathogen susceptibility is a strategy worth further studying. However, despite the major progress made in understanding phytohormones, there is still an enormous lack of understanding due to the continuous discovery of new hormones, new effects and interactions between all these hormones.

2 Plant Defense

The defense of plants against bacteria, fungi, insects, nematodes, etc. is very broad. It is based on structural barriers, chemicals and proteins. All these defenses can be constitutive or inducible. A constitutive part of plant immunity is the provision of structural barriers such as the cuticle and the cell wall. The cuticle is a layer of wax on the surface of leaves, protecting the leaf against the invasion of pathogens or damage by insects. The cell wall is a barrier making it hard for the pathogen to spread because lignin will fill the spaces between the cellulose, hemicellulose and pectin components. The inducible part of the immune system is activated upon pathogen recognition. Every plant cell has receptors detecting molecules or proteins of the pathogen. When a pathogen is detected, these receptors will induce multiple immune responses. For example, the cuticle and cell walls will become thicker and the stomata will close, by which the plant tries to prevent further invasion of pathogens. In other responses, the plant will start to produce chemicals and enzymes which are toxic or harmful for the microorganism or insect. The synthesis of these compounds is strictly regulated. Synthesis will only occur when a pathogen is detected because a lot of energy and nutrients, needed for growth and maintenance, are required for the production of these structures. Some compounds produced in infected areas can also alert other parts of the plant or even other plants by hormones, volatile structures or even electrical signals. When cells of these uninfected parts detect these signals, they will start with the production of toxic chemicals and thicken their cell wall. Plants can also reduce the area of infection by very strictly regulated self-destruction of some plant cells, called the hypersensitive response [6], [7].

2.1 Innate immune system

The immune system in plants is a very complex network of different receptors, which are able to detect the presence of pathogens. This system is divided into several phases. Figure 2.1 shows the 'zig-zag' model that describes the subsequent phases in plant immunity together with their state of susceptibility.

To start immune responses, it is crucial for plants to detect pathogens in their environment. The first recognition is performed by immune receptors at the host cell surface, called pattern recognition receptors (PRR). Most PRRs belong to the receptor-like kinases (RLK). A part of these membrane-bound proteins is located in the extracellular matrix and provides the detection of pathogen-associated molecular patterns (PAMP). These are molecules present in pathogens but not in the plant itself (e.g. flagellin, a protein present in the flagella of bacteria). Upon recognition of pathogens, plants will start a first basal immune response which will provide early protection against poorly adapted pathogens. The combination of all responses associated with the recognition of PAMPs is called the pattern triggered immunity (PTI), shown in figure 2.1 and 2.2a [8].

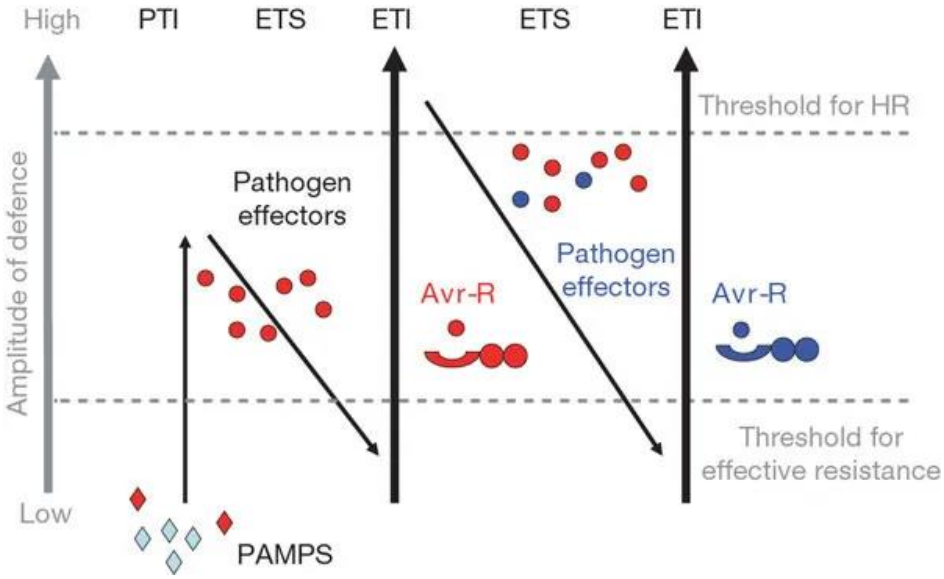

FIGURE 2.1: The 'zig-zag' model represents different parts of plant immunity together with their resistance capacity to pathogens. PAMPS, pathogen-associated molecular patterns; Avr-R, avirulence resistance protein; HR, hypersensitive response; PTI, pattern triggered immunity; ETS, effector-triggered susceptibility; ETI, effector-triggered immunity [8]

However, due to the coevolution of plants and pathogens, the latter try to interfere with PTI to promote infection by secreting proteins called effectors or avirulence proteins. This state is called effector-triggered susceptibility (ETS) (figure 2.1, 2.2b) As a response, plants have evolved a second layer of immunity, called the effector-triggered immunity (ETI), initiated when specific pathogen effectors are detected by a group of nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins, the coding products of the resistance (R) genes (ETI in figure 2.1, 2.2c). These receptor proteins can be divided into two major groups with a different N-terminal domain. The TIR class proteins, mainly present in dicots, have an N-terminal TIR (toll and interleukin receptor) domain. This domain is homologous to factors involved in mammalian immunity. In monocots, on the other hand, the coiled coil (CC) class is mainly present [9], [10].

The ETI consists of similar responses as the PTI, but here these responses are faster, more powerful and longer-lasting. Furthermore, in ETI, a threshold will be crossed resulting in the initiation of a hypersensitive response. This is the organised cell death of plant cells around the site of infection, controlled by the plant. By limiting the access to water and nutrients, the spread of pathogens to other parts of the plant is blocked. At last, pathogens sometimes produce new effectors through mutations or horizontal gene flow. As a consequence, there is a constant selection pressure for plants with new NBS-LRR proteins able to detect these new effectors (second ETS and ETI in figure 2.1) [8], [11].

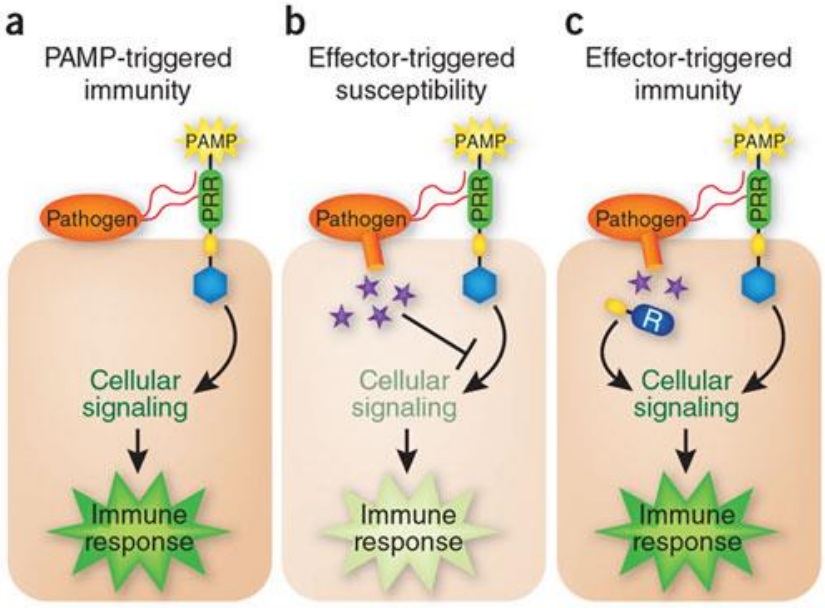

FIGURE 2.2: Different phases in plant immunity. a) Plant PRRs recognise specific pathogen PAMPs. These PRRs will trigger a signaling cascade leading to a basal defense response, PTI. b) Some pathogens can produce effector proteins (indicated as purple stars) that suppress PTI. This state is called the effector-triggered susceptibility. c) Plants can possess R proteins that will recognise effectors resulting in ETI. PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; R, resistance [12]

The PTI-associated defense pathways (also activated in ETI to a higher extent) include the production of Reactive Oxygen Species (ROS) such as H_2O_2, O_2^- and OH^-, the increase of the intracellular Ca^{2+} concentration, the activation of mitogen-activated protein kinases (MAPK), increasing expression of defense genes, synthesis of antimicrobial compounds and the accumulation of defense-related phytohormones.

The three most important phytohormones involved in plant immunity are jasmonic acid, ethylene and SA. These hormones will interact in a complex network of signals to make the plant less susceptible to pathogen attacks. In *Arabidopsis*, SA will trigger resistance against biotrophic and hemibiotrophic pathogens, while reactions induced by jasmonic acid and ethylene will do the same for necrotroph pathogens [13]. However, in rice and *Brachypodium distachyon*, the application of exogenous SA showed an increased resistance against a necrotrophic fungi, which means that the above-mentioned rule is not generally applicable for all plants [14]. The importance of SA is emphasized by its involvement in PTI as well as in ETI and the number of effectors trying to interfere with SA biosynthesis or by avoiding SA accumulation [11].

3 Salicylic Acid

Salicylic acid (SA, 2-hydroxybenzoic acid) is a small phenolic compound, produced in a wide range of prokaryotic and eukaryotic organisms. The chemical structure of this molecule is $C_6H_4(OH)COOH$ and figure 3.1 shows its structural formula. First, these phenolics were described as secondary metabolites not necessary for the survival of the plant. However, much research showed that SA is an important regulator of functions in plants. It is the trigger for some growth and development functions and abiotic stress responses. Despite this wide range of functions, the main role of SA is, together with jasmonic acid and ethylene, its involvement in the plant immune response. In complex signaling pathways, these hormones induce responses trying to make the plant resistant to a certain pathogen attack [15]. Beyond its function in plants, SA and its acetylated derivate, known as aspirin, are important pharmacological agents for humans [16].

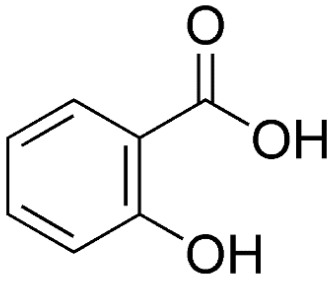

FIGURE 3.1: Structural formula of salicylic acid [17]

3.1 Involvement in growth and development

Salicylic acid is mainly known as a major plant hormone involved in pathogen defense. However, this compound is also involved in many different growth and development processes. The following functions of SA are described in literature for *A. thaliana*.

3.1.1 Seed germination

Seed germination requires interactions of different plant hormones (abscisic acid, jasmonic acid, gibberellic acid, ethylene, brassinosteroids, auxins, cytokinins and SA) as well as environmental factors. The concentration of SA plays an important role as well. SA will regulate levels of gibberellic acid and abscisic acid, two hormones playing an antagonistic role in regulating seed germination. There is a threshold SA concentration differing for plant species above which seed germination will be inhibited [18], [19], [20]. When SA concentration is below this threshold, a synergistic relationship between SA and gibberellic acid takes place, promoting seed germination. Once above this critical concentration, the relationship becomes antagonistic. This inhibition is due to SA-induced oxidative stress when hydrogen peroxide (H_2O_2) levels are highly elevated [21]. Under different abiotic stresses, low doses of applied SA can significantly improve seed germination by promoting the synthesis of essential proteins

for germination. The formation of some of these enzymes suggests that SA has a role in the initiation of an active seedling [19], [22].

3.1.2 Photosynthesis

SA can affect leaf thickness and chloroplast structure [23], stomatal closure [24], chlorophyll content [21] and activity of important photosynthetic enzymes (e.g. Rubisco) [25]. The concentration of SA plays an important role in photosynthesis. When high SA levels are detected, photosynthetic activity is reduced by lower Rubisco activity, lower chlorophyll content and a change in leaf anatomy leading to a decrease in leaf thickness. This conformational change will also affect the structure of the chloroplasts by swelling of the thylakoids, coagulation of the stroma and increasing their volume. The reduced photosynthetic capacity with high levels of SA is thus due to its effect on the thylakoid membrane and the light induced reactions happening there [23].

Another important effect of SA changing photosynthetic capacity is stomatal closure. This phenomenon is controlled by several phytohormones. The closure of the stomata is a necessary first step in plant immunity responses, because of the ease of pathogens entering via these structures. Besides abscisic acid, SA plays an important role in the function of the guard cells to control the closure of the stomata. Hence, when pathogens are detected, the immune system will carry out an SA-induced rapid stomatal closure to prevent these pathogens from entering the plant. This response leads to a lowered photosynthetic capacity because CO_2 cannot enter the plant either [22].

3.1.3 Growth

Various phytohormones are described to play a role in the growth of plants. However, SA is often not included in reviews concerning growth and development. SA can have different effects on the growth according to the concentration, the plant species and the developmental stage of the plant. In most cases, SA will inhibit growth to invest more energy into plant defense. However, when SA is present in a different concentration it can also stimulate plant growth. Very little is known about how SA will enhance growth but one of the possibilities is that SA, at the right concentration, improves the photosynthetic capacity which is beneficial for plant growth. On the other hand, several SA-inducible transcription factors are also receptive to auxins [26]. This link between both hormone signaling pathways suggests the role of SA is more complex. At last, mitogen associated protein kinases (MAPK) cascades and reactive oxygen species (ROS) are two SA-induced responses, mainly described as plant defense responses, which will also have an impact on plant growth [22].

3.1.4 Flowering and Senescence

The flowering of plants is also dependent on SA. The formation of new flower buds is promoted by SA. Studies have demonstrated that SA levels are strongly increased during or at the initiation of flowering [27].

The phenomenon of senescence is highly correlated with increased ROS and loss in photosynthetic activity, suggesting that a high SA concentration will also be involved. Once several environmental and developmental signals are present, the regulation of senescence-associated genes is a major change during senescence. The SA-signalling pathway has a role in this control of gene expression and cell death occurring at the end of senescence [28].

3.2 Involvement in abiotic stress responses

Next to its function in growth and development, SA is involved in a wide range of abiotic stress responses. SA is shown to be a key regulator in the transcription of some abiotic stress response genes such as genes for heat shock proteins, antioxidants, etc. [29]. For example, when an *Arabidopsis* plant is exposed to ozone (O_3), several defense-related genes are expressed. Some of these genes are also expressed during the hypersensitive response, a biotic stress response regulated by SA. Hydrogen peroxide (H_2O_2), formed out of ozone, is a very phytotoxic product. Detection of H_2O_2 induces SA accumulation that is required for the expression of some ozone-induced genes to break down the phytotoxic components such as H_2O_2 [30], [31]. Also during UV stress, SA will alter the expression of genes in *Arabidopsis*. When there is high UV radiation present, reactive oxygen species (ROS), promoted by SA, will play an important role in the UV signaling pathway which will ultimately lead to the downregulation of some photosynthetic genes [32], [33].

Another example is when plants are growing in a soil where high concentrations of heavy metals are present. These plants live under metal stress. Although some of these metals are essential micronutrients responsible for many important processes, an excess of these metals can negatively influence very diverse functions in the plant [34]. SA will induce plant responses trying to detoxify the heavy metals. This SA involvement is shown for lead in rice [35], for cadmium in *Zea mays* [36] and barley [37], for copper in *Phaseolus vulgaris* [38] and for some other heavy metals in different plant species [39], [40].

When exogenous SA is applied in very low doses to plants, some stress resistance can already be seen. Low concentrations of SA are not enough to induce ROS-production but are sufficient to induce expression of stress tolerance genes, decreasing stress by eliminating ROS. Optimal doses of SA (0.1 – 0.5 mM) will lead to a regulated increase of ROS levels by inhibiting antioxidant enzymes. When regulated ROS accumulation takes place, this will serve as a secondary stress signal to activate other protective stress proteins. This can happen during stress periods and is shown for salt stress [41], [42], [43] and drought stress [44] with *Arabidopsis thaliana* mutants deficient in certain enzymes critical for the production of SA. These mutants could not produce SA and were in every case more susceptible to the investigated stress because, in mutants where no SA is produced, the accumulation of ROS can be too high, which will lead to more severe damage. The accumulation of very high levels of SA will cause the oxidative burst, the rapid release of ROS in the cells, leading to cell death [45].

At last, temperature stress can also be countered by SA. Some plants only have a small temperature interval in which they will grow well. Outside of these optimal temperatures, heat and cold stress will occur. In both cases, the growth of plants in these suboptimal temperatures could be related to their levels of SA [46]. In plants capable of raising their temperature above the surrounding air temperature, also called thermogenic plants, SA is the main regulator of heat production. This is done by activating alternative respiration in the mitochondria. Unlike the commonly used cytochrome respiratory pathway, this pathway produces ATP in only one step. The energy coming from the other steps is released in the form of heat [16], [47].

3.3 Involvement in biotic stress responses

Salicylic acid is a multifunctional hormone, which can easily be placed with the major phytohormones such as auxins and cytokinins, according to its role in a whole range of processes. However, this hormone is mainly known for its role in pathogen defense. Because of extensive research, a lot of SA-induced actions playing a role in plant defense have been discovered [7].

The involvement of SA in biotic stresses is shown in several experiments. Infection of barley with *Fusarium graminearum* shows an SA concentration 15 times higher compared to non-infected barley plants [48]. Also, infection of *Nicotiana tabacum* with tobacco mosaic virus results in a strong increase in the SA levels [49].

3.3.1 Reactive Oxygen Species

Besides its role in abiotic stress responses, ROS play an important role in the resistance to biotic stresses. It has been shown that SA will alter the cellular redox state of a plant cell, by changing the ratio of the oxidised and reduced form of a redox couple. This is done by regulation of the activity of the mitochondrial alternative oxidase pathway. This pathway could also be used in the mitochondria for the production of energy but it has a lower efficiency than the citric acid cycle. An additional disadvantage of this pathway is the formation of ROS molecules. Low concentrations of SA will stimulate the production of ROS at low levels. These molecules will serve as secondary signals activating other biological processes. The accumulation of SA will increase H_2O_2 levels in the cell, initiating systemic acquired resistance or inhibiting pathogen growth. On the other hand, high doses of SA will lead to the production of high concentrations of ROS. These molecules are toxic in high concentrations and will cause oxidative stress and even cell death, called the hypersensitive response [16], [50].

3.3.2 *Activation of defense genes*

When a PAMP or effector is recognised by the cell, the accumulation of SA will change gene expression. This accumulation will activate a range of pathogenesis-related genes (PR-genes). One of the most important SA receptors which will regulate the expression of these genes is Non-expressor of pathogenesis-related genes 1 (NPR1). Under (hemi-)biotrophic pathogen attack, SA will bind to NPR1, leading to immune signaling. The activation of PR genes works through two different pathways: an NPR1-dependent and an NPR1-independent pathway. However, the importance of NPR1 in immunity is proven by SA treatment on *A. thaliana npr1* mutants. These mutants, despite receiving the necessary SA, will not be able to activate immunity [16]. Furthermore, the NPR1-dependent pathway is already studied the most. The general mode of action of this pathway is shown in figure 3.2.

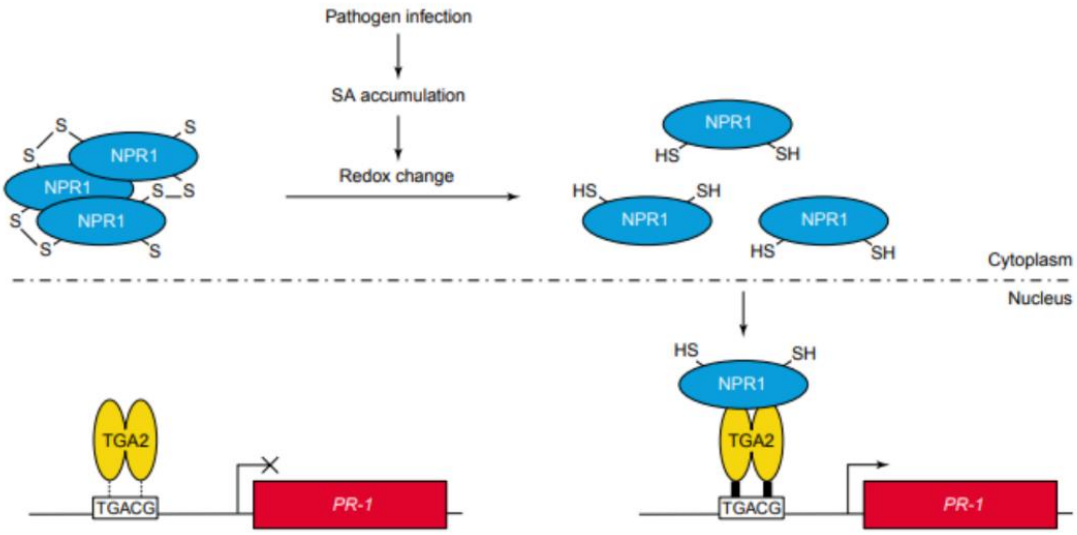

FIGURE 3.2: The process of the NPR1-dependent pathway. In uninfected parts of the plant, NPR1 is present as an oligomer bound by disulphide bonds. Upon pathogen effect, the SA accumulation will alter the redox state releasing active NPR1 monomers. These monomers migrate to the nucleus where they bind with TGA-transcription factors which leads to the activation of certain PR-genes [51]

NPR1 is always present in the cytosol of plant cells. However, when there is no accumulation of SA, NPR1 is found in its inactive form. This is an oligomer of NPR1 proteins, bound by intermolecular disulphide bonds. When infection occurs, SA will accumulate and this will alter the oxidative state of the cell. The change of redox state will reduce the disulphide bonds of this oligomer, releasing active NPR1 monomers. Released monomers are much smaller than the oligomeric form, making it possible for them to enter the nucleus through the nuclear pores. In the nucleus, the NPR1 monomers will bind to TGA-transcription factors, leading to the expression of certain immune-associated PR-genes [7], [52], [53]. Besides the binding of TGA-factors, NPR1 will also regulate the expression of WRKY-factors [54]. In *A. thaliana*, 74 WRKY-factors were found with most of them playing a role in immune responses. An

orthologue of NPR1 in monocots was found in *Oryza sativa* and is called NPR1 homolog 1 (NH1). It is shown in rice that WRKY-factors can also activate genes independent of NH1 to stimulate immune responses [55], [56].

The NPR1 protein concentration is regulated by SA and NPR1 paralogs NPR3 and NPR4. SA will regulate NPR1 phosphorylation, which will facilitate its proteasome-mediated degradation. Also, interaction with NPR3 and NPR4 will target NPR1 for degradation. This is shown in *npr3, npr4* double mutants. In these mutants elevated levels of NPR1 were detected. The degradation activated by NPR3 and NPR4 is SA-concentration dependent because of the difference in binding affinity for SA between NPR3 and NPR4 [54], [57], [58]. Figure 3.3 shows the interactions of NPR1 with NPR3 and NPR4 at different SA concentrations.

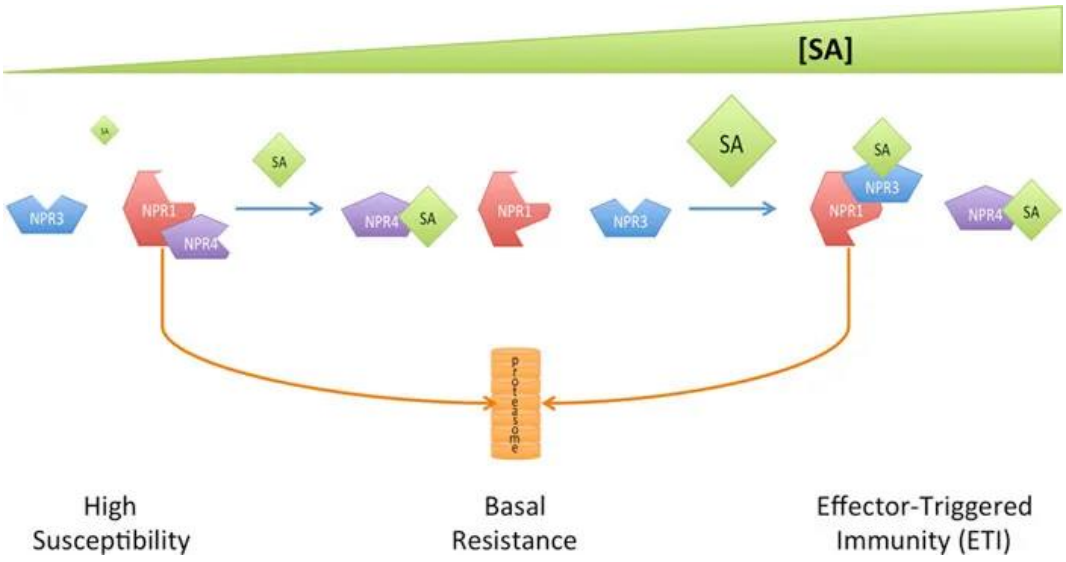

FIGURE 3.3: Interactions of NPR1 with NPR3 and NPR4 at different SA concentrations. Low concentration of SA, NPR1 is unavailable to induce PR-genes due to the interaction with NPR4, leading to degradation. After infection, SA levels rise and NPR4 will bind to SA, releasing NPR1 to induce PR-genes (basal resistance). At very high levels of SA, SA will promote the interaction of NPR3 and NPR1, leading again to NPR1 turnover and ETI [58].

Due to these complex interactions of NPR1 with its analogues, it is possible for SA to stimulate different responses when it is present in different concentrations. The SA concentration finetunes the NPR1 homeostasis which will help the plant specify the kind of disease resistance. Studies have shown that accumulation of NPR1 is necessary for basal defense gene expression, whereas proteasome-mediated turnover of NPR1 is crucial for ETI. The high susceptibility in SA-deficient plants is thus due to the unrestricted binding of NPR4 to NPR1 leading to no expression of PR-genes while in wild-type plants, basal levels of SA will bind to NPR4, allowing NPR1 to activate expression of PR-genes [57], [58].

3.3.3 Hypersensitive response

Hypersensitive response (HR) is an immune response mostly associated with ETI against (hemi-) biotrophic pathogens. When the plant initiates ETI after recognizing an effector, this response can be activated by SA. This is shown for the *Mi-1* gene in tomato. This gene will be expressed by SA and initiate HR immediately after recognition of *M. incognita*, before the nematode can form feeding sites [59]. For this, the plant will program some cells around the infection site to die. By this, the pathogen inside the plant will not be able to spread further in the plant and will die as well. Because of the sacrifice of some cells, the plant will prevent other parts from being infected. Because this response involves the death of cells, it is very well controlled. A complex network of different signals will initiate HR. These signals are the same as the ones activating other defense responses in ETI, such as ROS-production, NO and SA. The first signs of HR are visible a few hours after infection. First, there are changes in the actin cytoskeleton and nucleus. Immediately after this, there is a stop in the cytoplasmatic streaming and the plasma membrane will lose its permeability. At last, the cell will shrink completely and die [60], [61], [62].

3.3.4 Systemic acquired resistance

Another very important role of SA in plant immunity is the regulation of systemic acquired resistance (SAR). This is a defense mechanism of the plant where elevated resistance is detected towards a broad spectrum of pathogens in non-infected parts of the plant. Also, when the SAR pathway is initiated, the plant can secrete volatile structures to other plants surrounding the infected plant to increase resistance [63]. The changes involved in SAR are based on the upregulation of the so-called SAR-genes. These genes can stimulate lignin deposition in the cell wall to prevent pathogen spread or they code for pathogenesis-related (PR) proteins which are sometimes antimicrobial [64]. Because SAR is based on differential expression of PR genes, NPR1 and SA are key regulators of this immune response [65]. When a mutation in the PAL pathway of tobacco plants shuts the biosynthesis of SA down, these plants will not be able to induce SAR and will be more susceptible to pathogen attack [66]. Furthermore, when examining a transgenic *A. thaliana* plant capable of the production of salicylate hydroxylase, an enzyme degrading SA, SAR is not induced [67].

To induce defense in non-infected leaves or neighbouring plants, the infected plant has to produce a signal molecule. Production of methyl salicylate (MeSA), a modification of SA where a methyl group is attached, is strongly increased in leaves of *Arabidopsis* infected with *Pseudomonas syringae* [68]. The modification into MeSA makes SA inactive but very volatile which makes it possible to diffuse through the plasma membrane into the air outside the plant. In *Arabidopsis*, most of the produced MeSA is uncontrollably emitted into the atmosphere and only a small part is retained in the cells [68]. Due to MeSA in the surrounded air after pathogen attack, other parts of the infected plant and surrounding plants will be warned that a pathogen is present. If necessary, MeSA can rapidly be converted again to SA which will lead to elevated resistance [7], [69]. Figure 3.4 shows how SA and MeSA will contribute to SAR.

Besides the warning of MeSA to surrounding plants, another molecule, pipecolic acid (Pip), is thought to be the signal molecule transported inside the plant. This is shown in *Arabidopsis* mutants deficient in the biosynthesis of Pip, which showed a complete loss of their ability to induce SAR. When these plants were treated with Pip, SAR responses were restored [70]. More specifically, it is found that its modification, N-hydroxypipecolic acid (NHP), functions as the actual inducer of SAR [71]. The O-glycosylated form of NHP, N-OGlc-Pip, most likely serves as the compound able to travel through the plant to systemic leaves, where it can rapidly convert back to NHP to induce SAR here [72]. Interestingly, two known transcription factors involved in the biosynthesis of SA, SARD1 and CBP60g, also promote the production of Pip and NHP upon pathogen attack [73].

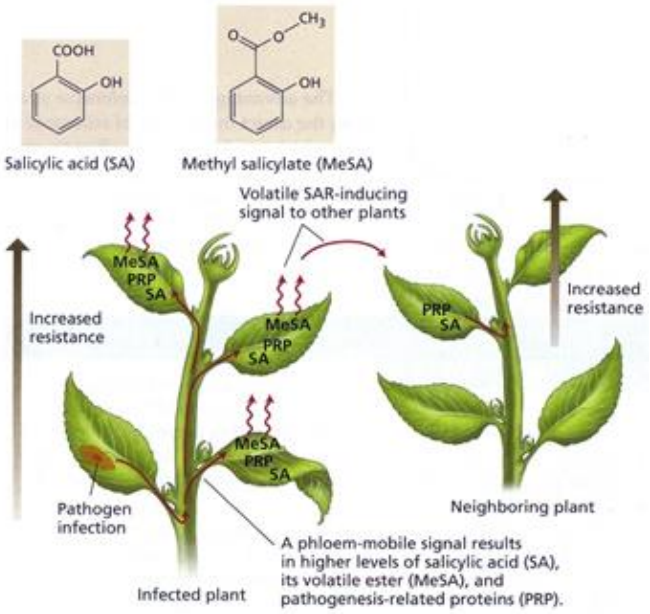

FIGURE 3.4: How MeSA and SA will contribute to SAR. MeSA can diffuse in the air to warn neighbouring plants where it can actively be converted back to SA, leading to elevated resistance [74].

When a pathogen is detected, the SAR-genes are expressed. Due to this expression, the plant obtains a primed state. This state is a huge advantage when the plant is attacked again by pathogens. When a new attack occurs, the plant will induce a much faster and much stronger immune response. Interestingly, the primed state can be epigenetically inherited by further generations. This has been proven by infection of an *Arabidopsis* plant and analysis of its offspring, where elevated resistance against pathogens was detected [75]. Figure 3.5 shows a curve illustrating the strength of defense in primed plants compared to un-primed plants.

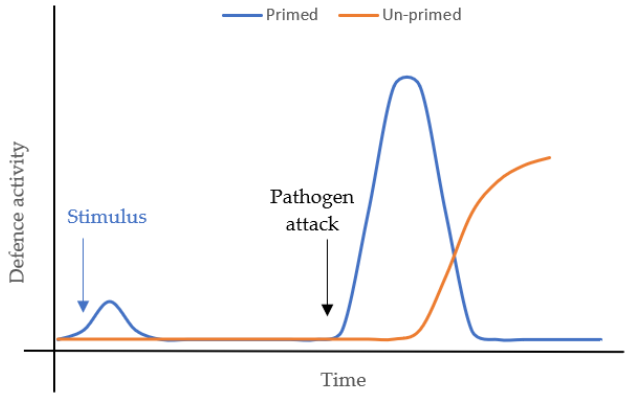

FIGURE 3.5: Strength of immune response in primed plants compared to un-primed plants. When the SAR-genes are expressed, a next attack will be fought with a faster and much stronger immune response.

3.4 Applications

Salicylic acid can be used by humans as a medicine for several causes such as acne, psoriasis and warts [76]. Also, aspirin or acetylsalicylic acid is a well-known pain reliever for reducing fever, minor headaches and other pains [77].

SA is a well-studied compound for resistance engineering in plants. Due to the huge amount of pathogens, researchers are constantly obligated to find new ways to make plants resistant. The application of pesticides was a first step in controlling them. However, these compounds are sometimes toxic and are the cause of severe problems in the environment. For these reasons, it is beneficial to focus on the innate immune system. This can be achieved by spraying SA in the right concentration on the plant to induce local and systemic acquired resistance against a broad range of pathogens [78]. Although the direct application of SA can enhance pathogen resistance, it is not the only molecule able doing this. Other compounds such as vitamins, chitosans, oligogalacturonides, volatile organic compounds, azelaic and pipecolic acid, thiamine, etc. are also able to induce, for example, the SA pathway and put the plant in a (chemically induced) primed state [79], [80].

Furthermore, genetic engineering can play an important role in enhancing the naturally occurring immune responses. Understanding metabolism, homeostasis and transport of compounds involved in these systems is of utmost importance. A first way of enhancing immunity is by making transgenic plants. Most genetically modified organisms (GMO) used today are made resistant to herbicides or insects. Transgenic plants against diseases are not fully distributed yet, but research on this topic is continually increasing [81]. A first strategy of making more disease-resistant GMOs is by introducing PR-genes, with a direct antimicrobial function. Upon pathogen attack, SA will affect the NPR1 pathway and this will lead to activation of the PR-genes, including the introduced ones. However, the proteins resulting from these genes can sometimes be toxic or allergens for humans. Therefore, new strategies are being developed [82].

A new, promising strategy is introducing an effector-gene in the plant together with a complementary resistance gene, controlled by a promotor induced upon pathogen attack. By doing this, the plant will express the effector protein and the resistance protein upon pathogen infection. This R-protein will detect the introduced effector and start immune responses, probably the hypersensitive response, by which the plant will be resistant to the pathogen [83]. Other targets for transgenic plants are NPR1 or the WRKY-factors. By overexpression of these genes, enhanced resistance was observed. However, the constitutive activation of these genes will lead to a lower fitness (less seed production, smaller plants, etc.), proving the importance of the pathogen-induced promotors to express these genes only when necessary [84], [85].

4 Biosynthesis of salicylic acid

Due to the importance of SA in responses against pathogen infection, its role in these responses is well known. However, there is still a lack of information about the biosynthesis pathway of SA, while insight in this is important to understand how SA is formed upon pathogen attack and how pathogens could interfere with the biosynthesis.

Unravelling the biosynthesis pathways of SA started nearly half a century ago. At this moment, two important biosynthesis routes are described: the isochorismate synthase (ICS) pathway (figure 4.1) and the phenylalanine ammonia-lyase (PAL) pathway (figure 4.2). Both pathways start from chorismate, produced by the shikimate pathway and a necessary precursor for several aromatic amino acids, such as phenylalanine, tyrosine and tryptophan [86]. Both of these pathways are not completely defined yet and certain enzymes are still not determined. Also, the relative importance of both pathways is different between plant species. Both pathways can have equal importance in the synthesis of SA as is the case in soybean [87]. In *A. thaliana* the most important pathway is the ICS pathway which produces about 95% of the SA [47], [88] while in rice, probably the PAL pathway has a higher significance [89], [90]. Furthermore, the production of SA differs between species and even within a single plant, differences can be seen. In rice, the basal SA concentration is higher in shoots compared to roots with rice leaves having one of the highest SA concentrations of all plant species [89], [91].

4.1 ICS pathway

The ICS pathway was first identified in *Pseudomonas* species. These bacteria are capable of synthesizing SA for the production of the siderophore pseudomonine by a two-step pathway. The first reaction is the conversion of chorismate to isochorismate and is catalysed by the enzyme isochorismate synthase (ICS). The second reaction of the pathway forms SA out of isochorismate and this reaction is catalysed by isochorismate pyruvate lyase (IPL) [47], [92]. Soon after this discovery, homologous *ICS* genes were found in plants catalysing the same reaction as in bacteria. The number of these genes is variable between plant species, but most plants have only one or two copies [88], [93].

Due to the homologous *ICS* gene in plants, it was expected for a long time that plants produced SA in the same way as bacteria. However, up until today no plant gene encoding for a protein similar to the bacterial IPL has been found. Recent studies further clarify the second step of the ICS pathway in *Arabidopsis*. For this, isochorismate is exported from the chloroplast into the cytosol by a member of the chloroplast transporter family, enhanced disease susceptibility5 (EDS5). The cytosolic aminotransferase avrPphB susceptible3 (PBS3) will further catalyse the conjugation of glutamate to isochorismate leading to the formation of isochorismate-9-glutamate. At the moment, the *PBS3* gene has only been found in *Arabidopsis*. The involvement of three proteins controlling the pathway in different cell compartments minimizes evolutionary forces and pathogens interference [94]. One hypothesis is that isochorismate-9-glutamate will spontaneously decompose into 2-hydroxy-acryloyl-N-glutamate and SA. An alternative to the spontaneous decomposition of isochorismate-9-glutamate is the enzyme-mediated reaction of this structure into SA. The gene *enhanced pseudomonas susceptibility1* (*EPS1*) encodes for a BAHD (the first letter of the four first discovered enzymes in this group) acyltransferase in the cytosol which will catalyse this reaction [95]. Further evidence for these steps in the ICS pathway is provided by *pbs3* and *eps1* mutants in *Arabidopsis*. Both mutants showed a similar phenotype being an enhanced susceptibility towards *Pseudomonas syringae* and a lower SA accumulation upon infection with this pathogen [95]. Figure 4.1 shows the reactions of the ICS pathway.

The ICS pathway plays a very important role in SA biosynthesis in *Arabidopsis thaliana*. In this plant two chloroplast-associated *ICS* genes (*AtICS1* and *AtICS2*) were identified, where the ICS1 enzyme is the most important one. This is shown by experiments performed on *ics1* and *ics1 ics2* mutants. When these plants are exposed to a UV treatment, the concentration of UV-induced SA will be 90% lower compared to a wild type plant. On the other hand, when an *ics2* mutant is exposed to the same treatment, no significant difference between wild type plant and mutant is observed. Other evidence demonstrating the importance of this pathway is that pathogens secrete enzymes metabolizing chorismate or isochorismate, promoting virulence by suppressing SA accumulation [7], [88], [96], [97].

In *Oryza sativa*, only one *ICS* gene (*OsICS*) is found. At first, it was thought that the activation of this gene by the OsWRKY6 transcription factor was leading to SA accumulation [98]. However, in rice, the *ICS* gene is believed to play a minor role in SA biosynthesis. It can be noted that the activity of the rice ICS enzyme is very low compared to the activity of its homologue in *Arabidopsis*. Furthermore, the SA accumulation can also be a response of the WRKY transcription factor activating other genes, like *PAL* [93], [98].

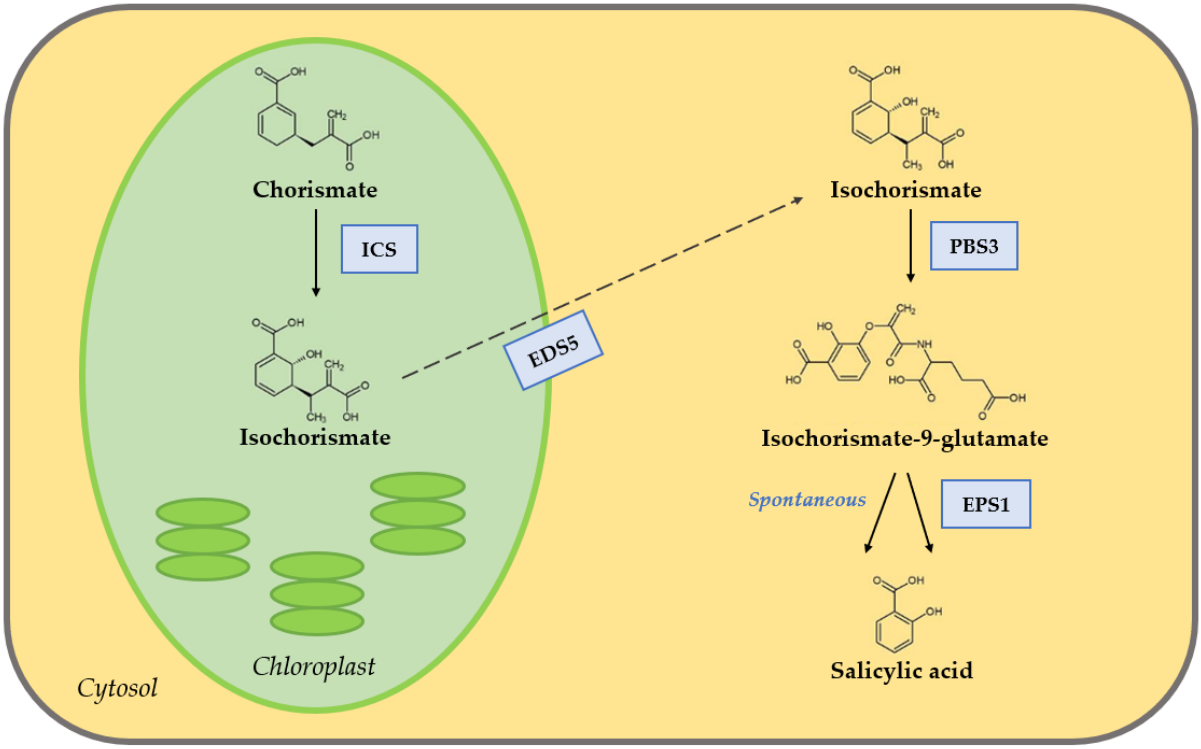

FIGURE 4.1: The reactions of the ICS pathway in Arabidopsis and the place in the cell where these reactions take place. Dashed arrows are transportation steps while full arrows are reactions. Enzymes and the EDS5 transporter are presented in a blue box. The reaction of isochorismate into salicylic acid is not mediated by an IPL enzyme as is the case in bacteria. Instead, isochorismate is transported to the cytosol where the subsequent steps are performed. ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; EDS5, enhanced disease susceptibility5; PBS3, avrPphB susceptible3; EPS1, enhanced pseudomonas susceptibility1.

4.2 PAL pathway

Before the discovery of the ICS pathway in bacteria, another pathway producing SA in plants was already identified. The PAL pathway was first described by using isotope feeding experiments. Here, plants were fed with different molecules containing the radioactive isotope ^{14}C. By following these isotopes, several intermediates were identified and a potential pathway was constructed.

This pathway also starts from chorismate. It is still unknown if chorismate stays in the chloroplast or is first transported to the cytosol. In the first step, chorismate is converted to prephenate by the enzyme chorismate mutase (CM). In most plant species, multiple *CM* genes have already been identified. The following multiple-step reaction will convert prephenate further into phenylalanine [99].

Phenylalanine is converted to trans-cinnamic acid (tCA) by the PAL enzyme. The PAL enzyme is present in a lot of plant species, but the number of *PAL* genes encoding this enzyme differs per plant species and the expression can differ between tissues [100]. The PAL enzyme is inhibited by its own product, tCA, which means when enough tCA is produced PAL is inhibited and the pathway will not proceed [101]. Despite PAL being an enzyme catalysing only the reaction into tCA, in some cases, like in maize, it can also have a secondary tyrosine

ammonia-lyase (TAL) activity [102], [103]. This means that some plant species are able to proceed with a similar pathway with tyrosine instead of phenylalanine. With isotope feeding experiments possible further intermediates in the PAL-pathway are identified, such as ortho-coumaric acid and benzaldehyde. However, this is still not certain yet because no enzymes catalysing these possible reactions are found, nor the conversion into SA is unravelled yet [104].

From the PAL pathway, there is a lot of branching leading to end products other than SA. For example, cinnamic acid is a precursor for the synthesis of a huge number of plant substances, including lignin, tannins, flavonoids, pigments, many of the flavor components of spices, and various alkaloids, such as morphine and colchicine. Phenylalanine can also be used in other pathways, such as the biosynthesis of tyrosine [99].

Mutant experiments in *Arabidopsis* identified another gene involved in the PAL pathway, abnormal inflorescence meristem1 (*AIM1*). The gene is called *AIM1* because a knock-out mutation showed a malfunction in the development of inflorescence and flowers in *Arabidopsis* [105]. At this moment, this member of the multifunctional protein (MFP) family has been identified in several plant species such as *Arabidopsis*, rice, *Brassica napus*, tobacco, etc. [106], [107], [108]. This protein family is mainly involved in fatty acid metabolism but is also an essential factor for the metabolism of amino acids such as tryptophan, tyrosine, etc. and of SA, as it is necessary for the production of benzoic acid [109], [110]. AIM1 acts as a beta-oxidation enzyme for one of the reactions of tCA into benzoic acid. However, tCA is not the only ligand AIM1 can use, also fatty acids are a possible target for this enzyme, which makes knock-out mutants in this gene very hard to interpret. However, if only SA levels are studied, these mutants can give important information about the SA biosynthesis.

In the last step of this pathway benzoic acid has to be converted to SA. For this reaction, no enzyme has been identified yet, but it is presumed that a benzoic acid hydroxylase will catalyse this reaction. In 1995, a study reported the involvement of benzoic acid 2-hydroxylase, a P450 monooxygenase, in catalysing the last step of the PAL pathway in tobacco [111]. However, since this report, similar results have not been published anymore. Figure 4.2 shows the reactions of the PAL pathway.

The number of *PAL* genes also differs between plant species. *Arabidopsis* has only four *PAL* genes. As already mentioned, in this species, the PAL pathway plays a minor role in the biosynthesis of SA. Mutant studies on single, double or triple mutants in these genes showed no significant differences in SA concentration between the mutants and wild type plants after infection with *Pseudomonas* species. However, the quadruple mutant showed a 50% lower concentration of SA after infection and an increased *Pseudomonas* susceptibility compared to a wild type plant [104]. These results prove the joint effort of both pathways in the production of SA. Another conclusion is that the ICS pathway is the most important one in the synthesis of SA in *Arabidopsis*. This conclusion is mainly based on the fact that the *PAL* quadruple mutant will cause severe developmental defects. These defects could have an impact on the ICS-derived SA. For this reason, the *PAL* quadruple mutant is not well suited to examine SA accumulation [104].

In rice, on the other hand, several *PAL* genes are already described by studies on stress responses [112]. Sequence homology compared to the *PAL* genes in *Arabidopsis* annotated nine genes as *PALs* in rice. Some *PAL* genes are more involved in defense responses than others. For example, when *PAL4* (in the article the gene is called *PAL6* because the authors used another nomenclature system) is knocked-out, rice plants will have a decrease in PAL activity up to 77% and consequently be more susceptible to the fungus *Magnaporthe oryzae*. Due to these results, *PAL4* can be considered as very important in the *PAL* gene family [91]. *PAL6* is also important for the accumulation of SA in rice because knock-out mutants in this gene show a decrease of 60% in their SA levels. This decrease of SA is present, despite the three times higher expression of *ICS* compared to wild type rice plants. This suggests that in rice, the PAL pathway is the major biosynthetic route of SA [91]. The nomenclature of the *PAL* genes in rice is not very simple. Authors give these genes a name based on homology to the *AtPAL* genes, the chromosome where they are located, etc. To specify which nomenclature is used in this masters dissertation table A.1 in the appendix shows the names given to the *PAL* genes together with their locus number.

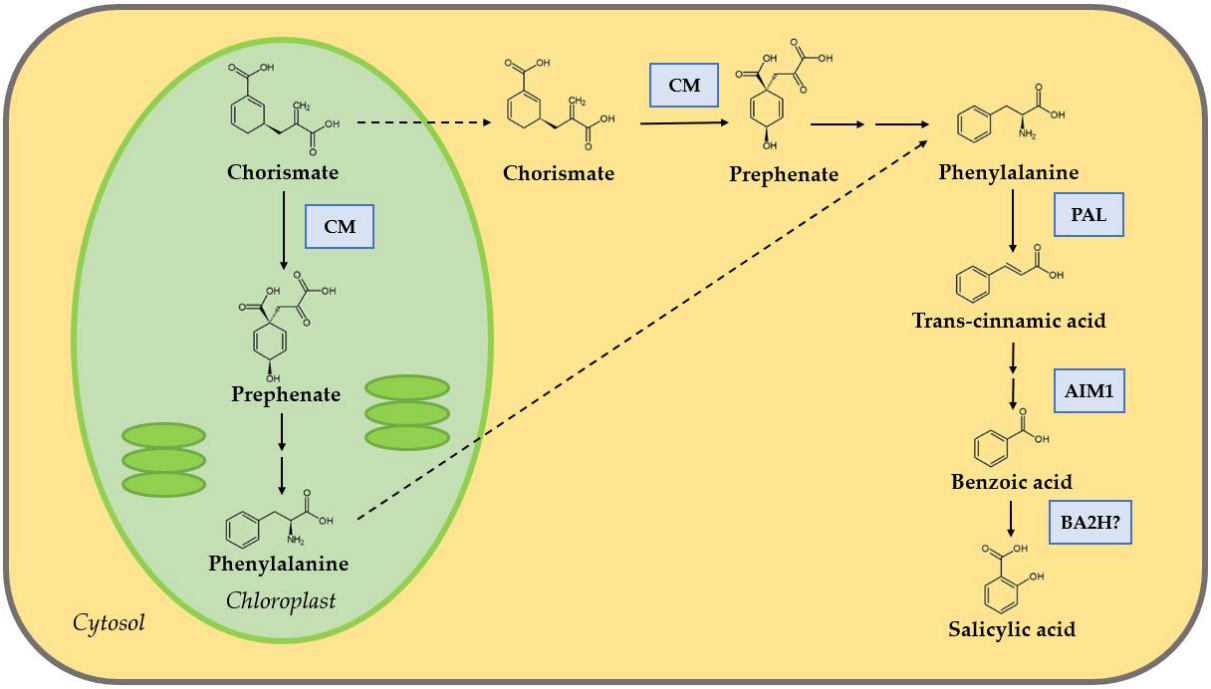

FIGURE 4.2: The reactions of the PAL pathway and the place in the cell where these reactions take place. Dashed arrows are transportation steps while full arrows are reactions. Multiple reactions between two intermediates are shown as two consecutive arrows. Enzymes are presented in a blue box. At the moment, it is not known if the reactions of chorismate to phenylalanine take place in the chloroplast, in the cytosol or in both. CM, chorismate mutase; PAL, phenylalanine ammonia-lyase; AIM1, abnormal inflorescence meristem1; BA2H, benzoic acid 2-hydroxylase.

It is shown that *PAL9* does not have PAL activity. Instead, this gene is involved in reactions as a tyrosine aminomutase enzyme. Because of this, its name has been changed into TAM1 [113]. Consequently, the PAL activity had to be confirmed for all other *PAL* genes. Until now, seven of the other rice *PAL* genes (*OsPAL1*, *OsPAL2*, *OsPAL3*, *OsPAL4*, *OsPAL5*, *OsPAL6* and

OsPAL7) have been co-localized with known QTLs (quantitative trait loci), a locus involved in a quantitative phenotypical trait for disease resistance, which is a strong indication of PAL-activity of these genes [114].

There is a complex interaction between the *PAL* genes upon pathogen infection. Depending on the infecting pathogen, a distinct *PAL* expression pattern can be seen. This was shown in a heterozygous *pal4* mutant because the homozygous mutant died, leading to the conclusion that a homozygous *PAL4* knock-out mutation was lethal [114]. However, mutants were obtained by chemical treatment making it questionable that the lethal effect is only due to a mutation in *PAL4*. The heterozygous mutant plant was infected with three different pathogens, resulting in different expression levels of the *PAL* genes [114]. This study also identified *OsPAL4* as the key contributor to resistance against several pathogens. Also, another study shows the importance of *OsPAL4* in pathogen infection and here, the homozygous mutant was not lethal (this article uses another nomenclature for the *PAL* genes, *OsPAL6* in the article is *OsPAL4* in the nomenclature used in this project) [91], [114].

In comparison to *Arabidopsis*, inflorescence and flowers are not disrupted in an *aim1* knock-out rice mutant. This plant, however, showed a reduced root meristem, revealing a different activity of this gene in rice [115]. Here, *AIM1* also belongs to the MFP family. The rice genome encodes four members of this family that can be divided into the AIM1 clade and the MFP2 clade based on sequence alignments and phylogenetic analyses [115], [116]. *AIM1* is the only gene in the AIM1 clade and as in *Arabidopsis*, it probably catalyses the reaction of tCA to benzoic acid in the PAL pathway while the other MFP family members most likely have a preference for long-chain acyl-CoAs as substrates [117]. The involvement of *AIM1* in the PAL pathway is shown by the *aim1* mutant showing a 70% decrease in SA concentration, a tCA concentration 6 times higher and a benzoic acid concentration 2 times lower compared to wild type rice [115].

4.3 Modifications

After biosynthesis of SA, this active phytohormone is present in the cytosol where it can regulate a whole range of immune responses. SA can also undergo several modifications, of which most make SA inactive, to help regulating the active SA levels. These modified forms provide rapidly accessible active SA, when converted back to the active compound, and facilitate the transport through the plant. Maintaining low levels of active SA when no pathogen attack takes place is critical for plants because activation of immunity systems reduces plant fitness by reducing the available energy for growth and development [32].

One of the major modifications is a glucose conjugation at the hydroxyl group of SA resulting in salicylic acid glucoside (SAG). When SAG is formed, it is actively transported from the cytosol into the vacuoles of plant cells, where it is present in large quantities [118], [119]. In the vacuoles, SAG is an inactive storage form that can be hydrolysed again to active free SA when a cell is attacked by a pathogen. The transportation of SAG to the vacuole decreases the end-product inhibition of the enzymes responsible for the conversion. Also, but to a lesser extent, salicylic acid glucose ester (SGE) formation happens by glucose conjugation at the carboxyl

group. The formation of SAG and SGE happens in the cytosol by glucosyltransferases and is pH-dependent. In *Arabidopsis,* UGT74F2 will catalyse the formation of SAG and SGE while UGT74F1 is only involved in the synthesis of SAG. Concentrations of SAG and SGE are thus dependent on the intracellular levels of these enzymes and the presence of a relevant substrate [90], [120]. Figure 4.3a shows the glucosylation of SA.

Salicylic acid can also be methylated by a SA carboxyl methyltransferase (SAMT), forming methyl salicylate (MeSA). This phloem-mobile structure has an increased membrane permeability and volatility, making it a very important long-distance transport molecule in SAR. When this compound arrives in the target cell, it is converted back to active SA that will induce pathogen resistance responses. Also, the destination of MeSA can be in a neighbouring plant, where again the conversion to free SA will result in resistance of plants surrounding an infected plant [90], [121]. Evidence supporting the hypothesis MeSA acts as a volatile signal triggering SAR is already found in tobacco, potato and *Arabidopsis* [122], [123], [124]. MeSA can also be further glycosylated to form MeSA glucoside (MeSAG). The exact function of this compound is not known yet, but one possibility is that MeSAG serves as a non-volatile storage form that can quickly be converted to MeSA [90]. Figure 4.3b shows the methylation of SA and the further glucosylation to MeSAG.

Another modification is amino acid conjugation, but this modification is less well characterised. Only a few proteins are identified in *Arabidopsis*, actively conjugating an amino acid to SA. GH3.5 overexpression shows a 3.5 times higher level of SA-Asp, the conjugation of aspartate to SA, upon pathogen infection [125]. Experiments, where plants were treated with exogenous SA-Asp, showed that *PR-1* was not induced, meaning this is an inactive form of SA. At the moment, it is thought this conversion is involved in the catabolism of SA because the conjugation of aspartate to auxin (IAA-Asp) leads to the degradation of this hormone [90], [126]. Figure 4.3c shows the amino acid aspartate conjugation to SA.

Next, a possible modification is sulfonation where a sulphotransferase will catalyse the transfer of a sulphuryl group to SA. This system is able to activate or inactivate hormones in mammalian cells. In *Arabidopsis*, this modification can activate and inactivate SA [90]. Figure 4.3d shows the sulfonation of SA.

A final modification is hydroxylation which leads to the formation of dihydroxybenzoates (DHBA) such as 2,3-DHBA and 2,5-DHBA (also known as gentisic acid). Studies have shown that SA can remove hydroxyl radicals by binding resulting in a non-enzymatic formation of both structures [127], [128]. However, most of the DHBA is formed enzymatically via the ICS pathway. In an *ics1* mutant of *Arabidopsis,* pathogen-induced production of 2,3-DHBA was completely suppressed and production of 2,5-DHBA showed a significant reduction. However, it is still unknown if the direct precursor is SA or isochorismate [90], [129].

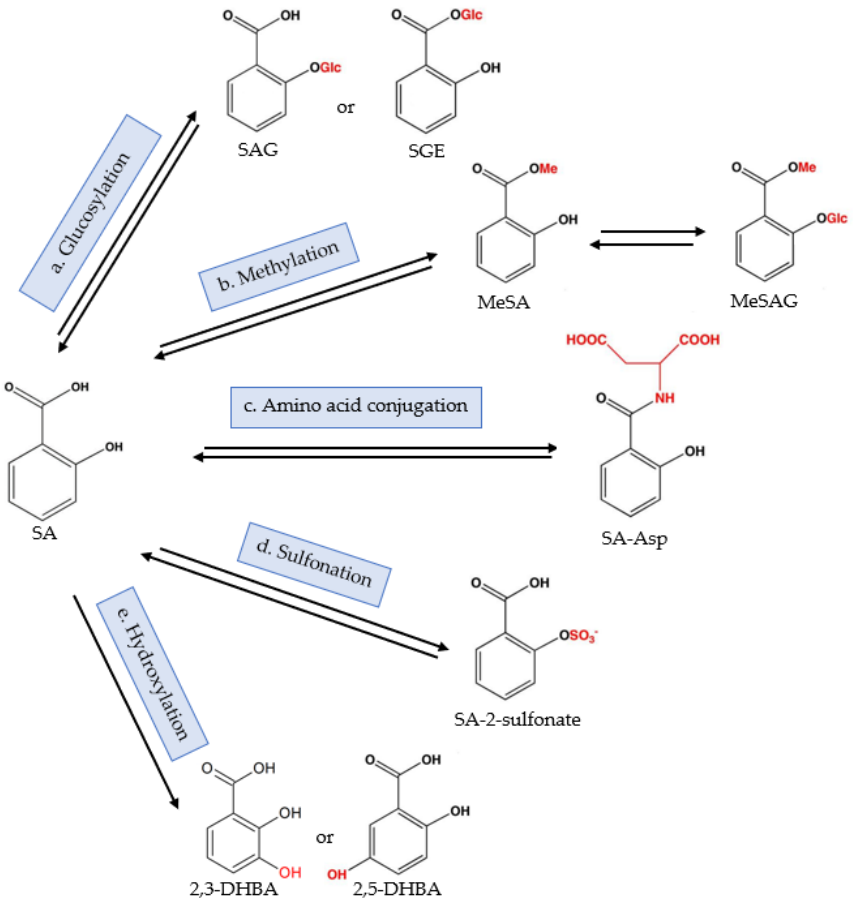

FIGURE 4.3: Possible modifications of SA. a) SA can be glucosylated into SAG or SGE depending on the location of the glucose-group. These structures are inactive storage forms, transported into the vacuole. b) SA can be methylated to MeSA. This compound is an important volatile molecule for defense in surrounding plants. c) An amino acid, mostly aspartate, can be conjugated to SA. This modification is most probably involved in the catabolism of SA. d) SA can be sulfonated to form SA-2-sulfonate. This modification is able to activate or inactivate SA. e) A hydroxylation of SA leads to the formation of 2,3- or 2,5-DHBA. SAG, salicylic acid glucoside; SGE, SA glucose ester; MeSA, methyl salicylate; MeSAG, MeSA glucoside; Asp, aspartate; DHBA, dihydroxybenzoic acid.

5 Engineering of rice plants

5.1 Rice as a model organism

In this project, all experiments will be performed on rice, more specifically *Oryza sativa Kitaake*. The choice for using rice and not wheat or barley, which are two more important crops in Belgium, is because rice can be considered as a model organism for monocots. Because *Arabidopsis thaliana* is the first used model organism, a lot of hypotheses about the signaling and biosynthesis of SA have first been confirmed in this species.

Twenty-five years ago, scientists agreed to perform most of their plant research on *A. thaliana*. This was needed to combine several disciplines in plant science such as biology, genetics, etc.

Because of the extensive research of all disciplines, the metabolic pathways and mechanisms of this plant are unravelled the most [130]. However, *Arabidopsis* is a dicot plant and many mechanisms unravelled in this plant are hard to generalise to the whole plant kingdom. Therefore, rice is moving up as the most important model organism in monocots. The choice for *Oryza sativa* was based on its small diploid genome, short generation time, small size and being the most important crop over the world. With these two organisms, it is believed that many mechanisms are going to be elucidated and extrapolated to other plant species [131].

5.2 Salicylic acid in rice

There are some interesting differences between the concentration of SA in rice and other plant species. Levels of SA, when no pathogen is present, are low in *A. thaliana* but rise to a concentration three times higher when a pathogen is detected. On the other hand, rice plants have high basal SA concentrations in their leaves but these levels stay relatively unchanged upon pathogen attack. A possible function of these high SA concentrations, when no infection occurs, is as antioxidant protecting rice from water stress when grown in flooded fields. Another major difference is that SA plays an important role against biotrophic pathogens in *Arabidopsis*, while it has an effect against both (hemi-)biotrophs and necrotrophs in rice [7], [132]. Furthermore, as already mentioned, the relative importance of the ICS and the PAL pathway for the biosynthesis of SA is different between plant species. In *A. thaliana*, the most important route is the ICS pathway, while in rice it is believed that the PAL pathway plays a more important role [88], [90]. Both pathways can also equally contribute to the biosynthesis of SA, as is the case in soybean [87].

5.3 CRISPR/Cas9

In this project, to create knock-out rice mutants, the relatively new technique of CRISPR/Cas9 will be used. With this method, it is possible to generate a mutation on a previously specified site in the genome. The development of this technique was based on the naturally occurring CRISPR/Cas9 in bacteria as a defense mechanism against viruses. In bacteria, clustered regularly interspaced short palindromic repeats (CRISPR) are arranged in the genome; these are short viral inserted DNA fragments (20 base pairs), separated by short palindromic repeats. The RNA transcribed from these regions can associate with specific nucleases, the Cas proteins (CRISPR-associated proteins). When a virus attack is ongoing, the virus will release its DNA in the host cell where the Cas proteins will form a complex with this invading DNA. In this complex, the Cas protein will cleave the viral DNA in smaller pieces. Some of these pieces will be inserted in the CRISPR region of the bacterial genome. However, because the viral DNA is inserted in the bacterial genome, the bacterial Cas protein must be able to make a distinction between the bacterial and viral DNA. For this reason, invading DNA must contain a specific, 3 - 5 base pairs long sequence, called a protospacer adjacent motif (PAM) acting as an extra security check before cleavage. When the same virus will infect the bacteria again, the whole CRISPR region is transcribed, forming a long CRISPR-RNA (crRNA). Another RNA, the trans-activating crRNA (tracrRNA), will lead the Cas protein to the crRNA,

leading to the formation of the CRISPR ribonucleoprotein complex (crRNP). This long RNA is then divided into several short crRNAs to form mature CRISPR complexes. When complementary viral DNA is present in the cell, this complex will bind with it and trigger the cleavage of the viral DNA [133], [134], [135]. Figure 5.1 shows how CRISPR works in bacteria as a defense mechanism.

After the discovery of CRISPR/Cas in bacteria, researchers did a lot of efforts for adapting this technique so it could be used in biotechnology. Today, CRISPR is one of the most used methods to create double-stranded DNA breaks which can lead to knock-out mutants in a specific site of the plant genome. Here, one needs to design a specific guide RNA (gRNA), a fusion of a crRNA and tracrRNA. This gRNA will bind with a Cas9 protein and, because of the sequence specificity, it will guide this protein to a target sequence in the genome. Every site in the genome can be chosen by making the gRNA specific for that region, provided that the site of choice is preceded by a PAM. In the case of Cas9, this PAM sequence is 5'-NGG-3' where N can be any nucleotide. When the gRNA binds to the complementary sequence in the genome, the Cas9 will produce a blunt end double-stranded break. The plant will react on this break by activating its DNA repair system consisting of homology-directed repair (HDR) and non-homologous end-joining (NHEJ) repair. When the DNA break is repaired by the latter, errors can be made. This is due to not using a template when repairing the breaks. Because most of the breaks will be repaired by the non-homologous variant, because it is much faster, errors can be made. These errors include insertions and deletions. When CRISPR is performed, one is hoping to get a frameshift in the chosen gene. A frameshift occurs when an insertion or deletion takes place (not a multiple of three nucleotides), causing an early stop codon or a conformational change in the coded protein and thus a loss of function. When such a frameshift is observed, a stable knock-out of the chosen gene is present [136], [137].

To obtain the gRNA and Cas9 protein inside the plant, plant transformation based on *Agrobacterium tumefaciens* is used. This bacteria will insert part of its genome, called the T-DNA, inside the plant genome. A vector containing the gene for Cas9 and a gene encoding a specific gRNA containing a protospacer motif is brought into *A. tumefaciens*. This bacteria will be used to infect the plant, resulting in the insertion of the T-DNA in the plant genome. When this occurs, the plant is able to produce its own Cas9 proteins and the specific gRNA. If that gRNA is designed to cleave a specific plant gene, the plant will generate a knock-out of that gene [138], [139].

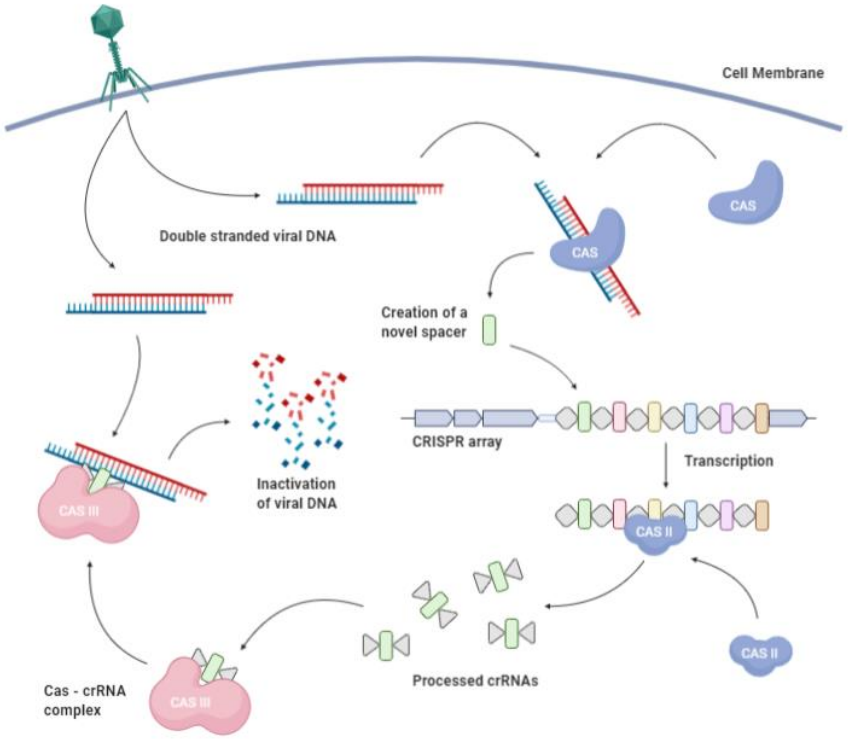

FIGURE 5.1: CRISPR/Cas as a defense mechanism in bacteria against viruses. When viral DNA enters the bacteria, it will be recognised by Cas proteins which will break it down. Some of these DNA fragments will be inserted in the bacterial genome. When the same virus attacks again, crRNA will bind to a Cas protein and target the viral DNA for degradation.

6 Nematodes

Nematodes or roundworms are used in this project in the infection experiments. Although only a part of the discovered species is plant-parasitic, a lot of research has been performed on these pathogens due to their influence in agriculture. Nematodes lower yields and are very difficult to control in crop fields.

Some examples on rice are the root-knot nematode *Meloidogyne graminicola* and the migrating nematode *Hirschmanniella oryzae*. Both are important pathogens that will lower rice yield by up to 25%. These nematodes will first use a stylet to penetrate the cell walls of roots while secreting cell wall degradation enzymes, such as cellulases. Root-knot nematodes secrete effectors that alter the metabolism of cells, which will lead to the formation of giant cells. These giant cells are used by the nematode as feeding sites. Other nematodes keep migrating in the cortex and feed on cortical cells which leads to necrosis of these roots and a reduction in plant growth [140], [141].

While the plant will try to overcome the nematode attacks by producing toxic compounds, the nematodes secrete effector proteins to neutralise the plant immune responses, e.g. peroxidases that will break down ROS. A lot of research is happening on the interaction between the plant immune system and these effectors [142].

B

Practical study

B.1 Objective

In *Arabidopsis*, it is already known for a long time that the ICS pathway, and not the PAL pathway, is the main route for the biosynthesis of SA. However, conclusions about the relative importance of both pathways in rice are less clear. The main objective of this masters dissertation is elucidating the role of the PAL pathway in the biosynthesis of SA in *Oryza sativa Kitaake*. More specifically, a closer look will be taken to the genes *PAL1, PAL2, PAL3, PAL4, PAL5, PAL6, PAL7, PAL8* and *AIM1* (locus numbers can be found in appendix table A.1). These genes are possibly responsible for the production of enzymes crucial in the PAL pathway. *PAL9* is left out of this project due to its role in the production of a tyrosine aminomutase enzyme.

To examine the role of the PAL pathway and more specifically the role of every above-mentioned gene, a comparison will be made between plants unable to express a gene (the knock-out CRISPR mutants), plants that will overexpress that same gene (the overexpression transgenic plants) and wild type plants as control. In the end, these plants will be examined based on their SA levels and resistance to the root-knot nematode *Meloidogyne graminicola* and a leaf pathogen. However, due to the large time range to transform and grow the rice plants, only the transformation has been performed and optimized in this project. In the CRISPR mutants, this leads to plants that still have to be made homozygous for the inserted mutation. Once these homozygous mutants are obtained, these plants will be examined for the characteristics mentioned above.

Further, infection experiments with the nematode *M. graminicola* will be performed on rice plants treated with α-aminooxy-β-phenylpropionic acid (AOPP), an inhibitor of the PAL enzyme. These plants are then examined based on their symptoms to observe the resistance against these pathogens compared to plants, which are not treated with the inhibitor. At last, also qPCR of several *PAL* genes was performed on infected and non-infected plants. However, this will not be discussed in this project because the high variance of the samples led to no significant differences and no clear conclusion.

B.2 Material and methods

This chapter will provide the followed methodology including protocols, compositions of media, etc. The first part will list all used media with their composition. Next, the methodology to obtain transformed *Agrobacterium* or generation of the knock-out rice mutant and the rice overexpression line is provided. After these chapters, the rice transformation protocol using these *Agrobacteria* is given. At last, the set-up for the PAL enzyme inhibition experiments is included.

1 Composition of media and solutions

The composition of the medium is every time specified for a volume of 1 l except the SAP medium. Keep all poured plates at 4°C until use.

1.1 N6D medium: callus generation from seeds

1) Add the following components together in a 1 l bottle:
   - 4 g Duchefa CHU (N6) medium
   - 300 mg VWR casamino acids
   - 2.875 g L-proline
   - 30 g sucrose
   - Add distilled water to 1 l
2) Adjust pH to 5.8 with a 1M NaOH-solution
3) Add 4 g Duchefa gelrite
4) Autoclave
5) From now on work in the laminar flow bench
6) Add 1 ml of a 2 mg/ml 2,4-D stock solution:
   - Weigh 20 mg of 2,4-D in a 15 ml falcon tube
   - Add 500 µl of 100% ethanol to dissolve the 2,4-D
   - Add distilled water to 10 ml
   - Filter sterilize under the flow
   - Keep the stock solution in -20°C
7) Pour plates

1.2 2N6-AS medium: cocultivation for callus and *Agrobacterium*

1) Add the following components together in a 1 l bottle:
   - 4 g Duchefa CHU (N6) medium
   - 300 mg VWR casamino acids
   - 30 g sucrose
   - 10 g glucose
   - Add distilled water up to 1 l
2) Adjust pH to 5.2 with a 1M NaOH-solution

3) Add 4 g Duchefa gelrite
4) Autoclave
5) From now on work in the laminar flow bench
6) Add 1 ml of a 2mg/ml 2,4-D stock solution
7) Add 1 ml of a 20 mg/ml acetosyringone stock solution
    - Weigh 200 mg of AS in a 15 ml falcon tube
    - Add 7 ml of 100% ethanol to dissolve acetosyringone
    - Add 3 ml of distilled water
    - Filter sterilize in the laminar flow bench
    - Keep the stock solution at -20°C
8) Pour plates

1.3 AAM medium: liquid medium for *Agrobacterium* growth

1) Add the following components together in a 1 l bottle:
    - 5 ml 200x MAA stock solution
        - Add to 1 l of distilled water: 40 mg Fe-EDTA, 10 mg $MnSO_4$, 2 mg $ZnSO_4$, 0.025 mg $CuSO_4$, 0.025 mg $CoCl_3$, 0.75 mg KI, 3 mg H_3BO_3, 0.25 mg Na_2MoO_4, 1 mg nicotinic acid, 1 mg pyroxidine HCl and 1 mg thiamine HCl
    - 50 ml 20x AA stock solution
        - Add to 1 l of distilled water: 7.5 mg glycine, 176.7 mg L-arginine, 900 mg L-glutamine and 300 mg L-aspartic acid
    - 250 mg $MgSO_4.H_2O$
    - 150 mg $CaCl_2.2H_2O$
    - 150 mg $NaH_2PO_4.2H_2O$
    - 3 g KCl
    - 500 mg VWR casamino acids
    - 68.5 g sucrose
    - 36 g glucose
    - 1 ml acetosyringone
    - 200 µl L-glutamine (73 mg/ml)
    - Add distilled water up to 1 l
2) Filter sterilize in the laminar flow bench

1.4 RE-III medium: callus regeneration

1) Add the following components together in a 1 l bottle:
    - 4.4 g Duchefa MS salts including vitamins
    - 2 g VWR casamino acids
    - 30 g sucrose
    - 30 g glucose
    - Add distilled water up to 1 l

2) Adjust pH to 5.8 with a 1M NaOH-solution
3) Add 4 g Duchefa gelrite
4) Autoclave
5) From now on work in the laminar flow bench
6) Add under the flow:
    - 20 µl NAA
    - 1 ml kinetin
    - 1 ml Duchefa ticarcillin/clavulanate (further referred to as timentin)
7) Pour plates

1.5 MS medium: plant growth

1) Add the following components together in a 1 l bottle:
    - 4.4 g Duchefa MS salts including vitamins
    - 4 g Duchefa gelrite
    - Add distilled water up to 1 l
2) Autoclave
3) From now on work in the laminar flow bench
4) Pour in tubes

1.6 Hoagland solution: nutrient solution for plants.

1) Add the following components to a 1 l bottle:
    - 2 ml 2.5M KNO_3
    - 2 ml 0.5M KH_2PO_4
    - 2 ml 2.5M $Ca(NO_3)_2$
    - 2 ml 1M $MgSO_4$
    - 2 ml micronutrient solution
    - 3 ml $FeSO_4$ and EDTA disodium salts solution
    - Add water to 1 l

1.7 LB medium: *E. coli* growth

1) Add the following components together in a 1 l bottle:
    - 25 g Duchefa LB broth
    - Add distilled water up to 1 l
2) If the medium is made for pouring plates: add 15 g of VWR agar powder
3) Autoclave
4) If necessary add an antibiotic and eventually pour plates

1.8 SAP medium: growth of rice plants for inhibition experiments

1) Add the following components to a bucket:
   - 3 l distilled water
   - 28 g DCM Aquaperla water crystals
2) Incubate for 2 h at room temperature until the polymer is dissolved. Blend the polymer flakes until a gelatinous mix is obtained
3) Add 25 kg Sibelco sand to a big box. Add the blended polymer and mix well
4) Let the sand dry at room temperature for about two weeks

2 Transformation of *Agrobacterium* for the generation of CRISPR mutants

These protocols serve to design and obtain a vector that later will be used in the transformation of rice plants to create the knock-out CRISPR mutants. For this, a chosen guide RNA (gRNA) is inserted into a vector. For the generation of the CRISPR mutants, the pBb7-B1-ccdB-B1-OSU3-NosT-zCAS9-ZmUBIL vector (obtained from the VIB center, Ghent, Belgium), further referred to as the VIB vector, is chosen. This adapted vector will then be inserted into competent *E. coli* TOP10 cells allowing amplification of the vector and to easily transform *Agrobacterium* with the VIB vector. To confirm the correct transformation of the *E. coli* cells and later the *Agrobacterium* cells, colony PCR is performed. Based on these results a positive colony is chosen of which the vector is extracted to send it for sequencing. If these results are positive, a glycerol stock of the positive colony is made and this is stored at -80°C until it is used in further steps.

This protocol is specific for the used VIB vector. If another vector is used for transformation, it can be a cloning system not based on restriction or restriction sites, the creation of the right overhangs of the gRNA, the used antibiotics and the designed primers might be altered.

2.1 Oligonucleotide design for gRNA

2.1.1 Design of the gRNA

For each gene of interest, in this project the *PAL* genes and *AIM1*, a gRNA is generated. This gRNA is inserted in the VIB vector and will, once inserted in the rice plant, lead the Cas9 enzyme to a specific sequence of the plant genome which will be cut by the enzyme. The choice for a correct gRNA will be made with the help of the online tool CRISPR-P [143].

1) Go to the online tool CRISPR-P. Choose *Oryza sativa* (MSU) as the target genome. Add the locus tag of the gene wherefore a gRNA is generated. Locus tags can be found in appendix table A.1.
2) Select a gRNA from the output list
   - Avoid a gRNA located in an intron or a UTR
   - Avoid a *Bsa*I restriction site in the gRNA, as this enzyme will be used for cloning
   - Preferably, choose a gRNA at the beginning of the first exon
   - Choose a gRNA with a low off-target score, more specifically with a few non-homologous base pairs in the seed sequence, the ten bp after the PAM sequence

3) Take the optimal 17-20 bp long spacer sequence
4) Generate the reverse complement of the spacer sequence using an online tool [144]
5) Add 'GGCA' to the original spacer sequence at the 5' side and 'AAAC' to the reverse complement sequence at the 3' side. This will generate complementary overhangs matching the *Bsa*I digested VIB vector
6) Order the oligonucleotides, for example:

Sequence 1: 5'-GGCAXXXXXXXXXXXXXXXXXXX-3'
Sequence 2: 3'-XXXXXXXXXXXXXXXXXXXXCAAA-5'

2.1.2 In vitro efficiency of the designed gRNA

This protocol serves to verify if the designed gRNA will have good efficiency *in vitro*. If the efficiency is not good *in vitro*, the efficiency *in planta* will probably also be very low. Positive results, however, will not give certainty for good efficiency of the gRNA *in planta*. This protocol only serves to exclude a gRNA for further use.

First, the transcription of gDNA is performed:

1) Add the following components into a PCR tube:
   - 2.5 µl VWR 10 x key buffer
   - 1 µl pICH86966_AtU6p vector
   - 1 µl 25mM $MgCl_2$
   - 0.5 µl 5 mM dNTPs
   - 0.5 µl forward oligonucleotide
   - 0.5 µl reverse oligonucleotide
   - 0.2 µl VWR Taq polymerase
   - 18.8 µl RNase free water
2) Use the following PCR program to obtain gDNA:
   - 5 min at 95°C
   - 31 cycles
     - 30 sec at 95°C
     - 60 sec at 56°C
     - 30 sec at 72°C
   - 2 min at 72°C

After this, the preparation of the DNA template for cleavage is performed.

3) Find a restriction enzyme that only cuts once in the plasmid backbone and not in the gene of interest. In this case, the restriction enzyme is ScaI
4) Add the following components to a PCR tube:
   - 2 µl Thermo Fisher Tango buffer
   - 10 µl pGEM-T vector with the gene of interest
   - 1 µl Thermo Fisher restriction enzyme ScaI
   - 7 µl RNase free water

5) Incubate for 2 h at 37°C to cleave the plasmid

Confirm the transcription of the gDNA together with the cleavage of the plasmid on an agarose gel. For the gDNA, a band of around 170 bp should be visible, while for the cleavage of the plasmid, a clear band must appear instead of a smear.

After confirmation, transcription of gRNA is performed:

6) Add the following components to a PCR tube:
   - 2 µl Bioké T7 buffer
   - 10 µl gDNA template
   - 2 µl 0.5 mM NTPs
   - 1 µl RNase inhibitor
   - 2 µl Bioké T7 RNA polymerase
   - 4 µl RNase free water
7) Incubate 2 h at 37°C to transcribe gDNA into gRNA with the T7 RNA polymerase

When the gRNA is obtained, the Cas9 *in vitro* cleavage is performed:

8) Add the following components to a PCR tube:
   - 3 µl Bioké Cas9 buffer
   - 5 µl gRNA
   - 1 µl Bioké Cas9 nuclease
   - 16 µl RNase free water
9) Incubate for 10 min at 25°C
10) Add 5 µl of the linearized plasmid to the mix
11) Incubate for 1 h at 37°C
12) Confirm the cleavage on an agarose gel. Add a well with only the template as a negative control. If the bands of the Cas9-cleaved plasmid are sharp compared to the negative control, the gRNA has good efficiency to cleave *in vitro* and this gRNA sequence can be used for further steps. However, if no distinction can be made with the negative control, another gRNA has to be designed.

2.2 Ligation of the gDNA into the VIB vector

To insert the chosen gDNA into the VIB vector, the ordered oligonucleotides have to form an oligo-duplex. The VIB vector must be digested with a restriction enzyme after which the double-stranded oligo-duplex can ligate into the vector.

2.2.1 Digestion of vector by BsaI

1) Add the following components together in a PCR tube to restrict the vector:
   - 2000 ng VIB-Cas9 vector
   - 2 µl Bioké tango buffer
   - 2 µl Bioké *Bsa*I restriction enzyme
2) Incubate at 37°C for 2-4 h
3) Heat inactivation of *Bsa*I by incubating at 85°C for 10 min

2.2.2 *Prepare oligo-duplex*

1) Add the following components together in a PCR tube to prepare the oligo-duplex:
   - 1 µl forward oligonucleotide
   - 1 µl reverse oligonucleotide
   - 1 µl Thermo Fisher 10x T4 DNA ligase buffer
   - 7 µl H_2O
2) Incubate the PCR tube in a thermal cycler using the following program:
   - 60 min at 37°C
   - 10 min at 95°C
   - Cool down to 25°C at 0.1°C/sec

2.2.3 *Ligation of the oligo-duplex into the digested vector*

1) Make a 1:200 dilution of the oligo-duplex for ligation
2) Add the following components to a PCR tube to ligate the oligo-duplex into the VIB vector:
   - 2.5 µl *Bsa*I digested vector
   - 1 µl diluted oligo-duplex
   - 0.5 µl Thermo Fisher 10x T4 DNA ligase buffer
   - 1 µl Thermo Fisher T4 ligase
3) Incubate overnight at 4°C

2.3 Transformation of competent *E. coli* cells

The ligated vector will now be inserted into competent *E. coli* TOP10 cells. This is done to amplify the vector by simply growing the bacteria. Another reason is that the transformation of *Agrobacterium* can easily be done by triparental mating when the vector is present in TOP10 cells. For this, *E. coli* TOP10 cells have to be made chemically competent after which these competent cells will be transformed by the heat shock protocol. The used competent cells were already available in the lab.

1) Add the following components to a tube and leave on ice for 10 - 30 min:
   - 50 µl of competent *E. coli* TOP10 cells
   - 4 µl ligated VIB vector
2) Hold for 45 sec in a thermo block of 42°C. Cool down on ice for 1 min
3) Add the mixture to 1 ml of LB medium and let it shake for 1 h at 37°C
4) Plate 150 µl of the cells on an LB + spectinomycin (spec) 100 plate and close the plate with parafilm
5) Grow the colonies overnight at 37°C

2.4 Confirmation of the transformed cells

To prove that the *E. coli* colony used further is correctly transformed, a colony PCR is performed. Based on these results, a positive colony is chosen. The vector of the chosen colony is then extracted and sent for sequencing. If the sequencing results are also positive, a glycerol stock of the positive colony is made to maintain the transformed cells until further use.

2.4.1 Colony PCR of E. coli

1) Touch 8 of the colonies on the plate with a separate tip. Touch with this tip another LB + spec100 plate, the master plate and place the tip into a PCR tube filled with 10 µl of distilled water
2) Turn the tip around a few times to dissolve the cells in the water and denature the samples for 10 min at 95°C
3) Add the PCR mix to the cells:
    - 3 µl VWR 10x key buffer
    - 0.5 µl dNTPs
    - 0.5 µl forward primer
    - 0.5 µl reverse primer
    - 15.4 µl distilled water
    - 0.1 µl VWR Taq DNA polymerase
4) Use the VIB-Cas9 forward and reverse primers
5) Use the following PCR program:
    - 35 cycles
    - 35 sec at 95°C
    - 35 sec at 56°C
    - 1 min at 72°
6) Perform electrophoresis on a 1.5% agarose gel for 20 minutes on 135 V
    - Make a gel solution by adding 6 g Thermo Fisher ultrapure agarose to 0.4 l of 0.5x TAE-buffer and heat in the microwave until everything is dissolved
    - Store the gel solution at 70°C
    - Pour the gel solution into a tray with a comb
    - After the gel is solidified, pull out the comb and lay the gel in the electrophoresis tank
    - Add loading dye to your samples and load into the wells, add a ladder and run for 20 min at 135 V
7) Lay the gel for 15-20 min in 0.3 µg/ml ethidium bromide
8) Visualize the gel under UV light with the Geldoc system and select the positive colonies

2.4.2 Sequencing of the vector

To sequence the vector, it must first be extracted from the TOP10 *E. coli* cells. The extraction is performed with the Thermo Scientific GeneJET Plasmid Miniprep Kit.

To sequence the extracted vector, a sample will be sent to an independent company. For this, the concentration of the vector needs to be between 100 ng/µl - 150 ng/µl.

1) Measure the concentration using the Nanodrop device
    - Wash the sensor with 2 µl of distilled water
    - Measure 2 µl of distilled water as a blank
    - Measure 2 µl of the sample
2) Dilute the sample with distilled water to obtain the desired concentration
3) Add 10 µl of the diluted vector and 4 µl of a chosen primer. The choice for a primer is based on the region of interest to be sequenced. It is best to take a primer around 100 bp (base pairs) before, or in the case of a reverse primer behind, the region of interest
4) Send the sample to the company LGC Genomics, where it will be sequenced

2.4.3 Glycerol stock of positive E. coli

When the results of the sequencing are positive for the chosen *E. coli* colony, a glycerol stock of this colony is made to maintain it.

1) Fill a falcon with 5 ml of LB + spec100 medium. Touch the positive colony on the master plate with a tip and place this tip in the medium.
2) Grow the culture overnight at 37°C
3) Add in a small tube 500 µl of the grown cell and 500 µl of a 50% glycerol solution.
4) Store glycerol stock in -80°C

2.5 Transformation and validation of *Agrobacterium*

2.5.1 Triparental mating

To transform *Agrobacterium* with the VIB vector, triparental mating is used. This method will allow the transformed *E. coli* to transfer the vector to *Agrobacterium* with the help of an *E. coli* helper strain.

1) Grow *Agrobacterium* EHA
    - Make 5 ml of LB medium + Rifampicillin 25
    - Add *Agrobacterium* EHA from glycerol stock
    - Grow overnight (or for 2 days) at 28°C in the dark
2) Grow *E. coli* helper cells
    - Make 5 ml of LB medium + Kanamycin 50
    - Add *E. coli* helper from glycerol stock
    - Grow overnight at 37°C
3) Grow *E. coli* with construct
    - Make 5 ml of LB medium + Spec100
    - Add transformed *E. coli* with the construct of interest from glycerol stock
    - Grow overnight at 37°C
4) Mix 100 µl of each of the three bacterial cultures and dry a 100 µl drop of the mixed culture on an LB + $MgCl_2$ plate
5) Incubate overnight at 28°C in the dark
6) Scrape the outer border of the dried drop and dissolve in LB medium. Mix well

7) Make a dilution series by adding 100 µl of the previous solution to 900 µl of LB medium. Do this until there are five samples
8) Plate 150 µl of each sample on a separate LB + Rif25 + Spec100 plate and incubate for 3 days in the dark.

2.5.2 Colony PCR of Agrobacterium

1) Dissolve 8 separate colonies in 15 µl of H_2O and denature for 10 min at 95°C
2) Collect 10 µl per sample into a new PCR tube
3) Perform the colony PCR as mentioned in 2.4.1 from here on

2.5.3 Sequencing of the extracted vector

This step is performed the same as mentioned in 2.4.2 for *E. coli*

2.5.4 Glycerol stock of positive Agrobacterium

1) Fill a falcon with 5 ml of LB + spec100 + rif25 medium. Touch the positive colony on the master plate with a tip, place this tip in the medium and grow for 2 days at 28°C
2) Add in a small tube 500 µl of the grown cell and 500 µl of a 50% glycerol solution
3) Store glycerol stock in -80°C

3 Transformation of *Agrobacterium* for the generation of overexpression lines

To obtain overexpression lines for rice, *Agrobacterium* has to be transformed with the gene of interest. For this, cDNA is synthesised from extracted RNA of a wild type plant. From this cDNA, a *PAL* gene or *AIM1* is amplified. These genes are then separately integrated into a pGEM-T vector which will then undergo Gateway cloning to transfer the gene to the destination vector pUBIL. This last vector will then be used in triparental mating to transform *Agrobacterium*.

3.1 cDNA synthesis

3.1.1 RNA extraction

For the RNA extraction, a wild type rice plant is grown. When enough plant material can be harvested, the RNA of this plant is extracted using the Qiagen RNeasy Kit according to the manufacturer's protocol. After extraction, the concentration is measured using a Nanodrop device.

3.1.2 DNase treatment

1) Add the following components to a PCR tube:
   - 3.6 µl buffer with $MgCl_2$
   - 1 µl Thermo Fisher riboblock RNase inhibitor
   - 3 µl Thermo Fisher DNase
   - x µl (3µg = 3000 divided by the measured concentration) RNA
   - 28.4 - x µl RNase-free water

2) Incubate for 30 min at 37°C. Add 2 µl 50 mM EDTA and incubate for 10 min at 65°C to stop the reaction

3.1.3 cDNA synthesis

First strand cDNA synthesis is performed with the Tetro cDNA Synthesis Kit.

1) Prepare priming premix on ice by adding the following components to an RNase-free reaction tube:
   - 12 µl DNase treated RNA
   - 1 µl 10µM oligo dT primer
   - 1 µl 10 mM dNTP mix
   - 4 µl 5x RT buffer
   - 1 µl Ribosafe RNase inhibitor
   - 1 µl Tetro Reverse Transcriptase
2) Mix gently by pipetting and incubate for 2 h at 45°C
3) Terminate the reaction by incubating for 5 min at 85°C, chill on ice and add 80 µl of distilled water. Store at -20°C

3.2 Isolation of a single gene

The following protocols serve to isolate a single gene from the cDNA that will be inserted into a vector. For this, a PCR is performed with gene-specific primers. After gel electrophoresis, a single band of the right size can be extracted and sent for sequencing.

3.2.1 Primer design

To isolate the gene of interest, specific primers bordering the whole gene must be designed. To do this, two online sites are used: phytozome [145] to show the sequence of the genes and primer 3 [146] to design the primers.

1) Go to phytozome and choose keyword search. Select *Oryza sativa* as target organism and enter the locus number of the gene of interest (see appendix table A.1)
2) Choose in the tab sequences for transcript sequence and paste the sequence into primer3. Change the product size range in the length of the gene, for example, 2000-2500 and pick primers.
3) Find the generated primers in the sequence on phytozome
   - Make sure the primers will amplify the whole gene
   - Prefer primers located in the UTR regions for optimal specificity

3.2.2 Gradient PCR Taq polymerase

1) Add the following components to each tube of a PCR strip:
   - 0.5 µl cDNA
   - 2 µl VWR 10x key buffer
   - 0.5 µl dNTPs
   - 0.5 µl forward primer
   - 0.5 µl reverse primer

- 15.9 µl distilled water
- 0.1 µl VWR Taq DNA polymerase

2) Use the following PCR program:
   - 35 cycles
   - 30 sec at 95°C
   - 30 sec gradient from 48°C to 68°C
   - 2 min 20 sec at 72°C

3.2.3 Velocity PCR

1) Add the following components to each tube of a PCR strip:
   - 5 µl Bioline 5x Hi-Fi reaction buffer
   - 2.5 µl dNTPs
   - 1.5 µl cDNA
   - 2 µl forward primer
   - 2 µl reverse primer
   - 0.75 µl DMSO
   - 0.2 µl Bioline High-fidelity Velocity Taq polymerase
   - 11.05 µl distilled water

2) Use the following PCR program:
   - 31 cycles
     - 30 sec at 95°C
     - 30 sec gradient from 48°C to 68°C
     - 90 sec at 72°C

3.2.4 All-in Taq PCR

1) Add the following components to each tube of a PCR strip:
   - 0.5 µl forward primer
   - 0.5 µl reverse primer
   - 0.5 µl cDNA
   - 12.5 µl HighQu Red All-in Taq mix
   - 11 µl PCR water
2) Use the following PCR program:
   - 31 cycles
     - 30 sec at 95°C
     - 30 sec gradient from 48°C to 68°C
     - 45 sec at 72°C

3.2.5 Q5 PCR

1) Add the following components to each tube of a PCR strip:
   - 5 µl 5x Q5 reaction buffer
   - 0.5 µl 10 mM dNTPs
   - 1.25 µl forward primer
   - 1.25 µl reverse primer

- 1.5 µl cDNA
- 0.25 µl Q5 High-fidelity DNA polymerase
- 15.25 µl nuclease-free water

2) Use the following PCR program:
   - 30 sec at 98°C
   - 35 cycles
   - 10 sec at 98°C
   - 20 sec gradient from 50°C to 72°C
   - 1 min 20 sec at 72°C

3.2.6 Gel extraction

When the visualised gel of one of the above mentioned PCRs shows a band of the appropriate size, it will be selected. If in the lane of the selected band, multiple bands are present the selected band is extracted from the gel, otherwise the PCR product can directly be used. To do this, all PCR product is loaded into the agarose gel. To extract the DNA, the GeneJET Gel Extraction Kit is used. After this, the product is sequenced for validation.

3.3 Gateway cloning

These protocols serve to insert the gene of interest into the pUBIL vector that will be used to transform *Agrobacterium*. At first, if the used polymerase does not do this directly, a polyA-overhang is synthesized at the gene to insert it in the pGEM-T vector. The following BP reaction transfers the gene into the pDONR221 vector after which it will be transferred to the pUBIL vector by the LR reaction.

3.3.1 Ligation into the pGEM-T vector

1) Add the following components to a PCR tube to synthesize the polyA-overhang:
   - 7 µl gel extraction product
   - 1 µl 2mM dATP
   - 1 µl VWR 10x key buffer
   - 1 µl VWR Taq polymerase
2) Incubate for 45 min at 70°C
3) Add the following components to a PCR tube:
   - 2.4 µl extracted gene with polyA-overhang
   - 4 µl 2x ligation buffer
   - 0.8 µl pGEM-T vector
   - 0.8 µl Thermo Fisher ligase
4) Incubate for at least 16 h at 4°C
5) Send for sequencing as described in 2.4.2
6) Perform a PCR on the positive product
   - Use primers with attb-sites to attach these attb-sites to your product
   - Use the following PCR program:
   - 30 sec at 95°C

- 5 cycles
    - 30 sec at 48°C
- 30 cycles
    - 30 sec at ideal annealing temperature for specific primer
- 40 sec at 72°C

3.3.2 BP reaction

1) Add the following components to a 1.5 ml tube:
    - 7 µl attb-PCR product
    - 1 µl pDONR221 vector
    - 8 µl Thermo Fisher TE buffer pH8.0
    - 2 µl Thermo Fisher BP Clonase II
2) Mix well by vortexing briefly and centrifuge shortly
3) Incubate reaction overnight at 16°C
4) Add 1 µl of Proteinase K to terminate the reaction. Vortex briefly and incubate for 10 min at 37°C followed by 10 min at 95°C

If specific primers are designed to isolate the gene of interest with attb sites, these primers can be used in one of the PCRs in 3.2. If this succeeds previous steps in 3.3 can be skipped. PCRs using these primers were performed in this project.

3.3.3 LR reaction

1) Add the following components to a 1.5 ml tube:
    - 7 µl product BP reaction
    - 1 µl pUBIL vector
    - 8 µl Thermo Fisher TE buffer pH8.0
    - 2 µl Thermo Fisher LR Clonase II
2) Mix well by vortexing briefly and centrifuge shortly
3) Incubate reaction overnight at 16°C
4) Add 1 µl of Proteinase K to terminate the reaction. Vortex briefly and incubate for 10 min at 37°C followed by 10 min at 95°

3.4 Transformation of *Agrobacterium*

When *E. coli* is obtained containing the pUBIL vector, these cells can be used for the transformation of *Agrobacterium*. The transformation is executed by triparental mating and is the same as specified in 2.5.1. Also, the confirmation of the transformed *Agrobacterium* and the preparation of a glycerol stock is exactly as mentioned in 2.5.2 to 2.5.4.

4 *Agrobacterium*-mediated transformation of rice

When transformed *Agrobacterium* is acquired, the actual *Agrobacterium*-mediated transformation of rice plants can be started. This protocol is the same for the generation of CRISPR mutants and overexpression lines and is shown in figure 4.1. Only the choice between the *Agrobacterium* containing the CRISPR construct or the one containing the overexpression vector will differ. For the knock-out mutants, the VIB *Agrobacterium* obtained as in chapter 2 will be used while for the overexpression lines the *Agrobacterium* obtained as in chapter 3 will be used.

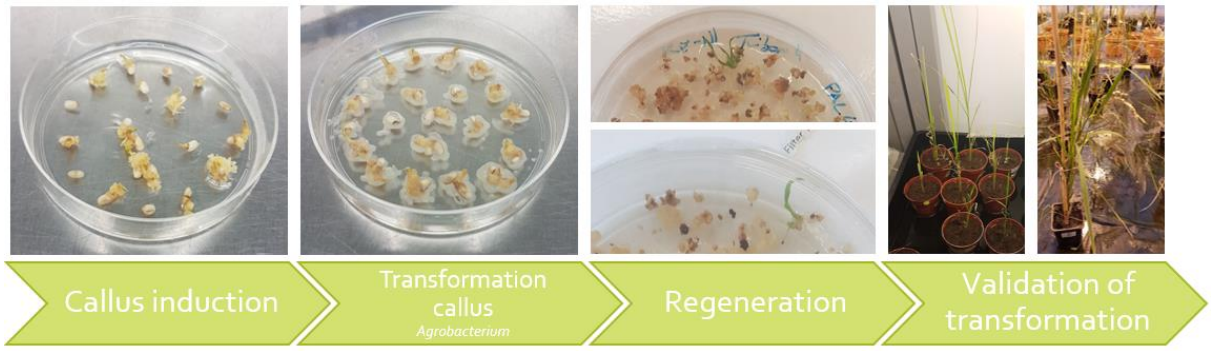

FIGURE 4.1: Agrobacterium-mediated rice transformation protocol. First, callus is induced out of rice seeds. This callus will then be transformed with Agrobacterium. New rice plants will regenerate out of the transformed callus. When these plants are high and strong enough, a sample can be taken to validate a correct transformation

4.1 Rice transformation

All steps in this protocol are performed under a vertical laminar air flow.

4.1.1 Scutellum derived callus proliferation

1) Prepare 0.4 l of N6D medium and pour plates
2) Dehusk ± 200 rice seeds and put them in a 50 ml falcon tube. Sterilize the seeds
   - Fill a 50 ml falcon tube with 70% ethanol and another one with distilled water, 1 guest medical bleach tablet and 1 drop of Tween 20
   - Add the 70% ethanol to the seeds and wash for 4 min by inverting. Work in the laminar flow bench from now on. Remove the ethanol, wash with 5 ml of the bleach solution and remove again
   - Add the remainder of the bleach solution and wash for 25 min on a rotator
   - Wash the seeds 10 times with autoclaved distilled water and let them dry on sterile paper
3) Inoculate the seeds on the N6D plates and close with micropore tape
4) Incubate for two weeks at 32°C under continuous light conditions

4.1.2 Agrobacterium plating

This step is performed 3 days before the callus inoculation with *Agrobacterium* (12 days after previous steps).

1) Prepare 0.4 l of LB + Rif25 + Spec100 medium and pour plates
2) Inoculate *Agrobacterium* from glycerol stock on a plate and close with parafilm
3) Grow *Agrobacterium* for 3 days in the dark at 28°C

4.1.3 Callus inoculation with Agrobacterium and co-cultivation

1) Prepare 0.1 l of AAM medium and 0.4 l of 2N6-AS medium. Pour 2N6-AS plates
2) Scrape *Agrobacterium* from the plate with a 1 ml tip and dissolve in 20 ml of AAM medium. Make a solution of 100 µl bacterial suspension and 900 µl of AAM medium
3) Check the OD600 with a spectrophotometer for this solution. Use 1 ml of AAM medium as a blank. Dilute the original bacterial suspension to an OD600 of 0.1 (keep in mind the measured solution was already diluted 10 times)
4) Collect your calli in a 50 ml falcon. Add the *Agrobacterium* solution of OD600 0.1 and incubate for 90 sec while inverting. Discard the liquid and let the calli dry on sterile paper
5) Transfer the calli to 2N6-AS plates and close with micropore tape. Co-cultivate for 3 days in the dark (wrap in aluminium foil) at 28°C

4.1.4 Regeneration

1) Prepare 0.4 l of N6D medium. After autoclaving add, apart from 2,4-D, 400 µl of timentin (300 mg/ml) stock solution and 400 µl of BASTA (30 mg/ml) solution and pour plates.
2) Autoclave 500 ml of distilled water, add 833 µl of Timentin. Wash the calli 10 times with this water. Wash the last time for 20 minutes while inverting. Blot the seeds dry on autoclaved paper
3) Transfer the calli to N6D + Tim + BASTA plates and close with micropore tape. Incubate for two weeks at 32°C under continuous light
4) Prepare 0.4 l of RE-III medium and pour plates. Transfer the calli to RE-III plates and close with micropore tape. Incubate at 32°C under continuous light
5) From now, transfer calli every two weeks to new RE-III plates

The following step can be performed when a plant, grown out of callus, touches the upper part of the petri dish.

6) Prepare 0.4 l MS medium and pour it into tubes. Transfer the callus of the grown plant into a tube. Seal with micropore tape and incubate at 32°C under continuous light

The following steps can be performed when the plant touches the upper part of the tube.

7) Autoclave soil. Make 10 l Hoagland solution. Fill pots with autoclaved soil and label all pots
8) Pull the plants carefully out of the MS tube. Wash the roots and remove all MS medium. Put the plant in the soil
9) Put all pots on a tray and fill with water until ± 2 cm height. Add 10 ml Hoagland per plant to the tray. Place all plants in 28°C room with 16/8 day/night regime.

4.2 Verification of transformed plants

When the potentially transformed plants are high and strong enough, a leaf sample can be taken into an Eppendorf tube. Out of these samples, the DNA is extracted which is then used for a PCR verifying the transformation. There are two different DNA extraction methods provided because the standard plant DNA extraction did not give positive extractions.

4.2.1 Standard plant DNA extraction

1) Prepare 0.1 l of rice extraction buffer by adding the following:
   - 200 mM Tris-HCl of pH 7.5
   - 250 mM NaCl
   - 25 mM EDTA
   - 0.5% SDS
2) Add 400 µl of the rice extraction buffer to each sample and grind samples thoroughly with a pestle. Incubate for 30 min at 95°C
3) Add 50 µl of chloroform and centrifuge for 5 min at 14000 rpm. Transfer 200 µl of the aqueous phase into a new Eppendorf tube and add 200 µl of isopropanol
4) Centrifuge 10 min at 14000 rpm and discard the supernatant. Wash with 100 µl ethanol and let dry
5) Dissolve DNA pellet in 30 µl of water and incubate for 1 h at 45°C

4.2.2 Plant DNA extraction using CTAB

1) Prepare the CTAB solution by adding following into a falcon:
   - 0.2 g CTAB
   - 1 ml 1M Tris HCl pH7.5
   - 2.8 ml 5M NaCl
   - 0.4 ml 0.5M EDTA
   - 5.8 ml distilled water
   - Put first the water in a falcon, measure the amount of CTAB and add to the water under the fume hood. Shake and put the solution at 65°C. Add the other components and put back at 65°C. Prepare fresh CTAB buffer every time.
2) Prepare EtOH/NaOAc solution by adding following into a falcon:
   - 4.5 ml 76% EtOH
   - 0.5 ml 2M NaOAc
3) Prepare EtOH/NH_4OAc solution by adding following into a falcon:
   - 4.95 ml 76% EtOH
   - 0.05 ml 1M NH_4OAc
4) Prepare chloroform/isoamylalcohol solution by adding following into a falcon:
   - 24 ml chloroform
   - 1 ml isoamylalcohol
5) Add liquid nitrogen and the sample into a mortar and crush the sample using a pestle. Immediately after crushing, add 1 ml of CTAB solution at 65°C and vortex until everything is in suspension. Leave 90 min at 65°C and mix every 15 min. Cool down 10 min at room temperature

6) Add 500 µl of the chloroform/isoamylalcohol solution and mix by inverting for 5 min. Centrifuge for 10 min at 13000 rpm and transfer aqua phase into a new 2 ml tube
7) Precipitate with 1 ml of isopropanol and mix very well. Centrifuge for 10 min at 13000 rpm and pour off all isopropanol
8) Wash with 500 µl of the EtOH/NaOAc solution and incubate for 20 min at room temperature
9) Vortex and centrifuge for 10 min at 13000 rpm. Pour of the wash solution, add 500 µl of the EtOH/ NH_4OAc solution and incubate for 10 min at room temperature
10) Centrifuge for 10 min at 13000 rpm and remove all ethanol with a tip. Dry for 20 min at 37°C
11) Dissolve the DNA in 30 µl of water and store at -20°C

4.2.3 Genomic PCR

First genomic primers have to be designed in a similar way as specified in 3.2.1. However, because the PCR is performed on the whole plant genome, introns are included in the sequence. For this, instead of choosing the transcript sequence, choose now the genomic sequence and choose primers located in introns for specificity.

1) Add the following components to a PCR tube:
    - 2 µl DNA template
    - 2 µl VWR 10x key buffer
    - 0.5 µl dNTPs
    - 0.5 µl forward primer
    - 0.5 µl reverse primer
    - 14.4 µl distilled water
    - 0.1 µl VWR Taq polymerase
    - Use the VIB primers to detect the insertion of the VIB vector
        - Primers will detect the inserted Cas9 gene of the vector, which is not originally present in the plant.
    - Use gene-specific genomic primers as a control for DNA extraction (this product can eventually be sent for sequencing if the PCR with VIB primers is positive)
    - If wanted, add a positive control to make sure the PCR was well performed
2) Use the following PCR program for both VIB and genomic primers:
    - 30 sec at 95°C
    - 30 sec at 56°C
    - 1 min at 72°C

4.2.4 Sending for sequencing

If the genomic PCR with VIB primers showed a band, the PCR product of that sample with the genomic primers was sent for sequencing to verify the transformation and the possible frameshift mutation. For details see also 2.4.2.

5 Inhibition experiment

In the first part of this inhibition experiment, rice plants are grown under the exact same conditions. After two weeks, half of the plants will be treated with an inhibitor of the PAL enzyme or a control treatment with water. One day after this treatment, all plants will be infected with the nematode *Meloidogyne graminicola*.

FIGURE 5.1: Inhibitor infection experiment protocol. First, rice seeds are germinated and grown for two weeks. Eleven days after germination half of the plants are treated with AOPP, a PAL enzyme inhibitor. Three days after AOPP treatment, all plants are infected with Meloidogyne graminicola. Two weeks dpi the root length is measured and all roots are stained with Raspberry Red. After this, galls and stained nematodes are counted.

5.1 Growing rice plants

1) Dehusk the number of needed rice seeds and sterilize the seeds as specified in 4.1.1
2) Dissolve 3mg thyram per g seeds in distilled water and add to the rice seeds to protect the seeds for contamination. Let incubate for 2 minutes.
3) Place a sterile paper into a petri dish. Inoculate the rice seeds on the paper and pour the rest of the thyram solution on the seeds. Add some more water to places where little water is present. Close the petri dish with parafilm and wrap in aluminium foil.
4) Incubate for four days in the dark at 32°C
5) Place the needed amount of tubes in a tray. Wrap some plastic foil in each tube. Fill the tubes with 20 ml of sand. Add SAP medium until the tube is filled half and add distilled water until water is dripping from the tube. Add SAP medium until about 1 cm above the tube and water again until it is dripping from the tube.
6) Wait until all water is moved into the medium. Carefully place the roots of the germinated seed into the SAP medium, but let the shoot above the medium
7) Wrap plastic foil around the tray to create humidity and incubate for 3 days at 28°C
8) Remove the plastic foil and give 10 ml of Hoagland solution to each plant. Incubate in the same room for 2 days
9) Add 10 ml of distilled water to each plant and incubate for 2 days
10) Add 10 ml of Hoagland solution to each plant and incubate for 3 days
11) Repeat step 8 to 10 until 2 days before the actual infection

5.2 Nematode extraction

These steps are performed three days before the actual infection (11 days after the germination of the seeds). Make sure to grow a *Meloidogyne* culture months before the infection experiment.

1) Remove the shoots from the *Meloidogyne* culture rice plant and wash the roots very carefully. Make sure the roots are washed very thoroughly
2) Cut the roots in pieces as small as possible (1 mm) to allow the nematodes to escape from the roots
3) Cover a 200 µm sieve with tissue paper and place it in a tray. Place the roots on top and add tap water to the tray until the roots are partly submerged in the water
4) Let incubate for 72 h at room temperature. After this time, the nematodes will have migrated into the tap water.

The following steps are performed on the day of the rice infection.

5) Collect the tap water and run it three times through a 20 µm sieve to collect nematodes
6) Count the nematodes under a microscope and dilute the solution to a concentration of 250 nematodes/ml

5.3 Inhibitor (AOPP) treatment

These steps are performed 24 hours before the actual infection (13 days after the germination).

1) Per eight plants, make 50 ml of 100 µM AOPP from a 100 mM stock solution
2) Spray the solution on the plants as uniform as possible, let dry under the fumehood
3) Return the plants to the growth room

5.4 Infection

These steps are performed 14 hours after the germination of the seeds.

1) Inoculate every plant with the same amount of nematodes. Make two small holes in the soil with a 1 ml pipet tip on each side of the plant. Add 750 µl of the prepared nematode solution into each hole and close them back up.
2) Incubate for 2 weeks at 28°C. Water the plants with 10 ml Hoagland 3 days after the inoculation. From now on water the plants as specified in 5.1.

5.5 Symptom evaluation

1) Two weeks past inoculation, the plants are harvested and cut to obtain only the roots
2) Root length and weight of all plants is measured
3) Color the roots with raspberry red by boiling them in a solution for 3 minutes
4) Transfer the roots to 6-well plates and add glycerol/HCl solution. Incubate for one week at room temperature on a plate shaker for destaining
5) Count nematodes and galls under the microscope
6) Analysis is performed using Excel and RStudio. Standard deviations are used for the error bars and significance is tested with a two-sided t-test or two-way Anova

B.3 Results and discussion

1 CRISPR mutants

To generate the knock-out CRISPR mutants, first, a suitable gRNA sequence must be found to cleave the gene of interest in the plant. The DNA-sequence for this gRNA is ligated into a vector specifically designed to generate CRISPR mutants in plants. This vector will then, by heat shock transformation, be inserted into *E. coli* and further, by triparental mating, into *Agrobacterium*. Rice plants will then be transformed with the help of this transformed *Agrobacterium*.

1.1 gRNA design

To create the CRISPR rice mutants, the first important step is designing good gRNAs for each gene of interest. To design these gRNAs, some important factors should be taken into consideration.

At first, the most important factor is the need for a PAM sequence at the 3' side of the oligonucleotide. By this restriction, the number of possible sequences is limited. In this project, the Cas9 endonuclease enzyme is used which needs a 5'-NGG-3' PAM sequence (with G: guanine, N: any nucleotide). The online tool CRISPR-P [143], will generate all possible gRNAs, 20 bp sequences followed by the PAM sequence, for the gene of interest. An output of the possible gRNAs for *AIM1* by CRISPR-P is given in figure 1.1. Although the need for a PAM sequence is the only actually limiting factor, there are some other factors needed to take into account to enhance the chance of a good CRISPR-induced mutation.

For the selection of a good gRNA, the location within the gene is important. The gRNA has to be located in an exon; the coding sequence of a gene. When the gRNA cleaves the gene, it is expected that an insertion or deletion will take place, causing a frameshift mutation. If this happens in an exon, it will lead to an alternative transcript, most probably leading to an unfunctional protein. Furthermore, it is most favourable that the gRNA is located as early as possible in the first exon of the gene. A frameshift mutation in a later exon could allow the protein to be partly or even completely functional because part of the mRNA can still be translated correctly, while a mutation at the beginning of the gene will completely disrupt the folding of the coded protein or it will induce a premature stop codon.

Another factor is the off-target score of the gRNA. Normally, the 20 bp gRNA will lead the Cas9 enzyme to a unique sequence in the plant genome. However, due to the tolerance for mismatches, off-target effects can still occur. The value of this score is given in CRISPR-P for every possible sequence (see figure 1.1) and has to be as low as possible to minimize these off-target effects. Especially for the *PAL* genes, this is of utmost importance because of their high homology. When a gRNA is used with a high off-target score for the generation of a knock-out rice mutant, results would be strongly biased at the end of the experiments. This bias would be caused by the uncertainty whether a different result is due to the cut in the gene of

interest or due to the cut in an off-target gene. However, it is shown that the seed sequence, the ten base pairs upstream of the PAM sequence, is important for specificity and the chance of an off-target cut can be highly reduced when the seed sequence of the off-target site differs in some base pairs.

Figure 1.1 shows the output of CRISPR-P for all possible gRNAs for *AIM1*. The upper part of the figure shows the location of all possible gRNA sequences. In the table on the left, all gRNAs are ranked based on their off-target score. Further, more information about each gRNA and their off-target score is provided on the right. Here, the location and seed sequence for the off-target sites is provided. For example, for *AIM1*, the third gRNA (presented as a yellow box in the upper part and as a blue box in the lower part of the figure) is used because it has a high on-score (0.8865) and because it is located in the coding sequence (CDS, exons), more specifically in the first exon, of the gene. It can be seen that this sequence has 47 off-target sites. Although some off-target sites are present in the CDS of the genome, this gRNA is chosen because there are a lot of mismatches in the seed sequence of each off-target site (presented as red nucleotides) lowering the chance of being targeted by the Cas9/gRNA complex.

FIGURE 1.1: CRISPR-P output for gRNAs for AIM1. In the upper part of the figure, the location of all gRNAs can be found. In the lower part left, all gRNAs are ranked, based on their on-target score (the first gRNA has the highest on-target score). Next to the actual gRNA sequence, also the region is provided. To have a knock-out mutation, the gRNA must be present in the coding sequence (CDS) of the gene. For AIM1, the third gRNA is selected. In the lower part right, more information about the off-target score of the chosen gRNA is given. Here, it can be seen that for this gRNA there are 47 off-target sites with some located in the CDS of other genes. However, because of the number of mismatches (red nucleotides) in the seed sequence of every off-target site, this gRNA is selected.

Once the sequence of the gRNA is chosen, the forward sequence and its reverse complement are synthesized because the gDNA will be ligated into a double-stranded vector. These two oligonucleotides will later be used to dimerise and form the oligo-duplex for the gDNA present in the plasmid. The gDNA sequences used for the different genes can be found in table 1.1. However, before ordering these oligonucleotides, four bases must be attached to the oligos. All forward oligos have to start with the sequence GGCA while all reverse oligos have to start with AAAC. These four bases must be attached because upon digestion of the vector with the restriction enzyme *Bsa*I, complementary overhangs will be generated, making it possible for the oligo-duplex to ligate in this vector.

TABLE 1.1: designed 5'-3' gRNA sequences for all genes used in this project

Gene	gRNA sequence
OsPAL1	GGACCCGCTCAACTGGGGCG
OsPAL2	GCGCCCTCGATCTTCACCAG
OsPAL3	GCGCCCTCGATCTTTACCAG
OsPAL4	TCATGCCATTGGCAGAAACG
OsPAL5	GGACCCCTCGATCTTCACCA
OsPAL6	CATGGCTGCCCGCGTCGCCG
OsPAL7	GGCGCCCTCGATCCGCACCA
OsPAL8	CCATCCTCTTCACCTCCTCG
OsPAL9	CGGCCGCCAGAACTCCGCGG
OsAIM1	ATTGAAGGAGAAGTACGCGG

1.2 Ligation into the VIB vector

When the ordered oligonucleotides are dimerised, the oligo-duplex can be ligated into a vector. To generate CRISPR knock-out mutants, the pBb7-B1-ccdB-B1-OSU3-NosT-zCAS9-ZmUBIL vector, further referred to as the VIB vector, is used. This vector is specifically designed to induce knock-out mutations in plants via *Agrobacterium*-mediated transformation. Figure 1.2 shows the VIB vector with its components. At first, the gRNA 'gene' is inserted by using the restriction enzyme *Bsa*I and ligate the gDNA between two *Bsa*I restriction sites (indicated in figure 1.2). This enzyme will cut out the *CmR* gene, a chloramphenicol resistance gene present to make a vector stock in *E. coli*, and the *ccdB* domain. For the *E. coli* strain, later used to transform with this vector (TOP10), the latter gene is lethal. So if the transformation was successful but no restriction (and insertion of the gRNA) occurred this gene will still be present in the vector which causes the death of the *E. coli* cell, making it easier to screen for transformants with the inserted gDNA.

Next, the grey boxes are the left border (LB) and the right border (RB). These sites indicate the ends of the part of the vector that at the end of the transformation will be integrated into the plant genome. It can be seen in figure 1.2 that *Agrobacterium* will insert around 11.000 bp into the plant. The genes present in the part of the vector that will not be transferred to the plant genome are mainly antibiotic resistance genes, making it possible to select colonies in *E. coli* as well as in *Agrobacterium*. For example, the red box indicated with Sm/SpR is a resistance gene for streptomycin and spectinomycin. In this project, spectinomycin was used to select transformed colonies.

The genes present between both borders will be transferred to the plant and are mainly associated with the generation of a CRISPR-induced cleavage. At first, the *CAS9* gene, preceded by a maize *UBIL* promotor can be seen. This gene will code for the CAS9 enzyme which will be led by the gRNA to a specific region in the plant genome (in this case a *PAL* gene) where it will cleave this region of the genome. In this project, when rice plants are validated if they are well transformed, a PCR using primers for this *CAS9* gene is used. Further, the *CmR* and *ccdB* domain are replaced by the dimerised gDNA oligonucleotides and this is preceded by a U3 small RNA promotor. When the gRNA is expressed in the plant together with the CAS9 enzyme, this will hopefully lead to CRISPR-induced knock-out mutations. At last, the red box indicated with Bar is a BASTA herbicide resistance gene used to screen for transformed rice plants. When the part of the vector is transferred to the plant, this gene will be present in the plant causing resistance to the herbicide.

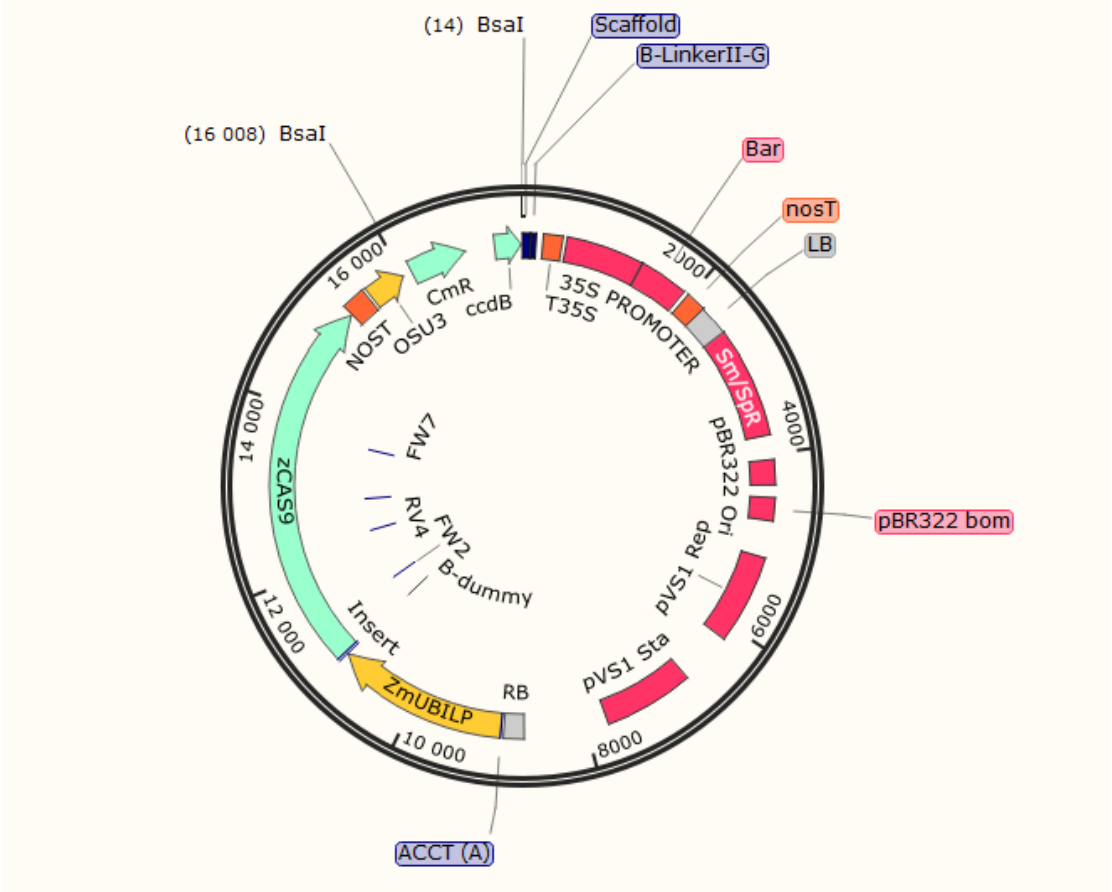

FIGURE 1.2: The pBb7-B1-ccdB-B1-OSU3-NosT-zCAS9-ZmUBIL or VIB vector with its components.

1.3 Transformation of *E. coli*

For an easy amplification, the ligated VIB vector is inserted into competent *E. coli* cells. Also because of the insertion into *E. coli*, it is possible to transform *Agrobacterium* with an easy procedure called triparental mating (see 1.4). The *E. coli* cells are transformed by the heat shock procedure.

To screen for transformed *E. coli* strains, all cells are plated on LB medium with spectinomycin. Most likely, colonies growing on this plate are transformed because the VIB vector possesses a resistance gene. However, to be certain, a colony PCR is performed. For this, the PCR is performed with a primer specific for the VIB vector and one for the gRNA sequence. With these primers, it is possible to directly check if the VIB vector is present inside the cell and if the gDNA duplex is correctly ligated into the vector. Figure 1.3 shows the electrophoresis gel of the colony PCR for *PAL2* (left) and *PAL3* (right). *PAL2* has only four tested colonies because only these four did grow on the antibiotic plate. It can be seen that nearly all tested colonies are transformed with the ligated VIB vector. The red asterisks show the colonies used in further steps. Similar figures with positive colonies were obtained for all genes of interest (*PAL1-8*, *AIM1*).

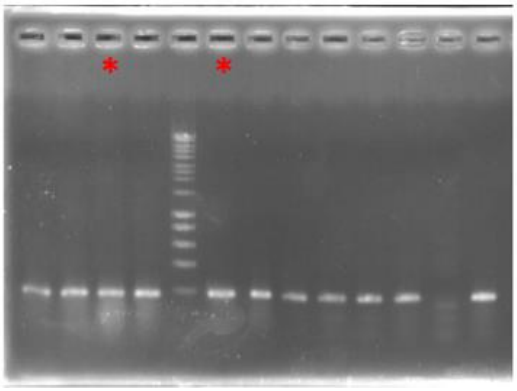

FIGURE 1.3: Electrophoresis gel after colony PCR for PAL2 (to the left of the ladder) and PAL3 (to the right of the ladder). The expected band size is 190 bp. All tested colonies, except one, were transformed with the recombinant VIB vector. The red asterisks indicate the colonies used in further steps.

To ensure the chosen colony was correctly transformed with the recombinant vector, DNA was extracted from the *E. coli* cells. The concentration of this DNA was then measured with a Nanodrop device. For sequencing, a concentration of 100-150 ng/µl is required. If concentrations are too low, DNA extraction has to be performed again while too high concentrations need to be diluted with water. When the right concentration is obtained, a primer is added to the sample. This primer will be the starting point of the sequencing. The location of the primer is very important because at some distance the peaks will not be accurate anymore. In this project, primers around 100 bp upstream of the ligated gDNA sequence are used. After sequencing, the results can be examined.

1.4 Transformation of *Agrobacterium*

Once it is shown that the transformation of the *E. coli* cells is well performed, these cells can be used to transform *Agrobacterium* cells. This is done by the triparental mating procedure and can be seen in figure 1.4a. In this method, the plasmid of interest, the VIB vector (blue) is present in an *E. coli* strain, called the donor strain. With the help of an *E. coli* helper strain (yellow), this plasmid will be transferred to the *Agrobacterium* strain (red). This is due to the location of genes responsible for conjugation, the transfer of a plasmid to another bacterial cell, located on the *E. coli* helper plasmid. This cell will pass its plasmid by conjugation to the donor strain where it will induce conjugation again and, in some cases, the VIB vector will be transferred to *Agrobacterium.* This was done by mixing even amounts of the three strains, making a drop on a $MgCl_2$ plate and incubating this for one day. After this incubation, a situation as in figure 1.4b is obtained. Here, the *Agrobacterium* is moved to the outer border of the drop where it can be seen as a slightly more white border (see figure 1.4b). This border is then scraped off and dissolved in growth medium.

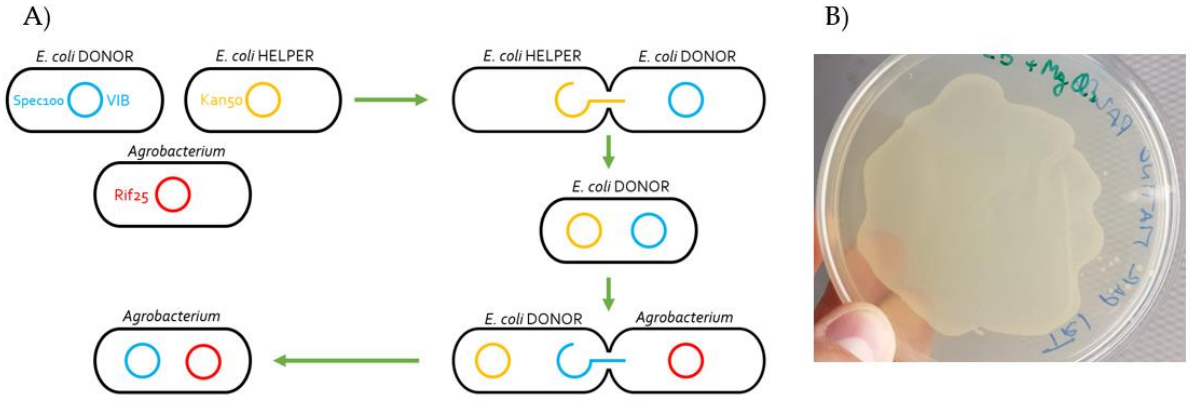

FIGURE 1.4: Triparental mating A) mechanism of triparental mating where the E. coli helper strain will induce conjugation to transfer the plasmid of interest, the VIB vector (blue), to Agrobacterium. B) Situation obtained after incubation where the Agrobacterium has moved to the outer border of the drop where it can be seen as a slightly more white border.

However, not all *Agrobacterium* present will be transformed. To screen for the transformed cells, a 10x dilution series is made. Every dilution is then plated on an LB plate with rifampicin to select for *Agrobacterium* strains and spectinomycin to select for the insertion of the VIB vector. These plates were incubated for three days and after this, the plate where colonies have grown sufficiently but separate colonies still can be picked is chosen. To ensure the *Agrobacterium* that will be used to transform the rice plants possesses the VIB vector with the right insert, a colony PCR is performed, the DNA is extracted and sequenced. Figure 1.5 shows the electrophoresis gel after colony PCR of the chosen *Agrobacterium* colonies for *PAL7* (left) and *PAL8* (right). For this PCR the same primers were used as with the PCR for *E. coli*. It can be seen that all tested colonies are positive for the insert. The red asterisks show the colonies used in further steps. Similar figures and thus positive colonies were obtained for all genes of interest (*PAL1-8, AIM1*).

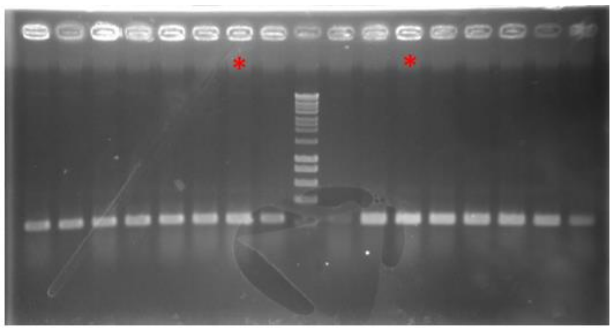

FIGURE 1.5: Electrophoresis gel after colony PCR for PAL7 (to the left of the ladder) and PAL8 (to the right of the ladder). The expected band size is 190 bp. All tested colonies were transformed with the recombinant VIB vector. The red asterisks indicate the colonies used in further steps.

Here again, the DNA was extracted from the cells and sequenced to ensure that the *Agrobacterium* possesses everything needed for the plant transformation. The primers used for sequencing were the same as was the case for the sequencing of *E. coli*.

1.5 *Agrobacterium*-mediated rice transformation

To obtain a CRISPR mutant, the rice plants need to be transformed. This is done by *Agrobacterium*-mediated transformation. *Agrobacterium tumefaciens*, also called *Rhizobium radiobacter*, is a plant pathogen causing crown gall disease in thousands of plant species [147]. However, this pathogen was found to be very useful in plant biotechnology. Upon infection, *A. tumefaciens* will insert part of its tumor-inducing (Ti) plasmid, the T-DNA, into the plant where this DNA is stably integrated into the plant genome. Surrounding this T-DNA there are two conserved 25 base pairs border sequences to cut the T-DNA out of the Ti-plasmid and integrate it in the plant genome. The most interesting characteristic in biotechnology of this T-DNA is that the sequence between these border sequences does not matter for the integration into the plant. In other words, if *Agrobacterium* is transformed with genes of interest inside the T-DNA, these genes will be inserted in the plant genome leading to a transformed plant [148]. The transformation of *Agrobacterium* served to do this. The VIB vector contains the two needed border sequences with between the gene for the CAS9 enzyme, the gRNA duplex and a herbicide resistance gene. This DNA will be integrated into the plant genomic DNA, leading in some cases to a knock-out of the gene of interest.

A first step in the rice transformation protocol is the proliferation of callus. Callus is non-differentiated plant tissue. When a plant regenerates from a single transformed callus cell, every plant cell will contain the inserted DNA.

In this protocol, the callus that is grown for two weeks, is inoculated with *Agrobacterium* which will insert part of its DNA into the non-differentiated plant cell genome. These calli will then be transferred to a growth medium plate containing the herbicide BASTA to screen for the transformation. Non-transformed callus cells did not obtain this part of the VIB vector and will die because of the herbicide, while transformed plants did get the herbicide resistance

gene from the VIB vector and will be able to regenerate on these plates. In this project, rice transformation is performed for nearly all genes of interest (*PAL1-4, PAL6-8, AIM1)*.

However, not all regenerated plants are transformed. To examine the number of actually transformed plants, a sample is taken from all plants, the DNA is extracted and a genomic PCR is performed. This PCR uses a primer that can only bind to a region inside the transferred vector. When plants show a band on this gel, the plant is most likely transformed. However, to be certain, the PCR product is sequenced to prove that the *CAS9* gene is present in the cell. Although the transformation was successful for these plants, it is still not known if the gRNA/CAS9 complex will efficiently cleave the gene of interest. Therefore, another PCR on this DNA is performed with primers around the predicted site of cleavage. This PCR product is then sequenced to see if a cleavage leading to an insertion or deletion has taken place. Table 1.2 shows for each examined gene the number of plants regenerated, transformed and the amount that contains a frameshift mutation. Further, for plants hopefully mutated in the mentioned gene, more information about these numbers are provided in the following paragraphs.

TABLE 1.2: Number of plants regenerated from transformed callus, plants actually transformed and plants containing a frameshift mutation

Gene	Regenerated plants	Transformed plants	Plants with frameshift
OsPAL1	16	3	-
OsPAL2	0	-	-
OsPAL3	0	-	-
OsPAL4	8	3	-
OsPAL6	33	4	-
OsPAL7	0	-	-
OsPAL8	0	-	-
OsAIM1	40	21	0

For four of the genes, plants were regenerated from the callus. These plants were then investigated to see if they are actually transformed. However, for the other constructs, no rice plants were regenerated. For *PAL2* and *PAL3* no plants have grown out of the callus before the callus died. This is maybe because some component in the medium was added in too small or too high quantity and the contamination of some plates with the pink bacteria, an organism present in rice seeds, that are not killed by the sterilization of the seeds. On the other hand, for *PAL7* and *PAL8*, there was an overgrowth of *Agrobacterium* which killed the callus before growth of the plants. Even after several transfers to new plates with a higher content of timentin, the *Agrobacterium* could not be killed and calli on these plates died.

From the regenerated plants that were high and strong enough, a leaf sample was taken and the DNA from this sample was extracted using a quick protocol. A PCR with a primer specific for the insert had to indicate the transformed plants. However, after multiple PCRs, the use of different primers and several DNA extractions, there was still no positive result for any of the genes. For this reason, the DNA extraction was tested by doing a PCR on all samples with primers for two household genes, *EIF5C* and *Exp Nar*. Also, this gel showed only a few positive

results (see figure 1.6) which led to the conclusion that none of the DNA extractions were successful. Therefore, another protocol was used to examine the plants, namely the CTAB extraction protocol.

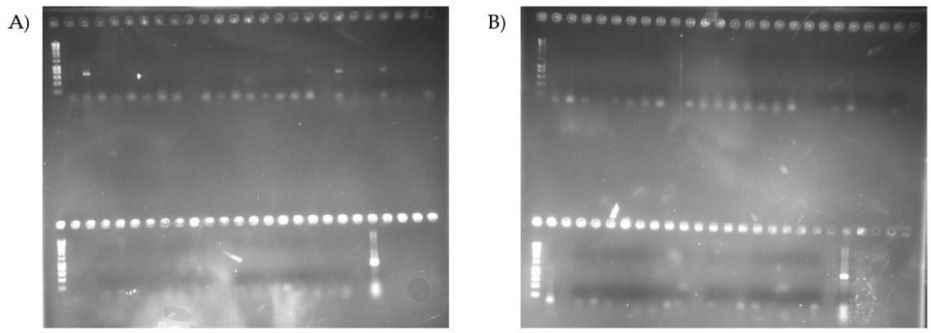

FIGURE 1.6: Genomic samples tested with primers for two household genes: A) EIF5C and B) Exp Nar for samples for PAL6 (top lanes), PAL1 (bottom left) and PAL4 (bottom right). The last lane is a positive control.

Although for most genes the original DNA extraction method failed, for *PAL6* it showed already four transformed plants as is shown in figure 1.7a. Unfortunately, these plants died for unknown reasons (a wild type plant did survive under the same conditions) before it was shown that they were transformed. Also, due to several PCRs to check for transformation, the DNA sample was finished and it could not be checked for a frameshift mutation. Of the plants that were still alive, DNA was also extracted using the CTAB method. However, no additional transformed plants were found. Using the CTAB extraction protocol, it is further shown that for *PAL1* and *PAL4* three of the regenerated plants were transformed as shown in figure 1.7b and 1.7c respectively. However, for the same reasons as with *PAL6* plants, more information about a possible frameshift mutation could not be provided. For *AIM1*, there were a lot of transformed plants (see figure 1.7d) and, contrary to the plants for the other genes, these plants did survive until transformation was proven.

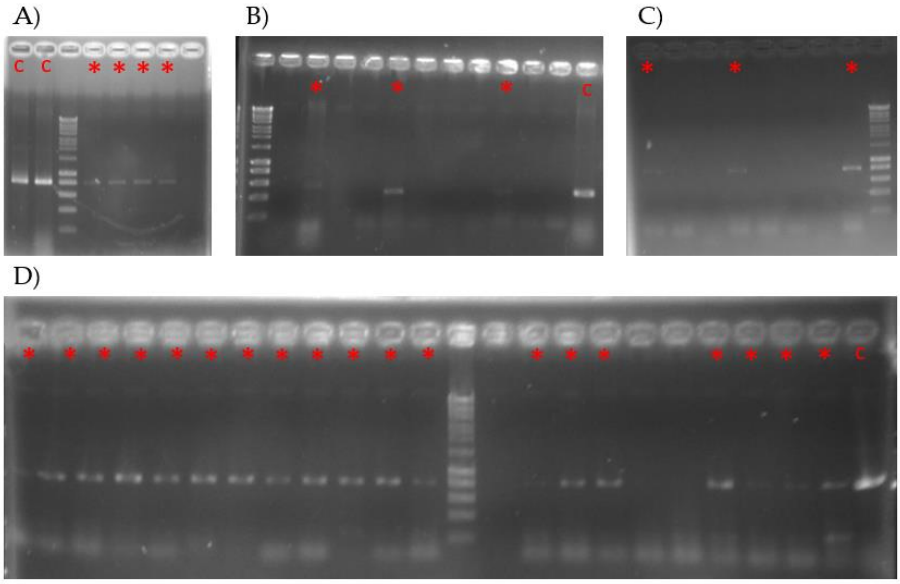

FIGURE 1.7: Genomic PCR with primer specific for the construct as a test for the transformation of the plant for A) PAL6, B) PAL1, C) PAL4 and D) AIM1. Red asterisks show the positive samples and a red 'c' shows the positive control to test if the PCR had performed well.

On the survived *AIM1* plant samples, another PCR was performed with primers around the predicted site of cleavage in the *AIM1* gene and this PCR was sequenced. Unfortunately, after analysis of the 21 sequenced samples with the online tool TIDE [149], it was found that in none of the samples a cleavage or frameshift occurred. An example of the outcome for a sample of *AIM1* can be found in figure 1.8. In this figure, the alignment (A) shows no mutations or gaps which means there are no insertions or deletions. Also, the graph represents this, where it can be seen that in 85% of the cases no insertion or deletion is present. The total efficiency is provided and here this is only 5.1%. Similar results were obtained for the other 20 samples.

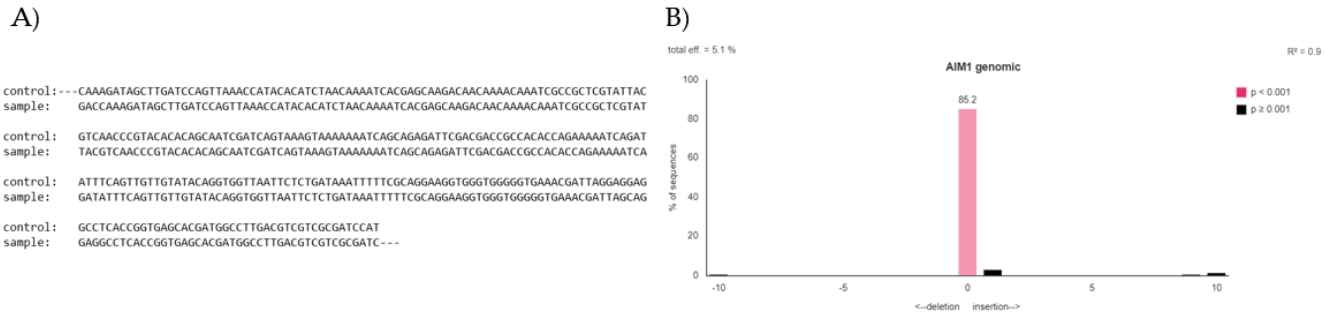

FIGURE 1.8: Output of the online tool TIDE for an AIM1 sample. A) It can be seen that there are no mutations or gaps in the alignment and B) also the graph shows that there are no insertions or deletions present.

Because the efficiency of the cleavage was so low for all *AIM1* samples, where none of the 21 samples showed a mutation or frameshift, the efficiency of the gRNA was tested *in vitro*. The hypothesis here was that if the gRNA had a low efficiency to guide the CAS9 enzyme to the specific sequence *in vitro* it would not be able to do this *in planta* as well. Therefore, four new gRNAs were designed and these were tested *in vitro* together with the one used for the generated transformed plants. Figure 1.9 shows the gel of the *in vitro* cleavage of a plasmid and thus the efficiency of the designed gRNAs. The red arrow indicates the gRNA used in previous experiments. It can be seen that the used gRNA was not able to cleave the plasmid *in vitro* leading to the assumption that it will not cleave the gene *in planta* either. It can also be seen that the third gRNA has much better efficiency. In further research experiments, *Agrobacterium* has to be transformed with a construct containing this gDNA and this strain must then be used to make new CRISPR rice mutants. However, a good efficiency *in vitro* is no proof for the efficiency *in planta*, this procedure serves only to exclude gRNAs with a low *in vitro* efficiency. Further research will prove if the other gRNA will cleave the gene *in planta*.

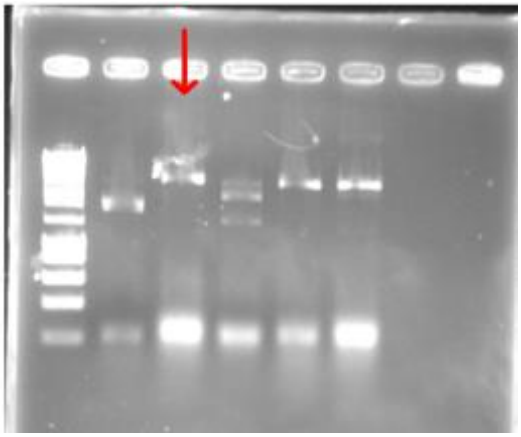

FIGURE 1.9: In vitro efficiency test for different gRNAs. The red arrow indicates the gRNA used in previous experiments. This gRNA was not able to cleave a plasmid in vitro which lead to the expectation that it won't cleave in planta as well. On the other hand, the third gRNA is able to cleave a plasmid in vitro. This one can be used to generate new CRISPR rice mutants.

2 Overexpression lines

To infer the function of the genes of interest in the SA biosynthesis, the knock-out CRISPR mutants have to be compared with control wild type plants and plants overexpressing one of these genes. To obtain these overexpression lines, RNA is extracted from a wild type plant from which the single gene is isolated. The cDNA for this gene is inserted into a vector in *E. coli*, after which it is transferred by the gateway cloning protocol to a destination vector which will be used to transform *Agrobacterium*. In an identical way as with the CRISPR mutants, the transformed *Agrobacterium* will be used to transform rice plants.

2.1 Gene of interest isolation.

The first step in the isolation of a single gene is the extraction of RNA from a wild type plant to make complementary DNA (cDNA). The use of cDNA is preferred to the use of genomic DNA because cDNA starts from RNA which does not contain introns. For this reason, cDNA will contain all fully functional genes without the extra nucleotides from the introns. Also, transformation of *E. coli*, *Agrobacterium* and rice will become more difficult for longer genes. RNA from three different plants was extracted to make three different cDNA-preparations; cDNA1, cDNA2 and cDNA3.

Because the obtained cDNAs are a mixture of a lot of genes, a PCR with specific primers is performed to isolate the gene of interest. At first, this was done for cDNA1. The primers used in these PCRs are designed in a way that the whole gene can be amplified and are mostly located in the (untranslated) UTR regions of the genes because of high homology, especially for the *PAL* genes. In the first instance, isolation of *PAL4* and *PAL6* was tried with a high fidelity velocity Taq polymerase. This high-fidelity polymerase was preferred because it has

proofreading activity, minimising mistakes in the gene of interest. However, following several gradient PCRs using this polymerase, no bands were visible after electrophoresis. For this reason, the cDNA and the used primers were tested. Figure 2.1 shows the gel from the cDNA test. A normal PCR was performed on the three cDNAs with primers for two household genes with high expression. It can be seen that cDNA1 did not show bands for both primers, leading to the discarding of this cDNA.

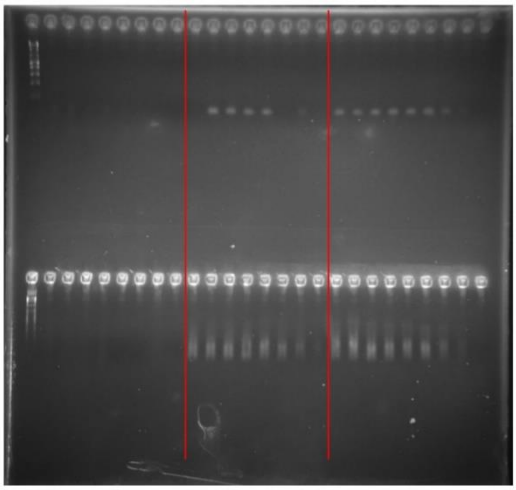

FIGURE 2.1: Test of the three cDNAs (cDNA1 left, cDNA2 middle and cDNA3 right) with primers for two household genes: EIF5C (top) and Exp Nar (bottom). cDNA1 did not show amplification for both primers and was discarded. cDNA3 had the nicest bands and was used in further steps.

Because cDNA3 had the best bands, this cDNA was used to perform the PCR again with *PAL4*-specific primers. However, this gel did also not show any positive bands. Therefore, several PCR protocols were used with different polymerases; high-fidelity velocity Taq polymerase, all-in polymerase mix, Q5 high-fidelity polymerase and normal Taq polymerase. After several gradient PCRs for *PAL4* with all different polymerases, no positive band could be extracted because no bands were present or the band of interest was to close to the other bands, which made gel purification impossible. However, after more gradient PCRs with velocity Taq polymerase for *PAL4*, an attempt was done to purify the gene out of the gel. Gel extraction succeeded for *PAL4* and *AIM1*. Figure 2.2 shows the gel for *PAL4* and indicates the extracted bands.

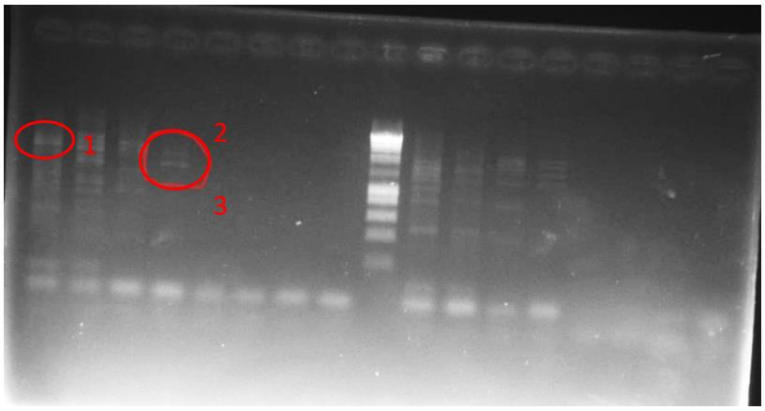

FIGURE 2.2: Electrophoresis gel for PAL4 (±2000 bp) with velocity Taq polymerase. The bands indicated with red numbers are purified and used in further steps.

2.2 Gateway cloning

The indicated bands in figure 2.2 were purified from the gel after which an adenosine overhang is generated. The A-overhang is needed for the ligation into the pGEM-T vector. Ligation in the pGEM-T vector and transformation of *E. coli* with this vector ensures that the purified DNA fragment can later be amplified by growing transformed *E. coli* cells. This vector can be bought, but only linearized with tyrosine overhangs. Because velocity Taq polymerase does not add an adenosine at the end of the product, this is done after the purification. When the A-overhangs are added, the gene is ligated into the pGEM-T vector. This vector is then sequenced to verify if the complete gene with no mutations is present in the vector. After this, another PCR is performed on this vector with primers containing attb-sites. With this PCR, the attb-sites will be attached to the PCR product. This product is then used to start the gateway cloning protocol showed in figure 2.3.

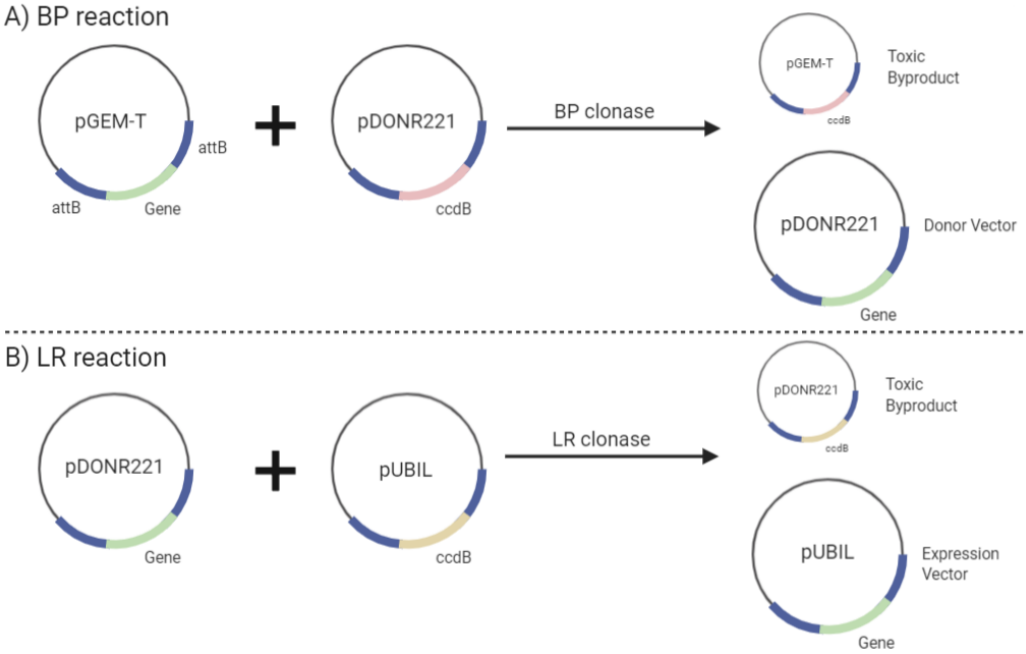

FIGURE 2.3: Schematic overview of the gateway cloning protocol. The gene of interest is inserted in the donor vector by the BP reaction and later in the expression vector by the LR reaction. After each reaction, the obtained vector is used to transform E. coli and a colony PCR is performed to validate the transformation. In the end, the expression vector is used to transform Agrobacterium via triparental mating. The ccdB gene is a lethal gene for the E. coli TOP10 cells used in this project.

The BP reaction is the first of two reactions in the gateway cloning protocol. By adding the pDONR221 vector and the enzyme BP Clonase, the gene of interest is transferred from the pGEM-T vector to the pDONR221 vector. This vector is then used for the heat shock transformation of *E. coli* after which a colony PCR will validate the transformation (figure 2.4a). When positive colonies are obtained, the vector is extracted again and used in the second LR reaction. In this reaction, the gene of interest is transferred to the destination vector, pUBIL, by adding this vector with the donor vector and LR Clonase. After this reaction, the vector is used again to transform *E. coli* cells and transformation is again validated with a colony PCR(figure 2.4b).

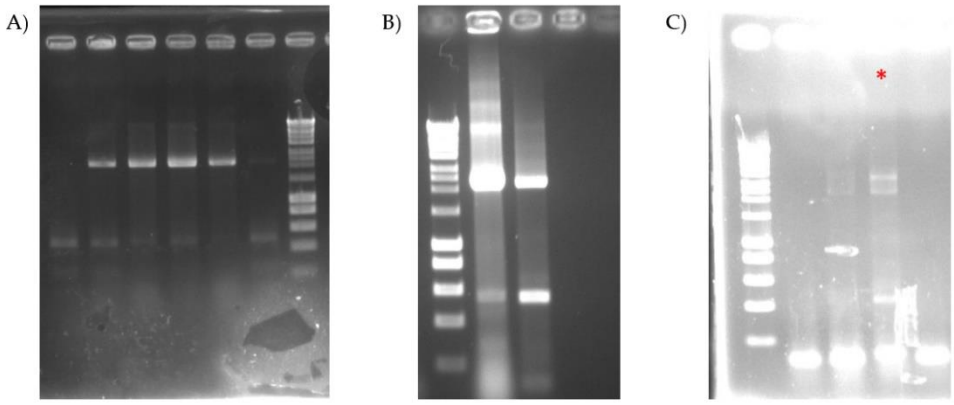

FIGURE 2.4: Gel electrophoresis pictures of colony PCRs after each reaction of the gateway cloning for AIM1. A) colony PCR after the BP reaction where E. coli is transformed with the pDONR221 vector. B) colony PCR after the LR reaction where E. coli is transformed with the pUBIL vector. C) colony PCR of transformed Agrobacterium via triparental mating.

When *E. coli* is obtained containing the pUBIL vector with the gene of interest, these cells can be used to transform *Agrobacterium* by triparental mating (figure 2.4c). This procedure is identical to the triparental mating used for the transformation of *Agrobacterium* for the CRISPR mutants as described in section 1.4. A transformed *Agrobacterium* was obtained for the *AIM1* construct. Furthermore, the transformed *Agrobacterium* will be used to transform rice plants in the same way as has been done for the generation of the CRISPR mutants. However, due to a lack of time, no transformation series for *AIM1* could be started.

3 Inhibition experiment

To infer the role of the PAL enzyme in infection with *M. graminicola*, half of the plants were treated with an inhibitor of the PAL enzyme: AOPP. After this, all plants were inoculated with an equal number of nematodes. After two weeks, root (figure 3.1a) and shoot (figure 3.1b) lengths were measured, the root weights were measured and galls were counted (figure 3.1c). After this, the roots were stained and then destained to make the nematodes visible. All nematodes were counted and classified according to their life cycle stage (figure 3.1d).

A two-way anova analysis was performed on the root and shoot lengths (figure 3.1a and b). Both treatments either with AOPP compared to water and with nematode infection compared to non-inoculated led to a significant difference in root length (p-values can be found in figure A.2 in appendix). However, shoot length is not significantly affected by a different treatment or the presence of infection. The bars labelled with the same letter are not significantly different according to a two-way anova test. It can be seen that there is a significant decrease in root length in infected plants compared to non-infected in both treatments. On the other hand, there is also a significant increase between water-treated and AOPP-treated roots. A possible explanation is that treatment with AOPP can alter also other functions in the roots which will

increase the root growth. Another possibility is that by inactivation of the relatively important enzyme for the biosynthesis of SA, the rice plant will not invest as much energy in defense against the nematode as the control plants do, but it will invest this energy in the growth of the roots.

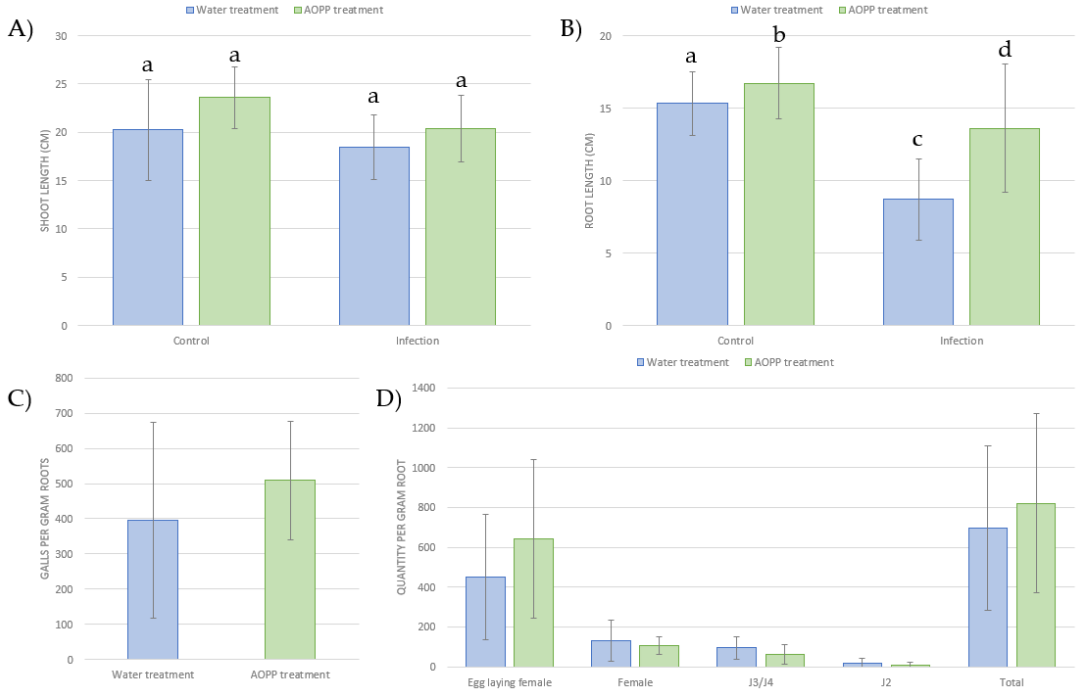

FIGURE 3.1: Results of the PAL enzyme inhibition experiment. Blue bars indicate the control groups (water treatment) while green bars indicate AOPP-treated groups. Error bars indicate the standard deviation of the presented group. The bars labelled with the same letter are not significantly different according to a two-way anova test. A) Length of shoots of the water- and AOPP-treated groups. No significant differences can be seen between any groups. B) Length of roots of the water- and AOPP-treated groups. Both treatments as well as infection affect the root length significantly. C) The number of formed galls per gram of roots. No significant conclusions could be made due to high variability. D) The number of nematodes per life cycle stage per gram of roots. No significant conclusions could be made due to high variability.

Further, the root weight was measured (table A.3 in appendix shows the weight of the roots) and the formed galls and nematodes per life cycle stage were counted on all infected roots. The quantity of galls and nematodes is presented per gram of roots (figure A.1 in appendix shows the counted nematodes per root system). Although the large standard deviations make it impossible to make a significant conclusion (two-sided t-tests were performed, but no significance on the 0.05 significance level was found), there seems to be a trend that the nematodes can more easily form galls when the PAL enzyme is inhibited, indicating an increased susceptibility towards the nematodes. Similar results can be seen when the nematodes were counted. Here, a trend can be seen that in total, more nematodes were able to invade the plant when the PAL enzyme was inhibited, which possibly proves again the increased susceptibility of the plants. On the other hand, most of the nematodes in the AOPP-treated plants were already developed into egg-laying females, which could indicate that nematodes can more easily develop in the roots where the PAL enzyme is inhibited. However, again due to large variability, no significant conclusions could be made.

B.4 Conclusion

It can be concluded that in this project the first steps in a greater series of experiments have been performed to unravel the importance of the PAL-pathway for the biosynthesis of salicylic acid in rice.

For the generation of the CRISPR knock-out mutants, a CRISPR construct was designed for each gene of interest (*OsPAL1-8*, *OsAIM1*) and for all these genes, transformed *Agrobacteria* were obtained. These strains are conserved in a glycerol stock at -80°C and can immediately be used again when new rice transformations are started. Also, for nearly all genes of interest, rice transformation was started. It is shown that rice transformation for CRISPR-mutants had a low yield of regenerated plants. In this project, some of the bottlenecks of the rice transformation have been resolved as detailed below. This information can be used to improve future rice transformation experiments.

1. Antibiotics to kill off all *Agrobacterium* after callus transformation must be applied in (relatively) high concentrations and if the bacteria are still able to grow, all callus must immediately be transferred to new plates.
2. Next, the original protocol mentioned the transfer of the callus to new RE-III plates every two weeks until the regenerated plants could be transferred to the MS medium. However, it was seen that the callus suffered from being transferred too often. To resolve this, thicker plates were poured and only if the medium was nearly dried out, a transfer was done.
3. Also, it was seen that some of the primers available in the lab for the detection of the rice transformants did not give good results, so for each gene better working primers have been designed. For example, for *PAL6,* four pairs of primers were designed and combinations of all primers were tested to find out which couple of primers was the best to use (see table A.2, PAL6_genomic_F2 and PAL6_genomic_R).
4. Most of the CRISPR *PAL4* plants died due to a spider mite contamination in the growth room while other *PAL1*, *PAL4* and *PAL6* CRISPR plants died of unknown reasons. However, it was observed that the plants transferred to the ILVO greenhouse had a higher chance of surviving than in the plant growth rooms.
5. Because the performed DNA extraction protocol failed, it is still unknown whether the dead plants of *PAL6*-CRISPR were transformed and if plants of *PAL1*-CRISPR and *PAL4*-CRISPR contained a frameshift mutation. For this reason, DNA extraction using another method (CTAB) was tried which seemed more successful.
6. At last, for AIM1 it was seen that all transformed plants had no frameshift mutation. Therefore, from now on, before starting a rice transformation series, the gRNA will be tested *in vitro* to exclude non-functional gRNAs used in further steps *in planta*.

For the generation of the overexpression lines, the *Agrobacterium* containing the *AIM1* gene has been obtained. Also, three candidate *PAL4* genes were isolated and ligated in pGEM-T vectors until further use. The isolation of the genes showed low efficiency and again, some bottlenecks have been resolved. All the primers used for future experiments have been tested and if needed new primers were designed. From now on, synthesized cDNA from extracted RNA has to be tested with the two pairs of primers for the household genes *EIF5C* and *Exp Nar* to

validate if RNA extraction and cDNA synthesis was performed well. Further, the PCR protocol for gene isolation was adjusted until clear bands of the appropriate length were found. Several polymerase mixes were tested and it was shown that the high-fidelity Velocity Taq polymerase was the only mix from which good results were obtained.

At last, from the PAL enzyme inhibition experiments, some hypotheses could be made. A possible hypothesis is that when the PAL enzyme is inhibited, the plant will probably invest less energy in defense against the nematodes and it will invest this energy in normal root growth, leading to an increase in root length when AOPP is sprayed on the infected plants compared to water-treated infected plants. Interestingly, it is also shown that AOPP acts as an inhibitor for auxin biosynthesis [150], [151]. As auxin is involved in many growth functions of the plant, this experiment could be redone using another inhibitor to investigate if similar results are obtained. Furthermore, a first indication of enhanced susceptibility towards *M. graminicola* is provided by these results. Although none of the differences were significant, an increase in the formed galls and invaded nematodes was seen. Also, there is a possibility that the inhibition of the PAL enzyme increases the developmental capacity of the nematodes. A last hypothesis is because nematode infection needs auxin, inhibition of auxin biosynthesis leads to less infection while inhibition of the PAL enzyme probably can stimulate infection, leading to small differences between both groups. However, to make these conclusions, significant differences must be found. For this, the inhibition experiments have to be performed again on more plants to decrease the effect of the variability.

B.5 Further research

This project aimed at improving the first steps in a larger analysis. In the future, several experiments will have to be performed to actually infer the importance of the PAL-pathway in rice.

Transformed *Agrobacterium*, obtained in this project will be used to make new CRISPR rice transformation lines for all genes of interest without the bottlenecks handled in this project. For this, at first, an *in vitro* gRNA test will be performed for all genes. This has been done for *AIM1*, for which it is already known which gRNA will be used in the following transformation line. Hereafter, new CRISPR lines for all genes will be generated. The validation of the transformations can also be performed quicker because of the new DNA extraction method and new primers tested in this project. These plants will be grown for seed production to obtain homozygous mutant plants. Furthermore, the *Agrobacterium* containing the *AIM1* gene will be used for a new overexpression transformation series while all other genes will be isolated using the Velocity Taq polymerase mix and used to transform *Agrobacterium*. When the overexpression lines are available, these plants will be compared with wild type plants and the CRISPR mutant for the corresponding gene based on a metabolome analysis (with most attention for levels of SA). Further, all plants will be infected with the nematode *Meloidogyne graminicola* and a leaf pathogen to examine if a change in the expression of that gene will alter the infection capacity of the pathogen in roots or shoots. At last, when combining the data obtained from the metabolome analysis for levels of SA and the susceptibility in roots and shoots, a hypothesis can be made about the importance of the examined gene in the biosynthesis of SA and the role in plant resistance towards root or leaf pathogens.

The qPCR results of the infection experiment with the AOPP inhibitor are not discussed because no conclusion could be made due to large error bars of control- as well as infected samples. In future experiments, the qPCR on different *PAL* genes will be redone to infer the role of these genes in infection. The inhibitor experiment showed large error bars. Therefore, the experiment should be repeated using more rice plants to reduce the effect of the variability between plants. Furthermore, the infection experiments in this project were carried out using *M. graminicola*. Also, the analysis can be performed with a leaf pathogen to have a clear distinction of a pathogen infecting roots or shoots.

At last, this future research will be expanded to include also the importance of the ICS pathway in the biosynthesis of SA in rice. For this, all methodology will be repeated for genes responsible in the ICS pathway. Furthermore, it has been found that nematodes are also able to secrete CM or ICM enzymes. It will be examined in what way these effectors will interfere with the plant ICS pathway and how this will lead to enhanced susceptibility towards the nematode.

References

[1] H. Lefevere, L. Bauters, and G. Gheysen, "Salicylic Acid Biosynthesis in Plants," *Front. Plant Sci.*, vol. 11, Apr. 2020.

[2] L. L. Dilworth, C. K. Riley, and D. K. Stennett, "Plant Constituents: Carbohydrates, Oils, Resins, Balsams, and Plant Hormones," in *Pharmacognosy: Fundamentals, Applications and Strategy*, Elsevier Inc., 2016, pp. 61–80.

[3] "SparkNotes: Plants: Essential Processes: Plant Hormones." [Online]. Available: https://www.sparknotes.com/biology/plants/essentialprocesses/section3/. [Accessed: 28-Oct-2019].

[4] H. Kende and J. A. D. Zeevaart', "The Five 'Classical' Plant Hormones," American Society of Plant Physiologists, 1997.

[5] "The role of new generation phytohormones: An overview | PharmaTutor." [Online]. Available: https://www.pharmatutor.org/articles/role-of-new-generation-phyto-hormones-overview. [Accessed: 09-Nov-2019].

[6] B. C. Freeman and G. A. Beattie, "An Overview of Plant Defenses against Pathogens and Herbivores."

[7] L. Lietaer, "Is there a role for salicylic acid in plant defence against Rhodococcus fascians?," 2017.

[8] J. D. G. Jones and J. L. Dangl, "The plant immune system," *Nature*, vol. 444, no. 7117. pp. 323–329, 16-Nov-2006.

[9] D. E. K. Tarr and H. M. Alexander, "TIR-NBS-LRR genes are rare in monocots: Evidence from diverse monocot orders," *BMC Res. Notes*, vol. 2, 2009.

[10] B. J. DeYoung and R. W. Innes, "Plant NBS-LRR proteins in pathogen sensing and host defense," *Nature Immunology*, vol. 7, no. 12. pp. 1243–1249, Dec-2006.

[11] H. Cui, E. Gobbato, B. Kracher, J. Qiu, J. Bautor, and J. E. Parker, "A core function of EDS1 with PAD4 is to protect the salicylic acid defense sector in Arabidopsis immunity," *New Phytol.*, vol. 213, no. 4, pp. 1802–1817, Mar. 2017.

[12] C. M. J. Pieterse, A. Leon-Reyes, S. Van Der Ent, and S. C. M. Van Wees, "Networking by small-molecule hormones in plant immunity," *Nature Chemical Biology*, vol. 5, no. 5. Nature Publishing Group, pp. 308–316, 2009.

[13] N. Denancé, A. Sánchez-Vallet, D. Goffner, and A. Molina, "Disease resistance or growth: The role of plant hormones in balancing immune responses and fitness costs," *Frontiers in Plant Science*, vol. 4, no. MAY. Frontiers Research Foundation, 24-May-2013.

[14] Y. Kouzai *et al.*, "Salicylic acid-dependent immunity contributes to resistance against Rhizoctonia solani, a necrotrophic fungal agent of sheath blight, in rice and Brachypodium distachyon," *New Phytol.*, vol. 217, no. 2, pp. 771–783, Jan. 2018.

[15] R. Bari and J. D. G. Jones, "Role of plant hormones in plant defence responses," *Plant Molecular Biology*, vol. 69, no. 4. pp. 473–488, Mar-2009.

[16] D. A. Dempsey and D. F. Klessig, "How does the multifaceted plant hormone salicylic acid combat disease in plants and are similar mechanisms utilized in humans?," *BMC Biol.*, vol. 15, no. 1, Mar. 2017.

[17] "Salicylic acid ACS reagent, ≥99.0% | Sigma-Aldrich." [Online]. Available: https://www.sigmaaldrich.com/catalog/product/sigma/s6271?lang=en&region=BE. [Accessed: 28-Oct-2019].

[18] L. Guan and J. G. Scandalios, "Developmentally related responses of maize catalase genes to salicylic acid,"

Proc. Natl. Acad. Sci. U. S. A., vol. 92, no. 13, pp. 5930–5934, Jun. 1995.

[19] L. Rajjou *et al.*, "Proteomic investigation of the effect of salicylic acid on arabidopsis seed germination and establishment of early defense mechanisms," *Plant Physiol.*, vol. 141, no. 3, pp. 910–923, Jul. 2006.

[20] Z. Xie, Z. L. Zhang, S. Hanzlik, E. Cook, and Q. J. Shen, "Salicylic acid inhibits gibberellin-induced alpha-amylase expression and seed germination via a pathway involving an abscisic-acid-inducible WRKY gene," *Plant Mol. Biol.*, vol. 64, no. 3, pp. 293–303, Jun. 2007.

[21] M. V. Rao, G. Paliyath, D. P. Ormrod, D. P. Murr, and C. B. Watkins, "Influence of salicylic acid on H2O2 production, oxidative stress, and H2O2-metabolizing enzymes. Salicylic acid-mediated oxidative damage requires H2O2," *Plant Physiol.*, vol. 115, no. 1, pp. 137–149, 1997.

[22] M. Rivas-San Vicente and J. Plasencia, "Salicylic acid beyond defence: Its role in plant growth and development," *Journal of Experimental Botany*, vol. 62, no. 10. pp. 3321–3338, Jun-2011.

[23] A. N. Uzunova and L. P. Popova, "Effect of salicylic acid on leaf anatomy and chloroplast ultrastructure of barley plants," *Photosynthetica*, vol. 38, no. 2, pp. 243–250, 2000.

[24] M. Melotto, W. Underwood, J. Koczan, K. Nomura, and S. Y. He, "Plant Stomata Function in Innate Immunity against Bacterial Invasion," *Cell*, vol. 126, no. 5, pp. 969–980, Sep. 2006.

[25] T. V. Pancheva and L. P. Popova, "Effect of salicylic acid on the synthesis of ribulose-1,5-bisphosphate carboxylase/oxygenase in barley leaves," *J. Plant Physiol.*, vol. 152, no. 4–5, pp. 381–386, 1998.

[26] H. G. Kang and K. B. Singh, "Characterization of salicylic acid-responsive, Arabidopsis Dof domain proteins: Overexpression of OBP3 leads to growth defects," *Plant J.*, vol. 21, no. 4, pp. 329–339, Feb. 2000.

[27] M. E. Abreu and S. Munne-Bosch, "Salicylic acid deficiency in NahG transgenic lines and sid2 mutants increases seed yield in the annual plant Arabidopsis thaliana," *J. Exp. Bot.*, vol. 60, no. 4, pp. 1261–1271, Mar. 2009.

[28] K. Morris *et al.*, "Salicylic acid has a role in regulating gene expression during leaf senescence," *Plant J.*, vol. 23, no. 5, pp. 677–685, 2000.

[29] F. Ahmad, A. Singh, and A. Kamal, "Salicylic acid-mediated defense mechanisms to abiotic stress tolerance," in *Plant Signaling Molecules: Role and Regulation under Stressful Environments*, Elsevier, 2019, pp. 355–369.

[30] M. V. Rao and K. R. Davis, "Ozone-induced cell death occurs via two distinct mechanisms in *Arabidopsis* : the role of salicylic acid," *Plant J.*, vol. 17, no. 6, pp. 603–614, Mar. 1999.

[31] Y. K. Sharma, J. León, I. Raskin, and K. R. Davis, "Ozone-induced responses in Arabidopsis thaliana: The role of salicylic acid in the accumulation of defense-related transcripts and induced resistance," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 93, no. 10, pp. 5099–5104, May 1996.

[32] C. Nawrath, S. Heck, N. Parinthawong, and J.-P. Métraux, "EDS5, an Essential Component of Salicylic Acid-Dependent Signaling for Disease Resistance in Arabidopsis, Is a Member of the MATE Transporter Family," *Plant Cell*, vol. 14, pp. 275–286, 2002.

[33] S. A.-H.-Mackerness, S. L. Surplus, B. R. Jordan, and B. Thomas, "Effects of Supplementary Ultraviolet-B Radiation on Photosynthetic Transcripts at Different Stages of Leaf Development and Light Levels in Pea (Pisum sativum L.): Role of Active Oxygen Species and Antioxidant Enzymes.," *Photochem. Photobiol.*, vol. 68, no. 1, pp. 88–96, Jul. 1998.

[34] N. H. Ghori *et al.*, "Heavy metal stress and responses in plants," *International Journal of Environmental Science and Technology*, vol. 16, no. 3. Center for Environmental and Energy Research and Studies, pp. 1807–1828, 14-Mar-2019.

[35] J. CHEN, C. ZHU, L. ping LI, Z. yang SUN, and X. bo PAN, "Effects of exogenous salicylic acid on growth and H2O2-metabolizing enzymes in rice seedlings under lead stress," *J. Environ. Sci.*, vol. 19, no. 1, pp. 44–49, Jan. 2007.

[36] A. Krantev, R. Yordanova, T. Janda, G. Szalai, and L. Popova, "Treatment with salicylic acid decreases the effect of cadmium on photosynthesis in maize plants," *J. Plant Physiol.*, vol. 165, no. 9, pp. 920–931, Jun. 2008.

[37] A. Metwally, I. Finkemeier, M. Georgi, and K. J. Dietz, "Salicylic acid alleviates the cadmium toxicity in barley seedlings," *Plant Physiol.*, vol. 132, no. 1, pp. 272–281, May 2003.

[38] F. Zengin, "Exogenous treatment with salicylic acid alleviating copper toxicity in bean seedlings," *Proc. Natl. Acad. Sci. India Sect. B - Biol. Sci.*, vol. 84, no. 3, pp. 749–755, Jan. 2014.

[39] J. L. Freeman, D. Garcia, D. Kim, A. Hopf, and D. E. Salt, "Constitutively elevated salicylic acid signals glutathione-mediated nickel tolerance in thlaspi nickel hyperaccumulators," *Plant Physiol.*, vol. 137, no. 3, pp. 1082–1091, 2005.

[40] Z. M. Yang, J. Wang, S. H. Wang, and L. L. Xu, "Salicylic acid-induced aluminum tolerance by modulation of citrate efflux from roots of Cassia tora L.," *Planta*, vol. 217, no. 1, pp. 168–174, May 2003.

[41] O. Borsani, V. Valpuesta, and M. A. Botella, "Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in Arabidopsis seedlings," *Plant Physiol.*, vol. 126, no. 3, pp. 1024–1030, 2001.

[42] A. Molina *et al.*, "Involvement of endogenous salicylic acid content, lipoxygenase and antioxidant enzyme activities in the response of tomato cell suspension cultures to NaCl," *New Phytol.*, vol. 156, no. 3, pp. 409–415, Dec. 2002.

[43] J. G. Scandalios, "Oxygen Stress and Superoxide Dismutases'."

[44] S. Munné-Bosch and J. Peñuelas, "Photo- and antioxidative protection, and a role for salicylic acid during drought and recovery in field-grown Phillyrea angustifolia plants," *Planta*, vol. 217, no. 5, pp. 758–766, Sep. 2003.

[45] S. Yuan and H.-H. Lin, "Role of Salicylic Acid in Plant Abiotic Stress," 2008.

[46] I. M. Scott, S. M. Clarke, J. E. Wood, and L. A. J. Mur, "Salicylate accumulation inhibits growth at chilling temperature in Arabidopsis," *Plant Physiol.*, vol. 135, no. 2, pp. 1040–1049, Jun. 2004.

[47] Z. Chen, Z. Zheng, J. Huang, Z. Lai, and B. Fan, "Biosynthesis of salicylic acid in plants," *Plant Signal. Behav.*, vol. 4, no. 6, pp. 493–496, Jun. 2009.

[48] Q. Hao *et al.*, "Isochorismate-based salicylic acid biosynthesis confers basal resistance to Fusarium graminearum in barley," *Mol. Plant Pathol.*, vol. 19, no. 8, pp. 1995–2010, Aug. 2018.

[49] D. Ogawa, N. Nakajima, S. Seo, I. Mitsuhara, H. Kamada, and Y. Ohashi, "The phenylalanine pathway is the main route of salicylic acid biosynthesis in Tobacco mosaic virus-infected tobacco leaves," *Plant Biotechnol.*, vol. 23, no. 4, pp. 395–398, 2006.

[50] Q. Hayat, S. Hayat, M. Irfan, and A. Ahmad, "Effect of exogenous salicylic acid under changing environment: A review," *Environmental and Experimental Botany*, vol. 68, no. 1. pp. 14–25, Mar-2010.

[51] C. M. J. Pieterse and L. C. Van Loon, "NPR1: The spider in the web of induced resistance signaling pathways," *Current Opinion in Plant Biology*, vol. 7, no. 4. pp. 456–464, Aug-2004.

[52] "TGA Transcription Factors - Georg-August-Universität Göttingen." [Online]. Available: https://www.uni-goettingen.de/en/tga+transcription+factors+/368268.html. [Accessed: 07-Dec-2019].

[53] Z. Mou, W. Fan, and X. Dong, "Inducers of plant systemic acquired resistance Regulate NPR1 function through redox changes," *Cell*, vol. 113, no. 7, pp. 935–944, Jun. 2003.

[54] C. Seyfferth and K. Tsuda, "Salicylic acid signal transduction: The initiation of biosynthesis, perception and transcriptional reprogramming," *Frontiers in Plant Science*, vol. 5, no. DEC. Frontiers Media S.A., 09-Dec-2014.

[55] D. Yu, C. Chen, and Z. Chen, "Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression," *Plant Cell*, vol. 13, no. 7, pp. 1527–1539, 2001.

[56] M. Shimono *et al.*, "Rice WRKY45 plays a crucial role in benzothiadiazole-inducible blast resistance," *Plant Cell*, vol. 19, no. 6, pp. 2064–2076, Jun. 2007.

[57] Z. Shi, S. Maximova, Y. Liu, J. Verica, and M. J. Guiltinan, "The salicylic acid receptor NPR3 is a negative regulator of the transcriptional defense response during early flower development in Arabidopsis," *Mol. Plant*, vol. 6, no. 3, pp. 802–816, 2013.

[58] M. Moreau, M. Tian, and D. F. Klessig, "Salicylic acid binds NPR3 and NPR4 to regulate NPR1-dependent defense responses," *Cell Res.*, vol. 22, no. 12, pp. 1631–1633, Dec. 2012.

[59] C. Branch, C. F. Hwang, D. A. Navarre, and V. M. Williamson, "Salicylic acid is part of the Mi-1-mediated defense response to root-knot nematode in tomato," *Mol. Plant-Microbe Interact.*, vol. 17, no. 4, pp. 351–356, 2004.

[60] L. A. J. Mur, P. Kenton, A. J. Lloyd, H. Ougham, and E. Prats, "The hypersensitive response; The centenary is upon us but how much do we know?," in *Journal of Experimental Botany*, 2008, vol. 59, no. 3, pp. 501–520.

[61] E. Lam, N. Kato, and M. Lawton, "Programmed cell death, mitochondria and the plant hypersensitive response," *Nature*, vol. 411, no. 6839. pp. 848–853, 14-Jun-2001.

[62] M. C. Heath, "Hypersensitive response-related death," in *Programmed Cell Death in Higher Plants*, Springer Netherlands, 2000, pp. 77–90.

[63] M. Wenig *et al.*, "Systemic acquired resistance networks amplify airborne defense cues," *Nat. Commun.*, vol. 10, no. 1, pp. 1–14, Dec. 2019.

[64] K. E. Hammond-Kosack and J. D. G. Jones', "Resistance Gene-Dependent Plant Defense Responses," American Society of Plant Physiologists, 1996.

[65] D. Tripathi, G. Raikhy, and D. Kumar, "Chemical elicitors of systemic acquired resistance—Salicylic acid and its functional analogs," *Current Plant Biology*, vol. 17. Elsevier B.V., pp. 48–59, 01-Jan-2019.

[66] J. A. Pallas, N. L. Paiva, C. Lamb, and R. A. Dixon, "Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus," *Plant J.*, vol. 10, no. 2, pp. 281–293, Aug. 1996.

[67] K. Lawton, K. Weymann, L. Friedrich, B. Vernooij, S. Uknes, and J. Ryals, "Systemic acquired resistance in Arabidopsis requires salicylic acid but not ethylene," *Mol. Plant-Microbe Interact.*, vol. 8, no. 6, pp. 863–870, 1995.

[68] E. Attaran, T. E. Zeier, T. Griebel, and J. Zeier, "Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in Arabidopsis," *Plant Cell*, vol. 21, no. 3, pp. 954–971, Mar. 2009.

[69] S. W. Park, E. Kaimoyo, D. Kumar, S. Mosher, and D. F. Klessig, "Methyl salicylate is a critical mobile signal for plant systemic acquired resistance," *Science (80-.).*, vol. 318, no. 5847, pp. 113–116, Oct. 2007.

[70] H. Návarová, F. Bernsdorff, A. C. Döring, and J. Zeier, "Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity," *Plant Cell*, vol. 24, no. 12, pp. 5123–5141, Dec. 2013.

[71] M. Hartmann *et al.*, "Flavin Monooxygenase-Generated N-Hydroxypipecolic Acid Is a Critical Element of Plant Systemic Immunity," *Cell*, vol. 173, no. 2, pp. 456-469.e16, Apr. 2018.

[72] Y. C. Chen *et al.*, "N-hydroxy-pipecolic acid is a mobile metabolite that induces systemic disease resistance in Arabidopsis," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 115, no. 21, pp. E4920–E4929, May 2018.

[73] M. Hartmann and J. Zeier, "N-hydroxypipecolic acid and salicylic acid: a metabolic duo for systemic acquired resistance," *Current Opinion in Plant Biology*, vol. 50. Elsevier Ltd, pp. 44–57, 01-Aug-2019.

[74] "Plant Physiology | Digital Textbook Library." [Online]. Available: https://www.tankonyvtar.hu/en/tartalom/tamop425/0010_1A_Book_angol_01_novenyelettan/ch03s05.html. [Accessed: 08-Dec-2019].

[75] A. Slaughter, X. Daniel, V. Flors, E. Luna, B. Hohn, and B. Mauch-Mani, "Descendants of primed Arabidopsis plants exhibit resistance to biotic stress," *Plant Physiol.*, vol. 158, no. 2, pp. 835–843, Feb. 2012.

[76] "Salicylic acid topical Uses, Side Effects & Warnings - Drugs.com." [Online]. Available: https://www.drugs.com/mtm/salicylic-acid-topical.html. [Accessed: 19-Dec-2019].

[77] "Aspirin: Health benefits, uses, and risks." [Online]. Available: https://www.medicalnewstoday.com/articles/161255.php. [Accessed: 19-Dec-2019].

[78] Y. M. Koo, A. Y. Heo, and H. W. Choi, "Salicylic acid as a safe plant protector and growth regulator," *Plant Pathology Journal*, vol. 36, no. 1. Korean Society of Plant Pathology, pp. 1–10, 2020.

[79] P. Aranega-Bou, M. de la O Leyva, I. Finiti, P. Garcfa-Agustfn, and C. Gonzalez-Bosch, "Priming of plant resistance by natural compounds. Hexanoic acid as a model," *Frontiers in Plant Science*, vol. 5, no. OCT. Frontiers Media S.A., p. 488, 01-Oct-2014.

[80] W. K. Huang, H. L. Ji, G. Gheysen, and T. Kyndt, "Thiamine-induced priming against root-knot nematode infection in rice involves lignification and hydrogen peroxide generation," *Mol. Plant Pathol.*, vol. 17, no. 4, pp. 614–624, May 2016.

[81] D. B. Collinge, H. J. L. Jørgensen, O. S. Lund, and M. F. Lyngkjær, "Engineering Pathogen Resistance in Crop Plants: Current Trends and Future Prospects," *Annu. Rev. Phytopathol.*, vol. 48, no. 1, pp. 269–291, Jul. 2010.

[82] D. B. Collinge, O. S. Lund, and H. Thordal-Christensen, "What are the prospects for genetically engineered, disease resistant plants?," in *Sustainable Disease Management in a European Context*, Springer Netherlands, 2008, pp. 217–231.

[83] M. H. Stuiver and J. H. H. V. Custers, "Engineering disease resistance in plants," *Nature*, vol. 411, no. 6839. pp. 865–868, 14-Jun-2001.

[84] S. J. Gurr and P. J. Rushton, "Engineering plants with increased disease resistance: How are we going to express it?," *Trends in Biotechnology*, vol. 23, no. 6. pp. 283–290, Jun-2005.

[85] H. Cao, X. Li, and X. Dong, "Generation of broad-spectrum disease resistance by everexpression of an essential regulatory gene in systemic acquired resistance," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 95, no. 11, pp. 6531–6536, May 1998.

[86] V. Tzin and G. Galili, " The Biosynthetic Pathways for Shikimate and Aromatic Amino Acids in Arabidopsis thaliana ," *Arab. B.*, vol. 8, p. e0132, Jan. 2010.

[87] M. B. Shine *et al.*, "Cooperative functioning between phenylalanine ammonia lyase and isochorismate synthase activities contributes to salicylic acid biosynthesis in soybean," *New Phytol.*, vol. 212, no. 3, pp. 627–636, Nov. 2016.

[88] C. Garcion *et al.*, "Characterization and biological function of the Isochorismate Synthase2 gene of Arabidopsis," *Plant Physiol.*, vol. 147, no. 3, pp. 1279–1287, Jul. 2008.

[89] P. Silverman *et al.*, "Salicylic Acid B iosynt hesis, Conjugat ion, in Rice' and Possible Role," 1995.

[90] D. A. Dempsey, A. C. Vlot, M. C. Wildermuth, and D. F. Klessig, "Salicylic Acid Biosynthesis and Metabolism," *Arab. B.*, vol. 9, p. e0156, Jan. 2011.

[91] L. Duan, H. Liu, X. Li, J. Xiao, and S. Wang, "Multiple phytohormones and phytoalexins are involved in disease resistance to Magnaporthe oryzae invaded from roots in rice," *Physiol. Plant.*, vol. 152, no. 3, pp. 486–500, Nov. 2014.

[92] J. Mercado-Blanco, K. M. G. M. Van Der Drift, P. E. Olsson, J. E. Thomas-Oates, L. C. Van Loon, and P. A. H. M. Bakker, "Analysis of the pmsCEAB gene cluster involved in biosynthesis of salicylic acid and the siderophore pseudomonine in the biocontrol strain Pseudomonas fluorescens WCS374," *J. Bacteriol.*, vol. 183, no. 6, pp. 1909–1920, Mar. 2001.

[93] S. Yokoo *et al.*, "Comparative analysis of plant isochorismate synthases reveals structural mechanisms

underlying their distinct biochemical properties," *Biosci. Rep.*, vol. 38, no. 2, Mar. 2018.

[94] D. Rekhter *et al.*, "Isochorismate-derived biosynthesis of the plant stress hormone salicylic acid," *Science (80-.).*, vol. 365, no. 6452, pp. 498–502, 2019.

[95] M. P. Torrens-Spence *et al.*, "PBS3 and EPS1 Complete Salicylic Acid Biosynthesis from Isochorismate in Arabidopsis," *Mol. Plant*, vol. 12, no. 12, pp. 1577–1586, Dec. 2019.

[96] Z. Chen, Z. Zheng, J. Huang, Z. Lai, and B. Fan, "Biosynthesis of salicylic acid in plants," *Plant Signaling and Behavior*, vol. 4, no. 6. Taylor & Francis, pp. 493–496, Jun-2009.

[97] C. Nawrath and J. P. Métraux, "Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation," *Plant Cell*, vol. 11, no. 8, pp. 1393–1404, Aug. 1999.

[98] C. Choi *et al.*, "Molecular characterization of Oryza sativa WRKY6, which binds to W-box-like element 1 of the Oryza sativa pathogenesis-related (PR) 10a promoter and confers reduced susceptibility to pathogens," *New Phytol.*, vol. 208, no. 3, pp. 846–859, Nov. 2015.

[99] H. Yoo *et al.*, "An alternative pathway contributes to phenylalanine biosynthesis in plants via a cytosolic tyrosine:phenylpyruvate aminotransferase," *Nat. Commun.*, vol. 4, no. 1, pp. 1–11, Nov. 2013.

[100] A. I. Reichert, X. Z. He, and R. A. Dixon, "Phenylalanine ammonia-lyase (PAL) from tobacco (Nicotiana tabacum): Characterization of the four tobacco PAL genes and active heterotetrameric enzymes," *Biochem. J.*, vol. 424, no. 2, pp. 233–242, Dec. 2009.

[101] J. W. Blount, K. L. Korth, S. A. Masoud, S. Rasmussen, C. Lamb, and R. A. Dixon, "Altering expression of cinnamic acid 4-hydroxylase in transgenic plants provides evidence for a feedback loop at the entry point into the phenylpropanoid pathway," *Plant Physiol.*, vol. 122, no. 1, pp. 107–116, 2000.

[102] J. Rösler, F. Krekel, N. Amrhein, and J. Schmid, "Maize phenylalanine ammonia-lyase has tyrosine ammonia-lyase activity," *Plant Physiol.*, vol. 113, no. 1, pp. 175–179, Jan. 1997.

[103] C. L. Cass *et al.*, "Effects of PHENYLALANINE AMMONIA LYASE (PAL) knockdown on cell wall composition, biomass digestibility, and biotic and abiotic stress responses in Brachypodium," *J. Exp. Bot.*, vol. 66, no. 14, pp. 4317–4335, 2015.

[104] J. Huang *et al.*, "Functional analysis of the Arabidopsis PAL gene family in plant growth, development, and response to environmental stress," *Plant Physiol.*, vol. 153, no. 4, pp. 1526–1538, Aug. 2010.

[105] T. A. Richmond and A. B. Bleecker, "A defect in β-oxidation causes abnormal inflorescence development in arabidopsis," *Plant Cell*, vol. 11, no. 10, pp. 1911–1923, Oct. 1999.

[106] E. L. Rylott *et al.*, "The Arabidopsis thaliana multifunctional protein gene (MFP2) of peroxisomal β-oxidation is essential for seedling establishment," *Plant J.*, vol. 45, no. 6, pp. 930–941, Mar. 2006.

[107] S. Arent, C. E. Christensen, V. E. Pye, A. Nørgaard, and A. Henriksen, "The multifunctional protein in peroxisomal β-oxidation: Structure and substrate specificity of the Arabidopsis thaliana protein MFP2," *J. Biol. Chem.*, vol. 285, no. 31, pp. 24066–24077, Jul. 2010.

[108] "AIM1 - Gene - NCBI." [Online]. Available: https://www.ncbi.nlm.nih.gov/gene/?term=AIM1. [Accessed: 02-Apr-2020].

[109] J. D. Bussell, M. Reichelt, A. A. G. Wiszniewski, J. Gershenzon, and S. M. Smith, "Peroxisomal ATP-binding cassette transporter COMATOSE and the multifunctional protein ABNORMAL INFLORESCENCE MERISTEM are required for the production of benzoylated metabolites in arabidopsis seeds," *Plant Physiol.*, vol. 164, no. 1, pp. 48–54, Jan. 2014.

[110] K. Kirschner and H. Bisswanger, "Multifunctional proteins," 1976.

[111] J. León, V. Shulaev, N. Yalpani, M. A. Lawton, and I. Raskin, "Benzoic acid 2-hydroxylase, a soluble oxygenase from tobacco, catalyzes salicylic acid biosynthesis," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, no. 22, pp. 10413–10417, Oct. 1995.

[112] X. Z. Yu, W. J. Fan, Y. J. Lin, F. F. Zhang, and D. K. Gupta, "Differential expression of the PAL gene family in rice seedlings exposed to chromium by microarray analysis," *Ecotoxicology*, vol. 27, no. 3, pp. 325–335, Apr. 2018.

[113] J. Yan *et al.*, "The tyrosine aminomutase TAM1 is required for β-tyrosine biosynthesis in rice," *Plant Cell*, vol. 27, no. 4, pp. 1265–1278, Apr. 2015.

[114] B. W. Tonnessen *et al.*, "Rice phenylalanine ammonia-lyase gene OsPAL4 is associated with broad spectrum disease resistance," *Plant Mol. Biol.*, vol. 87, no. 3, pp. 273–286, Dec. 2014.

[115] L. Xu *et al.*, "ABNORMAL INFLORESCENCE MERISTEM1 functions in salicylic acid biosynthesis to maintain proper reactive oxygen species levels for root meristem activity in rice," *Plant Cell*, vol. 29, no. 3, pp. 560–574, Mar. 2017.

[116] E. L. Rylott *et al.*, "The Arabidopsis thaliana multifunctional protein gene (MFP2) of peroxisomal β-oxidation is essential for seedling establishment," *Plant J.*, vol. 45, no. 6, pp. 930–941, Mar. 2006.

[117] S. Arent, C. E. Christensen, V. E. Pye, A. Nørgaard, and A. Henriksen, "The multifunctional protein in peroxisomal β-oxidation: Structure and substrate specificity of the Arabidopsis thaliana protein MFP2," *J. Biol. Chem.*, vol. 285, no. 31, pp. 24066–24077, Jul. 2010.

[118] J. V. Dean and J. D. Mills, "Uptake of salicylic acid 2-O-β-D-glucose into soybean tonoplast vesicles by an ATP-binding cassette transporter-type mechanism," *Physiol. Plant.*, vol. 120, no. 4, pp. 603–612, Apr. 2004.

[119] J. V. Dean, L. A. Mohammed, and T. Fitzpatrick, "The formation, vacuolar localization, and tonoplast transport of salicylic acid glucose conjugates in tobacco cell suspension cultures," *Planta*, vol. 221, no. 2, pp. 287–296, May 2005.

[120] A. M. G. Thompson, C. V. Iancu, K. E. Neet, J. V. Dean, and J. Y. Choe, "Differences in salicylic acid glucose conjugations by UGT74F1 and UGT74F2 from Arabidopsis thaliana," *Sci. Rep.*, vol. 7, 2017.

[121] V. Shulaev, P. Silverman, and I. Raskin, "Airborne signalling by methyl salicylate in plant pathogen resistance," *Nature*, vol. 385, no. 6618, pp. 718–721, Feb. 1997.

[122] S. W. Park *et al.*, "Use of a synthetic salicylic acid analog to investigate the roles of methyl salicylate and its esterases in plant disease resistance," *J. Biol. Chem.*, vol. 284, no. 11, pp. 7307–7317, Mar. 2009.

[123] P. M. Manosalva, S. W. Park, F. Forouhar, L. Tong, W. E. Fry, and D. F. Klessig, "Methyl esterase 1 (StMES1) is required for systemic acquired resistance in potato," *Mol. Plant-Microbe Interact.*, vol. 23, no. 9, pp. 1151–1163, Sep. 2010.

[124] A. C. Vlot *et al.*, "Identification of likely orthologs of tobacco salicylic acid-binding protein 2 and their role in systemic acquired resistance in Arabidopsis thaliana," *Plant J.*, vol. 56, no. 3, pp. 445–456, Nov. 2008.

[125] Z. Zhang *et al.*, "Dual regulation role of GH3.5 in salicylic acid and auxin signaling during arabidopsis-Pseudomonas syringae interaction," *Plant Physiol.*, vol. 145, no. 2, pp. 450–464, Oct. 2007.

[126] A. W. Woodward and B. Bartel, "Auxin: regulation, action, and interaction.," *Ann. Bot.*, vol. 95, no. 5, pp. 707–35, Apr. 2005.

[127] C. Y. Chang, Y. H. Hsieh, K. Y. Cheng, L. L. Hsieh, T. C. Cheng, and K. S. Yao, "Effect of pH on Fenton process using estimation of hydroxyl radical with salicylic acid as trapping reagent," *Water Sci. Technol.*, vol. 58, no. 4, pp. 873–879, Sep. 2008.

[128] Z. Maskos, J. D. Rush, and W. H. Koppenol, "The hydroxylation of the salicylate anion by a fenton reaction and Γ-radiolysis: A consideration of the respective mechanisms," *Free Radic. Biol. Med.*, vol. 8, no. 2, pp. 153–162, Jan. 1990.

[129] M. Bartsch *et al.*, "Accumulation of isochorismate-derived 2,3-dihydroxybenzoic 3-O-β-D-xyloside in Arabidopsis resistance to pathogens and ageing of leaves," *J. Biol. Chem.*, vol. 285, no. 33, pp. 25654–25665, Aug. 2010.

[130] M. Koornneef and D. Meinke, "The development of Arabidopsis as a model plant."

[131] W. A. Rensink and C. R. Buell, "Arabidopsis to rice. Applying knowledge from a weed to enhance our understanding of a crop species," *Plant Physiology*, vol. 135, no. 2. pp. 622–629, Jun-2004.

[132] D. De Vleesschauwer, J. Xu, and M. Höfte, "Making sense of hormone-mediated defense networking: From rice to Arabidopsis," *Frontiers in Plant Science*, vol. 5, no. November. Frontiers Media S.A., pp. 1–15, 11-Nov-2014.

[133] "CRISPR/Cas." [Online]. Available: http://www.vib.be/nl/biotech-basics/Pages/CRISPRCas.aspx. [Accessed: 23-Dec-2019].

[134] J. E. Garneau *et al.*, "The CRISPR/cas bacterial immune system cleaves bacteriophage and plasmid DNA," *Nature*, vol. 468, no. 7320, pp. 67–71, Nov. 2010.

[135] G. Gasiunas, R. Barrangou, P. Horvath, and V. Siksnys, "Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, no. 39, Sep. 2012.

[136] F. A. Ran, P. D. Hsu, J. Wright, V. Agarwala, D. A. Scott, and F. Zhang, "Genome engineering using the CRISPR-Cas9 system," *Nat. Protoc.*, vol. 8, no. 11, pp. 2281–2308, 2013.

[137] J. A. Doudna and E. Charpentier, "The new frontier of genome engineering with CRISPR-Cas9," *Science (80-.).*, vol. 346, no. 6213, p. 1258096, Nov. 2014.

[138] J. Miao *et al.*, "Targeted mutagenesis in rice using CRISPR-Cas system," *Cell Research*, vol. 23, no. 10. pp. 1233–1236, Oct-2013.

[139] R. Xu *et al.*, "Gene targeting using the Agrobacterium tumefaciens-mediated CRISPR-Cas system in rice," *Rice*, vol. 7, no. 1, p. 5, Dec. 2014.

[140] T. Kyndt *et al.*, "Transcriptional reprogramming by root knot and migratory nematode infection in rice," *New Phytol.*, vol. 196, no. 3, pp. 887–900, Nov. 2012.

[141] J. Hofmann, A. E. N. El Ashry, S. Anwar, A. Erban, J. Kopka, and F. Grundler, "Metabolic profiling reveals local and systemic responses of host plants to nematode parasitism," *Plant J.*, p. no-no, Mar. 2010.

[142] A. Haegeman, S. Mantelin, J. T. Jones, and G. Gheysen, "Functional roles of effectors of plant-parasitic nematodes," *Gene*, vol. 492, no. 1. pp. 19–31, 15-Jan-2012.

[143] "CRISPR-P v2.0." [Online]. Available: http://crispr.hzau.edu.cn/CRISPR2/. [Accessed: 06-Mar-2020].

[144] "Reverse Complement." [Online]. Available: https://www.bioinformatics.org/sms/rev_comp.html. [Accessed: 14-Mar-2020].

[145] "Phytozome v12.1: Home." [Online]. Available: https://phytozome.jgi.doe.gov/pz/portal.html. [Accessed: 20-Mar-2020].

[146] "Primer3 Input." [Online]. Available: http://primer3.ut.ee/. [Accessed: 20-Mar-2020].

[147] "crown gall | Description, Symptoms, & Control | Britannica." [Online]. Available: https://www.britannica.com/science/crown-gall. [Accessed: 06-Mar-2020].

[148] H.-H. Hwang, M. Yu, and E.-M. Lai, "Agrobacterium-Mediated Plant Transformation: Biology and Applications," *Arab. B.*, vol. 15, no. 15, p. e0186, Jan. 2017.

[149] "TIDE | Web tool." [Online]. Available: https://tide.deskgen.com/. [Accessed: 14-Apr-2020].

[150] T. Higashide, M. Narukawa, Y. Shimada, and K. Soeno, "Suppression of elongation and growth of tomato seedlings by auxin biosynthesis inhibitors and modeling of the growth and environmental response," *Sci. Rep.*, vol. 4, no. 1, pp. 1–7, Apr. 2014.

[151] K. Soeno *et al.*, "Auxin Biosynthesis Inhibitors, Identified by a Genomics-Based Approach, Provide Insights into Auxin Biosynthesis," *Plant Cell Physiol.*, vol. 51, no. 4, pp. 524–536, Apr. 2010.

Appendix

Table A.1: Nomenclature of the used rice genes in this masters dissertation combined with their locus number.

Name	Locus
OsPAL1	*LOC_Os02g41630*
OsPAL2	*LOC_Os02g41650*
OsPAL3	*LOC_Os02g41670*
OsPAL4	*LOC_Os02g41680*
OsPAL5	*LOC_Os04g43760*
OsPAL6	*LOC_Os04g43800*
OsPAL7	*LOC_Os05g35290*
OsPAL8	*LOC_Os11g48110*
OsPAL9	*LOC_Os12g33610*
OsAIM1	*LOC_Os02g17390*

Table A.2: Sequences and primers used in this project

Name	Primer
gRNA oligonucleotides	
PAL1_gRNA_F	GGCAGGACCCGCTCAACTGGGGCG
PAL1_gRNA_R	AAACCGCCCCAGTTGAGCGGGTCC
PAL2_gRNA_F	GGCAGCGCCCTCGATCTTCACCAG
PAL2_gRNA_R	AAACCTGGTGAAGATCGAGGGCGC
PAL3_gRNA_F	GGCAGCGCCCTCGATCTTTACCAG
PAL3_gRNA_R	AAACCTGGTAAAGATCGAGGGCGC
PAL4_gRNA_F	GGCATCATGCCATTGGCAGAAACG
PAL4_gRNA_R	AAACCGTTTCTGCCAATGGCATGA
PAL5_gRNA_F	GGCAGGACCCCTCGATCTTCACCA
PAL5_gRNA_R	AAACTGGTGAAGATCGAGGGGTCC
PAL6_gRNA_F	GGCACATGGCTGCCCGCGTCGCCG
PAL6_gRNA_R	AAACCGGCGACGCGGGCAGCCATG
PAL7_gRNA_F	GGCAGGCGCCCTCGATCCGCACCA
PAL7_gRNA_R	AAACTGGTGCGGATCGAGGGCGCC
PAL8_gRNA_F	GGCACCATCCTCTTCACCTCCTCG
PAL8_gRNA_R	AAACCGAGGAGGTGAAGAGGATGG

PAL9_gRNA_F	GGCACGGCCGCCAGAACTCCGCGG
PAL9_gRNA_R	AAACCCGCGGAGTTCTGGCGGCCG
AIM1_gRNA_F	GGCAATTGAAGGAGAAGTACGCGG
AIM1_gRNA_R	AAACCCGCGTACTTCTCCTTCAAT
Plant genomic insertion or deletion test	
PAL1_Genomic_F	CTACCTTCTCCTCCGGCTCT
PAL1_Genomic_R	TTACCTGAGGAGCTCGACTTG
PAL2_Genomic_F	GCTTCCAACTCGAGCTCTTC
PAL2_Genomic_R	GAGCTTGCGATCGATTTGTC
PAL3_Genomic_F	GAAGAGCCACACATCCCAGT
PAL3_Genomic_R	GCAATCTTAAACGCCTCAGC
PAL4_Genomic_F	TCTCGACGCTTTCTGTGCTA
PAL4_Genomic_R	TGCCGAGAAAACAAGCAAAT
PAL5_Genomic_F	CCAAGAAGAGCACACAGCAG
PAL5_Genomic_R	CAAGTGGCACGCAGTTCTAA
PAL6_Genomic_F	CACAACTCCTCCACCTCCAT
PAL6_Genomic_R	TCCACACAAGCTCTCACCAG
PAL6_Genomic_F2	CAACCACCACTCGTTACAGC
PAL6_Genomic_R2	GTCACCGCCACAGAGTTTG
PAL6_Genomic_F3	CATGAACAGCATGATGAACG
PAL6_Genomic_R3	AAGAAGCCGTGCTGGATG
PAL6_Genomic_F4	ACACATCGATCTCCCCTCTG
PAL6_Genomic_R4	TCTTCGCCAGCATCATGTAG
PAL7_Genomic_F	TCCATTGATCACTCGCTCTG
PAL7_Genomic_R	GGTGAGGTGGTCGGTGTACT
PAL8_Genomic_F	ACCTCGAGGAGGTGAAGAGG
PAL8_Genomic_R	CCCACCCAATGAACGAATTA
PAL9_Genomic_F	TGTTAGCTGGACGACCTGTG
PAL9_Genomic_R	CCGGCGTTGAGGTATCTTTA
AIM1_Genomic_F	CCCGGATAAATACCCCAACT
AIM1_Genomic_R	GAGTTCCAGACCACCAGGAA
Plant transformation test	
VIB_F	CAAGCCTGAGAACATCGTCA
VIB_R	GGCGATCATCTTCCTCACAT
In vitro test	
AIM1.3_gRNA_F	GGCAGGGATTGAAGGAGAAGTACG
AIM1.3_gRNA_R	AAACCGTACTTCTCCTTCAATCCC
AIM1.4_gRNA_F	GGCAGCTGGAGGCAAATTTTGTGG
AIM1.4_gRNA_R	AAACCCACAAAATTTGCCTCCAGC
AIM1.5_gRNA_F	GGCAGGATGCAGCGCATTGACGGG
AIM1.5_gRNA_R	AAACCCCGTCAATGCGCTGCATCC
Amplification in vitro test	
AIM1.1_Invitro_F	GCGGCCTCTAATACGACTCACTATAGGGATTTACTTCCT TAAGCACAAGTTTTAGAGCTAGAAATAGCAAG
AIM1.2_Invitro_F	GCGGCCTCTAATACGACTCACTATAGGGATTGAAGGAG AAGTACGCGGGTTTTAGAGCTAGAAATAGCAAG

AIM1.3_Invitro_F	GCGGCCTCTAATACGACTCACTATAGGGGGATTGAAG GAGAAGTACGGTTTTAGAGCTAGAAATAGCAAG
AIM1.4_Invitro_F	GCGGCCTCTAATACGACTCACTATAGGGGCTGGAGGCA AATTTTGTGGGTTTTAGAGCTAGAAATAGCAAG
AIM1.5_Invitro_F	GCGGCCTCTAATACGACTCACTATAGGGGGATGCAGCG CATTGACGGGGTTTTAGAGCTAGAAATAGCAAG
AIM1_Invitro_R	TGTGGTCTCAAGCGTAATGCCAACTTTGTAC
Gene isolation overexpression lines	
PAL6_OX_F	CACAACTCCTCCACCTCCAT
PAL6_OX_R	TCTTCTCGTCGGTCCTCTGT
AIM1_OX_F	GCCTCCCGGATAAATACCCC
AIM1_OX_R	CTGCCTTCCCCCTTACGTTT
AIM1_OX_F2	TCGAACGAACGAAGAACCCT
AIM1_OX_R2	GTTCGGAAAACCGGAAAGGC
Gene isolation with attb sites	
PAL4_OX_attb_F	GGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAGTG CGAGAACGGCCG
PAL4_OX_attb_R	GGGACCACTTTGTACAAGAAAGCTGGGTGTCAGCAGAT TGGCAGGGGCT
PAL6_OX_attb_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCCACA ACTCCTCCACCTCCAT
PAL6_OX_attb_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCTTCTCGT CGGTCCTCTGT
AIM1_OX_attb_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCGG GGGCGATCCGCGT
AIM1_OX_attb_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACATGC GTGACCTCGACG
Sequence test overexpression lines	
PAL6_Seqcheck_1	AACACTCTGCTCCAGGGGTA
PAL6_Seqcheck_2	GCGATCGGTAAGCTCATGTT
AIM1_Seqcheck_1	GGAAGGGAAAGAAGGTGGTC
AIM1_Seqcheck_2	ACTTCTCAAGGGTGCATTGG
Check transformation *E. coli* and *Agrobacterium*	
VIB-Cas9_F	CACTGGGTACGTTGGAAACC
VIB-Cas9_R	GCTGCGTAAGCCTCTCTAACC
Test cDNA	
EIF5C_F	AGGAACATGGAGAAGAACAAGG
EIF5C_R	CAGAGGTGGTGCAGATGAAA
Exp_Nar_F	CACGTTACGGTGACACCTTTT
Exp_Nar_R	GACGCTCTCCTTCTTCCTCAG

TABLE A.3: Weights of the examined roots in the inhibition experiments

	Weight (g)	
	Water treatment	**AOPP treatment**
1	0.087	0.108
2	0.048	0.067
3	0.065	0.056
4	0.057	0.058
5	0.054	0.164
6	0.062	0.157

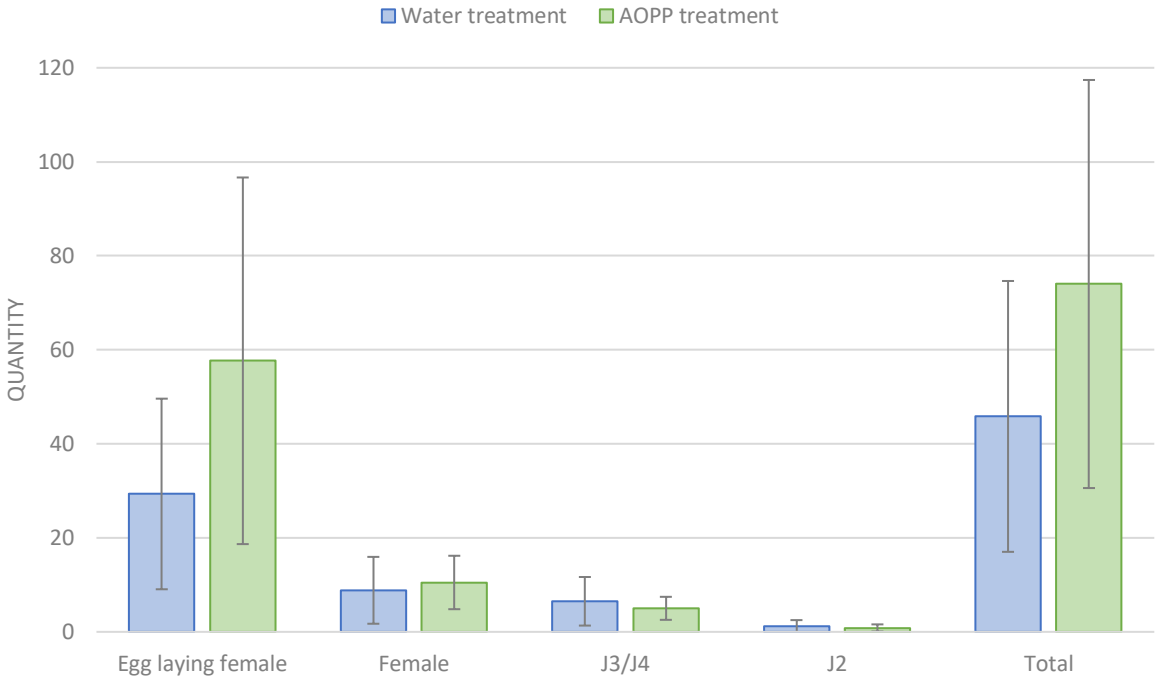

FIGURE A.1: Results of the PAL enzyme inhibition experiment. Blue bars indicate the control groups (water treatment) while green bars indicate AOPP-treated groups. Error bars indicate the standard deviation of the presented group. T-tests were performed on all groups. The number of nematodes is presented per life cycle stage in the root systems. No significant conclusions could be made due to high variability.

Shoot

```
            Df Sum Sq Mean Sq F value Pr(>F)
Treatment    1  42.69   42.69   3.066 0.0952 .
Infection    1  35.89   35.89   2.578 0.1240
Residuals   20 278.42   13.92
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Root

```
            Df Sum Sq Mean Sq F value  Pr(>F)
Treatment    1  68.94   68.94   6.783 0.01696 *
Infection    1 131.13  131.13  12.902 0.00182 **
Residuals   20 203.26   10.16
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

FIGURE A.2: p-values after two-way anova analysis for the root and shoot lengths in the inhibition experiment