Universitat de Girona
Escola Politècnica Superior

Incoming Erasmus Student
Final Degree Project

Degree course: Eng. Tècn. Agrícola Indústries Agroalim. Pla 1999

Title: Application of real-time PCR to assess transgene copy numbers in GM plants and to investigate plant gene expression

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Application of real-time PCR to assess transgene copy numbers in GM plants and to investigate plant gene expression

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EPS Advisor: Maria Pla
Odisee Mentor: Patrcick Demeyere

Universitat de Girona
Eng. Química, Agrària i Tecn. Agroalimentària
Àrea de Tecnolgia dels Aliments

June 2015

Information work placement

Period: 9th of February 2015 till 12th of June 2015

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Escola Politecnica Superior (P1)
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ABSTRACT

Quantitative real-time polymerase chain reaction (qPCR) is a powerful method to compare specific DNA levels across different sample populations. In combination with reverse transcription (RT) it is the most common method for either characterising or confirming gene expression patterns. This is due to the high sensitivity, specificity, accuracy and reproducibility of the method. Quantitative DNA and RNA analysis needs an appropriate control gene for accurate normalisation of data. For gene expression analysis, an ideal endogenous control gene, also called a reference or housekeeping gene, is one that is stably expressed within the samples to be compared, regardless of tissue differences, experimental conditions or treatments.

Different genes had been qPCR analysed in this study with different objectives. On the one hand, the objective was to determine the transgene copy number in genetically modified rice where only one copy of the inserted transgene was desirable; and on the other hand, RT-qPCR had been applied for studies of gene expression in *Prunus perisca* subjected to different treatments and conditions to induce genes related to plant defence mechanisms.

ACKNOWLEDGEMENTS

I wish to record my appreciation and thanks to my mentors and colleagues of the Department of food technology for the intellectual interaction, friendly kind atmosphere and assistance in various ways. It was a pleasure working with you all.

I thank Dr. Maria Pla, my supervisor and Anna Nadal for the critical reading and corrections on my final degree project and to help me for the preparation of it.

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Furthermore my gratitude to Patrick Demeyere who took care of the opportunity to go on Erasmus to Girona. I couldn’t imagine a better place and subject for my final degree project.

And on top of all I would like to thank my mother. Without her I wouldn't even be here. She is a great woman, who I adore. She supports me through good and bad and only wants the best for me.

My scientific knowledge has grown a lot. And although I made mistakes, or were my result not as expected, they said it was no big deal and that you never obtain the result you expect or want. It is important to know what you did wrong and otherwise you just try again.

A person who never made a mistake, never tried anything new (-A. Einstein)

TABLE OF CONTENT

LIST OF ABBREVIATIONS

AGE	agarose gel electrophoresis
cDNA	complementary DNA
C_t	threshold cycle
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
dNTP	deoxynucleotide triphosphate
dR	relative fluorescence
dsDNA	double stranded DNA
DsRed	discosoma red fluorescent protein
dUTP	deoxyuridine triphosphate
G/C	guanine/cytosine content
GM	genetically modified
GMP	genetically modified plant
$Hygro^R$	hygrobromicine
IRTA	Institut de Recerca i Technologia Agroalimentàries
MeSa	methyl salycilate
MeJA	methyl jasmonate
mRNA	messenger ribonucleic acid
NTC	non template control
PCR	polymerase chain reaction
qPCR	real-time polymerase chain reaction
ssDNA	single-stranded deoxyribonucleic acid
RNA	ribonucleic acid
RT	reverse transcriptase
RT-qPCR	reverse transcription coupled to real-time polymerase chain reaction
SD	standard deviation
T_0	primary transformants plantlets
T_m	melting temperature
UV	ultra violet

I. ABOUT THE PLACEMENT

This project is performed at the University of Girona (UdG) at the department of the research group Tecnologia Alimentària. The Food Technology group is a member of the Institute of Food and Agricultural Technology (INTEA) and also of the Department of Chemical and Agricultural Engineering and Agrifood Technology (EQATA) both situated at the Escola Politècnica Superior (P1) at campus Montilivi.

The INTEA aims to promote research and teaching activities related to food and agricultural knowledge, specially related to the needs of the area of Girona and Catalunya. It has two main objectives. First of all, promoting research, development and transfer of technology related to primary material production and food transformation to companies in the agronomical and food sector. Secondly, promoting continuous specialised training in all aspects related to technology and alimentary processes (biocat, 2015)

The department of EQATA is the unit of the University of Girona that has the objective of teach and research in chemical, environmental, agricultural and food engineering and technology (UdG, 2015)

II. INTRODUCTION

PCR or Polymerase Chain Reaction is a method for amplifying deoxyribonucleic acid (DNA). With this technique, small amounts of a specific DNA sequence can be replicated very rapidly and cyclically; and thereby amplified to such an extent that the DNA becomes easy to detect, to study and use for any given purpose.

The cost of instruments and reagents is well within reach of individual laboratories, assays are easy to perform, capable of high throughput and combine high sensitivity with reliable specificity.

Unlike conventional PCR, which is a qualitative end-point assay, real-time PCR (qPCR) allows accurate quantification of amplified DNA in real time during the exponential phase of the reaction. It is possible to achieve accurate and biologically meaningful quantification if meticulous attention is paid to the details of every step of the qPCR assay, starting with sample selection, acquisition and handling through assay design, validation and optimisation.

Current real-time PCR technology has applications in a huge range of scientific areas including molecular biology, microbiology, genetics, diagnostics, clinical laboratories, forensic science, environmental science, food science, hereditary studies, paternity testing, and many other areas of the life sciences. This study presents the use of qPCR to assess (i) transgene copy numbers in genetically modified plants previously obtained in the laboratory; and (ii) expression of a number of stress-responsive genes in *Prunus persica* , in response to various abiotic factors.

II.1 GENERAL PRINCIPLE OF POLYMERASE CHAIN REACTION

PCR is a revolutionary method developed by Kary Mullis in the 1980s. PCR is a cyclic, exponential *in vitro* DNA amplification process where the phenomenon of *in vivo* DNA replication is imitated. In performing a PCR reaction, several critical constituents are needed. Firstly, the sample that contains the DNA to amplify (complementary DNA, cDNA, if RNA has to be analysed) is required. Secondly, two different and specific oligonucleotides (primers) that will bind to opposite strands flanking the amplification target on the template DNA, must be available. Then deoxynuleoside triphosphates (dNTPs) and the enzyme DNA polymerase are also essential components. PCR is based on using the ability of DNA polymerase to elongate a new strand of DNA complementary to the offered template strand and this is repeated up to 40-50 times resulting in exponential amplification of the target (**Figure II.1**). This way, PCR amplifies a specific DNA sequence flanked by two primer sequences. When the target nucleic acid is RNA, reverse transcriptase is included in the PCR reaction to convert RNA to cDNA. The whole procedure is known as reverse transcription (RT) coupled to PCR (RT-PCR) (Halliday *et al.*, 2010).

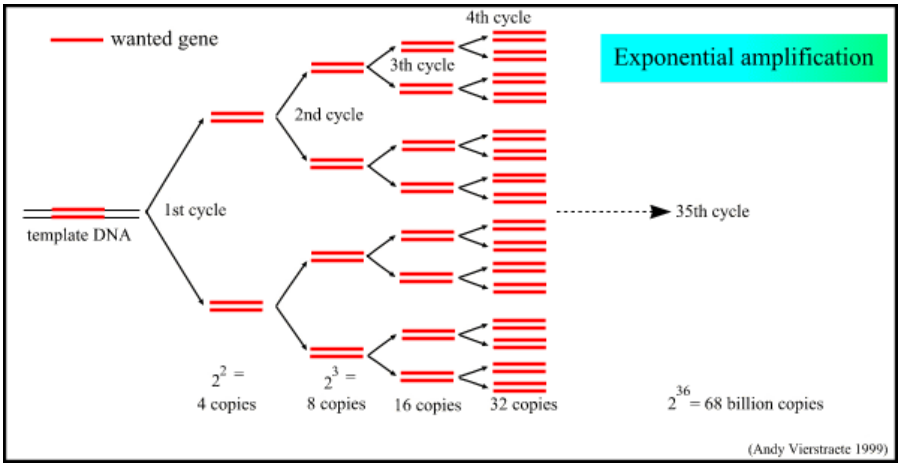

Figure II.1 The exponential amplification by PCR of a template DNA

II.1.1- Components of the PCR

A PCR reaction mix consists of target DNA, two primers (forward and reverse), heat-stable DNA polymerase, deoxynuleoside triphosphates (dNTPs including dATP, dCTP, dGTP and dTTP), and a buffer usually containing Mg^{2+}, an essential component for the correct efficiency and specificity of the reaction (Halliday *et al.*, 2010).

The DNA polymerase

PCR reactions are usually carried out with the Taq DNA polymerase, an enzyme originally isolated from the thermophilic bacterium *Thermus aquaticus*. The half-life is 40 minutes at 95°C. The DNA polymerase extends a DNA chain in the 5' → 3' direction, so this is the direction of the DNA synthesis. This enzyme has no proof reading activity (correction of incorporated errors) and the reliability depends strongly on the concentration of Mg2+ and dNTP's. Nowadays the Taq DNA polymerase is supplied by several companies of molecular biological products (Marmenout, 2014).

The deoxyribonucleoside triphosphates (dNTPs)

The four dNTPs (deoxyadenosine triphosphate (dATP), deoxycytosine triphosphate (dCTP), deoxyguanine triphosphate (dGTP) and deoxyuridine triphosphate (dUTP)) have to be added equimolar. The optimal concentration depends on the length of the PCR amplification, the concentration of the primers and $MgCl_2$ and the stringency of the reaction. Concentrations of dNTPs are usually from 100 to 200 µM although the reliability of the Taq polymerase is higher with lower concentration.

The oligonucleotide primers

Primers are short oligonucleotides of known sequence that are complementary to the 3'-ends of the target DNA (**Figure II.1.1**). The design of these primers is very important since the specificity of the primers determines the accuracy of the PCR assay. Appropriate primers have a length of 14 to 40 nucleotides and the content of G/C is in the rage of 40 to 75%. The designed primers cannot display complementarity between them or self-complementarity in order to exclude primer dimerization or formation of secondary structures. It is preferable to use primer pairs with the same melting temperature (Tm) and at equimolar concentrations, between 0.1 and 1 µM is suitable (Marmenout, 2014).

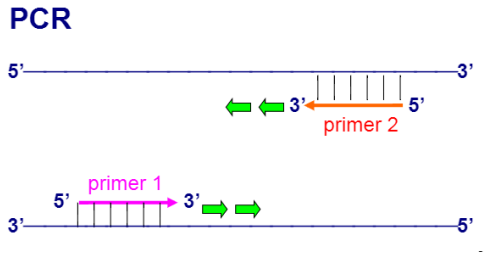

Figure II.1.1 DNA synthesis with two different and specific oligonucleotides (primers) that bind to opposite strands flanking the amplification target on the template DNA.

The template DNA: purity and concentration

For the many different applications of the PCR, many different methods of isolating and preparing the template DNA exist, mostly depending on the source of the DNA. It is important to note that the outcome of a PCR is dependent on the quality and integrity of the template DNA. It is wise to purify template DNA using a product or method that is specifically designed to purify template DNA for use in PCR. Poor quality of DNA template or non-target background DNA can influence the specific annealing of the primers. This can result in nonspecific amplification and possible misinterpretation of results. The amount of input template DNA is also of crucial importance in PCR and a common mistake is to add too much template DNA to the PCR reaction (James, 2010)

II.1.2- Temperature and time profile for the PCR cycles

Any PCR essentially involves a number of cycles and every cycle needs three different phases of temperature: (i) denaturation of the dsDNA at 92 to 96 °C, (ii) annealing of the primers with the complementary part in the template at 55 to 72 °C and (iii) extension of the primers from 5' to 3' end by incorporation of nucleotides at 72°C (i.e. the optimal temperature for the heat stable DNA polymerase activity) (**Figure II.1.2**) (Halliday *et al.*, 2010).

PCR amplification is automated and performed on thermocyclers programmed to heat and cool to different temperatures for varying lengths of time. Today, a large variety of such cyclers are available and different companies commercialized its devices [i.e. Applied Biosystems (Forster, USA), Bio-Rad (California, USA), Roche (Indianapolis, USA) etc.]. Some of these also offer real-time detection of the amplification reaction and will be discussed below.

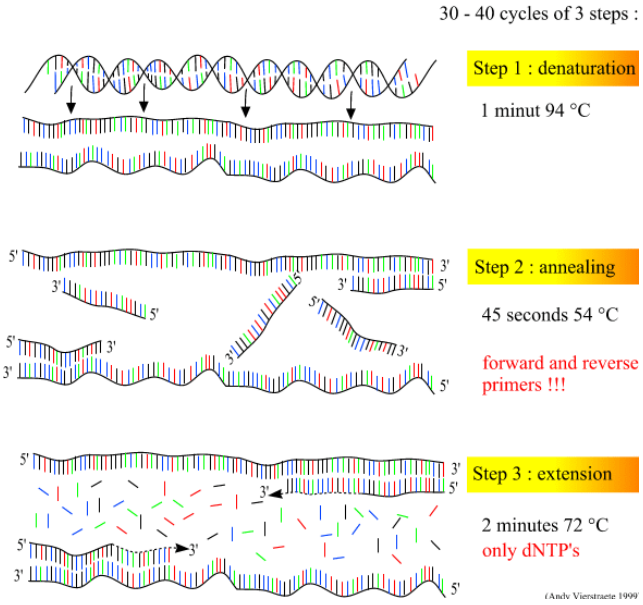

Figure II.1.2 Three major steps and temperatures stages in a PCR cycle (Vlierstrate, 2009).

II.1.3- Amplification and product detection

During denaturation, the DNA template is heated (94–96◦C) to separate the two DNA strands. The temperature is then cooled (50–65◦C) during the annealing step to allow the specific primers to hybridize to the 2 ends of the separated DNA strands. The annealing temperature chosen for the PCR reaction depends on the length and composition of the primers. Finally, during the extension at 72ºC, the Taq DNA polymerase catalyses the elongation of the primers by incorporating the complementary dNTPs to the generated amplicon. The extended primers form two new strands of target DNA for the next PCR cycle. In theory, the amount of target DNA should double after each PCR cycle. Repeated cycles of amplification lead in theory to an exponential synthesis of a DNA fragment of which the ends are defined by the 5'ends of the primers in the reaction. The exponential synthesis can be expressed with the formula: $(x \cdot 2^n - 2n)$. Where n is the number of cycles, 2n the primary and secondary reaction products with indefinite length and x the number of copies of the original template (**Figure II.1.1**).

Detection of amplified DNA product is visualized following separation of DNA segments within an agarose or polyacrylamide gel under the influence of an electric current (electrophoresis). Addition of a dye such as ethidium bromide or SYBR Safe, that intercalate to dsDNA, either during electrophoresis or in a bath following electrophoresis, allow visualization of the PCR product through excitation of the bound dye by irradiation with UV light. The length of the PCR product, which is an indication of its identity, is calculated by comparison against a molecular weight with size ladder of DNA fragments of known length.

In conventional PCR the result of amplification is only seen after the PCR is completed (end-point detection) and the amplicons are analysed by agarose gel electrophoresis (AGE). In the case of real-time PCR, detection of amplification does not occur at the end-point since the amplicon is detected and quantified during the reactions and the analysis it is not followed by AGE (see below, II.3).

II.2 CONVENTIONAL PCR: ADVANTATGES AND LIMITATIONS

Conventional PCR assays typically take 4–5 h from PCR setup to analysis of product following electrophoresis. This method is simple and relatively cost efficient and has been widely used by laboratories since the earliest examples of published PCR assays. The considerable increase in analytical sensitivity and specificity of PCR-based assays compared to conventional diagnostic tests are their major advantage, however, there are also limitations. The cost of performing molecular tests is high in comparison to traditional diagnostic tests and re-imbursement is often low, particularly for assays developed “in-house”. Additionally, laboratories performing these assays need to invest considerable costs in dedicated “DNA-free” laboratory space and equipment. DNA fragments are very easily aerosolized and dispersed on air currents and on the clothes and skin of laboratory staff and can contaminate subsequent PCR assays resulting in the production of false positive results. This is essential to minimize contamination of subsequent specimens by PCR amplicons that can lead to false positive results. This must be monitored by the inclusion of non-template or water controls in every PCR. The development of closed-tube, real-time PCR technologies and melting curve analysis has greatly reduced the risk of contamination and false positive results. DNA degradation or PCR inhibitors can lead to false negative results and this must be monitored by the inclusion of internal positive controls (James, 2010).

II.3 REAL-TIME PCR OR QUANTITATIVE PCR

In real-time PCR or quantitative PCR (qPCR), exactly the same procedure as in conventional PCR happens but with two major differences: first the amplified DNA is fluorescently labelled and second, the amount of fluorescence is monitored during the whole PCR process detected in ‘real time’, during the reaction. The higher the initial number of DNA molecules in the sample, the faster the fluorescence will increase during the PCR cycles, an example is given in **Figure II.3**. In other words, if a sample contains more targets the fluorescence will be detected in earlier cycles. The cycle in which fluorescence can be detected is termed threshold cycle (Ct for short) and is the basic result of qPCR: lower Ct values mean higher initial copy numbers of the target. This is the basic principle of quantitative approach that real-time PCR offers**.**

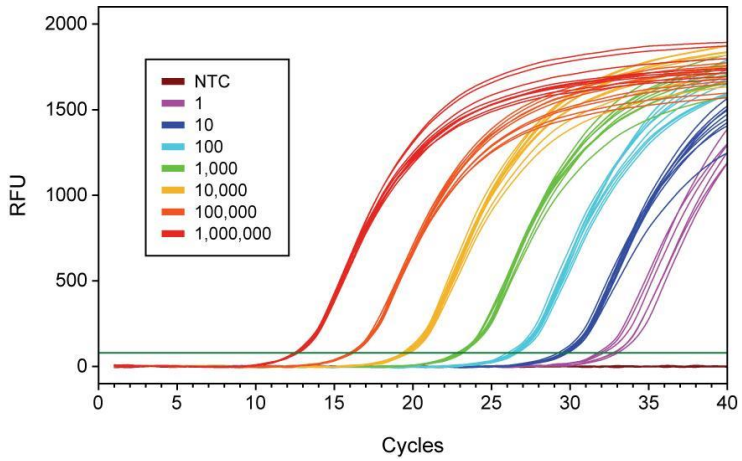

Figure II.3 Example of a real-time PCR amplification plot. NTC: non template control. The numbers indicate the number of targets in the reaction.

As an improvement of conventional PCR, real-time PCR assays can provide a quantitative result. PCR product is retained within the reaction tube and so cannot contaminate subsequent PCR assay samples and the potential for false positive results is greatly reduced but not eliminated as any tube breakage following amplification could result in subsequent contamination events. Moreover, the assays can be completed in 1-2 hours.

During the PCR, DNA amplification is exponential, eventually reaching a linear plateau. Real-time PCR monitors this exponential amplification via software driven detection system that collects and plots amplification data throughout the PCR. At any point during the exponential amplification phase for a reaction the amount of DNA can be calculated (**Figure II.3**), then through a simple mathematical calculation the initial concentration of DNA in that reaction can also be determined.

II.3.1- Chemistries of detection

Real-time PCR was first described using ethidium bromide as the non-specific reporter molecule. Ethidium bromide and SYBR Green I are fluorescent, double-stranded DNA binding dyes. SYBR Green I has become the most frequently used dye in qPCR because it fluoresces 50 times brighter than ethidium bromide when bound to dsDNA. At the elongation phase of the qPCR, after the Taq DNA polymerase has elongated the target sequence, the SYBR Green I dye binds to the dsDNA product. The longer the PCR product becomes, the more SYBR Green I dye binds to the product. Light then excites the SYBR Green I and the fluorescent emission at a second wavelength is detected as the signal for real-time PCR monitoring (**Figure II.3.1)**.

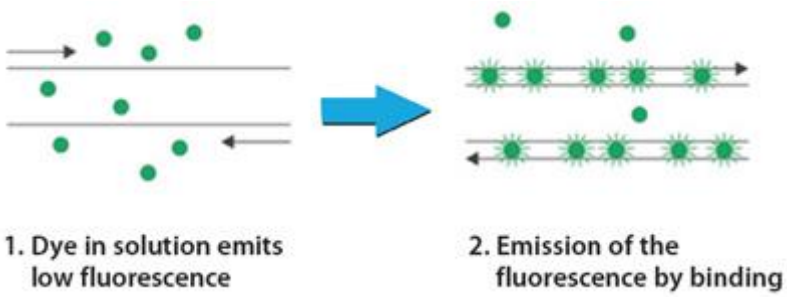

Figure II.3.1 Performance of SYBR Green I (Sigma-Aldrich, 2015)

The principle drawback with DNA binding dye-based detection is that both specific and non-specific products generate signal. For this reason, real-time PCR has developed also probe-based assays to increase specificity. The assays include in the reaction the two primers and the probe. Two main types of hybridization probes are hydrolysis probes (also named TaqMan probes) and molecular beacons. Each system has its own unique characteristics, but the strategy for each is similar; they must link a change in fluorescence to amplification of DNA (Josefsen, 2002).

Non-specific dyes such as SYBRR green, YO-PRO 1, SYTO9 and more recently BOXTO (TATAA Biocenter, Sweden) are relatively inexpensive and do not require additional oligoprobe design. Also they are not affected by mutations in target sequence which may impair the binding of specific probes thereby influencing the final result. SYBRR green is the most widely used chemistry, and provides the simplest and most economical format for detecting and quantifying PCR products. It is present in the reaction mix at the start, and binds to the minor groove of double stranded DNA, emitting 1.000-fold greater fluorescence than when it is free in solution. Thus, as amplification product accumulates, fluorescence increases. The disadvantage is that it will bind to any double-stranded DNA in the reaction, including non-specific reaction products such as primer-dimer, which may give false-positive results or an overestimation of the target concentration (Sloots *et al*., 2010). The advantage of probes are the added value of specificity, however they are much more expensive.

II.3.2- Kinetics of real-time PCR

The kinetics of a PCR reaction plotted graphically has a distinctive shape, with four distinct phases; (a) the background; (b) the early phase; (c) the exponential growth phase; and (d) the *plateau* phase (**Figure II.3.2.1**). In the early phase the oligonucleotides hybridise to the target sequence and the PCR reaction has commenced, but at an undetectable level. In the exponential growth phase the target is amplified in an exponential manner and the fluorescence levels become detectable above the background. In the plateau phase the reagents of the reaction have been consumed or have deteriorated and the PCR reaction is no longer operating efficiently. The exponential phase is the important stage of qPCR. During this phase accurate quantification of the target DNA is possible by monitoring the emission of fluorescence. Each cycle produces an increase in number of amplicon in proportion to amplification efficiency (Kubista *et al*., 2006).

The PCR efficiency can be calculated from the slope of the curve and a formula has been stablished ($E = 10^{(-1/slope)} - 1$) (Rasmunsen *et al*., 2001). Therefore, once a standard curve has been established, unknown samples can be amplified by the same process and compared to the standards to determine the target concentration in the original sample.

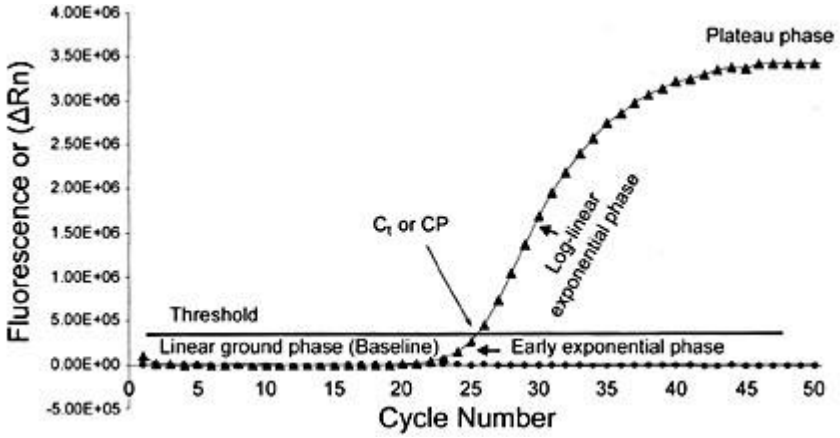

Figure II.3.2.1 Amplification curve of real-time PCR of log fluorescence versus cycle number showing the 4 phases of the PCR reaction.

The most common and easiest way to produce quantitative results is to create a standard curve using external standards. External standards are usually 10-fold serial dilutions of the target. The standards are usually plasmids but can be whole organisms or nucleic acid (Tavernier *et al*., 2004). These standards are amplified and detected using the same assay conditions as for samples. The PCR cycle at which product fluorescence intensity rises or is higher than the background is called the threshold cycle (Ct or CP). At this point the exponential PCR phase begins. Theoretically, the rate of amplification is maximal with PCR products doubling every cycle; hence quantification is performed at this stage. Following completion of the assay the Ct for each standard is determined and standard curve is prepared using instrument software. A typical standard curve is a plot of the cycle number Ct at the (Y-axis) versus the log of initial template amount (e.g. copy number or nanogram) (X-axis), derived from an assay based on serial dilutions. The standard curve is a least square fit line drawn through all dilutions (**Figure II.3.2.2**).

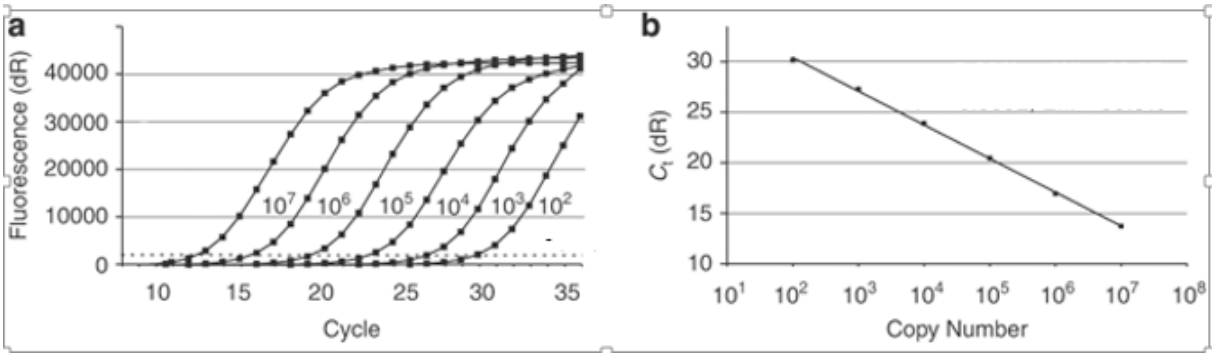

Figure II.3.2.2 Examples of (**a**) amplification plots of the qPCR standards (10-fold dilution series); relative fluorescence (dR) plotted against cycle number; (**b**) standard curve derived from amplification plots; threshold cycle C_t plotted against initial amount of template (copy number) (Wolff and Gemmell, 2008).

II.4 APPLICATIONS OF REAL-TIME PCR

The polymerase chain reaction and specifically the real-time PCR technology is a powerful tool used in many scientific disciplines and is also utilised for many applications such as research, food safety, veterinary and human *in-vitro* diagnostics (Huggett and O'Grady, 2014; Saunders and Martin, 2013; Filion 2012; and the references there in). In molecular biology, for example, PCR is used in research laboratories in DNA cloning procedures, Southern blotting, DNA sequencing and recombinant DNA technology. In clinical microbiology laboratories PCR is invaluable for the diagnosis of microbial infections, epidemiological studies and genetic testing. In food science PCR it has become increasingly important to the agricultural and food industries as a valuable alternative to traditional species specific detection methods (i.e. in authenticity studies, pathogen detection, assessment of starter cultures, etc.). PCR is also used in forensics laboratories and is especially useful because only a small amount of original DNA is required, for example, sufficient DNA can be obtained from a droplet of blood or a single hair.

In molecular biotechnology there are two important applications: the analysis of genomic DNA of all kind of organisms; and the analysis of RNA, that is, gene expression studies. It can be used to detect the presence of a given organism, its quantity or viability in the fields of pathogen detection, food safety, quality, medical and vet determinations, etc. In parallel of these applied uses, it is very widely used in basic research and developments. Two particular applications will be discussed that have been used in this work, namely the quantification of transgene copy numbers in a given genetically modified rice plant; and gene expression analysis in *Prunus persica* plants subjected to different stress conditions.

APPLICATION 1: QUANTIFICATION OF TRANSGENE COPY NUMBER

There are two key steps to obtain genetically modified plants (GMP). First step is the DNA insertion into the host genome and the second step is the regeneration of a transformed cell into a plant. There are different methods for genetic transformation to introduce a new DNA fragment into the plant genome: (1) *Agrobacterium tumefaciens* mediated transformation and (2) micro projectile bombardment of intact cells or tissues. Selection genes which allow making transgenic plants visually recognizable can be used, such as *hygromycin resistance* (*Hygro*^{R}) gene or *DsRed*, which codes for the fluorescent protein discosoma red fluorescent protein.

Different steps are required to obtain a GMP: 1) identification and isolation of the gene of interest; 2) preparation of a construct containing the gene of interest (with regulatory sequences) and a selection gene; 3) transformation of plant cells; 4) selection of transformed cells and regeneration of plants; and 5) acclimation and evaluation of the transformed plant. **Figure II.4** Illustrates the procedure of GM obtainment.

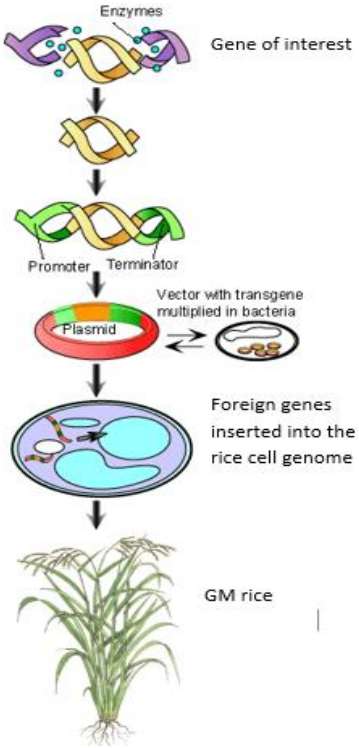

Figure II.4 General schematic of GM crop production

Genetic transformation has been widely exploited to study biology and development of plants as well as to obtain commercial crops with improved agronomic characters. Plants with one transgene are preferred for stable and high-level expression of transgene (Schubert *et al*., 2004). Therefore an early screening of transformants with one copy of transgene and transgene homozygotes in subsequent generations would be highly desirable and beneficial, especially for crop plants that require a lot of space and long time to grow.

Regardless of the scope of the transformation, when new transgenic plants are obtained an early and essential step is their molecular characterization. The reason for analysing many primary transformants (T_0) resides in the mechanism of integration itself: since the new DNA is inserted at random in the plant genome, plants with one to several integrated copies are obtained, and the multiple copies can be found in one or more chromosome locations. Usually plants where one or two integration events have occurred are those with the highest level of expression of the new gene. Low and sometimes unstable expression of transgenes has been related with high copy number and subsequent transgene silencing (Mason *et al*., 2002). It is therefore clear that the T_0 plantlets have to be analysed as soon as possible, so that only the most interesting ones are taken through the steps of acclimation in soil, flowering, seed production, etc.

Transgene copy number is defined as the number of copies of exogenous DNA insert(s) in the genome. For example, if the exogenous DNA fragment inserts only once at a single locus of the genome, it is a single copy transgenic event. The copy number is closely relevant to another concept, zygosity. If a single exogenous DNA insert exists as two identical alleles on the homologous chromosomes in the organism, it is a homozygous transgenic line with one copy of transgene. If the single exogenous DNA insert exists in only one of the homologous chromosomes, it is a hemizygous (syn. heterozygous) transgenic line with one copy of transgene. Both transgene copy number and zygosity are important in the genetic analysis of gene function (Yuan *et al*., 2007).

Quantitative real-time PCR technique is used to determine the copy number of transgenes and reference gene in transgenic rice lines. The method involves relative quantification of the genes of interest versus a reference gene known to be single copy, like β-actin (Plaffl, 2005). The objective is to significantly accurate estimate the transgene copy in the transformants, with the transgene copy number obtained as the ratio between the starting copy numbers of the transgene versus that of the β-actin gene (Vandesompele *et al*., 2002).

APLICATION 2: GENE EXPRESSION ANALYSIS

Gene expression analysis is the determination of the pattern of genes expressed at the level of genetic transcription, under specific circumstances or in a specific cell. The development and application of modern molecular biology have led to a better understanding of plant adaptations and responses to abiotic stress conditions, like salt stress, cold stress, high temperature stress, acid stress, alkaline stress and drought stress; and biotic stresses like pathogenic infections (See review Soda *et al*., 2015 and references therein). Plants can sense, process and respond to environmental stress and activate related-gene expression to increase their resistance to stress. Genes responsible for adaptation processes to every example of given stress have been identified in important model plants such as *Arabidopsis thaliana* and *Oryza sativa* (Fu-Tai Ni *et al*., 2009).

In case of gene expression studies, RNA is used as a template to synthesize cDNA. The RNA needs to be reverse transcribed into DNA (also termed complementary/copy DNA or cDNA) before it is amplified with real-time PCR. There is a term for this combined method: reverse transcription coupled to real-time PCR (RT-qPCR). For all samples, levels of both target and endogenous control genes (also named housekeeping gene) are assessed by RT-qPCR. Endogenous control gene expression is chosen to normalize input amounts. The data output is expressed as a fold-change or a fold-difference of expression levels between the target and the endogenous control gene (Huggett *et al*., 2005).

II.5 BACKGROUND OF THIS PROJECT

II.5.1- Production of antimicrobial peptides in plants as biofactories

In the recent years, transgenic plants have also been extensively used as biofactories to produce products of interest in the industrial, therapeutic and agricultural fields. Examples are the production of endoglucanases in rice seeds (Zhang *et al*., 2012); H1N1 vaccine in tabacco plants (Cummings *et al*., 2013); and Hepatitis B surface antigen in maize seeds (Hayden *et al*., 2012) (N. Company Thesis, 2014).

Antimicrobial peptides (AMP) have strong interest as a novel class of antimicrobial agents in the phytosanitary field and its production in plant biofactories can be considered an economically viable strategy (N. Company Thesis, 2014). The Biopeptide BP100 is a synthetic and strongly cationic α-helical undecapeptide with high, specific antibacterial activity against economically important plant-pathogenic bacteria (Badosa *et al*., 2007).

The Food Technology group at the University of Girona is working at the expression of recombinant AMPs in plants, specifically in rice. Transgenic rice lines producing active BP100 derivatives have been generated (Nadal *et al*., 2012; Company *et al*., 2014). The studies demonstrate that accumulation of recombinant BP100 can show some toxicity towards the host plant when a high peptide level is reached. To solve this problem, an alternative strategy based on strict control of transgene expression by using inducible promoters is developed.

In this study the molecular characterization of these transgenic plants have been analysed, specifically in the determination of the transgene copy number.

II.5.2- Analysis of expression of *Prunus persica*

Bacterial spot of stone fruits (BSSF) is a serious disease caused by *Xanthomonas arboricola* pv*. pruni* (*Xap*), a quarantine pathogen in the European Union. One of the common hosts is *Prunus persica* or peach tree.

Control of the disease is uniquely based on preventive application of copper derivatives. The limitations of copper use in agriculture (limited efficacy, negative environmental impact and pathogen resistance development) and the lack of alternative effective compounds, make the search for new sustainable control strategies to be implemented necessary.

This project aims at establishing the basis for the development of innovative strategies for integrated control. One of these strategies will include the effective use of plant defence inducers.

III. OBJECTIVES

This project focused on two distinct applications of real-time PCR (qPCR). In this context, there were two main objectives:

1. Molecular characterization of GM rice by determining the number of transgene copies inserted in the genome

   To that end, the following specific objectives were established:

   1.1. Extraction and quantification of genomic DNA from GM rice leaves
   1.2. Using the genomic DNA as template, qPCR based quantification of the amount of copies of three target genes: *DsRed-BP100* (tagged antimicrobial peptide sequence), *Hygro*^{R} (selection gene giving resistance to hygromycin) and β-*actin* (rice endogenous control gene)

2. qPCR based analysis of the mRNA levels of plant defence-related genes in leaves of *Prunus persica* treated with methyl salicylate, methyl jasmonate and harpins.

   To that end, the following specific objectives were established:

   2.1. Construction of plasmid template DNA for use as standard in three qPCR quantification assays. This required amplification and cloning of three target sequences (*pr5-tlp*, *pr2* and *pr4*)
   2.2. Optimization of three qPCR assays (*pr5-tlp*, *pr2* and *pr4*) by varying the concentration of the primers, using plasmid DNA as template
   2.3. Determination of the performance of three qPCR assays (*dfn1, nrp1 and erf1α*): linearity, efficiency, limit of detection (LOD) and limit of quantification (LOQ)
   2.4. Assessment of the expression levels of three plant defence-related genes (*dfn1, nrp1* and *erf1α*) in treated and control *Prunus persica* leaves

IV. MATERIAL AND METHODS

IV.1 DNA EXTRACTION FROM IN VITRO CULTURED GM RICE PLANTS

Prior to the real-time PCR assay, genomic DNA was extracted from leaves of transgenic rice. For each sample a leaf was grinded to a fine powder with liquid nitrogen. This powder was considered as homogenous and 1 spoon (~ 20 ng of plant material) of it was subjected to DNA extraction. The extractions were carried out with the Nucleospin Plant II kit (Machery-Nagel, USA). This commercial Kit is optimized for isolation of genomic DNA from various plants, and the extracts could be used directly as PCR template.

Materials:
- Nucleospin Plant II kit
- Centrifuge
- Thermal heating-block
- Eppendorf tubes 1,7mL

Protocol of the extraction:
Before starting with the extraction preheat the incubator to 65°C
1) To homogenize the sample grind the plant material plus liquid nitrogen well until fine powder. Refill mortar occasionally with nitrogen to keep the sample frozen.
2) For cell lysis transfer one spoon of the powder to a new tube with a precooled spatula and add 400 µL of Buffer PL1. Vortex the mixture thoroughly and incubate suspension for 20 min at 65°C (vortex 2 times during incubation)
3) For the filtration/clarification of the crude lysate place a NucleoSpin Fliter (violet ring) into a new collection tube (2 ml) and load the lysate onto the column.
4) Centrifuge 2 minutes at max. rpm, discard filter and collect the clear flow-through in a new tube.
5) To adjust the DNA binding conditions add 450 µL of Buffer PC and vortex mixture thoroughly.
6) Place a NucleoSpin Plant II Column (green ring) into a new Collection tube (2 ml).
7) Load with 700 µL lysate.
8) Centrifuge 3 minutes at 4000 rpm and discard filtrate.
9) Centrifuge 2 minutes at maximum rpm and discard filtrate.
10) To wash and dry the silica membrane add 400 µL Buffer PW 1 to the NucleoSpin Plant II Column.
11) Centrifuge 3 minutes at 4000 rpm and discard filtrate.
12) Centrifuge 1 minutes at maximum rpm and discard filtrate.
13) Add 600 µL Buffer PW 2 to the NucleoSpin Plant II Column.
14) Centrifuge for 3 minutes at 4000 rpm and discard filtrate.
15) Centrifuge for 1 minutes at maximum rpm and discard filtrate.
    - (Preheat ± 1 mL Buffer PE to 65°C)
16) Add 300 µL Buffer PW 2 to the NucleoSpin Plant II Column.
17) Centrifuge for 3 minutes at 4000 rpm and discard filtrate.
18) Centrifuge for 2 minutes at maximum rpm in order to remove wash buffer and dry the silica membrane completely.
19) To elute the DNA place the NucleoSpin Plant II Column into a new tube and pipette 50 µL Buffer PE (65°C) onto the centre of the membrane.
20) Incubate the NucleoSpin Plant II Column for 5 minutes at 65°C.
21) Incubate the NucleoSpin Plant II Column for 20 minutes at room temperature.
22) Centrifuge for 3 min at 4000 rpm and afterwards centrifuge again for 2 min at maximum rpm.
23) NanoDrop quantification: Quantify 2 µL of the obtained DNA sample with the NanoDrop ND-1000. spectrophotometer (See section IV.2).

IV.2 NANODROP: QUANTIFICATION OF THE TOTAL AMOUNT OF GENOMIC DNA

The quality and quantity of the extracted genomic DNA was analysed using a NanoDrop ND-1000 spectrophotometer (ThermoFisher, UK). DNA concentration was calculated in ng/µL at the absorbance of 260 nm, measured with a path length of 1 mm. Also the ratios of absorbance at 260/280 nm and 260/230 nm were measured to assess the purity and quality of the DNA. The presence of proteins which absorb at 280 nm and the presence of organic components or phenol used in the purification kit which absorb at 230 nm was evaluated. The expected values for the 260/280 ratio are commonly in the range of 1.8 – 2.0 and for the 260/230 ratio in the range of 2.0 – 2.2. Significant deviations from these values indicate possible contamination of the sample. The following protocol is used for NanoDrop quantification:

Materials:
- NanoDrop ND-1000 Spectrophotometer
- NanoDrop ND-1000 Software
- Laboratory wipe

Procedure of the quantification:
1) Start up the NanoDrop software and select the function "Nucleic Acids".
2) Load 2 µl of water onto the pedestal and click "OK" to initialize the NanoDrop machine.
Wipe the pedestal clean.
3) Load 2 µL of the elution buffer and click “Blank”.
4) Wipe the pedestal clean and apply 2 µL of the genomic DNA onto the pedestal. Click "measure". The apparatus measures the spectrum automatically.
5) Clean the pedestal after the measurement with a clean laboratory wipe and apply 2 µL of the next sample. Repeat this for the rest of the samples one by one, wiping the pedestal clean between each sample.

The obtained data was saved and all samples were afterwards individually diluted with water to a concentration of 50 ng/µL except for the samples with a stock concentration below 50 ng/µL. All DNA samples were stored at -20°C.

IV.3 REAL-TIME PCR: CONSTRUCTION OF SPECIFIC TEMPLATES FOR THE STANDARD CURVE

To quantify the number of copies of a specific target/gene by real-time PCR, a standard curve is needed, both, for transgene copy number quantification and for gene expression analysis.

IV.3.1 - Conventional PCR: obtainment of the specific DNA template

Conventional PCR technique was applied to obtain DNA template that would be used as standard curve in PCR quantitative assay. For PCR product amplification thermostable TaqDNA polymerase (Biotools, Germay) was used. A PCR product positive control was also performed to ensure PCR ran well. **In Tables IV.3.1.1 and IV.3.1.2**, PCR reagents and conditions are shown. Reactions were run on a PCR conventional device, the 96-Well GeneAmp®PCR System 9700 (Applied Biosystems, Foster City, USA)

Table IV.3.1.1 : PCR reaction reagents with Taq Biotools.

Reagent	Stock concentration	Final concentration	µL/reaction
Nuclease free water			36,80
Buffer 10x Biotools with Mg	10 x	1 x	5,00
dNTP's (TTP)	2,5 mM each	200 µM each	4,00
Taq Biotools (5 u / µL)			0,20
Mix/tube			46,00
Primer_for	10 000 nM	300 µL	1,50
Primer_rev	10 000 nM	300 µL	1,50
Template: gDNA(100ng/µL)			1,00
TOTAL			50,00

Table IV.3.1.2: PCR reaction thermocycling conditions with Taq Biotools..

	Time	Temperature (°C)
	3 min	95
24 cycles	30 s	95
	30 s	60*
	30 s	72
	10 min	72
	Hold	4

**Although, 60 ºC is one of the most commonly used temperature for this step (extension), this value depends on the melting temperature of each primer pair.*

IV.3.2 - PCR product analysis: agarose gel electrophoresis

This is a method in which nucleic acid fragments are separated by length, size or charge. It is an easy technique for analysis and purification of DNA fragments. The agarose gel provides a matrix with pores to allow migration of molecules. The size of the pores is related to the percentage of agarose in the preparation of the gel. In this case, fragments between 70-190 bp were amplified, and a 2% agarose concentration was used to analyse PCR products of this range.

Protocol for agarose gel electrophoresis:

1) Dissolve the desired 2 g of agarose (SIGMA, St. Louis, USA) in 100ml of buffer TAE 1x (TAE 50x Stock: TRIS, base, 484 g/l; glacial acetic acid, 57,1 ml/l; EDTA, 0,5 M; pH 8.0).
2) Heat the solution in the microwave, until agarose is completely dissolved. (no boiling).
3) Let the solution cool down to 50ºC.
4) Pour the agarose into a gel tray (BioRad, Hercules, USA) with the well comb in place and let it polymerizing for at least 30 minutes.
5) Take 5 µl of each PCR product and mix with 1 µl of the loading buffer xylencianol or bromurphenol, depending on the length of the PCR products and the % of agarose.
6) Carefully load 5 µL of molecular weight ladder (*1Kb* ADN Plus ladder, Invitrogen) into the first and last lane of the gel.
7) Carefully load the samples into the additional wells of the gel.
8) Run the gel at 120 mA during 20-40 min, until the dye line is approximately 75-80% of the way down the gel.

9) Turn OFF power supply, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
10) Place the gel into a container filled with 100 ml/10 µl TAE 1X previously mixed with 10 µl of SYBRSafe solution (Invitrogen, Carlsbad, USA), and leave to dye for 20-30 minutes.
11) Using a UV light emitting device, you can visualize the DNA fragments.

IV.3.3 - PCR product purification

Before PCR amplicons could be further used, it was necessary to purify them to ensure its purity, by removing any trace of primers, dNTP's, enzymes and any other reagents used in the PCR reaction. PCR products that showed only one band in the agarose gel electrophoresis analysis were purified with a commercial kit based on affinity columns (QIAquick QIAGEN kit, Hilden, Germany). This will enable the purification of fragments between 100 bp and 10 kb.

Protocol for the purification of amplicons:
It is recommended to start with at least 100 µl of PCR product. If that is not the case, add MilliQ water to obtain the desired volume.

1) Add 5 volumes of Buffer PB to 1 volume of the PCR sample(500 µL of buffer to 100 µL of the PCR sample) and vortex to homogenize well.
2) Place a QIAquick spin column in a provided 2 ml collection tube. To bind DNA, apply the sample to the QIAquick column (max V 650µL).
3) Centrifuge for 3 minutes at 4,000 rpm. Repeat if the sample is not on the bottom of thetube.
4) Centrifuge for 30 seconds at 13,000 rpm.
5) Discard flow-through. Place the QIAquick column back into the same tube.
6) To wash, add 750 µl Buffer PE to the QIAquick column.
7) Centrifuge for 3 minutes at 4,000 rpm. Repeat if the sample is not on the bottom of theTube.
8) Discard flow-through and place the QIAquick column back in the same tube.
9) Centrifuge the column for 1 minute at 13,000 rpm in order to remove all ethanol still present in the sample.
10) Place the QIAquick column in a new, clean siliconized eppendorf (low binding tubes).
11) Add 30 µl of Buffer EB (Elution Buffer) to the center of the QIAquick membrane.
12) Incubate for 15 minutes at room temperature.
13)To elute the DNA, centrifuge for 3 minutes at 4,000 rpm and repeat for 1 minute at 13,000rpm.
14) Quantify 2 µl of the sample in a NanoDrop spectrophotometer, to calculate the quantity and quality of the PCR products(see section IV.2).

IV.3.4 – Cloning and ligation of the PCR product into a plasmid

To clone the DNA fragment it was necessary to insert it in a vector (plasmid), and obtain a covalently bound hybrid. To multiply the number of copies, the recombinant plasmid had to be transformed into a microorganism (generally *E. coli*, which has been used in this experiment).

Ligation is used to fuse insert to a vector. In this work, the vector used for this procedure was the 3000 bppSpark®–TA DNA Cloning vector (CavanxBiotech, Córdoba, Spain), a vector based on a classical TA technology. This cloning system has the advantage of working without restrictionenzymes. Insert will attach to the vector through its poly-T ends, since DNA amplified by PCR with non-proofreading Taq DNA polymerases contains single 3'-adenine overhanging ends.

Ratios of insert to a vector from 5:1 to 1:1 provide good initial parameters. PSpark®–TA DNA Cloning vector has been optimized using an insert to vector ratio of 3:1. Optimal ratio of insert to vector is from 3:1 to 5:1. The calculations for a ratio 5:1 are as follow:

- $\frac{50 \text{ ng vector} \times 1000 \text{ pb insert} \times 5}{3000 \text{ pb vector} \times 1} \sim 50$ ng of insert vector

- $\frac{\text{pb insert} \times \text{ng vector} \times 5}{\text{pb vector} \times 1} =$ ng of insert amplicon

Protocol for ligation:
1) Briefly centrifuge the pSpark®–TA DNA Cloning vector and Control Insert DNA tubes to collect contents at the bottom of the tubes.
2) Vortex the 5x T4 DNA Ligase Buffer vigorously before each use.
3) Set up ligation reactions as described below:

Reagent	Cloning reaction	Control reaction	Background reaction
Water	5 µL	5 µL	6 µL
5x T4 DNA ligase buffer	2 µL	2 µL	2 µL
pSpark®–TA DNA cloning vector (50ng/µL)	1 µL	1 µL	1µL
diluted amplicon	1 µL	-	-
positive control insert	-	1 µL	-
T4 DNA ligase (5Weiss units/µL)	1 µL	1 µL	1 µL
Total volume	10 µL	10 µL	10 µL

4) Mix the reactions by pipetting slowly.
5) Incubate the ligation reactions one hour at 22ºC in either a thermoblock, a thermocycler, a water bath or at bench if room temperature is between 20-24ºC.
6) Incubate for 10 minutes at 65°C (heatshock)
7) Proceed to transformation bacterial transformation).

IV.3.5 - *E. coli* transformation

Bacterial cells had to be competent before exposing them to the DNA, with the objective to favour the crossing through the cell wall and membrane. These competent cells were then mixed with a solution of plasmids (which had the DNA fragment of interest integrated), and were formerly transitory permeabilised to produce pores in the cell membrane. This was done with a thermal shock. These cells were then cultivated in a solid medium in presence of an antibiotic (Ampicillin) corresponding to the resistance gene that was contained in the plasmid. In this way, only bacteria that had been successfully transformed would be able to form colonies. In this experiment *E.coli* XL1 Blue strain was used.

The protocol for transformation:
1) Thaw competent cells in ice. Do not vortex.
2) Add the plasmid to be transformed. Make two controls: transformed cells only with the plasmid and cells that have not been transformed. Add 15 µl of ligation reaction to each eppendorf with competent cells, and mix carefully.
3) Mix and incubate for 30 minutes in ice.
4) Incubate 90 seconds at 42ºC. Be careful to be precise in this step.
5) Place eppendorfs in ice for 1-2 minutes.
6) Add 800 µl of SOC or LB (without antibiotic).
7) Incubate 45 minutes at 37ºC in agitation at 300 rpm.
8) Spread 100 µL over plates containing LB with the selection antibiotic.
9) Centrifuge 5 minutes at 4,000 rpm Discard a part of the supernatant (100 µL of the suspension should be kept) and homogenize by hand.
9. Spread 100 µl over plates containing LB with the selection antibiotic.
10. Incubate plates at 37ºC.
11. Colonies should appear after 12-26 hours.

After transformation, a screening procedure was done to ensure the insert was present in the plasmid. Screenings were performed as conventional PCR's, using as template a suspension of bacteria colonies holding, in this case, pSpark transformed plasmids for each gene. Four replicates (four colonies of each plate) for each gene plus a one negative control were tested. (See section IV.3.1 Conventional PCR).

IV.3.6 – Plasmid DNA purificaiton

After the colony screening, the first colony of each plate was taken to do and overnight in liquid LB (lysogeny broth) medium. To purify the plasmid DNA from the *E.coli* suspensions a commercial kit was used (QIAGEN NucleoSpin Plasmid kit, Hilden, Germany). This procedure started with the lysis of the cells and was followed by a denaturation of nucleic acids and proteins under alkaline conditions. The later addition of potassium acetate precipitated proteins and chromosomal DNA, but allowed plasmids to renature and stay in solution, so it could be purified very easily.

Protocol for the purification of the plasmid (PSpark+amplicon):
1) Pour 5 mL of a saturated *E.coli* LB culture in a standard benchtop and centrifuge for 5 min at 4000 rpm.
2) Discard supernatant (remove as much liquid as possible)
3) For cell lysis add 250 µL Buffer A1 (stored in fridge) and vortex.
4) Add 250 µL Buffer A2 and mix gently by inverting the tube 8 times.
5) Incubate for 5 min at room temperature.
6) Add 300 µL Buffer A3 and mix thoroughly by inverting the tube 8 times.
7) Collect the lysate in new eppendorf.
8) To clarify the lysate centrifuge for 5 min at 13000 rpm.
9) For binding the DNA place a NucleoSpin Plasmid Column in a Collection tube (2mL) and load 750 µL of the supernatant onto the column.
10) Centrifuge for 3 min at 4000 rpm. Discard flow-through.
11) Centrifuge for 30 sec at 13000 rpm.
12) To wash and dry the silica membrane, add 600 µL Buffer A4 to the NucleoSpin Plasmid Column.
13) Centrifuge for 3 min at 4000 rpm. Discard filtrate.

14) Centrifuge for 2 min at 13000 rpm.
15) To elute the DNA, place the NucleoSpin Plasmid Column into a new siliconized tube and add 50 µL of Buffer AE**.**
16) Incubate for 15 min at room temperature.
17) Centrifuge for 5 min at 5000 rpm and repeat for 15 sec at 13000 rpm.
18) Nanodrop quantification (see section V.2)
19) Store samples at -20°C

IV.3.7 Preparation of plasmid dilutions

A standard curve was constructed with serial dilutions of a template (plasmid + amplicon) with known amount of the number of plasmid copies.. With the known amount of plasmid DNA in ng/µL, it was possible to calculate the number of copies with the following formula:

$$initial\ conc\ \left(\frac{ng}{\mu L}\right).\frac{1g}{10\text{^}9ng}.\frac{1mol}{bp(amplicon+sPark\ vector)\ .\ 325\frac{g}{mol}.2}.6{,}02\ .10^{23}molecules/mol$$

Where 325 g/mol is the average weight of a nucleotide and multiplied by two because of the double stranded DNA. With that solution a serial dilution could be made to obtain 1e9, 1e8, 1e7, 1e6, 1e5, 1e4, 1e3, 1e2 until 1e1 copies of plasmid per µL. The last six were implemented in the qPCR to calculate the standard curve.

IV.4 REAL-TIME PCR: OPTIMIZATION OF PRIMER CONCENTRATIONS

In the case of real-time PCR assays developed for gene expression analysis, specific primer pairs previously designed by the group were optimized in this work. Each primer pair was analysed at different concentrations (100-100, 300-300 or 900-900 nM) in order to select the optimal final concentration expecting to give the best performance criteria. As DNA template, 10^5 copies of plasmid harbouring a specific fragment of each gene was used. See section IV.3.6: Preparation of plasmid dilutions. Negative controls, i.e., reactions without DNA template, were also included for each primer concentration. These controls are very important when SYBR-Green based qPCR assays are developed.

All reactions, samples and controls, were run by triplicate on a 7500 Fast real-Time PCR (Applied Biosystems, Foster City, USA), and analysed with 7500 Fast System Software (see section IV.6). In **Table IV.4.1 and IV.4.2** qPCR reagents and protocol conditions are shown.

Table IV.4.1 : Quantitative PCR conditions for a final primer concentration of 100nM, 300nM and 900nM.

Reagent	Stock concentration	Final concentration	µL/reaction
Nuclease free Water			8,6
Primer_for	10 000 nM (10µM)	100 nM/ 300 nM/ 900 nM *	0,2
Primer_rev	10 000 nM (10µM)	100 nM/ 300 nM/ 900 nM *	0,2
MIX Syber Green	2 x	1 x	10,0
Mix/tube			19,0
template: pSpark + amplicon (10^5 copies)			1,0
TOTAL			20,0

**For optimization assay the three concentrations have to be tested for performance analysis. Final primer concentration depends on optimal primer concentration experimentally determined during the optimization assay.*

Table IV.4.2 : Quantitative PCR thermocycling conditions for optimization of primers.

	Time	Temperature (°C)
qPCR conditions 50 cycles	10 min	95
	1 min	60
	15 s	95
melting curve	15 s	95
	1 min	60
	slowly heated to	95

IV.5 REAL-TIME PCR: Performance of the assay

In the case of real-time PCR assays developed for gene expression analysis, the performance of the assay was experimentally evaluated in this work. Limit of detection (LOD), Limit of quantification (LOQ), and efficiency (E) of each qPCR assay was determined using as DNA template 10-fold serial dilutions of the corresponding plasmid (i.e., from 10^6 to 0.1 copies in the reaction, see section IV.3.6). The reactions were carried out according to the protocol described in section IV.6.

These values were determined according to the following concepts and formula (in the case of efficiency):

LOD: the smallest amount of copies that can be detected and distinguished from zero.

LOQ: the smallest amount of copies which can be measured and quantified with defined precision and accuracy.

Efficiency: $E= 10^{(-1/slope)}$; % Efficiency= (E-1) x 100%

IV.6 REAL-TIME PCR: QUANTIFICATION

Real-time PCR was run with 1 µL of DNA/cDNA template of the sample and 19 µL of mastermix in each well. Negative controls, i.e., reactions without DNA template, were also included for each primer concentration. The SYBR® Green PCR Master Mix is a convenient premix of the components (except primers, template and water) necessary to perform real-time PCR using SYBR® Green I Dye. The mix is optimized for SYBR® Green reactions and contains SYBR® Green I Dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference, and optimized buffer components.(Applied Biosystems, Forster, USA).

An initial denaturation at 95°C for 10 min was followed by 50 amplification cycles of denaturation at 60°C for 1min and annealing and extension at 95°C for 15s. The conditions are shown in **Table IV.6.1.4.** The fluorescence signals were measured after each annealing/elongation step.

Since qPCR assays are developed based on SYBR-Green detection method, a melting curve analysis was added at the end of the amplification step in order to discard unspecific amplifications. A melting curve is a graph that displays melt curve data from the amplicons of quantitative PCR runs. Change in fluorescence, due to a dye or probe interacting with double-stranded DNA, is plotted against temperature.

It was important to maintain separate areas and dedicated equipment and supplies for sample preparation, mastermix preparation, PCR setup, PCR amplification and analysis of PCR products.

IV.6.1 –Determination of the number of copies of the transgene in GM rice

For absolute quantitation normalized to an endogenous control, standard curves were prepared for both the transgenes and the endogenous gene. The Ct of an unknown sample was compared against a standard curve with known copy numbers. For each rice plant to be tested, the amount of transgene and endogenous gene was determined from the appropriate standard curve. Then the amount of transgene copies was divided by the amount of endogenous gene and a normalized transgene value was obtained. In this study β*actin* was the endogenous gene. *Hygro*^{R} and *DsRed-BP100* were the transgenes. The three specific genes were quantified for 16 different rice lines and all reactions, samples and controls, were run by triplicate on a 7500 Fast real-Time PCR and analysed with 7500 Fast System Software. An example of the distribution in the wells onto the qPCR plate, is given in **Table IV.6.1.5.**

Materials:

- Eppendorf tubes 1.7 ml
- Primers and reagents, presented in table 1, 2 and 3
- 7500 Fast Real Time PCR System (Applied Biostystems, Foster City, CA, USA)
- 7500 Fast System SDS Software (Applied Biosystem)
- Optical Adhesive Cover (Applied Biosystem)
- MicroAmp^{TM} Optical 96-Well Reaction Plate (Applied Biosysteem)
- Centrifuge 5810R with holder for 96-well plates

Procedure of performing the real-time PCR:

1) Preparation of the plasmid dilutions for the standard curve (see section IV.3.6)
2) Preparation of the mastermixes, composition showed in **Table IV.6.1.1, IV.6.1.2 and IV.6.1.3**
3) Distribute the mastermix in a 96-well plate, 19 µL per reaction
4) Add the genomic DNA or plasmid DNA, 1 µL per reaction, to the correct wells and mix by pipetting. Use tips with filter.
5) Seal the plate with an adhesive cover and centrifuge until reaching 1400 rpm
6)Load the plate into the 7500 Fast Real Time System apparatus and start the software. The thermocycler conditions are given in table 5. Also generate a melt curve after the PCR-run.
7) Analyze the results using the 7500 Fast System SDS software. Ct value of each sample is extrapolated into the standard curve to obtain the number of copies of each target in the sample.
8) Export the results to an excel document.
9) Calculate the number of copies of the inserted transgene in each plant using the following formula: # of copies of DsRed/ # of copies of actin and # of copies of Hygro/ # of copies of actin.

Table IV.6.1.1: DsRed mastermix composition (PCR7500) and example for 75 reactions

Reagent	Stock concentration	Final concentration	µl/reaction	µL/75 reactions
Deionized water			8,6	645
SyDsRed_for	10000 nM (10µM)	100 nM	0,2	15
SyDsRed_rev	10000 nM (10µM)	100 nM	0,2	15
MIX SybrGreen	2x	1x	10	750

Table IV.6.1.2: Hygro mastermix composition (PCR7500) and example for 75 reactions.

Reagent	Stock concentration	Final concentration	µl/reaction	µL/75 reactions
Deionized water			7,8	585
SYHygro_for	10000 nM (10µM)	300 nM	0,6	45
SYHygro_rev	10000 nM (10µM)	300 nM	0,6	45
MIX SybrGreen	2x	1x	10	750

Table IV.6.1.3: Actin mastermix composition (PCR7500) and example for 75 reactions.

Reagent	Stock concentration	Final concentration	µl/reaction	µL/75 reactions
Deionized water			8,6	645
Actin_for	10000 nM (10µM)	100 nM	0,2	15
Actin_rev	10000 nM (10µM)	100 nM	0,2	15
MIX SybrGreen	2x	1x	10	750

Table IV.6.1.4: Thermal cycling conditions for the real-time PCR reactions

	Time	Temperature (°C)	Phase
qPCR conditions 50 cycles	10 min	95	Initial denaturalization
	1 min	60	Denaturalization
	15 s	95	Annealing and elongation
melting curve	15 s	95	
	1 min	60	
	slowly heated to	95	

The 10 min, 95 °C step is required to activate the AmpliTaq Gold® DNA Polymerase.

Table IV.6.1.5 Columns 1-3, standard curve: DNA dilutions (10^6, 10^5, 10^4, 10^3, 10^2, 10^1) of DsRed plasmid; columns 4-12, distribution of the genomic DNA samples onto the qPCR plate. All the samples and points of the standard curve are analysed by triplicate. NTC: non template controls (without DNA).

	1	2	3	4	5	6	7	8	9	10	11	12
	DsRed	DsRed	DsRed	DsRed	DsRed	DsRed	DsRed	DsRed	DsRed	DsRed	DsRed	DsRed
A	1,00E+06	1,00E+06	1,00E+06	planta T2 Hemo 1. 5	planta T2 Hemo 1. 5	planta T2 Hemo 1. 5	planta T2 7.1.1.1	planta T2 7.1.1.1	planta T2 7.1.1.1	planta T2 15.2.1.1	planta T2 15.2.1.1	planta T2 15.2.1.1
	DsRed	DsRed	DsRed	DsRed	Hygro	Hygro	DsRed	DsRed	DsRed	DsRed	DsRed	DsRed
B	1,00E+05	1,00E+05	1,00E+05	planta T2 Hemo 2. 13	planta T2 Hemo 2. 13	planta T2 Hemo 2. 13	planta T2 8.2.2.2	planta T2 8.2.2.2	planta T2 8.2.2.2	planta T2 15.1.2.3	planta T2 15.1.2.3	planta T2 15.1.2.3
	DsRed	DsRed	DsRed	DsRed	Hygro	Hygro	DsRed	DsRed	DsRed	DsRed	DsRed	DsRed
C	1,00E+04	1,00E+04	1,00E+04	planta T2 ADH1. 15	planta T2 ADH1. 15	planta T2 ADH1. 15	planta T2 8.1.2.1	planta T2 8.1.2.1	planta T2 8.1.2.1	planta T2 15.2.2.2	planta T2 15.2.2.2	planta T2 15.2.2.2
	DsRed	DsRed	DsRed	DsRed	Hygro	Hygro	DsRed	DsRed	DsRed	DsRed	DsRed	DsRed
D	1,00E+03	1,00E+03	1,00E+03	planta T2 G3P. 9	planta T2 G3P. 10	planta T2 G3P. 11	planta T2 8.2.1.1	planta T2 8.2.1.1	planta T2 8.2.1.1	planta T2 5.1.1.2	planta T2 5.1.1.3	planta T2 5.1.1.4
	DsRed	DsRed	DsRed	DsRed	Hygro	Hygro	DsRed	DsRed	DsRed	DsRed	DsRed	DsRed
E	1,00E+02	1,00E+02	1,00E+02	planta T2 7.5.1.3	planta T2 7.5.1.3	planta T2 7.5.1.3	planta T2 8.3.1.2	planta T2 8.3.1.2	planta T2 8.3.1.2	NTC	NTC	NTC
	DsRed	DsRed	DsRed	DsRed	Hygro	Hygro	DsRed	DsRed	DsRed			
F	1,00E+01	1,00E+01	1,00E+01	planta T2 7.7.2.2	planta T2 7.7.2.2	planta T2 7.7.2.2	planta T2 8.3.2.1	planta T2 8.3.2.1	planta T2 8.3.2.1			

IV.6.2 – Analysis of gene expression in *Prunus perisca*

The protocol to quantify the levels of expression of genes was the same as described in section IV.6.1. The main difference was the type of template of the samples. In this case, cDNA was used instead of genomic DNA. The cDNA was obtained previously by the group through a reverse transcription reaction from total RNA extraction from *Prunus persica* leaves. In **Table IV.6.2** an example of the distribution of a plate during the gene expression analysis is shown.

Table IV.6.2 Distribution of the samples onto the plate for the analysis of expression of NPR1 gene. Lines A-C: dilutions from 10^6 to 10^{-1} of the standard curve. Lines D-H: samples with different treatments and controls. All the samples and points of the standard curve are analysed by triplicate. NTC: non template controls (without DNA).

	1	2	3	4	5	6	7	8	9	10	11	12
A	NPR1 1,00E+06	NPR1 1,00E+05	NPR1 1,00E+04	NPR1 1,00E+03	NPR1 1,00E+02	NPR1 1,00E+01	NPR1 1,00E+01	NPR1 1,00E+00	NPR1 1,00E+00	NPR1 1,00E-01	NPR1 FONG	NPR1 PUGO
B	NPR1 1,00E+06	NPR1 1,00E+05	NPR1 1,00E+04	NPR1 1,00E+03	NPR1 1,00E+02	NPR1 1,00E+01	NPR1 1,00E+01	NPR1 1,00E+00	NPR1 1,00E+00	NPR1 1,00E-01	NPR1 FONG	NPR1 PUGO
C	NPR1 1,00E+06	NPR1 1,00E+05	NPR1 1,00E+04	NPR1 1,00E+03	NPR1 1,00E+02	NPR1 1,00E+01	NPR1 NTC	NPR1 1,00E+00	NPR1 NTC	NPR1 1,00E-01	NPR1 FONG	NPR1 PUGO
D	NRP1 cDNA ME SA 1H R1	NRP1 cDNA ME SA 1H R1	NRP1 cDNA ME SA 1H R1	NRP1 cDNA ME SA 1H R2	NRP1 cDNA ME SA 1H R2	NRP1 cDNA ME SA 1H R2	NRP1 cDNA ME SA 3H R1	NRP1 cDNA ME SA 3H R1	NRP1 cDNA ME SA 3H R1	NRP1 cDNA ME SA 3H R2	NRP1 cDNA ME SA 3H R2	NRP1 cDNA ME SA 3H R2
E	NRP1 cDNA ME JA 625 1H R1	NRP1 cDNA ME JA 625 1H R1	NRP1 cDNA ME JA 625 1H R1	NRP1 cDNA ME JA 625 1H R2	NRP1 cDNA ME JA 625 1H R2	NRP1 cDNA ME JA 625 1H R2	NRP1 cDNA ME JA 625 3H R1	NRP1 cDNA ME JA 625 3H R1	NRP1 cDNA ME JA 625 3H R1	NRP1 cDNA ME JA 625 3H R2	NRP1 cDNA ME JA 625 3H R2	NRP1 cDNA ME JA 625 3H R2
F	NRP1 cDNA HARP 1H 1x R1	NRP1 cDNA HARP 1H 1x R1	NRP1 cDNA HARP 1H 1x R1	NRP1 cDNA HARP 1H 1x R2	NRP1 cDNA HARP 1H 1x R2	NRP1 cDNA HARP 1H 1x R2	NRP1 cDNA HARP 3H 1x R1	NRP1 cDNA HARP 3H 1x R1	NRP1 cDNA HARP 3H 1x R1	NRP1 cDNA HARP 3H 1x R2	NRP1 cDNA HARP 3H 1x R2	NRP1 cDNA HARP 3H 1x R2
G	NRP1 cDNA HARP 1H 2x R1	NRP1 cDNA HARP 1H 2x R1	NRP1 cDNA HARP 1H 2x R1	NRP1 cDNA HARP 1H 2x R2	NRP1 cDNA HARP 1H 2x R2	NRP1 cDNA HARP 1H 2x R2						
H	NRP1 cDNA control 1H R1	NRP1 cDNA control 1H R1	NRP1 cDNA control 1H R1	NRP1 cDNA control 1H R2	NRP1 cDNA control 1H R2	NRP1 cDNA control 1H R2	NRP1 cDNA control 3H R1	NRP1 cDNA control 3H R1	NRP1 cDNA control 3H R1	NRP1 cDNA control 3H R2	NRP1 cDNA control 3H R2	NRP1 cDNA control 3H R2

V. RESULTS AND DISCUSSION

V.1 ANALYSIS OF GM RICE PLANTS

The transgenic plants analysed in this project, were transformed with the (i) transgene coding for the antimicrobial peptide BP100 tagged with the fluorescent protein DsRed (*DsRed-BP100*) and the (ii) selection gene coding for the resistance to hygromycin (*Hygro*^{R}).

The real-time PCR assays for the determination of the transgene copy number had been designed and optimized previously for the Food and Technology group at University of Girona.

V.1.1- Genomic DNA purification and quantification

Total genomic DNA concentrations quantified in ng/µL together with the quality rations for each sample measured are shown in **Table V.1**. All DNA samples were quantified as described in the Materials and Methods (section IV.1) using the NanoDrop device.

Table V.1.1 The measured concentrations and quality ratios of the genomic DNA samples.

Plant code	concentration (ng/µL)	260/280	260/230
ADH1 15.1	671.29	2.07	2.24
ADH1 15.2	25.87	2.09	2.03
ADH1 15.3	15.43	2.36	2.14
ADH1 15.4	19.81	1.97	2.12
ENOL 7.1	486.57	2.12	2.29
ENOL 7.2	177.21	2.14	2.24
ENOL 7.3	222.47	2.14	2.23
G3P 9	198.09	2.14	2.25
HEMO1 5.1	47.67	2.07	2.01
HEMO1 5.2	252.76	2.17	2.29
HEMO2 13	360.85	2.15	2.31
MIT 8.1	19.68	2.01	1.49
MIT 8.2	214.89	2.13	2.31
MIT 8.3	18.21	1.9	1.24
MIT 8.4	163.59	2.13	2.23
MIT 8.5	174.48	2.1	2.17

Genomic DNA was extracted from all rice leaf samples, although for some of them (i.e. ADH1 15.2, ADH1 15.3, ADH1 15.4; and MIT 8.1 and MIT 8.3) the obtained concentrations were rather low. The lower the concentration was, the bigger was the influence of "contaminant" molecules like proteins from the sample itself or organic compounds from the kit of purification. This was the case of samples MIT 8.1 and MIT 8.3 displaying 260/230 ratios below the ideal range (i.e. 2-2.2). However and according to the experience of the group, these values should be suitable for further analysis. For samples where high concentrations were obtained, the quality ratios indicate that there is no significant contamination. In conclusion, all measured concentrations should allow further analysis by real-time PCR.

V.1.2- Determination of the transgene copy number by real-time PCR

Real-time PCR allowed the determination of the number of copies of the inserted transgene at an early stage of plant growth. Therefore, low or single copy transformation events may be selected by real-time PCR early in the transformation process.

The rice genome is 2n (12 + 12 chromosomes) and the leaf samples were taken from the first transgenic plant (T_0 plants). The T_0 plants were hemizygous, having only a copy of the inserted transgene in one of the chromosomes instead of the two copies of the endogenous gene. Since the transgene copy number is calculated according to the ratio between "copies of the transgene"/"copies of the endogenous gene" in each sample, the expected value in the case of T_0 plants was 0.5.

For each DNA sample three targets were analysed: the transgenic sequences *DsRed-BP100* and the *Hygro*^{R}; and the endogenous control *β-actin* gene. As it has been mention before, the number of copies of the transgene inserted in the genome of the transgenic plant was normalised by using an endogenous control, in this specific case the *β-actin* sequence. Consequently the actin gene was measured as well in all the samples. The gene should be similarly expressed in all analysed rice samples. In **Table V.1.2** the transgene copy numbers are summarised.

Due to the precision and sensitivity of the qPCR technique, a ratio in the range of 0.25 – 1 between the transgene and endogenous control coukd be considered acceptable; and this indicated that there should be one copy present. Otherwise when the value was between 1 and 2, it was difficult to precisely calculate if there was one or two copies since it had been reported that two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR (Bubner, 2004). Ratios of transgene (taking in consideration *DsRed-BP100* and *Hygro*^{R}) to actin copy numbers were close to 0.5 for samples ADH1 15.2, ADH1 15.3, ADH1 15.4, ENOL 7.1, G3P 9, HEMO1 5.1, and MIT 8.2 (light green values in the table) suggesting that they had a single copy of the inserted transgene. For the samples ADH1 15.1, MIT 8.3, MIT 8.4 and MIT 8.5 it should be considered that two copies were present in the genome (blue values in the table). All previous mentioned samples with a value between 0.25 and 2 were acceptable for further uses because only one or a very small number of copies was desirable. In the case of samples ENOL 7.3, HEMO1 5.2, HEMO2 13 and MIT 8.1 the ratio suggests that there were several copies of the inserted transgenes in the genome (yellow values in the table).

The sample with plant code ENOL 7.2 should be remarked because of the high number of copies (red value in the table). During the transformation there had been inserted more than 10 copies in its genome. Plants with high copy numbers are not appropriate for further stable regenerations because of the low and sometimes unstable expression of transgenes and subsequent transgene silencing (Mason *et al*., 2002).

Table V.1.2 Determination of transgene copy number by real-time PCR. Means and standard deviation of three experimental replicates are shown. Transgene DNA copy numbers were normalised with *β-actin* values. The colour code is to indicate single copy (light green), two copies (dark green), several copies (yelow) and more than ten copies (red) of the transgene copy number.

	Copy numbers per qPCR ± SD			Normalised transgene DNA level	
Plant code	***DsRed-BP100***	***Hygro^R***	*β-actin*	***Hygro^R/β-actin***	***DsRed-BP100/β-actin***
ADH1 15.1	6E+04 ± 3E3	5E+04 ± 1E3	4E+04 ± 8E2	1.3	1.4
ADH1 15.2	2E+04 ± 1E3	2E+04 ± 8E2	2E+04 ± 1E3	0.8	0.9
ADH1 15.3	6E+03 ± 2E2	5E+03 ± 3E2	9E+03 ± 7E2	0.6	0.7
ADH1 15.4	1E+04 ± 9E2	8E+03 ± 5E2	2E+04 ± 1E3	0.5	0.7
ENOL 7.1	3E+04 ± 2E3	2E+04 ± 3E2	7E+04 ± 2E3	0.3	0.4
ENOL 7.2	8E+05 ± 3E4	5E+05 ± 2E4	5E+04 ± 1E3	9.3	14.7
ENOL 7.3	2E+05 ± 6E3	7E+04 ± 2E3	7E+04 ± 3E3	1.0	2.8
G3P 9	2E+04 ± 3E3	1E+04 ± 5E2	6E+04 ± 2E2	0.3	0.3
HEMO1 5.1	3E+04 ± 4E3	1E+04 ± 4E3	4E+04 ± 3E2	0.4	0.9
HEMO1 5.2	2E+05 ± 8E3	8E+04 ± 2E3	7E+04 ± 2E3	1.1	2.3
HEMO2 13	1E+05 ± 3E3	8E+04 ± 2E3	6E+04 ± 1E3	1.3	2.4
MIT 8.1	5E+04 ± 3E3	3E+04 ± 9E2	2E+04 ± 2E3	1.7	3.0
MIT 8.2	8E+04 ± 2E2	4E+04 ± 6E2	7E+04 ± 1E3	0.7	1.1
MIT 8.3	2E+04 ± 8E2	1E+04 ± 8E2	2E+04 ± 9E2	0.8	1.4
MIT 8.4	1E+05 ± 2E3	8E+04 ± 2E3	6E+04 ± 1E3	1.4	1.9
MIT 8.5	8E+04 ± 6E3	6E+04 ± 2E3	5E+04 ± 3E3	1.3	1.6

V.2 GENE EXPRESSION ANALYSIS IN *PRUNUS PERSICA*

V.2.1- PLASMID TEMPLATE OBTAINMENT FOR GENERATING A STANDARD CURVE

As it has been mention before, for the quantification of a specific target/gene by real-time PCR a standard curve was needed. In the case of the analysis of GM rice plants, the standard curves for the transgenes (*DsRed-BP100* and *Hybro^R*) and endogenous gene (*β-actin*) had been previously obtained for the group and this specific step has not been discussed in this document. In the case of gene expression analysis all steps are performed. The aim was to contribute to the obtainment of standard curves for the analysis of expression of *pr1b*, *pr5-tlp*, *pr2* and *pr4* pathogenesis-related (PR) proteins involved in plant defence.

V.2.1.1- Amplification of specific PCR products

For each specific gene, conventional PCR amplification was performed and the PCR product was analysed by agarose gel electrophoresis (AGE). The objective was to confirm that, with the specific primers, a unique band was amplified and that the amplicon size was as expected. Genomic DNA (previously obtained for the group) was extracted from leaves of *Prunus persica* and used as template to obtain PCR products. Analysis of the PCR products by AGE confirmed that bands were rather faint and re-amplification was carried out using PCR product as a template. PCR amplification is not always a 100% efficient, and the efficiency can be decreased by different causes. It is well known that some factors like primer and magnesium concentration, temperature, may affect PCR performance, even though the same reagent concentration and the same PCR conditions were used. This is the reason why some PCRs reactions produce an intense band while others result in faint bands. This means that fewer amounts of product are obtained in the latter, and PCR re-amplification (using PCR product as template) was subsequently used to obtain a higher amplicon quantity. After re-amplification, each specific PCR product could be clearly visualised and had the expected length (**Figure V.2.1.1**).

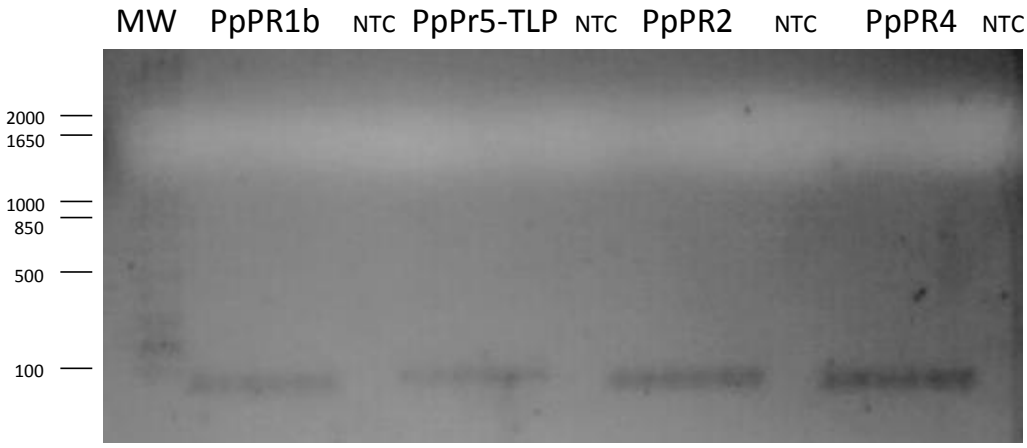

Figure V.2.1.1 PCR products obtained by re-amplification. Analysis by 2% agarose gel of triplicate reactions. Negative controls are indicated as NTC (1 reaction).

The obtained PCR products were purified with a commercial kit and quantified by spectrophotometry using a NanoDrop device. DNA concentrations and quality parameters are shown in **Table V.2.1.1.** The values of quantity and quality ratios indicated that they were in the expected intervals which confirm that all of them were adequate for further analysis

Table V.2.1.1 Concentration and quality values of the purified PCR products.

Sample	DNA conentration (ng/µL)	260/280	260/230
PpPR1b	32.9	1.83	1.91
PpPR5-TLP	27.0	1.82	1.74
PpPR2	41.1	1.87	2.20
PpPR4	42.5	1.86	2.12

V.2.1.2- Cloning and bacterial transformation

The amplified DNA fragments were directly ligated into a *pSpark*®plasmid, a commercial TA cloning vector, using the commercial kit (see section IV.3.4- Ligation). The insert and vector DNA amounts to be ligated should be in the range of 3:1 or 5:1 on a molar basis. For all genes enough ng/µl of purified PCR product was obtained for a 5:1 insert-vector ratio. Positive and negative controls were also performed. Positive control included all the ligation reagents plus a 600 bp DNA fragment (provided by the kit) which is known to have almost perfect ligation efficiency, to ensure reaction worked well. The negative control had no insert DNA, only water, so colony formation after transformation would show either contamination of extraneous DNA or binding of the plasmid sticky ends which forms a circular vector.

The results of the ligation reactions were both effective and free of re-circularised plasmids, except for the plates named PpPR1b where no colony growth was observed after incubation.

A screening based on PCR was performed to ensure that the transformed bacteria held the right inserted amplicon in the vector. The results of this screening and its visualization on agarose gel electrophoresis are shown in **Figure V.2.1.2.**

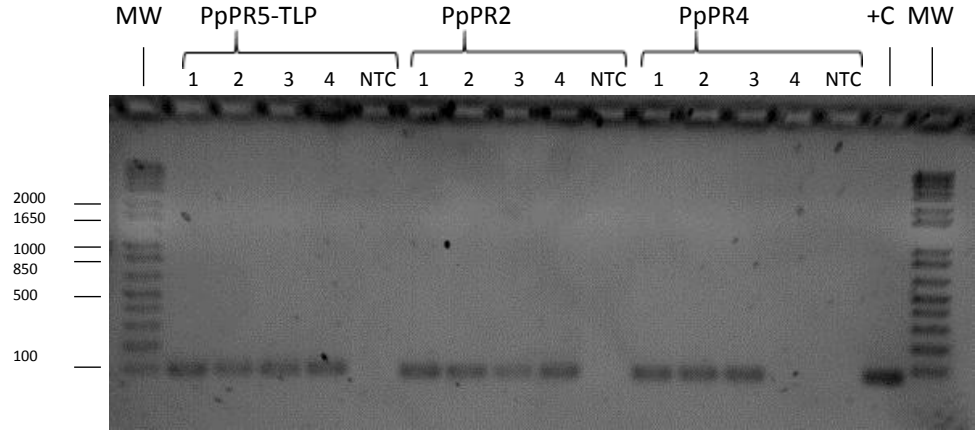

Figure V.2.1.2 Specific PCR products obtained with the screening of *E. coli* transformed colonies. Analysis by 2% agarose gel electrophoresis. For each transformation, four independent colonies and a NTC were analysed. MW: molecular weight (in base pairs); +C: positive control.

For each line one specific band and no smear was visualized and had the expected length, except for the fourth colony of PpPR4 transformation. In this case, no amplification was obtained. For the non template control, there was no amplification which means that there was no contamination of gDNA in the mastermix or primer-dimer conformation. For the positive control (+C) a brighter band was obtained because there was a lot of amplification, which means that the AGE run correctly and that the reagents of the kit worked properly. For each gene the first colony was taken to use in the next steps.

V.2.1.3- Plasmid DNA purification and quantification

A single colony carrying *pSpark*® with each insert was inoculated in liquid LB medium and overnight cultures were carried out. Purification of the plasmid DNA from suspensions of cells containing each gene fragment was performed, using a commercial kit (see section IV.3.6 plasmid DNA purification). Purified plasmid DNAs were then quantified using the NanoDrop device. Results are shown in **Table V.2.1.3.**

Table V.2.1.3. Concentration and quality of purified plasmid DNA.

Sample	DNA conentration (ng/µL)	260/280	260/230
PpPR5-TLP	92.5	1.86	1.20
PpPR2	226.6	1.94	2.02
PpPR4	140.4	1.92	1.88

The quantity and quality of these plasmid DNAs were adequate to use as template in the optimization of qPCR assays and to obtain standard curves for the quantification of gene expression levels.

V.2.2 OPTIMIZATION OF REAL-TIME PCR ASSAYS TARGETING THE SELECTED GENES

Primers were designed for annealing under standard conditions, thus the initial option is to consider standard reaction conditions for all real-time PCR's while optimizing the primer concentration in each specific reaction. To that end, three different primer concentrations were tested for each target sequence: 100, 300 and 900 nM of each primer (see section IV.4). The results obtained with 10^5 copies of target plasmid DNA per reaction are shown in **Table V.2.2**. Examples of the amplification and dissociation curves are illustrated in **Figure V.2.2**.

Table V.2.2 Summary of the real-time PCR optimisation results. Non template controls (NTC) are only shown for 100-100 nM primer concentration. NTC for 300-300 and 900-900 nM resulted negative (no amplification).

Gene	Primer concentration (nM)	Average Ct value ± SD	Average amplicon Tm
PpPR5-TLP	100-100	32.60 ± 0.079	83.6 °C
	300-300	31.43 ± 0.178	
	900-900	30.93 ± 0.277	
	NTC	46.35 ± 0.329	81.3 °C
PpPR2	100-100	30.55 ± 0.154	79.4 °C
	300-300	29.25 ± 0.104	
	900-900	29.41 ± 0.240	
	NTC	no amplification	-
PpPR4	100-100	29.99 ± 0.076	75.7 °C
	300-300	29.28± 0.204	
	900-900	29.73 ± 0.067	
	NTC	42.91 ± 0.161	79.0 °C

Since the Ct value is measured during the exponential phase of PCR, when reagents are not limited, real-time PCR can be used to reliably and accurately calculate the initial amount of template present in the reaction. A large amount of template present at the start of the amplification reaction means that relatively few amplification cycles will be required to accumulate product to give a fluorescent signal above background.

The results showed Ct values between 29 and 33 at all primer concentrations (100-100, 300-300 or 900-900 nM) for all three DNA targets. If primer concentrations are too low, the amplification will stop too early, if they are too high, they normally lead to primer dimer effects. Amplification can be reached with a low number of cycles, thus all reactions seemed to work properly (**Figure V.2.2**). Optimal primer concentration is set at the minimal primer concentrations allowing good amplification rates, so resulting in the lowest Ct value. For each system, the lowest concentration of forward and reverse primers giving a high endpoint fluorescence and low Ct value was chosen as the optimal primer concentrations. In view of the results, 100-100 nM for forward and reverse primers was chosen as the optimal concentrations. In these reactions, 10^5 copies of target DNA gave Ct values close to 30.

The melt-curve analysis function of the real-time PCR device can be used to distinguish specific from non-specific PCR products. A single Tm peak indicated a single PCR product. This was the result for all three real-time PCR assays. As an example, **Figure V.2.2** shows PpPR2 amplification plot and melting-curves obtained with 10^5 initial target molecules.

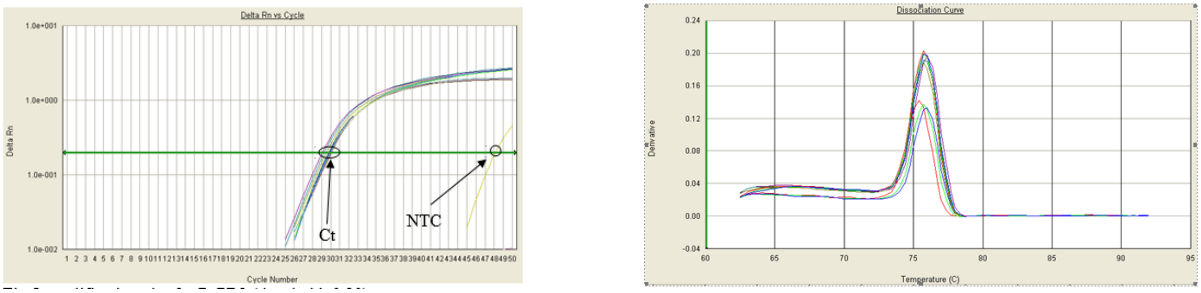

Figure V.2.2 Example of amplification and melting curve results for PpPR2. Different concentrations of primers (100-100, 300-300 and 900-900 nM) were used to amplify 10^5 target molecules.

Non template controls (NTC) produced no significant amplification curves in any developed real-time PCR assays, which indicated a lack of unspecific amplification using these reactions. Ct values above 40 did not interfere with the interpretation of the results and are most probably due to unspecific interactions among primers at very late stages of the real-time PCR. When the Ct values were close to 30 in the absence of template DNA it could have been a problem, but interpretation of positive and false positive results was expect to be easy due to the very different Tm values of the two products.

V.2.3 –PERFORMANCE OF THE REAL-TIME PCR ASSAYS TARGETING THE SELECTED GENES.

From now on and for organizational reasons, I participated in the analysis of another plant defence genes: defensin 1 (*dfn1),* NAS-1 receptor for the plant defence (*nrp1) and* ethylene response factor 1a (*erf1a*).

Serial ten-fold dilutions of every plasmid (10^6 to 10 molecules per reaction) were analysed by real-time PCR with the established primer optimal conditions (i.e. 100 nM). The linearity (R^2), efficiency (E), limit of detection (LOD) and limit of quantification (LOQ) are shown in **Table V.2.3.1**. **Figure V.2.3** shows an example of the *nrp1* amplification plot and regression curves. In **Table V.2.3.2** the Ct and standard deviation (SD) values for each gene and concentration are shown, which were important when defining LOD and LOQ especially since SD values determine whether the data points are spread out (high SD) or not (low SD) over a large range of values. In this case, the average are given by the standard curve and the data points are the replicates.

Table V.2.3.1 Performance parameters of the optimised real-time PCR assays.

Gene	Slope	Intercept	R^2	LOD	LOQ	E (%)
dfn1	-3.54	39.68	0.998	10	100	91.5
nrp1	-3.13	36.87	0.990	10	10	91.0
erf1a	-3.37	39.25	0.999	10	100	97.8

The R^2 value of a standard curve represents the linearity, how well the experimental data fit the regression line. Linearity gives a measure of the variability across assay replicates and whether the amplification efficiency is the same for different starting template copy numbers. A significant difference in observed Ct values between replicates will lower the R^2 value.

For all real-time PCR reactions an absolute value of $R^2 > 0.990$ was reached, which is considered highly adequate. The efficiencies for all reactions were calculated on the basis of the slope of the standard curve. They were all above 91% which was an optimal result for the real-time PCR reaction for all three target genes. This meant that at least 91% of the target DNA molecules are duplicated per PCR cycle.

Table V.2.3.2 Ct and SD values for each gene and at each template concentrations from 10^6 to 10 copies.

Template amount (copies)	Average Ct	SD Ct
DFN1 10^6	18.21	0.099
DFN1 10^5	22.17	0.060
DFN1 10^4	25.70	0.056
DFN1 10^3	29.01	0.158
DFN1 10^2	32.51	0.430
DFN1 10	35.46	1.939
NRP1 10^6	17.56	0.100
NRP1 10^5	21.06	0.031
NRP1 10^4	24.24	0.034
NRP1 10^3	27.75	0.096
NRP1 10^2	31.39	0.182
NRP1 10	34.25	0.563
ERF1a 10^6	19.13	0.231
ERF1a 10^5	22.27	0.173
ERF1a 10^4	25.60	0.237
ERF1a 10^3	29.27	0.168
ERF1a 10^2	32.67	0.009
ERF1a 10	37.11	1.173

The determination of the limits of detection and quantification of an assay should be typically carried out with a minimum of 10-15 replicates per target DNA dilution. In this way, LOD and LOQ (95% probability) values could be calculated. However, the expected uses of the real-time PCR assays optimized along this study were not related to detection of small amounts of target sequences of their quantification close to the LOQ. Therefore three experimental replicates per concentration and per target were carried out.

With down to ten copies of DFN1, NRP1 and ERF1a target DNA was consistently detected in all replicates: thus, the LOD was set at ten copies.

Analysis of the mean Ct and SD in the different assays at 100 and 10 target molecules indicated that the LOQ is placed close to 100 target molecules due to high SD values of 10 copies analysis. Except for NRP1 where it was still possible to quantify 10 copies.

A B

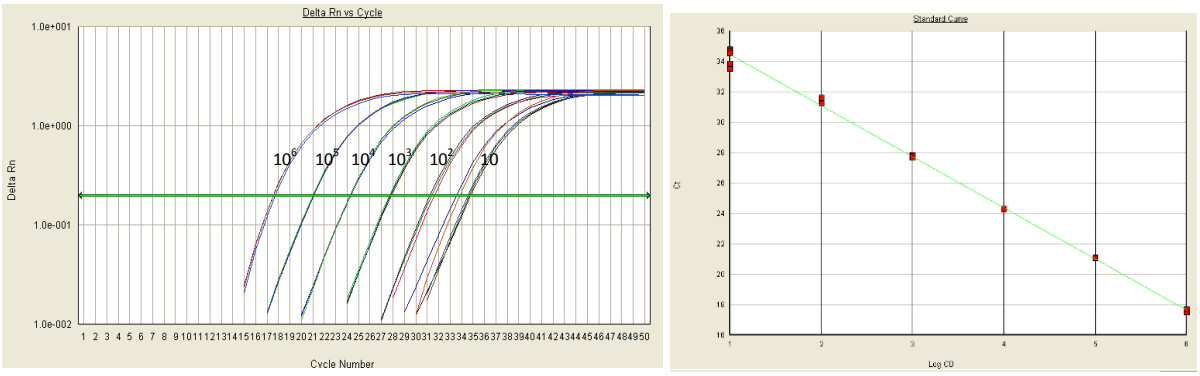

Figure V.2.3 Generation of a standard curve to assess the performance of the NRP1 specific qPCR assay. Amplification curves obtained from 10^6 till 10 target molecules of NRP1 plasmid (A). Standard curve with Ct values plotted against the log of the starting quantity of template DNA (B).

The developed real-time PCR assays proofed that a very fine tool for quantifying DNA, and the use of plasmid DNA as starting material to construct standard curves was adequate and will be used to quantify the amount of DFN1, NRP1 and ERF1a cDNA previously synthesized for the group from mRNA extracted from *Prunus perisca* plants subjected to methyl salicylate, methyl jasmonate and harpine for different times.

V.2.4- Analysis of gene expression by real-time PCR

Nrp1, *dfn1* and *erf1a* are genes supposed to be involved in plant defence response. One of the objectives of this project is to test if they were induced with the application of methyl salicylic acid, methyl jasmonic acid and harpins. The level of gene expression iss expressed by the FOLD of treatment versus control. It means how many times (after gene expression normalization) are the genes of interest overexpressed at the corresponding treatment versus the control. Samples named “Fungi infection” and “Aphid infection” corresponded to leaves obtained from plants infected with fungi and aphids. These were samples subjected to biotic stress, but the infection was moderate.

For each DNA sample four targets were analysed: the sequences *dfn1*, *nrp1* and *erf1a*; and the housekeeping gene, *Tef* (transcript elongation factor). The level of mRNA of each target was normalised by using an endogenous control, in this specific case the *Tef* sequence, which had been reported as an adequate housekeeping gene for *Prunus persica* (Zhaoguo *et al.,* 2009). The qPCR assay for the housekeeping gene was previously developed for the group and display highly linearity (R^2=0.996) and efficiency (E=99%) (C. Ruiz, personal communication). The gene was similarly expressed in all analysed samples with a Ct ranged from 18 to 21.

The values of "Normalized gene mRNA levels were obtained by dividing "the gene mRNA levels" with "the TEF mRNA levels" for each sample. To obtain the FOLD, the value of "Normalised mRNA levels" of the treatment was devided by the "Normalised mRNA levels" of the corresponding control. Samples of 1H were divided by the control of 1H. And samples of 3H by the control of 3H. In the case of Fungi infection and Aphid infection, the corresponding control is 3H. In Tables **V.2.4.1, V.2.4.2** and **V.2.4.3** the obtained results are show. For each sample three replicates were analysed and the average for each was calculated.

Table V.2.4.1 Average values of normalised DFN1 mRNA levels and fold

Sample Name	DFN1 mRNA levels	TEF mRNA levels	Normalized DFN mRNA levels	FOLD (treatment vs. control)
Fungi infection	3.02E+05	3.50E+05	8.65E-01	2.19
Aphid infection	8.80E+04	9.22E+04	9.54E-01	2.41
Metil Salycilate 2 mM 1H	1.02E+05	4.01E+04	2.54E+00	2.50
Metil Salycilate 2 mM 3H	2.70E+05	5.43E+05	4.97E-01	*1.26*
Metil Jasmonate 625 µM 1H	8.80E+04	9.27E+04	9.49E-01	*0.93*
Metil Jasmonate 625 µM 3H	3.15E+05	6.25E+05	5.04E-01	*1.27*
Hairpines 0.02% (w/v) 1H	1.96E+05	9.88E+04	1.98E+00	*1.93*
Hairpines 0.02% (w/v) 3H	1.88E+05	5.98E+05	3.15E-01	*0.80*
Hairpines 0.04% (w/v) 1H	3.59E+05	1.60E+05	2.24E+00	2.20
Control with water 1H	1.77E+05	1.75E+05	1.02E+00	*1.00*
Control with water 3H	2.01E+05	5.09E+05	3.95E-01	*1.00*

Table V.2.4.1 Values of normalised NRP1 mRNA levels and fold

Sample Name	NPR1 mRNA levels	TEF mRNA levels	Normalized NPR1 mRNA levels	FOLD (treatment vs. control)
Fungi infection	1.90E+04	3.50E+05	5.44E-02	2.00
Aphid infection	6.40E+03	9.22E+04	6.94E-02	2.54
Metil Salycilate 2 mM 1H	1.81E+04	4.01E+04	4.50E-01	*1.79*
Metil Salycilate 2 mM 3H	4.03E+04	5.43E+05	7.42E-02	2.71
Metil Jasmonate 625 µM 1H	2.31E+04	9.27E+04	2.49E-01	*0.99*
Metil Jasmonate 625 µM 3H	3.32E+04	6.25E+05	5.31E-02	1.89
Hairpines 0.02% (w/v) 1H	2.49E+04	9.88E+04	2.52E-01	*1.00*
Hairpines 0.02% (w/v) 3H	2.71E+04	5.98E+05	4.53E-02	*1.66*
Hairpines 0.04% (w/v) 1H	5.63E+04	1.60E+05	3.51E-01	*1.39*
Control with water 1H	4.40E+04	1.75E+05	2.52E-01	*1.00*
Control with water 3H	1.39E+04	5.09E+05	2.73E-02	*1.00*

Table V.2.4.1 Values of normalised ERF1a mRNA levels and fold

Sample Name	ERF1a mRNA levels	TEF mRNA levels	Normalized ERF1a mRNA levels	FOLD (treatment vs. control)
Fungi infection	6.33E+04	3.50E+05	1.81E-01	*1.53*
Aphid infection	8.67E+03	9.22E+04	9.40E-02	*0.79*
Metil Salycilate 2 mM 1H	2.10E+04	4.01E+04	5.25E-01	2.10
Metil Salycilate 2 mM 3H	9.03E+04	5.43E+05	1.66E-01	*1.41*
Metil Jasmonate 625 µM 1H	3.08E+04	9.27E+04	3.32E-01	*1.33*
Metil Jasmonate 625 µM 3H	7.24E+04	6.25E+05	1.16E-01	*0.98*
Hairpines 0.02% (w/v) 1H	2.49E+04	9.88E+04	2.52E-01	*1.01*
Hairpines 0.02% (w/v) 3H	1.23E+05	5.98E+05	2.06E-01	*1.74*
Hairpines 0.04% (w/v) 1H	4.49E+04	1.60E+05	2.80E-01	*1.12*
Control with water 1H	4.37E+04	1.75E+05	2.50E-01	*1.00*
Control with water 3H	6.02E+04	5.09E+05	1.18E-01	*1.00*

When the FOLD was between 0.5 and 2, it meant that there was no expression of the corresponding gene because the final mRNA level of the gene expression was equal, slowly smaller or higher than the initial mRNA level (values indicates in italics in the tables). This situation was represented by the majority of the results. The application of three hour, instead of one hour, do not always result in higher gene expression levels.

However, some interesting treatments must be considered. The gene *dfn1* was especially induced after fungi infection, aphid infection, application of metil salycilate (2 mM 1H) and hairpines (0.04% 1H). The gene *nrp1* is especially expressed by aphid and fungi infection and methyl salycilate (2 mM 3H). In plants under stress, salicylic acid (produced by the plant) bound directly to NRP1 receptor which in his turn activated other defense genes. So that is why after the application with methyl salycilate the *nrp1* gene has been activated in the plant and that the infections also lead to the regulations of nrp1 expression to induces the defense genes.

The last studied gene, *erf1a* is only induced by metil salycilate (2 mM 1H) and the induction is at the limit of 2 fold. Normally *erf1a* also should be expressed after treatment with methyl jasmonate and the pathogen infections, because these treatments should encode the transcription factor that regulates the expression of pathogen response genes that prevent disease progression. But in these experiments this changes in the expression levels had not been detected.

From these preliminary results, it can be concluded that the methyl salicylate did induce moderately the expression of genes related to resistance disease (i.e. *dfn1* and *nrp1*) but the duration of application was an important factor depending on the gene of interest. Although it will be necessary to carry out more experiments, this organic component could be a possible product to activate defense mechanisms of *Prunus persica* against invading pathogens such as fungi and bacteria.

VI. CONCLUSIONS

VI.1 ANALYSIS OF TRANSGENE COPY NUMBER IN GM RICE PLANTS

1. Genomic DNA from GM rice plants was successfully obtained and the concentration and quality values were suitable for qPCR analysis

2. Seven GM plants had a single copy of the transgene (i.e. 43% analysed plants); and four (25%) were estimated to have 2 transgene copies. They were suitable for further assays

3. Four additional plants had several transgene copies and a single plant (6%) had incorporated more than 10 transgene copies in the genome. They were not appropriate for further investigation

VI.2 GENE EXPRESSION ANALYSIS IN *PRUNUS PERSICA*

1. Amplification of the three target DNA fragments, *pr5-tlp*, *pr2* and *pr4* was successfully achieved by conventional PCR. All PCR products had the expected length and were cloned into *pSpark* plasmids

2. Plasmid DNA harbouring the specific target is an adequate template to generate the standard curve in qPCR quantification analyses

3. Three qPCR assays were optimized for quantification of the selected target genes (*pr5-tlp*, *pr2* and *pr4*). Optimal primer concentration was set at 100 nM for forward and reverse primers

4. Performance analysis of three qPCR assays (*dfn1, nrp1 and erf1a*) showed high linearity ($R^2 > 0.990$) and efficiency (> 91%). The LOD was placed at 10 target molecules in the 3 assays; and the LOQ was 10 target molecules for *nrp1* and 100 target molecules for *dfn1* and *erf1a*

5. Fungi and aphid infection induced the expression of the disease-resistance genes *dfn1* and *nrp1*. The same genes were moderately induced by methyl salicylate. The possible implications of these preliminary results would require further investigations

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