

SPATIOTEMPORAL STUDY OF POTATO
HYPERSENSITIVE RESPONSE-CONFERRED
RESISTANCE TO POTATO VIRUS Y

Tjaša Lukan

Doctoral Dissertation
Jožef Stefan International Postgraduate School
Ljubljana, Slovenia

Supervisor: Prof. Dr. Kristina Gruden, National Institute of Biology, Ljubljana, Slovenia and Jožef Stefan International Postgraduate School, Ljubljana, Slovenia
Co-Supervisor: Dr. Špela Baebler, National Institute of Biology, Ljubljana, Slovenia

Evaluation Board:

Prof. Dr. Marina Dermastia, Chair, IPS and National Institute of Biology, Ljubljana, Slovenia and Jožef Stefan International Postgraduate School, Ljubljana, Slovenia
Assoc. Prof. Dr. Maruša Pompe-Novak, Member, National Institute of Biology, Ljubljana, Slovenia
Dr. John Carr, Member, University of Cambridge, Cambridge, Great Britain

MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
JOŽEF STEFAN INTERNATIONAL POSTGRADUATE SCHOOL



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ČASOVNOPROSTORSKA ŠTUDIJA
PREOBČUTLJIVOSTNEGA ODGOVORA KROMPIRJA NA
OKUŽBO S KROMPIRJEVIM VIRUSOM Y

Doktorska disertacija

Supervisor: Prof. Dr. Kristina Gruden

Co-Supervisor: Dr. Špela Baebler

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Abstract

Plant pathogens are responsible for substantial economic losses. Understanding the exact mechanisms of plant response to pathogens is required for designing stress resilient plant varieties. One of our aims was to decipher a plant immune regulatory network through characterisation of the promoters in different potato genotypes. These could in the next stage be used for designing specific sensors of immune signalling which would allow precise monitoring of spatiotemporal dynamics of this process. Nucleotide alignments of the promoter sequences from selected genes involved in plant hormonal signalling showed high variability between, but also within the analysed genotypes. Accordingly, in silico analysis of the promoter variants resulted in the identification of diverse pathogen-inducible regulatory elements in the promoters of the selected genes. The activities of the promoter variants with the most promising combination of regulatory elements were analysed in transient and stable expression set-ups after promoters were fused with fluorescent proteins. To prepare a sensor which will enable simultaneous following of the activity of several promoters, different combinations of fluorescent proteins under different promoters first need to be introduced into the plant genome. As cloning multiple DNA fragments is still one of the main technological challenges in plant synthetic biology, we developed Plant X-tender, an extension of multigene assembly strategy AssemblX, based on overlap-dependend cloning methods and rare-cutting restriction enzymes. Plant X-tender consists of a set of plant expression vectors and the protocols for the most efficient cloning into the novel vector set. It covers different empty backbones with different selection markers to allow full design flexibility and includes *ccdB* counterselection to allow straightforward and highly efficient transfer of multigene constructs into the novel vector set. We have further validated the developed procedure for plant multigene expression in a transient set-up. In the third part of this thesis, we studied the involvement of cell death in hypersensitive response (HR)-conferred resistance to viral infection. We showed that potato virus Y can be detected outside the cell death zone in *Ny-1*-mediated HR in potato plants (cv. Rywal), observed as individual infected cells or clusters of infected cells outside the cell death zone. We confirmed that the cells at the border of the cell death zone harbour a viable virus that is able to reinitiate the infection. We further studied the dynamics of cell death zone expansion and occurrence of infected cells outside it. We compared the response of Rywal plants to their transgenic counterparts, impaired in salicylic acid accumulation, where the lesions develop but the spread of the virus is not restricted. We showed that the virus is detected outside the cell death zone in all developmental stages of HR lesions. We observed rapid lesion expansion and even faster virus spread in salicylic acid-depleted plants. On the other hand, the majority of analysed lesions slowly expand also in HR-conferred resistance opening the possibility that the infected cells are eventually engulfed by cell death

zone. Thus we suggest that the HR-associated cell death is separated from the resistance mechanisms which lead to potato virus Y restriction in *Ny-1* genetic background and propose that HR should be regarded as a process where the dynamics of events is crucial for the effectiveness of viral arrest.

Povzetek

Povzročitelji bolezni rastlin so vzrok za znatne ekonomske izgube. Razumevanje mehanizmov odgovora rastlin na škodljivce je ključno za pridobivanje odpornih genotipov. V prvem delu doktorata smo preučevali promotorje iz različnih genotipov krompirja, ki so vključeni v regulatorno omrežje imunskega odgovora. Te bi v naslednjem koraku lahko uporabili za pripravo specifičnih senzorjev signalizacije v imunskem odgovoru, kar bi omogočilo natančno spremljanje prostorsko-časovne dinamike tega procesa. Poravnave nukleotidnih zaporedij promotorjev izbranih genov, vključenih v rastlinsko hormonsko signalizacijo, so pokazale veliko variabilnost tako med genotipi, kot tudi znotraj istega genotipa. Prav tako smo v promotorjih izbranih genov z analizami in silico določili različne regulatorne elemente, ki se aktivirajo ob napadu povzročiteljev bolezni. Promotorje z ustrezno kombinacijo regulatornih elementov smo sklopili s fluorescenčnimi proteini in njihovo aktivnost določili po prehodni in stabilni transformaciji. Za pripravo senzorja, ki bi omogočal sočasno spremljanje aktivnosti več promotorjev, je potrebno v rastlinski genom vnesti različne kombinacije fluorescenčnih proteinov pod kontrolo različnih promotorjev. Ker je kloniranje več fragmentov DNA še vedno eden od tehnoloških izzivov v rastlinski sintezni biologiji, smo razvili Plant X-tender, ki vsebuje komplet rastlinskih ekspresijskih vektorjev in protokole za učinkovito kloniranje večgenskih konstruktov v pripravljene vektorje. Vektorji predstavljajo prazna ogrodja z različnimi selekcijskimi markerji, kar omogoča popolno fleksibilnost pri načrtovanju konstruktov. Poleg tega vsebujejo kaseto *ccdB*, ki omogoča preprost in učinkovit prenos večgenskih konstruktov v pripravljene vektorje. Metodo smo validirali z izražanjem genov po vnosu večgenskega konstrukta s prehodno transformacijo. V tretjem delu doktorske disertacije smo preučevali vlogo celične smrti v s preobčutljivostnim odzivom pogojeni odpornosti na okužbo s krompirjevim virusom Y pri krompirju. Pokazali smo, da se v rastlinah krompirja, pri katerih je preobčutljivostni odziv pogojen z genom *Ny-1* (sorta Rywal), virus nahaja izven območja celične smrti, kar smo opazili kot posamezne okužene celice ali majhne skupke okuženih celic. Potrdili smo, da celice na meji območja celične smrti vsebujejo virus, ki lahko povzroči ponovno okužbo. Nadalje smo preučevali dinamiko širjenja območja celične smrti in pojavljanje okuženih celic izven tega območja. Odziv v rastlinah krompirja sorte Rywal po okužbi z virusom smo primerjali z odzivom v rastlinah krompirja, pri katerih ne pride do kopičenja salicilne kisline. V teh rastlinah pride do pojava lezij, širjenje virusa pa ni omejeno. Pokazali smo prisotnost virusa izven območja celične smrti v vseh razvojnih stopnjah nekrotičnih lezij. Opazili smo hitro širitev lezij in še hitrejše širjenje virusa v rastlinah, pri katerih ne pride do kopičenja salicilne kisline. Poleg tega se večina analiziranih lezij počasi razširja tudi v rastlinah, pri katerih je odpornost pogojena s preobčutljivostnim odzivom, kar odpira možnost, da grejo okužene celice sčasoma v celično smrt. Rezultati kažejo na to, da je celična smrt v

preobčutljivostnem odgovoru ločena od mehanizmov odpornosti, ki vodijo do zaustavitve širjenja krompirjevega virusa Y v genetskem ozadju Ny-1. Predlagamo, da se s preobčutljivostnim odzivom pogojena odpornost obravnava kot proces, pri katerem je dinamika dogodkov ključna za učinkovitost zaustavitve širjenja virusa.

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Abbreviations

ABA	. . .	abscisic acid
ACC	. . .	1-aminocyclopropane-1-carboxylic acid
ACO	. . .	ACC oxidase
AM	. . .	assembly method
Amp	. . .	ampicillin
AOC	. . .	allene oxide cyclase
AOS	. . .	allene oxide synthase
AOX	. . .	alternative oxidase
AP2/ERF	. . .	APETALA2/ethylene responsive factor
as-1/ocs	. . .	activator sequence-1
ASX	. . .	AssemblX
AttR	. . .	Gateway cloning recombination sites
Avr	. . .	avirulence protein
bZIP	. . .	basic-region leucine zipper
CaMV	. . .	cauliflower mosaic virus
CAPE1	. . .	CAP-derived peptide 1
CBF	. . .	ET-responsive element-binding
CDPK	. . .	Ca-dependent protein kinase
CDF	. . .	cumulative distribution function
CDS	. . .	coding sequence
CHS	. . .	chalcone synthase
CMV	. . .	cucumber mosaic virus
COI1	. . .	coronatine insensitive 1
Cox	. . .	cytochrome oxidase
CP	. . .	coat protein
CPBS	. . .	colorado potato beetle saliva
CPEC	. . .	circular polymerase extension cloning
CRFD	. . .	cumulative relative frequency distribution
CTR1	. . .	Raf-like kinase
DIECA	. . .	diethyldithiocarbamic acid
dpi	. . .	days post inoculation
ECFP	. . .	enhanced cyan fluorescent protein
EGFP	. . .	enhanced green fluorescent protein
ER	. . .	extreme resistance
ET	. . .	ethylene
ETI	. . .	effector triggered immunity
ETS	. . .	effector triggered susceptibility
FP	. . .	fluorescent protein
GAs	. . .	gibberellins
GST	. . .	glutathione S-transferases
gDNA	. . .	genomic DNA
H2BRFP	. . .	histon sequence fused to red fluorescence protein
Hd3a	. . .	(mRFP1)
HiFi	. . .	Heading date 3a
HM	. . .	NEBuilder HiFi DNA assembly method
HR	. . .	homemade TOP10 electrocompetent <i>E. coli</i>
Hyg	. . .	hypersensitive response
ICS	. . .	hygromycin
INA	. . .	isochorismate synthase
JA	. . .	2,6-dichloroisonicotinic acid
JAZ	. . .	jasmonic acid

JERE	. . .	jasmonate-ZIM domain protein
Kan	. . .	jasmonate-responsive regulatory element
LB	. . .	kanamycin
LOD	. . .	Luria-Bertani medium; left border of T-DNA
MAMP	. . .	limit of detection
MC	. . .	pathogen-associated molecular patterns
mCherry	. . .	multicystatin
MCS	. . .	monomeric red fluorescent protein 1
MeJA	. . .	multiple cloning site
MeSA	. . .	methyl jasmonate
MR	. . .	methyl salicylate
mRFP1	. . .	molar ratio between the plasmid backbone and the
NO	. . .	insert
NPR1	. . .	monomeric red fluorescent protein 1
OPDA	. . .	nitric oxide
OPR3	. . .	nonexpresser of PR genes 1
p35S	. . .	12-oxophytodienoic acid
PAL	. . .	oxophytodienoate-reductase3
PAMP	. . .	cauliflower mosaic virus CaMV 35S promoter
PCD	. . .	phenylalanine ammonia-lyase
PCR	. . .	pathogen-associated molecular patterns
PD	. . .	programmed cell death
PIAMV	. . .	polymerase chain reaction
PMC	. . .	plasmodesmata
pNOS	. . .	plantago asiatica mosaic virus
PR	. . .	potato multicystatin
PRR	. . .	nopaline synthase promoter
PTI	. . .	pathogenesis-related
PVY	. . .	pathogen recognition receptor
qPCR	. . .	PAMP-triggered immunity
RT-qPCR	. . .	potato virus Y
RB	. . .	quantitative PCR
RBOH	. . .	reverse transcription quantitative PCR
Re	. . .	right border of T-DNA
R gene	. . .	respiratory burst oxidase homologue
ROS	. . .	selection marker conferring resistance in <i>E. coli</i>
Rp	. . .	and <i>A. tumefaciens</i>
SA	. . .	dominant resistance gene
SABP2	. . .	reactive oxygen species
SABP3	. . .	selection marker conferring resistance in plants
S-AdoMet	. . .	salicylic acid
SAMT	. . .	methyl esterase
SAR	. . .	carbonic anhydrase
SARE	. . .	S-adenosyl-methionine
SLIC	. . .	SA methyl transferase
SLiCE	. . .	systemic acquired resistance
SNP	. . .	SA-inducible regulatory element
SOD	. . .	sequence and ligation independent cloning
Spec	. . .	Seamless ligation cloning extract cloning method
t35S	. . .	single-nucleotide polymorphisms
TAR	. . .	superoxide dismutase
TCV	. . .	spectinomycin
TF	. . .	cauliflower mosaic virus CaMV 35S terminator
TM	. . .	cloning based on transformation-associated
TMV	. . .	recombination
TRX	. . .	turnip crinkle virus
URA3	. . .	transcription factor
USER	. . .	transformation method
VRC	. . .	tobacco mosaic virus
w/v	. . .	thioredoxin
YFP	. . .	yeast selection marker
		uracil-specific excision reagent cloning
		viral replication complex

weight/volume
yellow fluorescent protein

Chapter 1

Introduction

1.1 Plant Immune Response

Plants have evolved sophisticated mechanisms to perceive pathogen attack and trigger an effective immune response. Even though the plant immune system is less complex if compared to the well-studied immune system of mammals, it is highly specific and often enables a durable memory of the encountered pathogens (reviewed in Spoel and Dong, 2012). Plants lack a circulatory system and mobile immune cells specific for the vertebrates, but like all multicellular organisms, they possess an innate immune system, based on germ line-encoded components. Innate immune response responds to molecules common to different pathogens by recognizing pathogen-associated molecular patterns (PAMP) via receptor-like proteins known as pathogen recognition receptors (PRR). This immune response is known as PAMP-triggered immunity (PTI). In response to PTI, some pathogens have evolved effector proteins to overcome PTI. This process, referred to as effector-triggered susceptibility (ETS), allows the pathogen to establish a successful infection (reviewed in Jones and Dangl, 2006; Bigeard et al., 2015). As an additional layer of defense, plants have another, more specific branch of plant innate immunity, known as effector-triggered immunity (ETI). Here, the outcome of the attempted infection is determined by the genotypes of the host and the pathogen (Figure 1.1). Effectors that enable pathogens to overcome PTI, termed avirulence (Avr) proteins, are recognized by specific plant disease resistance (R) genes. R genes are conferring resistance in a variety of plant-pathogen interactions (Wu et al., 2014). The majority of R genes encode NB-LRR proteins, composed of nucleotide-binding and leucine-rich repeat, which are intracellular and effective only against (hemi)biotrophic pathogens (Lee and Yeom, 2015; Glazebrook, 2005). Direct interaction (gene-for-gene hypothesis) has been confirmed for some R genes and corresponding Avr proteins (Dodds et al., 2006; Ueda et al., 2006, Jia et al., 2000). On the other hand, some NB-LRR genes have co-opted cellular recognition co-factors, referred to as guarder, decoy or bait (naming is depended on the proposed recognition model), which mediate their interaction with Avr proteins (Moffett, 2009; Sarris et al., 2015). Over the years, the increasing number of sequenced plant genomes has enabled identification of NB-LRR resistance genes in various plant species, including *Arabidopsis* (*Arabidopsis thaliana*) (Meyers et al., 2003), potato (*Solanum tuberosum* L.) (Jupe et al., 2012; Lozano et al., 2012), tomato (*Solanum lycopersicum*) (Andolfo et al., 2013) and cassava (*Manihot esculenta*) (Lozano et al., 2015). For example, 159 NB-LRR genes were identified in *Arabidopsis* (Guo et al., 2011), while 755 NB-LRR genes were identified in *S. tuberosum* clone DM (Jupe et al., 2013). However, only a small

proportion of all known R genes have been cloned and characterized to date (Witek et al., 2016).

		Plant genotype	
		no R gene	R gene
Pathogen genotype	no Avr	Disease	Disease
	Avr	Disease	Resistance

Figure 1.1: Gene-for-gene resistance. The resistance depends on the genotypes of both the host and the pathogen. Resistance occurs if the pathogen possesses avirulence (Avr) protein, which is recognized by the host resistance (R) gene. Adapted from *Advances in Virus Research*, First edition, Chapter 1: Mechanisms of recognition in dominant R gene mediated resistance, Moffett, Pages 1-23, 2009.

Gene-for-gene activation is followed by the induction of rapid and transient redox potential changes (Matika and Loake, 2014), altered cytoplasmic and chloroplast calcium (Ca^{2+}) fluxes (Seybold et al., 2014) and activation of diverse signalling cascades in the host plant (Pieterse et al., 2009, Tsuda and Somssich, 2015), which will be described in more detail in the following sections.

1.1.1 Plant immune response against virus infection

A large number of plant viruses are potentially capable of infecting plants. Even though, the development of the disease is an exception rather than a common outcome as only a few of them can cause serious diseases. In most other cases, plant defense mechanisms prevent virus multiplication (Pallas and Garcia, 2011). To establish a successful infection, plant viruses have to overcome a defense system composed of several layers when invading the host cell. First they need to overcome the physical barrier of a cell wall to enter the host cells via mechanical inoculation or infection, usually mediated by the vectors (Ivanov et al., 2014).

The genome of the majority of plant viruses is positive sense single-stranded RNA (Pallas and Garcia, 2011). One of the largest families of this group is Potyviridae, with several members of significant economic importance, including the genus Potyvirus. Once the potyvirus enters the cell, the virus coat protein disassembles and releases the RNA into the cytosol (Figure 1.2). The viral RNA serves as mRNA which is translated by the host ribosomes into a polyprotein, which is then processed by viral proteases to yield different viral proteins. Resulting proteins form viral replication complexes (VCRs), which are transported

towards the cell wall and plasmodesmata to facilitate cell-to-cell movement. VRC-derived viral RNA can be translated and incorporated into progeny VRCs, allowing the RNA to participate in new rounds of viral replication (reviewed in Ivanov et al., 2014; Mäkinen and Hafrén, 2014).

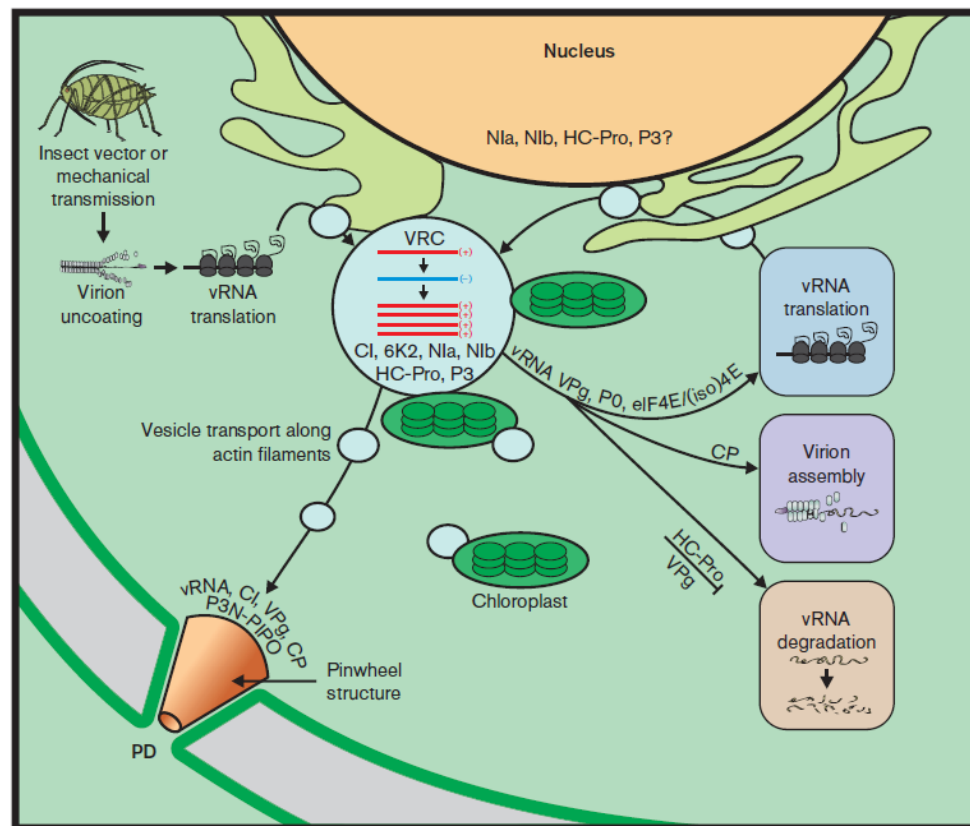


Figure 1.2: Replication cycle of the potyvirus. Virus enters the cell by mechanical inoculation or insect vector. Following virion uncoating, positive sense single-stranded viral RNA is translated into a polyprotein, which is processed by proteases. Resulting viral proteins are incorporated in the replication complexes (VRCs), which are transported to the plasmodesmata to participate in cell-to-cell movement. VRC-derived viral RNA can enter new cycles of viral replication, can be degraded or packed into virions. vRNA: viral RNA, CP: coat protein; PD: plasmodesmata, VRC: replication complex, eIF4E/(iso)4E: translation initiation factor, CI, 6K2, Nla, Nlb, HC-Pro, P3, VPg, CP and P3N-PIPO: viral proteins. Republished with permission of Microbiology Society, from Molecular and cellular mechanisms underlying potyvirus infection, Ivanov et al., Volume 95, 2014; permission conveyed through Copyright Clearance Center, Inc.

While viral RNA is multiplying and spreading, it is simultaneously exposed to the host innate immune responses (Mäkinen and Hafrén, 2014). One of the first is RNA silencing, triggered by the viral RNA (reviewed in Csorba et al., 2015; Figure 1.3, left panel). If compared to PTI triggered by non-viral pathogens, the detection of viral RNA occurs intracellularly, while the detection of non-viral MAMP/PAMP occurs extracellularly (de Ronde et al., 2014; Ding and Voinnet, 2007). However, several recent publications suggest the involvement of membrane-associated receptors, which recognize the dsRNAs and induce

typical PTI responses, separate from RNA silencing (Niehl et al., 2016).

In response to RNA silencing, viruses have evolved proteins which interfere with a specific part of the RNA silencing pathway to inhibit its effectiveness, also known as viral suppressors of RNA silencing (VSR) which allows the virus to establish a successful infection (reviewed in Calil and Fontes, 2017; Figure 1.3, middle panel). Next layer of defense involves R genes, which recognize viral proteins (corresponding to Avr proteins in other (hemi)biotrophic pathogens) and induce effector-triggered immunity (ETI) (reviewed in Moon and Park, 2016; Figure 1.3, right panel). Importantly, however, many of the viral proteins recognized by R genes are not VSRs and may not necessarily be analogous to the effector proteins of other pathogens (Moffet, 2009; Cheng and Wang, 2017). As in other (hemi)biotrophs, the major class of viral R genes represents the NB-LRR type of dominant resistance gene (reviewed in Carr et al., 2010; de Ronde et al., 2014). Avr determinants have been identified only for approximately one third of more than 200 known R genes directed against plant viruses (reviewed in de Ronde et al., 2014).

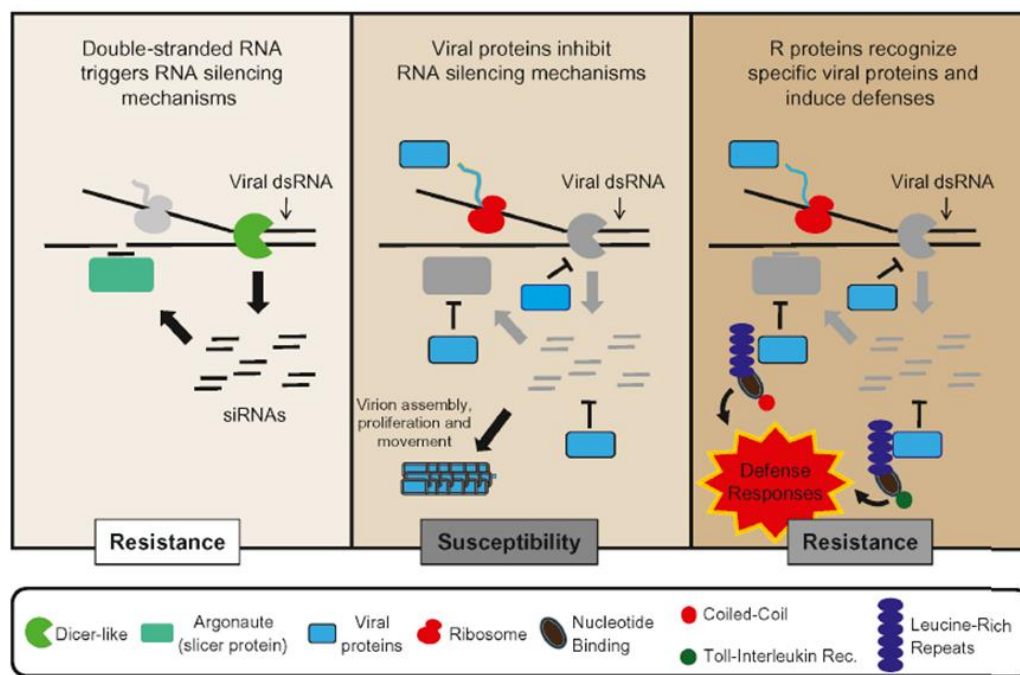


Figure 1.3: Plant immune response against viruses. Left: PAMP-triggered immunity (PTI). Once the virus has entered the plant cell, RNA silencing mechanisms result in the degradation of viral RNA to protect plants against most viruses. Middle: Some viruses have evolved effectors, which suppress RNA silencing on different levels of RNA silencing pathway. Right: In response to viral effectors some hosts have evolved resistance (R) proteins that recognize the effectors to induce effector-triggered immunity (ETI). Republished from *Advances in Virus Research*, First edition, Chapter 1: Mechanisms of recognition in dominant R gene mediated resistance, Moffett, Pages 1-23, 2009, with permission from Elsevier; permission conveyed through Copyright Clearance Center, Inc.

Activation of NB-LRR proteins can result in the induction of the programmed cell death (PCD), manifested as visible necrotic

lesions at the site of virus entry and prevention of further virus spread by activating downstream defense responses. This form of resistance confers extreme resistance (ER) or HR-conferred resistance. In the case of HR-conferred resistance, some viral accumulation is detected also outside the initially infected cell (Palukaitis and Carr, 2008). In some cases, R genes do not confer absolute resistance and low levels of virus replication can still be observed. These R genes are referred to as partial resistance genes (reviewed in de Ronde et al., 2014).

1.1.1.1 Potato response against potato virus Y

Potato is the fourth-most-important food crop in the world, with a yield of 377 million tonnes in 2016, and a continuous increase in the world production of tubers (<http://faostat.fao.org/>). The most harmful viral pathogen affecting cultivated potatoes is the potato virus Y (PVY), a member of *Potyvirus* genus from family *Potyviridae*. PVY has also been classified as one of the plant viruses with the highest economic and scientific importance (Scholthof et al., 2011). The infection of the potato field with PVY may reduce crop yields up to 100 % (Karasev and Gray, 2013).

As other members of family *Potyviridae*, its genome is a single stranded positive sense RNA. The genome is translated into a single polyprotein, which is proteolytically processed by virus-encoded proteinases resulting in the production of ten mature proteins. One of them is the capsid protein (CP), which encapsidates the viral RNA to form filamentous viral particles (Karasev and Gray, 2013). A conserved feature of potyviruses is the existence of a short ORF embedded within the P3-encoding region in a reading frame different from the polyprotein, which is used to express the P3N-PIPO protein (Reverse and Garcia, 2015).

The severity of the infection outcome and symptoms appearance depend on the potato cultivar, virus strain and environment. Following genetic definition of PVY strains we distinguish five original PVY strain groups PVY⁰, PVY^C, PVY^Z, PVY^N and PVY^E. Recombination between PVY⁰ and PVY^N resulted in the occurrence of PVY^{NTN}, currently known as the most aggressive PVY strain (Singh et al., 2008; Karasev and Gray, 2013). Different potato cultivars show different levels of sensitivity against PVY, from susceptibility to extreme resistance. The outcome of the infection is determined by the plant-virus interaction (Figure 1.4). In sensitive potato cultivars (e.g. cv. Igor, cv. Nadine), PVY can elicit a development of potato tuber necrotic ringspot disease, causing a decrease in the quality and quantity of potato production. Several potato cultivars also express tolerant response in which the virus is efficiently multiplying, but the disease symptoms do not appear (e.g. cv. Désirée, cv. Pentland Squire) (Lacomme et al., 2017). On the other hand, resistance to PVY in potato is conferred by two types of resistance genes, *Ry* genes in case of ER and *Ny* genes in case of HR (Kogovšek and Ravnikar, 2013). Symptoms do not develop in the ER response as the multiplication of the virus is limited to initially infected cell (e.g. cv. Sante, cv. White Lady, breeding clone PW363), while HR-conferred resistance results in development of local necrosis as a consequence of virus multiplication and spreading in the proximity of the initially infected cell (e.g. Rywal) (Lacomme et al., 2017). The best studied type of plant resistance to PVY is HR (Armengot et al., 2014). In potato cv. Rywal, HR is initiated by *Ny-1* resistance gene and is manifested by the formation of necrotic lesions on inoculated leaves three days post inoculation (dpi) (Szajko et al., 2008). Another major parameter affecting the infection outcome is the temperature. In several cases of HR-

conferred resistance, the virus is efficiently restricted only at lower temperatures, while the increase of temperature results in virus spread (Szajko et al., 2008).

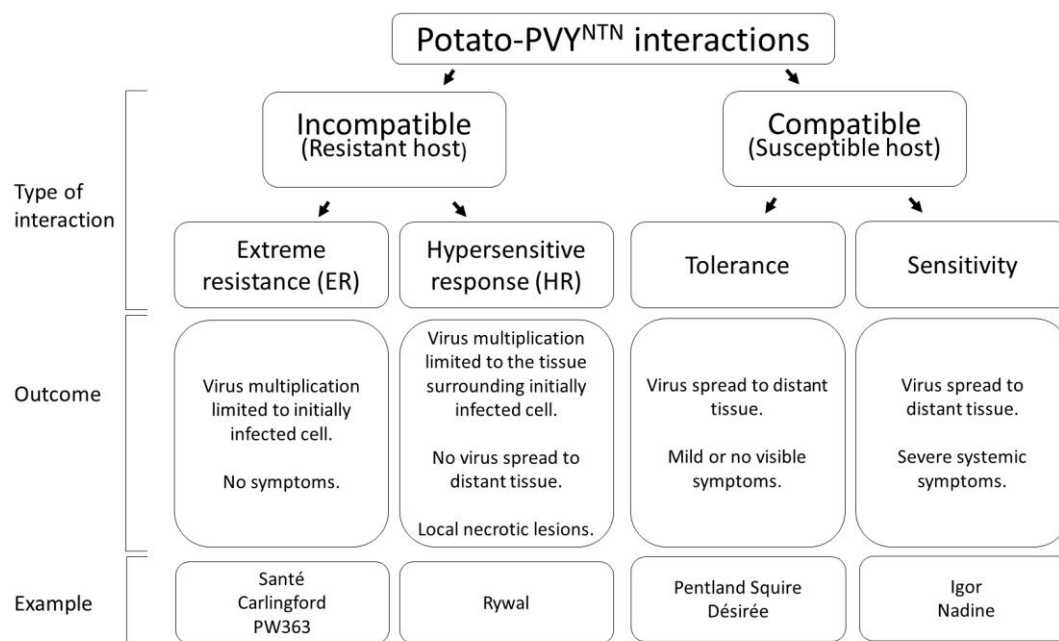


Figure 1.4: Outcomes of potato-PVY^{NTN} interaction. Outcomes depend on the host sensitivity and are manifested in different responses in terms of virus multiplication and symptom development in different potato genotypes. Adapted from Potato virus Y: biodiversity, pathogenicity, epidemiology and management, First edition, Chapter 3: Molecular and Cellular Events During Infection of Potato by PVY, Pompe-Novak and Lacomme, Pages 21-42, 2017.

1.1.2 Hypersensitive response

As already mentioned above, HR-conferred resistance is an effective defense response that restricts pathogen spread. It is often manifested as the formation of necrotic lesions at the site of pathogen entrance (Mur et al., 2008). Our understanding of the mechanisms that underlie HR-conferred resistance against pathogens is still incomplete. Following pathogen recognition by the R protein, transduction of the perceived signal to the inducers of cell death is engaged, PCD is induced and the pathogen is restricted to the site of inoculation (Figure 1.5; Singh and Upadhyay, 2013).

It has long been known that the salicylic acid (SA) plays a central role in downstream defense responses and is required for the localization of pathogens during HR. Although the role of SA in virus restriction is not as well understood as SA-mediated resistance to other (hemi)biotrophic pathogens (Carr et al., 2009), there are several examples confirming the involvement of SA in HR also in different plant-virus interactions. For example, SA is involved in inhibition of virus replication and spread in tobacco after TMV infection (Chivasa et al., 1997; Chivasa and Carr, 1998) and was shown to be a key component in the orchestration of the events restricting PVY spread in resistant potato cultivar Rywal (Baebler et al., 2014). In the above-mentioned studies, the importance of SA was determined by the use of NahG-transgenic plants, where the endogenous SA is degraded by

SA hydroxylase or by the use of inhibitors of alternative oxidase (AOX), which antagonizes SA-induced resistance to TMV.

Another characteristic feature of the HR is the rapid and intense production of reactive oxygen species (ROS) (Zurbriggen et al., 2010). The production of ROS in the apoplast in response to pathogens has been proposed to orchestrate the establishment of different defensive barriers against the pathogens (reviewed in Torres et al., 2006). Several reports demonstrated the involvement of respiratory burst oxidase homologue (RBOH) gene family in the production of apoplastic ROS during the defense responses as the main mechanism responsible for oxidative burst in several pathosystems (Torres and Dangl, 2005). Analyses of mutants and antisense lines have identified RBOH as the principal generator of ROS after pathogen attack in several compatible interactions, such as biotrophic bacterial or oomycete pathogens-*Arabidopsis* (Torres et al., 2002) and hemibiotrophic oomycete-*Nicotiana benthamiana* (Yoshioka et al., 2003). In addition, there are also reports of ROS being produced during compatible interactions involving virus, for example, TMV-*Nicotiana tabacum* interaction (Allan et al., 2001). The exact function of the ROS in resistance is still debated. Although ROS production is strongly correlated with the early defense response, RBOHD-induced oxidative burst could not be confirmed as directly associated with the HR-associated cell death and/or the restriction of the pathogen growth in some pathosystems (Torres et al., 2002; Love et al., 2005; Allan et al., 2001; Pogany et al., 2009). Moreover, reduced ROS production, obtained by silencing or eliminating RBOH, resulted in variable effects on pathogen growth and HR-associated cell death (reviewed in Torres et al., 2006; Torres and Dangl, 2005; Pogany et al., 2009).

Following ROS production, the change in redox potential activates Ca^{2+} influx, which functions upstream (regulation of RBOHD) and downstream (positive feedback on ROS production) of ROS production. Moreover, Ca^{2+} activates reversible protein phosphorylation through mitogen-activated protein kinase (MAPK) cascades and Ca^{2+} -dependent protein kinases (CDPK) (Buscail and Rivas, 2014). In addition, nitric oxide (NO) accumulation was proposed to be connected to HR (Ling et al., 2012).

Activation of this network of signal transduction has been associated with the expression of pathogen-responsive genes in several plant-pathogen interactions (Buscail and Rivas, 2014). These pathogen responsive genes encode glutathione S-transferases (GST), peroxidases, cell wall proteins, proteinase inhibitors, hydrolytic enzymes (chitinases, β -1,3-glucanases), PR proteins and phytoalexin biosynthetic enzymes, such as phenylalanine ammonia lyase and CHS (Kushalappa et al., 2016). Some of them possess antifungal or antibacterial activity in vitro and their expression leads to increased resistance to some pathogens (Singh and Upadhyay, 2013). In addition, rapid callose deposition in cell walls is also frequently associated with sites of pathogen incompatibility as it has been required to impede cell-to-cell movement of pathogen, including viruses in some plant-virus pathosystems (Zavaliev et al., 2011; Li et al., 2012). However, precise temporal and spatial coordination of induced signalling pathways is required to successfully kill the pathogen with minimal damage to the host tissue. In the initially infected cells, rapid responses lead to cell death, while in the surrounding cells, the defence is more transcriptionally dependent (Singh and Upadhyay, 2013).

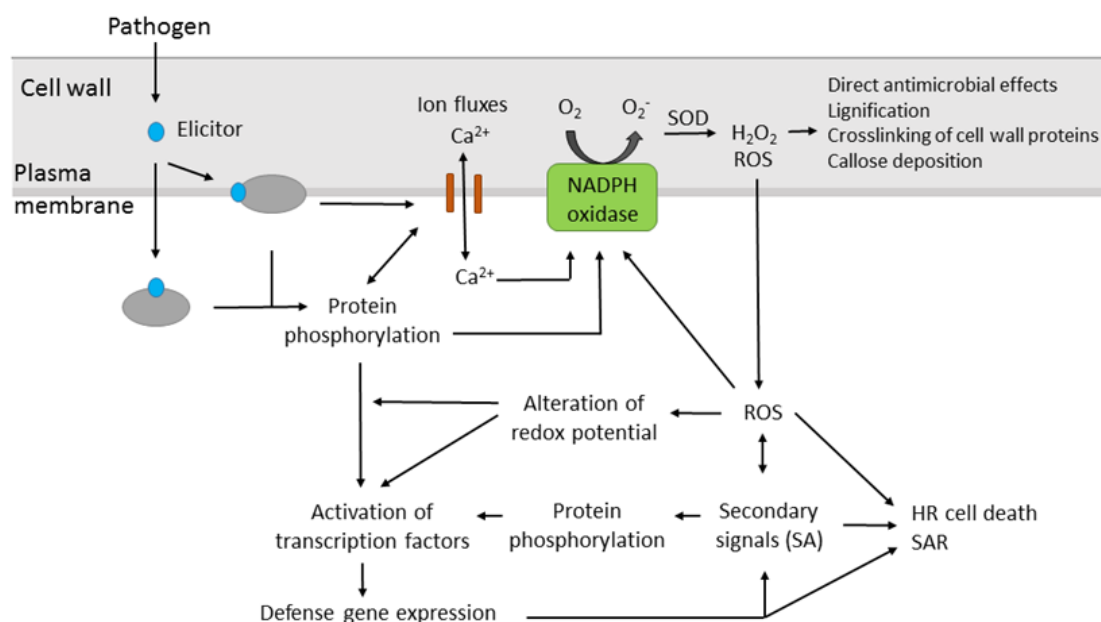


Figure 1.5: Signalling in the hypersensitive response is initiated by the gene-for-gene interaction. Interaction between avirulence (Avr) proteins (elicitor) and corresponding plant resistance genes (R genes) results in diverse and interconnected downstream signalling events initiated by protein phosphorylation and ion fluxes. NADPH oxidase (RBOH) generates reactive oxygen species (ROS) which results in redox potential changes. Several signalling cascades are activated, including salicylic acid signalling pathway, resulting in activation of transcription factors and expression of defense-related genes. These signals contribute to the development of HR-associated cell death and SAR. H₂O₂: hydrogen peroxide; SOD: superoxide dismutase; HR: hypersensitive response; SAR: systemic acquired resistance. Adapted from Singh and Upadhyay, 2013; Yang et al., 1997; Zurbriggen et al., 2010.

1.1.2.1 Programmed cell death in the hypersensitive response-conferred resistance

In plants, we distinguish two main types of PCD, vacuolar and necrotic. The PCD observed in the HR-conferred resistance combines the signs of both types and should be therefore considered as a separate type of PCD (Fomicheva et al., 2012). To study the role of HR-associated cell death in the restriction of the pathogen growth, several *Arabidopsis* and tobacco mutants have been produced (Greenberg and Yao, 2004; Morel and Dangl, 1997). Results showed that the role of cell death in resistance depends on the type of host-pathogen interaction. In general, it is more likely that the cell death contributes to the arrest of the pathogens that require living cells in order to replicate (Greenberg and Yao, 2004). This was shown for some (hemi)biotrophic pathogens. For example, the cell death was directly responsible for the restriction of oomycete multiplication (Gilroy et al., 2011; Hein et al., 2009). On the other hand, few examples in which the restriction of the (hemi)biotrophic pathogens spread was shown to be uncoupled from the HR-associated cell death were reported (reviewed in Künstler et al., 2016). For example, the attenuation of the HR-associated cell death by modifying *Arabidopsis* type I metacaspase regulatory

module (Coll et al., 2010) did not lead to enhanced biotrophic oomycete *Hyaloperonospora arabidopsidis* and hemibiotrophic bacteria *Pseudomonas syringae* proliferation. In addition, results obtained by the overexpression of the R-gene interactor RIN13 (Al-Daoude et al., 2005) or by the mutation of a cyclic nucleotide-gated ion channel DND1 (Yu et al., 1998; Clough et al., 2000) further confirmed that restriction of *P. syringae* growth could also occur in the absence of the HR-associated cell death.

1.1.2.2 The role of programmed cell death in plant-virus interactions

Similarly to some (hemi)biotrophic pathosystems mentioned above, the cell death was not directly responsible for the pathogen restriction also in some plant-virus interactions. NbMAPKKK α silencing (Komatsu et al., 2010) or introgression of CCD1 from *Nicotiana bigelovii* into *Nicotiana clevelandii* (Cawly et al., 2005) suppressed cell death without leading to enhanced plantago asiatica mosaic virus (PlAMV), potato virus X (PVX) and cauliflower mosaic virus (CaMV) proliferation. Treatment with ROS or antioxidant components resulted in the suppression of the HR-associated cell death without restricting TMV spread (Farkas et al., 1960; Hafez et al., 2012; Király, et al., 2008).

In addition to the direct evidence mentioned above, indirect data also suggest that HR-associated cell death can be dispensable for virus restriction in some pathosystems. The mutants with unaltered HR phenotype, but with an inability to restrict the pathogen growth confirm the theory that the HR-associated cell death may not be sufficient for pathogen arrest when other defenses are compromised (Greenberg and Yao, 2004). For example, the resistance against turnip crinkle virus (TCV) was compromised in eds5, pad4 and sid2 mutants without affecting HR-associated cell death (Chandra-Shekara et al., 2004). Similarly, the disabled interaction between the WRKY70 transcription factor and the RCY1 resistance protein (named after resistance CMV(Y)) deactivated cucumber mosaic virus (CMV) restriction of movement with no effect on HR-associated cell death (Ando et al., 2014).

1.1.3 Plant hormonal signalling pathways in immune response

As already mentioned above, recognition of the pathogen is followed by the activation of diverse signalling cascades in the host plant (reviewed in Pieterse et al., 2009). Among plant hormonal signalling pathways, SA, jasmonic acid (JA) and ethylene (ET) play crucial roles in mediating downstream transcriptional reprogramming resulting in effective defense responses (Tsuda and Somssich, 2014). These pathways do not function independently, but rather influence each other through a complex network. There is a growing body of literature suggesting the presence of both positive and negative regulatory interactions between these three pathways (Buscail and Rivas, 2014). However, NO, abscisic acid (ABA), gibberellins (GAs), auxins, cytokinins (CKs), brassinosteroids also function as important modulators of the plant immune signaling network (Pieterse et al., 2012).

Although the signalling pathways are strongly interconnected, SA signalling pathway has originally been known as crucial for the restriction of biotrophic pathogens, including viruses. On the other hand, the activation of defense responses following mechanical wounding and insect attack is mediated dominantly by jasmonates (Pieterse et al., 2012). This complex network evolved to allow plants to fine-tune their defense responses by activating the suitable combination of signaling pathways against the particular pathogen. The combinatorial plasticity between

pathways might further ensure that the particular signaling pathway is not activated in response to some pathogens if not needed (Shigenaga and Argueso, 2016).

1.1.3.1 Salicylic acid signalling pathway

The role of SA in plant defence was first mentioned in 1979 (White, 1979). In 1990, higher levels of SA were detected not only in the infected tissue but also in the systemic tissue (Malamy et al., 1990; Métraux et al., 1990). The response that develops in the distal, uninfected parts of plants after local pathogen infection is known as systemic acquired resistance (SAR) (reviewed by Fu and Dong, 2013).

SA biosynthesis can be triggered by PTI or ETI (Janda and Ruelland, 2015). Two pathways of SA biosynthesis have been proposed in plants, both originating from the chorismate, the end product of the shikimate pathway (Shah, 2002; Kumar, 2014). Several experiments have been performed to identify the genes involved in the signalling downstream of SA. In 1994, an *Arabidopsis* mutant, nonresponsive to inducers of SAR, was characterized and the corresponding gene was named nonexpresser of PR genes 1 (NPR1) (Cao et al., 1994, reviewed in Dong, 2004), and is to date also known as NIM1 (named after the non-inducible immunity phenotype of *nim1* mutants; Delaney et al., 1995) and SAIL1 (named after salicylic acid-insensitive *sail* mutant; Shah et al., 1997). Once synthesized, SA alters the cellular redox potential which triggers partial reduction of the NPR1, resulting in monomerisation of the NPR1 oligomer (Figure 1.6) (Janda and Ruelland, 2015). The monomerisation facilitates the NPR1 translocation from the cytoplasm into the nucleus (Wu et al., 2012). NPR1 paralogues NPR3 and NPR4 are SA receptors that bind SA with different affinities to mediate NPR1 degradation in an SA-regulated manner. High or low levels of SA initiate NPR4 interacts with NPR1, which leads to NPR1 degradation. On the other hand, when SA levels are intermediate, interaction between NPR1 and NPR3 is prevented, allowing NPR1 to accumulate and activate SA-dependent defenses (Fu et al., 2012). It was proposed that in the nucleus, NPR1 regulates downstream signalling through association with other proteins, as it contains protein-protein interaction domains (reviewed in Zhang et al., 2003 and Heather et al., 2012). Using yeast two-hybrid screens and other methods for studying interactions, several members of TGA (named after TGACGTC cis-element-binding protein) transcription factors family interacting with NPR1 have been identified in different species, namely AtTGA2 (also known as AHBP-1b; Zhang et al., 1999; Després et al., 2000; Fan and Dong, 2002), AtTGA3 (Zhou et al., 1999), AtTGA5 (Zhou et al., 1999; Kim et al., 2002), AtTGA6 (Zhang et al., 1999; Zhou et al., 1999; Kim et al., 2002), AtTGA7 (Heather et al., 2009), TGA2.2 from tobacco, which shows high similarity to the above-mentioned AtTGA2, AtTGA5 and AtTGA 6 (Niggeweg et al., 2000a, Zhang et al., 2003), TGA2.1 from tobacco, which shows lower similarity to AtTGA2 in comparison to TGA2.2 (Niggeweg et al., 2000a) and rTGA2.1, rTGA2.2 and rTGA2.3 from rice, which share 75 %, 76 % and 78 % identity with the TGA2 from *Arabidopsis* (Chern et al., 2001). However, to date, most studies have been focused on TGA family from *Arabidopsis*, which consists of 10 members classified into five clades. Clade II (TGA2/5/6) factors, which act immediately downstream of NPR1 was proposed as crucial for SA-dependent downstream responses (Zhang et al., 2003; Mou et al., 2003).

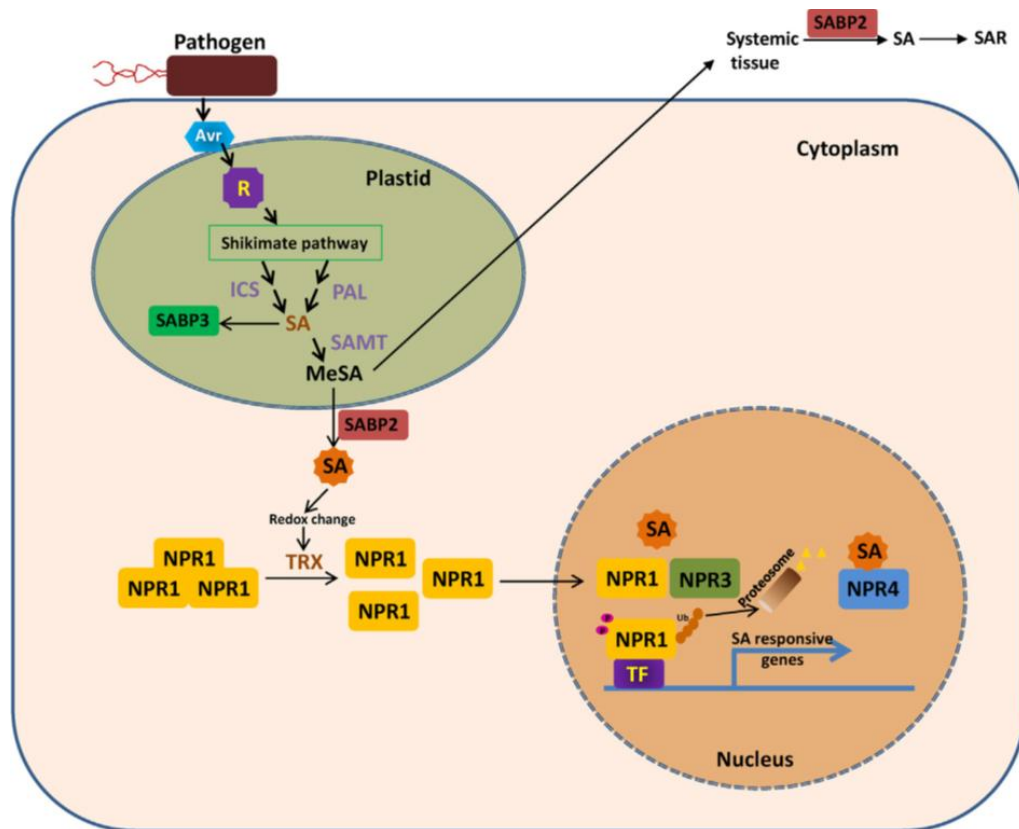


Figure 1.6: Salicylic acid (SA) signalling pathway. Pathogen attack induces biosynthesis of SA via ICS/PAL pathway in plastids. SAMT catalyzes conversion of SA to MeSA which diffuses into the cytoplasm where it is converted back to SA by the esterase activity of SABP2. SA induces NPR1 monomerisation through redox changes and its translocation into the nucleus. NPR3 and NPR4 bind SA with different affinities to mediate NPR1 degradation, which is dependent on SA concentration. High or low levels of SA initiate interaction between NPR4 and NPR1, which leads to NPR1 degradation, while intermediate SA levels disable interaction between NPR1 and NPR3, allowing NPR1 monomers to bind to TGA transcription factors, which activate expression of PR1 and other defense genes. ICS: isochorismate synthase, PAL: phenylalanine ammonia-lyase, SA: salicylic acid, SAMT: SA methyl transferase, MeSA: methyl salicylate, SABP2: methyl esterase, SABP3: carbonic anhydrase, TRX: thioredoxin, NPR: nonexpressor of pathogenesis related, TF: transcription factors from TGA transcription factors family, SAR: systemic acquired resistance. Republished with permission of Elsevier, from Salicylic acid signaling in disease resistance, Kumar, Volume 228, 2014; permission conveyed through Copyright Clearance Center, Inc.

TGA transcription factors enable transcriptional activation of the genes encoding pathogenesis-related proteins (PR) by binding to their promoters (Buscaill and Rivas, 2014; Seyfferth and Tsuda, 2014). PR genes, which are known as reporters of SA-dependent defences, were first mentioned in 1970 by (Van Loon and Van Kammen, 1970). In tobacco, increased endogenous levels of SA resulted in the expression of several PR genes in both inoculated and non-inoculated upper leaves after tobacco mosaic virus (TMV) infection (Malamy et al., 1990; reviewed in Breen et al., 2017). Moreover, exogenous application of SA or the SA functional analogue 2,6-dichloroisonicotinic acid (INA) induced PR gene expression which

resulted in pathogen resistance in different plant species (White, 1979; Van Loon et al., 1982; Mettraux et al. 1991; Vernooij, 1995; Görlach et al., 1996; Zhang et al., 2003). To date, a large amount of data has been available regarding PR genes, which have been identified in different plant species (Kunkel and Brooks, 2002). Among PR genes, which are classified into 17 families, PR1 genes are a dominant group of PR genes induced by pathogens or SA and therefore the most common marker for SA-dependent plant defense (van Loon et al. 2006). Clade II (TGA2/5/6) transcription factors are required and sufficient for SA-induced expression of PR1 genes (Zhang et al., 2003; Mou et al., 2003). Moreover, they are marker genes for systemic acquired resistance in different plant species, besides dicots, such as *Arabidopsis* and *N. tabacum*, also in monocots, such as rice (Mitsuhara et al., 2008). The PR1 family, which is strongly conserved, was present in all plant species investigated to date (reviewed in Van Loon et al., 2006). PR1 proteins are among the most abundant produced proteins during defense responses (reviewed in Breen et al., 2017). In 1970, the first PR1 gene reported as pathogen-responsive was PR1a from tobacco, upregulated after TMV infection (Van Loon et al., 1970). Since then, several studies have been focused on the roles of PR1 proteins in pathogen defence in various plant species and the results are summarized in several reviews (Van Loon and Van Strien, 1999; Van Loon et al., 2006; Fu and Dong, 2013; Breen et al., 2017). For example, among 22 PR1-type genes from *Arabidopsis*, only one (At2g14610) is activated after infection by the pathogenic bacterium *P. syringae*, insect attack (green peach aphid), or exogenous application of SA. Similarly, in *N. tabacum* only four out of 16 PR1-type proteins were induced after TMV infection, namely PR1a, PR1b, and PR1c and PR1g (reviewed in Van Loon et al., 2006). Expression of PR1b gene was also induced in sensitive and resistant potato cultivars after PVY infection (Baebler et al., 2011; Baebler et al., 2014). However, only a small proportion of all known PR1 genes has been studied in details, mostly from tobacco and *Arabidopsis*, while the information regarding other members is limited (Mitsuhara et al., 2008).

At least some of PR genes are not just the markers for activation of the SA signalling pathway, as they appear to be directly involved in the resistance against pathogens due to their antimicrobial activity. For example, overexpression of PR1 resulted in increased resistance to oomycetes, bacteria and fungi (reviewed in Breen et al., 2017). Moreover, results of recent studies have shown interactions between PR1 proteins and effectors from different fungal pathogens (Lu et al., 2014; Breen et al., 2016). On the other hand, direct role of PR proteins in the defense against virus infection has not been shown to date (reviewed in Breen et al., 2017). However, the role of PR1 against pathogens, including viruses, might be indirect as it was suggested that CAP-derived peptide (CAPE1), a peptide consisting of 11 amino acids derived from the C terminus of tomato PR1 protein, has a role as a defense-signalling molecule. Comparison of PR1 sequences from various plant species identified the conserved consensus motif in the CAPE1 peptide, suggesting the importance of CAPE-induced defense responses in different species (Chen et al., 2014). In addition, the results of the studies reviewed in Breen et al. (2017) suggested the involvement of a CAP domain of PR1 proteins also in sterol, ion and lipid binding.

Both positive and negative regulatory elements were found in the promoters of the PR1 gene from *Arabidopsis*, suggesting the involvement of TGA transcription factors in the activation and repression of PR1 expression (Lebel et al., 1998; Zhang et al., 2003; Kesarwani et al., 2007). This theory was confirmed by

several experiments on transgenic plants. For example, overexpression of the tobacco TGA2.2 diminished SA-inducible PR gene induction (Niggeweg et al., 2000a), while on the other hand Pontier et al. observed enhanced PR gene induction if AtTGA2 from Arabidopsis (homologue of tobacco TGA2.2) was overexpressed (Pontier et al., 2001). Moreover, in Arabidopsis NPR1 enhanced binding of the AtTGA2 to a positive (LS7) and a negative (LS5) regulatory element of the PR1 promoter (Després et al., 2000). Zhang et al. suggested that Clade II (TGA2/5/6) transcription factors from Arabidopsis repress the basal expression of PR1, while the increased PR1 expression may be the result of the loss of TGA factors which bind to the negative element in the PR1 promoter (Zhang et al., 2003; Blanco et al., 2009). Confirming these results, *in vivo* plant transcription assays revealed that TGA2 is a transcriptional repressor in both untreated and SA-treated cells (Rochon et al., 2006).

1.1.3.2 Jasmonic acid signalling pathway

JA is synthesized through three cycles of β -oxidation from 12-oxophytodienoic acid (OPDA) precursor after its translocation into the peroxisome (Schaller and Stintzi, 2009). Synthesized JA is conjugated with isoleucine to form the active hormone JA-Ile (Fonseca et al., 2009). JA-Ile is perceived by the JA receptor coronatine insensitive 1 (COI1) (Figure 1.7). Upon signal perception, COI1 recruits the negative regulator Jasmonate-ZIM domain protein (JAZ) for ubiquitination and degradation. In the absence of JA, this negative regulator represses the target transcription factor essential for activation of downstream JA-responsive genes. Interaction between COI1 and JAZ results in the release of target transcription factor from the complex JAZ-transcription factor. A free transcription factor can then induce the expression of JA-inducible genes (reviewed in Shan et al., 2012; Memelink, 2009).

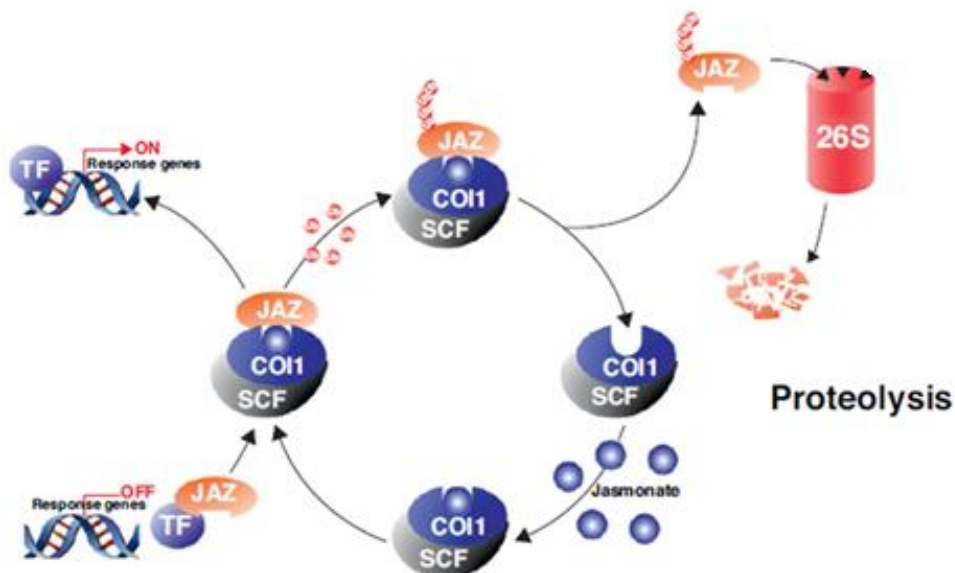


Figure 1.7: Jasmonic acid (JA) signalling pathway. JA receptor coronatine insensitive 1 (COI1) forms SCFCOI1 complex. In the absence JA-Ile, JAZ cannot bind SCFCOI1 complex. Instead, JAZ binds transcription factor such as MYC2 and represses expression of downstream JA-responsive genes. In the presence of JA-Ile, JA-Ile binds to SCFCOI1 complex, which then forms new platforms for

interaction with negative regulator JAZ. After JAZ degradation, transcription factor can activate the expression of downstream JA-responsive genes. SCFCO11: a multi-protein E3 ubiquitin ligase complex catalyzing the ubiquitination of proteins destined for proteasomal degradation, CO11: Coronatine insensitive 1, JAZ: Jasmonate-ZIM domain protein, TF: transcription factor such as MYC2, Jasmonate: Ja-Ile, Ub: ubiquitine. Republished with permission of Elsevier, from Comparison of phytohormone signaling mechanisms, Shan et al., Volume 15, 2012; permission conveyed through Copyright Clearance Center, Inc.

MYC2 from *Arabidopsis* was the first identified direct target of JAZ repressors (Chini et al., 2016). Since then, many other JAZ-interacting transcription factors from different families and species have been described, including MYC3, MYC4, MYC5, MYB21/24 and MYB75 from *Arabidopsis* (reviewed in: Shan et al., 2012; Chini et al., 2016) and MYC2 from tomato (reviewed in Du et al., 2017). In *Arabidopsis*, MYC2 directly binds to the G-box or G-related elements in their target promoters (Dombrecht et al., 2007) and is required for activation of many JA-responsive genes, particularly those involved in response to wounding and insect attack (Browse, 2009). Well-characterized JA-inducible genes are the JA biosynthetic genes allene oxide cyclase (AOC), allene oxide synthase (AOS), oxophytodienoate-reductase3 (OPR3) and lipoxygenase, several JAZ genes and defense genes such as proteinase inhibitors (Du et al., 2017).

Potato multicystatin (PMC) is a multi-domain cysteine proteinase inhibitor that functions in plant defence and as a storage protein. High concentrations of PMC were detected in potato leaves (up to 0.6 µg/g of fresh weight tissue), where it accumulated under conditions that induce the accumulation of other proteinase inhibitors linked to plant defense (Walsh and Strickland, 1993). Results from Orr et al. (1994) confirmed the proposed role of PMC in plant defense, as the growth of corn rootworm larvae was inhibited, if they were fed with PMC. Moreover, expression of potato proteinase inhibitors was induced by wounding and methyl-jasmonate (MeJA) (Hildmann et al., 1992).

1.1.3.3 Ethylene signalling pathway

The first step of ET biosynthesis is the conversion of S-adenosyl-methionine (S-AdoMet) to 1-aminocyclopropane-1-carboxylic acid (ACC) catalyzed by ACC synthase. In the next step, ACC is oxidized by ACC oxidase (ACO) to ET (Wang et al., 2002). The ET receptor is located at the endoplasmic reticulum membrane (Figure 1.8). Kinases and phosphatases play a significant role in ethylene signalling (reviewed in Xu and Zhang, 2014). In the absence of ethylene, the receptor represses ET-dependent downstream response by activating a Raf-like kinase CTR1. Binding of ET and copper as a cofactor deactivates ET receptor, which results in CTR1 deactivation (reviewed in Shan et al., 2012). The next known component downstream of CTR1 is EIN2, a positive regulator of ET responses. Inhibition of CTR1 is a signal for cleavage and translocation of the EIN2 C terminus into the nucleus, where it activates EIN3 and EIL1 (EIN 3-like) transcription factors. EIN3 and EIL1 activate expression of the ERF1 transcription factors, which in turn activate the expression of other ET-responsive genes (reviewed in Ju et al., 2012; Ju and Chang, 2012).

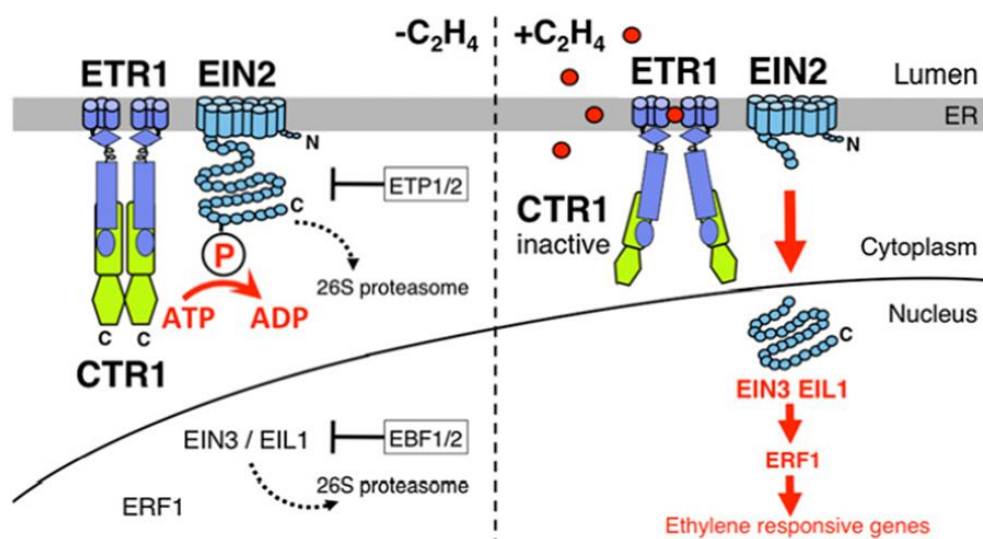


Figure 1.8: Ethylene (ET) signalling pathway. In the absence of ET, ET receptor ETR1 activates CTR1 kinase which phosphorylates the C-terminal domain of EIN2, preventing its translocation into the nucleus. Binding of ET to ETR1 deactivates CTR1. Inactive CTR1 allows EIN2 to function as a positive regulator of ET signalling pathway. EIN2 activates EIN3 and EIL1 (EIN3-like) transcription factors, which bind to the promoter of ERF1 gene to activate their transcription. ERF1 further activates the expression of other ET-responsive genes by binding to GCC box in their promoters. C_2H_4 : ethylene, ETR1: ethylene receptor, EIN2: ethylene-insensitive protein 2, CTR1: kinase, EIN3, EIL1 and ERF1: ethylene responsive transcription factors. Republished from *Advances in ethylene signalling: protein complexes at the endoplasmic reticulum membrane*, Ju and Chang, 2012.

In addition to ET, ERF proteins are also induced by other biotic and abiotic stresses, including pathogen infection, wounding, JA and ABA. They were originally characterized as transcription factors binding to the promoters of stress responsive genes, including pathogenesis-related (PR) genes (reviewed in Licausi et al., 2013). In addition, several ERF proteins control ET biosynthesis by binding to GCC-box in promoters of ACS and ACO genes, regulating their expression (reviewed in Gu et al., 2017).

1.1.4 Transcriptional regulation

Regulation of gene expression as a result of biotic and abiotic stress is mediated through transcriptional, post-transcriptional and post-translational changes (Hernandez-Garcia and Finer, 2014). To establish an effective defense response, cells require transcription factors to deploy their activity rapidly, transiently, spatially, and hierarchically (Moore et al., 2011). In immune response, the early signal transduction events may be distinct for various pathogen elicitors. However, many of these signals are integrated into one of a few terminal pathways that lead to the interaction between activated transcription factors and pathogen-responsive cis-regulatory elements in the promoters of defense-related genes (Buscaill and Rivas, 2014).

Cellular promoters are DNA sequences located upstream of gene coding regions (towards the 5' region of the positive sense

strand) which initiate transcription of the particular gene. Regulation of gene expression is controlled by physical interaction between cis-regulatory elements present within the promoter and transcription factors, proteins specific for binding to particular cis-regulatory elements (Figure 1.9; Hernandez-Garcia and Finer, 2014).

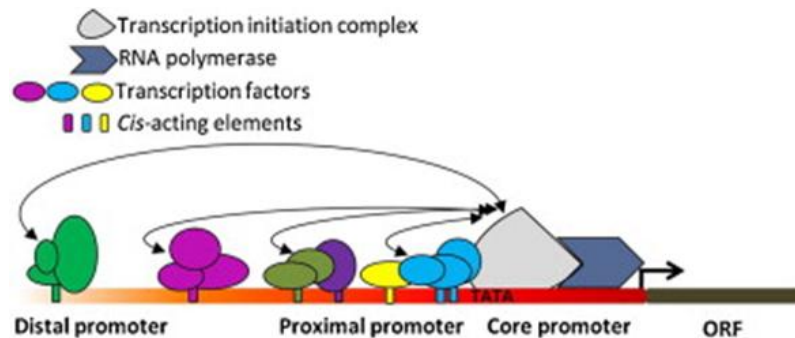


Figure 1.9: Transcriptional regulation of genes. Transcription factors bind to regulatory elements present within the distal and proximal promoter and interact with the transcriptional machinery attached to the core promoter. This complex regulates the transcription of the particular gene. Republished from Identification and validation of promoters and cis-acting regulatory elements, Hernandez-Garcia and Finer, 2014.

A core promoter is the minimal portion of the promoter required to properly initiate the transcription and is located approximately 40 bp upstream of the transcriptional initiation site. It contains general transcription binding sites (e.g. TATA box) and binding sites for the basic transcriptional machinery, including RNA polymerase II. Upstream of the core promoter region are the proximal and distal regions of the promoter. The length of the upstream region that contributes to the transcription is less uniform between different genes if compared to the core promoter, and the size of the fully functional promoter depends on the position of the cis-regulatory elements present in the proximal and distal regions (Lee and Young, 2000). The regulation of transcription is therefore dependent on the type, number, position and the availability of regulatory elements (Hernandez-Garcia and Finer, 2014). In addition, mutational analysis of individual regulatory elements confirmed the importance of combinatorial interplay between regulatory elements within promoter sequence. For example, mutations of conserved individual regulatory elements in the GluB-1 promoter resulted in significant reduction in promoter activity. Moreover, each regulatory element was unable to confer the transcriptional activity on its own (Wu et al., 2000).

1.1.4.1 Functional and nucleotide promoter diversity

The structure of promoters consists of transcription factor binding sites located within a background sequence of non-functional regions. Many studies have been focused on nucleotide polymorphism in coding regions in plants as single nucleotide polymorphisms (SNPs) and insertions/deletions are the basis of most differences between alleles. Therefore, the identification of SNP and insertions/deletions can be used in marker-assisted breeding (Kim et al, 2016). On the other hand, little is known

about the regulatory diversity within plant species. Functional changes in promoters can arise from mutations, pointing to promoter regions as a fundamental determinant of functional genetic variation. Mutation within the promoter regions can result in a loss or gain of function (Meaux et al., 2005). Therefore, sequence comparison is the first step towards the identification of the promoter with the desired function. However, it is difficult to predict which nucleotide changes are contributing to functional differences just by sequence comparison, as different changes can alter the properties of a binding site in different ways (Dermitzakis et al., 2003). Therefore, further functional evaluation through expression analysis is crucial for functional characterisation of the promoter.

A few studies focused on the role of variability in promoter regions for transcriptional activity. Results suggested that the variations in promoters such as insertions or deletions can affect the gene expression level. For example, investigation of the promoters of Heading date 3a (Hd3a) gene among different rice cultivars revealed 12-bp long insertions in the promoters of some cultivars. Genes under control of these promoters showed a higher expression level (Takahashi et al., 2008). Similarly, promoter diversity in ET-responsive element-binding (CBF) genes in different *Arabidopsis* ecotypes resulted in different expression profiles. For example, no expression of CBF1 gene was detected in Co-1 ecotype which contains 365-bp insertion in the promoter region, while in Ita-0 ecotype which contains different, 261-bp long insertion, the CBF1 expression was detected (Lin et al., 2008). The results from the studies focused on the role of variability in promoter regions of the PR genes will be explained in Section 1.1.4.2.1.

It has been shown that not only longer insertions or deletions, but also point mutations in the promoter region can affect promoter activity, especially those that emerge in the binding sites and are involved in the interaction with crucial transcription factors. Moreover, one nucleotide mutation can change the structural configuration of DNA and alters the interaction between the transcription factor and regulatory element (Bruckner et al., 1995). For example, fourteen nucleotide polymorphisms and seven insertions/deletions were found to be distributed within the promoter of chalcone synthase (CHS) genes in *Arabidopsis*. Comparison of promoter sequences in combination with expression analysis suggested a candidate point mutation that may explain different transcriptional activity in light response (Meaux et al., 2005). Similarly, association between single-nucleotide polymorphisms in the promoter region and the expression level was also determined for Hd3a, a gene involved in flowering time in rice (Takahashi et al., 2009), Rubisco activase from soybean (Shi et al., 2013) and CBF1 and CBF3 genes from *Arabidopsis* (Lin et al., 2008). These results suggest that the variation in the promoter (either insertions/deletions or SNPs) can affect promoter activity of different genes in different species.

1.1.4.2 Promoters of the genes involved in plant signalling pathways

Cis-regulatory element is a noncoding DNA sequence upstream of a gene required for proper spatiotemporal expression of that gene, often containing binding sites for transcription factors. A single pathogen elicitor may activate multiple transcription factors that interact with different cis-regulatory elements in the same or different promoters, leading to induction of one or more defense-related genes (reviewed in Kushalappa et al., 2016). Accordingly, the promoters of defense genes may contain different regulatory

elements and each contributes to one or more aspects of a complex expression profile. Promoters of a few of these pathogen-inducible genes have been characterized and functionally validated, which resulted in the identification of regulatory regions involved in plant immune response (Hernandez-Garcia and Finer, 2014; Birkenbihl et al., 2017). Functional dissection of promoter regions and their functional characterization uncovered common biotic stress-related cis-regulatory elements discussed below.

A large number of genes from different species involved in biotic stress regulatory network contain W boxes in promoter regions. Rushton and Somssich reported the existence of two types of W box elements, one contains the sequence TTGACC and the other is composed of two core TGAC elements (Rushton and Somssich, 1998). By date, different variations of W boxes are known, but they usually consist of (C/T)TGAC(C/T) nucleotides (Choi et al., 2015; Eulgem et al., 2000; Rushton et al., 1999; Yu et al., 2001). For example, W boxes from parsley PR1 promoters, characterized by TGAC core, differ slightly at the sequence level. Box W1 and W2 contain TTGACC elements, while Box W3 is composed of two TGAC elements (Rushton et al., 1996). However, all three W boxes are bound specifically by the members of WRKY transcription factors family, which is an extensive family of plant DNA-binding proteins containing zinc finger domain on the carboxy terminal side (reviewed in Phukan et al., 2016). In addition to PR gene promoters from parsley, W box sequences were also found in pathogen-inducible promoters of many other species. In addition, some W-boxes are also wound inducible (reviewed in Rushton et al., 2010). Importantly, W box motif was significantly enriched in the promoters of JA-responsive genes which are antagonized by SA, suggesting its involvement in SA/JA crosstalk (Van der Does et al., 2013).

Another regulatory element involved in biotic stress response is the activator sequence-1 (as-1 or ocs; CTGACGTAAGGGATGACGCAC). The as-1 element responds to various signals, including SA, JA and hydrogen peroxide. The as-1 elements are bound by the members of well-characterized basic-region leucine zipper (bZIP) transcription factors (reviewed in Alves et al., 2013). For example, some members of TGA transcription factors, isolated from tobacco and Arabidopsis, bind the as-1 element (reviewed in Gatz, 2013; Yang et al., 1997). Moreover, TGA directly target the as-1 element in the promoter of ET-responsive GCC box binding protein ORA59, suggesting that an important regulatory hub for the activation and SA suppression of ET-induced genes is as-1 dependent (Zander et al., 2014).

The next regulatory element, highly represented in the promoters of pathogen-inducible gene, is ET-responsive element GCC box identified by the core sequence AGCCGCC (Ohme-Takagi and Shinshi, 1995). So far we know several GCC-like elements with different patterns of pathogen induction (Gurr and Rushton, 2005) and even minor variations in the core sequence of this regulatory element can significantly alter the inducibility and expression of the corresponding gene (Rushton et al., 2002). For example, ERF subfamily members of the APETALA2/Ethylene-responsive element binding factor (AP2/ERF) family show the greatest affinity to the GCC sequence. To date, it was proposed that the GCC box plays a key role in the regulation of genes responsive to biotic stress, in particular to genes related to JA and ET signalling pathways (reviewed in Tsuda and Somssich, 2015). For example, binding of AP2/ERF transcription factors to the conserved GCC box in promoters of PR genes directs PR gene expression after ET and JA induction or pathogen attack (reviewed in Alves et al., 2014).

Similar regulatory elements to GCC box have been proposed to direct jasmonate-responsive expression (JERE; AGACCGCC) (Menke et

al., 1999) and fungal elicitors-induced expression (box S; AGCCACC) (Kirsch et al., 2000).

G-boxes (CACGTG), members of the family of ACGT-containing cis-regulatory elements are present in the promoters of many plant defense genes (Rushton and Somssich, 1998). Activation of G-box mediate responses to diverse stimuli, including elicitors and redox changes. It has been implicated in the expression of jasmonate-inducible proteinase inhibitor in tobacco (Kim et al., 1992). These cis-regulatory elements are binding sites of GBF family of transcription factors and often function in concert with H-boxes (Yang et al., 1997).

H-box (CGTACC) is an elicitor-responsive cis-regulatory element involved in the activation of some defense genes, including tobacco PR1 gene. This regulatory element is the type II MYB consensus sequence A(A/C)C(A/T)A(A/C)C, suggesting that it is bound by MYB (MRE) (Yang et al., 1997) and it was first identified in the chs15 promoter (Yu et al., 1993). Mutations in H-box or G-box reduced the resistance against TMV (Rushton and Somssich, 1998).

TCA element and SARE are the next two regulatory elements involved in plant biotic stress. TCA element (TCATCTTCTT) was found in promoters of more than 30 different stress-inducible genes, including tobacco PR genes, and is involved in the expression of PR-2d gene after TMV infection or SA-induction (Goldsbrough et al., 1993). SARE (TTCGACCTCC) is a SA-inducible regulatory element, which was identified in the tobacco PR2-d gene (Shah and Klessig, 1996).

1.1.4.2.1 Molecular characterization of the PR1 promoters

Among genes that are responsive to immune signalling, promoters of PR genes are best studied. The effects of mutations within the promoters of PR1 genes on the promoter activity in tobacco and Arabidopsis are summarised in Table 1.1. For example, the mutation in the 46-bp long element located inside the promoter of the tobacco PR2d gene (from -364 bp to -288 bp upstream of the PR2d transcription start), abolished the induction of the PR2d gene by SA (Shah and Klessig, 1996). In another study, the mutation in the 73 bp long fragment (X1 region containing GCC-box) of the PRb-1b promoter, located between positions -213 bp and -141 bp upstream of the transcription start site, abolished ET-responsiveness (Sessa et al., 1995). The length of different tobacco PR1 promoters, required for efficient gene induction and the crucial promoter regions have also been identified (Table 1). Analysis of a series of 5' end deletions of the PR2d promoter indicated that the cis-regulatory elements, crucial for the induction by SA and TMV, are localized in the region between -321 bp and -607 bp upstream of the transcription start site (Hennig et al., 1993). In another study, Uknes et al. (1993) demonstrated that 661 bp long PR1a promoter was sufficient to impair TMV and SA inducibility. Similarly, 689 bp long or longer upstream sequence of the PR1a gene was sufficient for the PR1a induction in TMV-inoculated leaves, systemically induced leaves of TMV-infected plants and leaves treated with SA. No such induction was detected if upstream sequences of 643 bp or shorter were used, which confirmed the localization of TMV- and SA-responsive elements between positions -643 and -689 upstream of the PR1a gene (Van de Rhee et al., 1990). In agreement with these results, analysis of PR1a promoter indicated that regulatory elements crucial for the induction by TMV are located between -335 bp and -906 bp upstream of the transcription start (Grüner et al. 1994), while functional analysis of the PR1a promoter performed by Strompen et al. (1993) identified even more defined region of 139

bp (from -691 bp to -553 bp) mediating expression of the gene under PR1a promoter in response to SA. Analysis of the above-mentioned region revealed two TGACG elements reminiscent of as-1. Similarly, Yang et al. (2000) identified cis-regulatory region in tobacco PR1a promoters that is responsive to SA treatment or TMV infection through deletion analysis of PR1a promoters. The region is located between -893 bp and -499 bp upstream of transcription site, which is in agreement with previously mentioned studies (Strompen et al., 1998; Uknes et al., 1993; Van de Rhee et al., 1990).

Besides tobacco, a few studies have also been performed on Arabidopsis. Lebel et al. identified the minimal promoter length required for induction of PR1 gene in Arabidopsis. The activity of the 4258 bp long promoter after INA treatment did not differ significantly from the 1974 bp, 1293 bp and 815 bp long promoters. Interestingly the activity of the 984 bp long promoter was lower in comparison to 815 bp long promoter, indicating the presence of negative regulatory elements at the position between -984 bp and -815 bp upstream of the transcription site. However, INA induction was abolished completely when promoters shorter than 621 bp were analysed. PR-box, cis-regulatory element, conserved among the promoters of some SAR genes, was found at position -632 to -627 and 8-bp long conserved motif, named -624 box, present also in the Arabidopsis PR1 promoter and tobacco PR1a and PR2d promoters, was identified (Lebel et al., 1998). Besides that, some other cis-regulatory elements have been connected to SA inducibility, for example, W1 box and W2 box in the promoters of PR1 genes (Rushton et al., 2002; Yang et al., 1999) or as-1 elements in the promoters of PR1a genes (Strompen et al., 1998).

Table 1.1: Crucial promoter regions of PR gene for ethylene, salicylic acid and tobacco mosaic virus responsiveness in tobacco and Arabidopsis.

Species	Gene	Crucial promoter	Responsiveness	Reference
tobacco	PRB-1b	141 bp - 213 bp	ET	Sessa et al., 1995
	PR2d	288 bp - 364 bp	SA	Shah and Klessig,
	PR2d	321 bp - 607 bp	SA, TMV	Henning et al.,
	PR-1a	1 bp - 661 bp	SA, TMV	Uknes et al., 1993
	PR-1a	643 bp - 689 bp	SA, TMV	Van de Rhee et al.,
	PR-1a	335 bp - 906 bp	TMV	Grüner et al. 1994
	PR-1a	553 bp - 691 bp	SA	Strompen et al.,
	PR-1a	499 bp - 893 bp	SA	Yang et al., 2000
<i>A.thaliana</i>	PR1	1 bp - 621 bp	SA	Lebel et al., 1998

* upstream of the transcription site

In addition to 5' end deletion analyses explained above, in silico analyses were performed to study promoters of PR genes from different species (Kaur et al., 2017).

1.2 Plant Synthetic Biology

Synthetic biology is an interdisciplinary field that aims at applying engineering principles to redesign existing natural biological systems or to design and construct new biological parts, devices or systems (Schwille, 2011). It started with bacteria but has been successfully applied to eukaryotic systems in the last few years (Slusarczyk and Weiss, 2012). In plants,

the field is still in its early stages and nowadays there are only a few published examples of plant-oriented applications (Liu and Stewart, 2015). The examples of those are reconstruction of complex biochemical pathways (Giuliano 2014; Lu et al. 2013), engineering synthetic signal transduction systems (Larrieu et al. 2015) and other high-impact goals in plant synthetic biology (Nemhauser and Torii 2016). However, cloning for delivery of multiple DNA fragments into the plant genome is still one of the main technological obstacles for achieving those goals (Liu and Stewart 2015).

1.2.1 Assembly and expression of multigene constructs in plants

Two crucial components are required for the development of an efficient multigene cloning strategy in plant synthetic biology: first, a method by which the foreign coding sequence (CDS) and regulatory elements, i.e. promoter and terminator sequences, can be assembled and inserted into the plant expression vector in a highly efficient and versatile manner and second, the availability of plant expression vectors capable of holding and introducing multiple expression cassettes into the plant genome. Although a remarkable variety of plant expression plasmids has already been developed, the basic design of many of these vectors is quite restrictive and rarely permits the cloning and transfer of more than a single expression cassette into plant cells (Altman and Hasegawa, 2011; Dafny-Yelin and Tzfira, 2007).

1.2.1.1 Classical restriction-based multigene cloning

Due to the increasing requirement for high throughput approaches to assemble complex designs, classical restriction-based cloning became limited. The conventional cloning strategies involve the use of type II restriction enzymes, modification of DNA ends and the ligation of DNA fragments which make the cloning expensive, time consuming and less efficient, especially in the case of high throughput settings. Moreover, these procedures often result in unwanted sequences at the junction sites. Another drawback of using conventional type II restriction enzymes for the cloning of large DNA fragments is the high occurrence of their recognition sites within expression cassettes. Although the latter drawback was overcome by exploiting the power of rare cutters for the assembly of multiple expression cassettes by designing the pAUX series of plasmids (Goderis et al. 2002) and their variations pSAT (Chung, Frankman, and Tzfira 2005; Tzfira et al. 2005), the system is still limited by the number of different commercially available rare-cutting restriction enzymes.

1.2.1.2 Recombination-dependent multigene cloning

To overcome the limitations of classical restriction-based cloning methods, the emergence of different recombination-dependent methods was exploited for multigene cloning applications. The first recombination methods were based on the Cre/loxP recombination system (reviewed in Gilbertson, 2003), which is derived from the bacteriophage P1. It consists of the 38.5-kDa Cre recombinase and the 34-bp loxP site. Cre binds to 13-bp long inverted repeats in the loxP site and catalyzes a crossover in the 8-bp spacer regions of two loxP sites. Since the invention of the first recombination methods based on this system (reviewed by Gilbertson, 2003) the number of elements that can be assembled was increased by combining this system with two rare-cutter endonucleases (Lin et al. 2003).

Another recombination-based cloning method, Invitrogen's Gateway technology, which is based on the bacteriophage-site-specific recombination system (Hartley et al., 2000) overcomes the shortcoming of classical Cre/loxP recombination system, since it allows delivery of DNA fragments from entry into multiple destination vectors with high specificity and efficiency (Hartley et al., 2000; Karimi et al., 2002, Karimi et al., 2007). Although the upgrade of the technique into Multisite Gateway enables delivery of up to three independent transcription units into plant cells (Sasaki et al. 2004; Vemanna et al. 2013), it is still limited in its flexibility due to a small number of available att sites. However, with MultiRound Gateway cloning, based on multiple rounds of LR recombination reactions, it is possible to assemble up to seven expression cassettes (Buntru et al. 2013; Chen et al. 2006). Despite many benefits of Gateway-based systems, there are still several drawbacks such as the introduction of unwanted cloning scars at the junction sites and high cost, especially for large-scale projects (Chee and Chin 2015).

1.2.1.3 Modular ligation-dependent methods

In recent years, significant efforts were dedicated to the development of simple and efficient cloning systems for standardized assembly of genetic modules, using defined rules (Canton et al., 2008; Ellis et al., 2011). High throughput modular ligation-dependent methods, efficient for multipart assemblies for expression in plants, are Golden Gate (Engler et al., 2008) and derived strategies i.e. MoClo (Weber et al. 2011), GoldenBraid (Sarrion-Perdigones et al. 2011) and GreenGate (Lampropoulos et al. 2013). These strategies are based on the use of type II restriction enzymes, which cut outside of their recognition sequence. With proper design of the cleavage sites, two fragments cut by type II restriction enzymes can be ligated into a product lacking the original restriction site. This method is therefore as efficient as recombination-based cloning technologies but yields recombinant plasmids that do not contain unwanted sequences in the final construct (Engler et al., 2008).

However, disadvantages of those methods are common to conventional cloning strategies, as they are based on the use of type II restriction endonucleases. For those, the main disadvantage is the high occurrence of recognition sites within expression cassettes, which becomes increasingly relevant in the case of large multigene constructs.

1.2.1.4 Overlap-dependent assembly methods

In contrast to Golden Gate and derived methods, overlap-dependent assembly methods, such as circular polymerase extension cloning (CPEC) (Quan and Tian 2009), uracil-specific excision reagent cloning (USER) (Nour-Eldin et al., 2010), Gibson assembly (Gibson et al. 2009), HiFi assembly (NEB), sequence and ligation-independent cloning (SLIC) (Li and Elledge, 2012), TAR (Kouprina and Larionov 2006) and seamless ligation cloning extract (SLiCE) (Zhang et al, 2012), are sequence-independent and therefore convenient and efficient when performing multigene cloning (reviewed in Patron, 2014). Moreover, they do not require premade standardised biological parts and can thus be used to easily clone and characterize novel functional parts. Therefore, the overlap-based assembly methods and Gibson assembly, in particular, are the most accepted methods within the synthetic biology community (Kahl and Endy 2013).

All overlap-dependent assembly methods require that the parts to be inserted are double-stranded, linear and that both ends

share identity to the fragment or plasmid vector sequence to which they will be adjacent in the final assembly. The optimal length of the overlap varies between assembly methods, ranging from six to 60 base pairs. Overlapping fragments are created by PCR amplification of each fragment, primed by custom oligonucleotides that have extensions with identity to the fragments required to be adjacent in the final assembly (reviewed in Patron, 2014).

Overlap-dependent assembly methods are an ideal modular assembly approach for engineering purposes, including plant engineering. But so far, they have not been widely adopted by plant biotechnology probably due to the lack of databases and software tools to facilitate the design of complex constructs as well as the lack of vectors and cloning parts which would build the basis for the assembly and delivery of multiple-gene cassettes into plant cells. Recently developed AssemblX (Hochrein et al. 2017) and implementation of plant grammar in GenoCAD (Coll et al., 2015) have laid a foundation for the set-up of overlap-dependent assembly methods in plants.

1.2.1.4.1 Software support for genetic constructs design

Despite several modular assembly methods developed in recent years, the plant biotechnology community has not widely adopted them yet, also due to the lack of software tools, which would enable supported design of the assembled constructs in plants. GenoCAD is a software tool for synthetic biology which allows fast design of genetic constructs based on the notion of genetic parts. The user is guided through the design process which enables the automatical assembly of genetic parts derived from the GenoCAD libraries into a final construct (Czar et al., 2009). GenoCAD has recently been updated with the implementation of plant grammar which includes a library of plant biological parts organized in structural categories and a set of rules describing how to assemble these parts into large constructs thus minimizing the risk of introducing errors (Coll et al., 2015). By the implementation of plant grammar into GenoCAD, a foundation for the set-up of overlap-dependent assembly methods in plants has been laid.

1.2.1.4.2 AssemblX cloning procedure

AssemblX toolkit is a recently developed cloning strategy for the assembly of subunits into a multigene structure, which exploits overlap-dependent assembly methods in combination with rare-cutting restriction enzymes. It contains vectors of three different levels and an accompanying online tool (Hochrein et al., 2017). Although the AssemblX toolbox is designed to support easy transfer to any organism, the Level 2 AssemblX vectors are currently available only for bacteria (*Escherichia coli*) and yeast (*Saccharomyces cerevisiae*) (Hochrein et al. 2017).

Specific overhang sequences first need to be introduced into subunits (e.g. promoter, CDS, terminator) by polymerase chain reaction (PCR) using custom-designed primers. These primers contain overlaps to the neighbouring fragment in their 5' region to allow the assembly into entry Level 0 vectors by using overlap-dependent cloning methods. The as-assembled gene cassettes can be combined into the multigene structure in a Level 1 AssemblX vector. To facilitate the transfer of assembly units between different assembly Levels without relying on PCR amplification, rare-cutting restriction enzymes are employed. A multitude of Level 0 and Level 1 vectors is available to allow the assembly of currently up to 25 expression cassettes in a user-defined order.

AssemblX toolkit is supported by the AssemblX web tool to facilitate the design of multigene cassettes and computation of primers (Hochrein et al. 2017).

1.2.2 Biosensors to detect plant immune responses

One of the emerging fields of synthetic biology is the design of biosensors (Samodelov and Zurbriggen, 2017). A biosensor is a tool that transmits a recognition event received by a biological receptor to a transformer which converts this recognition event into a signal that can be captured and interrogated. One common version of a biosensor is transcriptional biosensor, which follows the concept of regulation by transcription. In this version of biosensor, the recognition event is binding of transcription factor(s) to the promoter region, which activates the transcription (Sadanandom and Napier, 2010). The transcription is detected by fusing the gene for a reporter protein downstream of the promoter (Figure 1.10). While a variety of reporter proteins have been used, those encoding autofluorescent proteins are best suited for optical biosensors, where the signal can be interrogated by confocal microscopy (Rao, 2009).

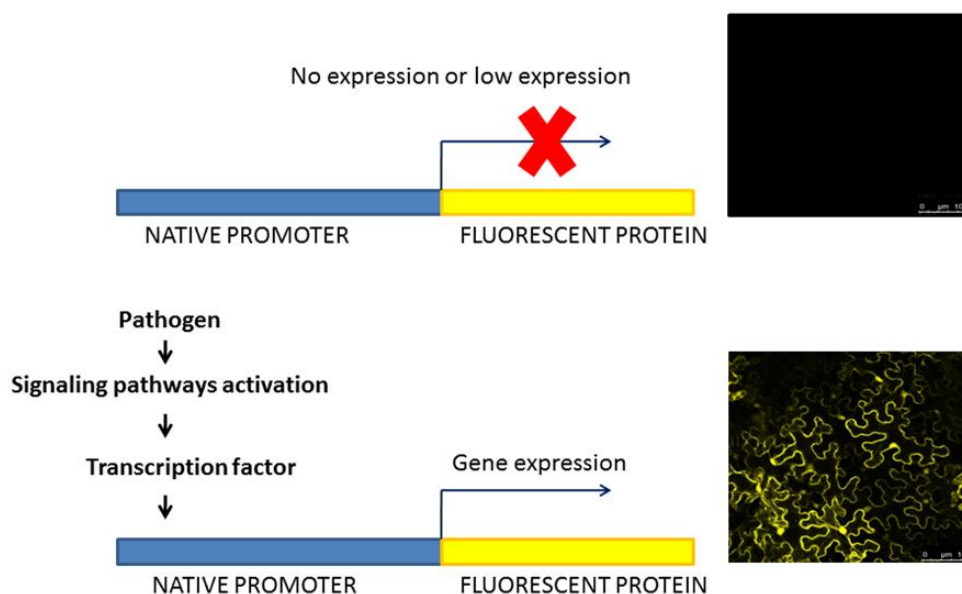


Figure 1.10: Use of transcriptional biosensor for studying biotic stress. A transcriptional biosensor is constructed by fusing the promoter of the gene of interest with a reporter gene coding for a fluorescent protein. After pathogen attack, signalling pathways are activated, which results in the expression of transcription factor(s). Transcription factor(s) bind to regulatory elements present in promoter region to activate transcription. Activation of the promoter, which controls reporter gene expression, leads to the synthesis of the reporter protein which can be detected by confocal microscope.

The main advantage of this version of biosensors is the ability to measure the signal in real time in living plant cells (Samodelov and Zurbriggen, 2017). Therefore, they are particularly useful to study the spatiotemporal distribution of phytohormones to get insight into the complex plant hormonal signalling pathways (Wells et al., 2013). To date, several genetically encoded fluorescent

sensors have been reported. Among them, only a few were designed to study plant immune response, including JA (Larrieu et al., 2015), Ca^{2+} (Toyota et al., 2008), ROS sensors (Jiang et al., 2006) and subcellular behaviour of multiple organelles in a biotrophic plant-microbe interaction (Ghareeb et al., 2016).

1.3 Research Hypotheses

Within this doctoral thesis we studied the processes leading to restriction of viral spread and multiplication during HR-conferred resistance. More specifically we studied the involvement of HR-associated cell death and ROS in virus restriction in HR-conferred resistance in potato and prepared the tools for the introduction of multigene constructs into the plant cells.

The research objectives were:

- 1) To get better insight into the mechanisms that underlie HR-conferred resistance against pathogens.
 - To decipher plant immune regulatory networks through promoter analysis.
 - To determine if the HR-associated cell death is separate from the resistance mechanisms which lead to PVY restriction in HR-conferred resistance in potato.
 - To study the involvement of redox sensitive oxygen species generated by RBOHD in the restriction of PVY in the HR-conferred resistance in potato.
- 2) To support faster introduction of synthetic biology into the plant science.
 - Generation and evaluation of the toolbox for cloning and delivery of multiple DNA fragments into the plant cells in a streamlined and highly efficient way.

We have tested the following hypotheses:

- 1) Promoters of several genes from the immune signalling harbour binding sites specific for the transcription factors involved in immune signalling.
- 2) Cell death is not restricting PVY spread in the HR-conferred resistance.
- 3) The introduction of AssemblX into plant synthetic biology will enable efficient and straightforward introduction of multigene constructs into plant cells.

The results responding to the first hypothesis are explained in the Results Section 3.2 and 3.3 and have not been published yet, the results confirming the second hypothesis are explained in the Results Section 3.4 and have been explained in Lukan et al. (2018a), while the results from the Results Section 3.1 correspond to the third hypothesis and have been explained in Lukan et al. (2018b). Unless otherwise specified, the work presented within this doctoral dissertation was performed by myself.

Chapter 2

Materials and Methods

2.1 *In silico* Analyses

2.1.1 Nucleotide BLAST

Basic local alignment search tool (blastn, Altschul et al., 1990) was used to search for the closest potato relative to AC01 gene from tomato (Solyc07g049530.2.1). blastn was not used to search for the closest potato relatives for other genes as the information regarding the candidates in potato was obtained from previously published data.

2.1.2 Analysis of promoter sequences

Promoter sequences of selected genes obtained from PW363, cv. Rywal and cv. Désirée were sequenced and compared to the available model genome sequences from PGSC database using AlignX (Lu, 2004). Pairwise comparison was created in order to show percent identity and differences between promoter sequences using CLC. Three databases, PlantCARE (Lescot et al., 2002), TRANSFAC (Wingender et al., 1996) and PLACE (Higo et al., 1998) were used to search for plant cis-regulatory elements and transcription factors specific for known transcription factor binding sites in promoter sequences.

2.1.3 *In silico* design of AssemblX constructs for expression in plants

When needed to be assembled from different subunits, the expression cassettes for AssemblX pL0A_0-1 Level 0 plasmid were designed *in silico* using the extended plant grammar implemented in GenoCAD (<http://www.genocad.com/>). Sequences were exported as GenBank format and imported into AssemblX web tool (www.assemblx.org) for the design and virtual assembly of multigene constructs and the primer design.

2.2 Cloning

2.2.1 Genomic DNA isolation

Genomic DNA (gDNA) was isolated from potato plants using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The concentrations and purity (260/280 and 269/230 absorption ratios) of the DNA samples were assessed using a NanoDrop ND1000 spectrophotometer (Nanodrop technologies).

2.2.2 Preparation of chemically competent *Escherichia coli* cells

Homemade chemically competent *E. coli* cells were prepared from TOP10 or *ccdB* Survival™ 2 *E. coli* strain (Thermo Fisher Scientific). 25 mL of overnight culture grown in Luria-Bertani (LB) medium (1 % (w/v) Bacto Tryptone, 0.5 % (w/v) Yeast Extract, 0.5 % (w/v) sodium chloride) was incubated at 37 °C until OD600 reached 0.4 – 0.6. The culture was centrifuged 10 minutes at 4000 rpm at 4 °C. The pellet was resuspended in 1.8 mL ice-cold CaCl₂-Tris-glycerol (final concentrations 50 mM CaCl₂, 10 mM Tris, 10 % glycerol), aliquoted, frozen in liquid nitrogen and stored at -80°C.

2.2.3 Preparation of electrocompetent *Escherichia coli* cells

Homemade electrocompetent *E. coli* cells were prepared from TOP10 or *ccdB* Survival™ 2 *E. coli* strain (Thermo Fisher Scientific). The culture was grown in LB medium until OD600 reached 0.4 – 0.6 as described above. The culture was cooled on ice for 10 minutes and then centrifuged 10 minutes at 5000 rpm at 4°C. The step was repeated twice after the cells were washed first in ice-cold sterile water and then in ice-cold sterile 10 % glycerol. Finally, after the last centrifugation step, the cells were resuspended in 1 ml of ice-cold sterile 10 % glycerol, aliquoted, frozen in liquid nitrogen and stored at -80°C.

2.2.4 Construction of sensors using Gateway recombination cloning

The location of the MYC2 (PGSC0003DMG400017535), TGA2 (PGSC0003DMG402023696), ACO2 (PGSC0003DMG400013894), PR1b (PGSC0003DMG400002028), PTI5 (PGSC0003DMG400010724) and MC (PGSC0003DMG400005950) genes within the potato genome was identified using Solanaceae Genomics Resource (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml) and Ensembl Plants portal (<http://plants.ensembl.org/index.html>) to identify promoter regions. Promoter regions of different lengths (500, 1000 and 1500 bp) were amplified from gDNA (100 ng) by touchdown nested PCR using Phusion® High-Fidelity DNA Polymerase (NEB) or VELOCITY DNA Polymerase (Bioline) with outer forward and reverse primers (F1.1/F2.1/F3.1 and R1) as shown in Figure 2.1 (1st PCR). PCR products were purified from the gel using Wizard® SV Gel and PCR Clean-Up System (Promega) and 1000-times diluted used as a template for the second PCR with inner primers (F1.2/F2.2/F3.2 and R2) as shown in Figure 2.1 (2th PCR). Primers are listed in Table 2.1 and the protocol for touchdown PCR is shown in Table 2.2.

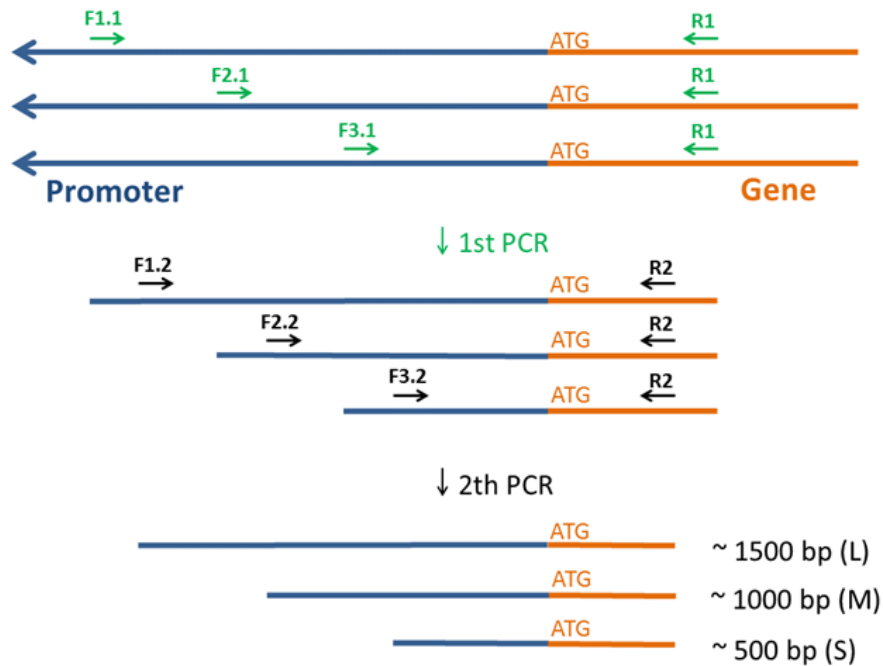


Figure 2.1: Amplification of the promoter regions of different lengths (approximately 500 (S), 1000 (M) and 1500 (L) bp) from genomic DNA. Promoter sequences of different lengths were amplified from gDNA with outer forward and reverse primers (F1.1/F2.1/F3.1 and R1). Following purification from the gel, PCR products were used as a template for the second PCR with inner primers (F1.2/F2.2/F3.2 and R2).

Table 2.1: Primers for the amplification of promoter regions from the genomic DNA. Gene identifiers are shown above.

Gene	Primer name	Sequence (5' → 3')
MYC2	F1.1	GCAACATTCTAATTCACCAACAAA
MYC2	F1.2	CTTTTAAACACTTTTGTGTTGGCC
MYC2	F2.1	CAACCTTTTAAATGTAAGTGTGGC
MYC2	F2.2	TTCCATAATGAGATAGCAACAAGGA
MYC2	F3.1	AAAAGATACTACTCCCTCTGTCCCA
MYC2	F3.2	CGACGATTTGGTCATGAAATCTTTA
MYC2	R1	CACAGCAGCAGAAGCAGAATTAGT
MYC2	R2	TTCCATCATAGACACGTTATCATCG
TGA2	F1.1	TTTAGTACTATACACATCCACCAACG
TGA2	F1.2	TCCGTTACAAATAATATGATTGAAGC
TGA2	F2.1	AAATTATACTCCGTTCCATTCAAA
TGA2	F2.2	TCCTTGACAATTCCTAATTCAATA
TGA2	F3.1	AAAAATAAATTCTGGGTTGTTGA
TGA2	F3.2	GAACCACTCCAACCTTGCTTATTT
TGA2	R1	TTCGATTAAACATTAGCACCATCTC
TGA2	R2	TATTTGTATTGTTCCCTTCAGTGC
ACO2	F1.1	CACTTGACGTTATTTTCTTACTCG
ACO2	F1.2	TGCAACTAACATACTTATGACTAATA
ACO2	F2.1	CATTAACAATCAAATCTTTTTTTTCGG
ACO2	F2.2	TTTCCATGGGGCAAATGTGC
ACO2	F3.1	ATAGGGGCGAAGCTAGAATGT
ACO2	F3.2	CTTTCCTTTAGTTAGGAGGATTGA
ACO2	R1	CACTTCTTGTAATGTCCCTTTGT
ACO2	R2C	TGGGATTCCATGGTTCACCAA
ACO2	R2T	TTCATGTGGAATTCATGGTTCA
PR1b	F1.1	GTCATTCTCCAAATCAAAATCAGTG
PR1b	F1.2	AAAATAGAAGTCAAGAAAAAATCTCC
PR1b	F2.1	TATTCGTAAATTCAACTTCAACCCTT
PR1b	F2.2	ATTCTCATTCGAAGCTGCCAG
PR1b	F3.1	TAGACTTTTTATTCCACCCAAAAATG
PR1b	F3.2	CAAGAATTACCTAGATTAAAGTATGAC
PR1b	R1	AAGATAGTCTTGGGGTGAATTTGGG
PR1b	R2	TACCATGAGACAAGTGAGTAACAA
MC	F1.1	AGCTAAAATCCAAATCCATCAAAT
MC	F1.2	GGCTAGAACCACCTTAAAATGAATAATT
MC	F2.1	TGAGTATACATTTCGATTTGAAGACG
MC	F2.2	CGAAGTGAGTTTGTATGTTTTGAAA
MC	F3.1	TTGCTACAAAAATTTGATACAAAACC
MC	F3.2	TTTCGATTCTTTTGACATATTACCC
MC	R1	ACTATTTGTTGCTTCACGTTCAAA
MC	R2	AGCAAGATCATCAAACCTCGACTTT

Table 2.2: Touchdown PCR cycling program for the amplification of the promoter regions. * denotes that the temperature is gradually decreased for 1 °C per cycle.

Phase	Step	Temperature	Time	No of cycles
	Initial denaturation	96°C	2 min	
Touchdown phase	Denaturation	96°C	30 s	15 cycles*
	Annealing	60°C	30 s	
	Extension	72°C	2 min 30 s	
Amplification phase	Denaturation	96°C	30 s	15 cycles
	Annealing	45°C	30 s	
	Extension	72°C	2 min 30 s	
	Final extension	72°C	5 min	

In the next step, PCR products were purified from the gel as explained above and cloned into the pJET 1.2 blunt cloning vector (Thermo Fisher Scientific) according to the manufacturer's protocol. In order to show the importance of different lengths for promoter activity, different lengths of promoters regions (see Table 2.1) were cloned into pJET for MYC promoters (see Table 2.5). For the promoters of other genes only one length was selected. Chemically competent *E. coli* were transformed with the constructs, plasmids were isolated from three bacteria colonies for the promoters of each gene using GenElute™ Plasmid Miniprep Kit (Sigma) and sequenced (GATC Biotech). The longest promoter regions (L in Table 2.1) were fully sequenced, using pJET forward and reverse primers (Thermo Fisher Scientific) and the primers listed in Table 2.3.

Table 2.3: Sequencing primers

Promoter	Primer name	Sequence
MYC2	MYCR600seq	AGTAAATTATTGATATTGAAGGAC
MYC2	MYCF840seq	TAAAGTATTAACGATTACAATAATAA
TGA2	TGAF1090seq	AAAATTATACAAAAATAAATTCTGGG
TGA2	TGAR1470seq	ACTTTAATACATACACCGCAT
TGA2	TGAR730seq	CCCAAAGTTTTTATGTTTTTCAC
ACO2	ACOF680seq	CAAAATGTGCAGCCCTAAA
ACO2	ACOR830seq	AGTTGGGACAGTTAGAATTTG
ACO2	ACOR1670seq	CAAAGAAGCCCCAATTTTC
PR1b	PR1bF1040seq	GTTGTAGAACATGTGTGAG
PR1b	PR1bR740seq	ATGCGATTCAGTGTTTACTT
MC	MCFPWseq	TACACGATAAGGAATGTAAAAGT
MC	MCRPWseq	GATTGTAACGATGGTGTAAAATC
MC	MCF902RyDeseq	GTTACAATCTTCGTCACTTTCTA
MC	MCR1009RyDeseq	TTCTTATCGTGTAGCTGGAT
MC	MCF710RyDeseq	GAATCCTATCACCTCGTAAAC
MC	MCR802RyDeseq	CAAAGAGAATGCAATACACTTAC
MC	MCF800PWseq	GAAAATCTTCAAMATCAAATTATTATC
MC	MCF735RyDeseq	AACGGTTTAAAAAGAATTAATTGTAA

Several database searches, mentioned in the *In silico* analysis section 2.1.2, were performed to analyze obtained sequences. For the fusion of the promoter region of the selected length with the

fluorescent reporter, promoter sequences in pJET were amplified using forward primers, designed with CACC tail at the 5' end (bold in Table 2.4) and reverse primers with overlapping sequence (lower case in Table 2.4), reverse complementary to the fluorescent protein (see Table 2.4 for the list of primers and Table 2.5 for the combination of primers for PCR). Different primers for the amplification of the promoters of the particular gene were designed according to the determined SNPs in the promoters of different potato cultivars.

Table 2.4: List of primers for the fusion of promoters with fluorescent proteins. Bold letters mark CACC tail, lower case letters mark an overlapping sequence, reverse complementary to the fluorescent protein. P: promoter region from PW363, R: promoter region from cv. Rywal, D: promoter region from cv. Désirée. Primers for the amplification of PR1b promoter are listed in Pezić et al., 2016.

Promoter	Primer	Sequence (5' → 3')
MYC2 (P/R/D)	A	CACC TGTTAGGGCATTGAGTCATTAATAGAAAA
MYC2 (P/R/D)	A2	CACC TTCTTCTCTAGCTTTACAATTCATTCC
MYC2 (P/R/D)	B	CATGTTATCCTCCTCGCCCTTGCTCACCatggtgggtaagctgtattcagtc
MYC2 (P/R/D)	C	ATGGTGAGCAAGGGCGAG
MYC2 (P/R/D)	D	TTACTTGTACAGCTCGTCCATGC
MC (P)	A	CACC ATTTTACACCATCGTTACAATCTTCGT
MC (R/D)	A	CACC TCAACTGTACACTTATTATTCACGGGAA
MC (P/R/D)	Gf	tcgcagtATGGTGAGCAAGGGCGAG
MC (P/R/D)	Gr	ctcaccatCACTGCGAATAATTAATAACTTAATTAAG
MC (P/R/D)	D	TTACTTGTACAGCTCGTCCATGCCGAGAGTGA
ACO2 (R)	A	CACC TGCAACTAACATACTTATGACTAATAAAATATWAATGG
ACO2 (R)	B	AGCTCCTCGCCCTTGCTCACCATTTTAAATTTCTTGAAGAAatggtgagcaagg gcgaggagct
ACO2 (R)	C	ATGGTGAGCAAGGGCGAG
ACO2 (R)	D	TTACTTGTACAGCTCGTCCATGC
TGA2 (P/R)	A	CACC TCCGTTACAAATAATATGATTGAAGCGAG
TGA2 (P/R)	B	GGCTATAAGAGTGGTACTTCTGGGatggtgagcaagggcgaggagctgttc
TGA2 (P/R)	C	ATGGTGAGCAAGGGCGAG
TGA2 (P/R)	D	TTACTTGTACAGCTCGTCCATGC

Table 2.5: Primer combinations for the amplification of the promoter regions with PCR. For the theoretical length of the amplified promoter and explanation of the marks L and M regions see Figure 2.1. Letters P, R and D stand for PW363, Rywal and Désirée, while the numbers 1, 2 and 3 stand for the colony number used for the isolation of the construct following pJET transformation. For example, P2 stands for the promoter region amplified from PW363 and isolated from the second colony after pJET transformation. Primer combinations for the amplification of PR1b promoter regions are listed in Pezić et al., 2016.

Promoter	Primer combination
MYC2 (P2_L)	A/B
MYC2 (P2_M)	A2/B
MYC2 (R2_L)	A/B
MYC2 (R2_M)	A2/B
MYC2 (R3_M)	A/B
MYC2 (R3_M)	A2/B
MYC2 (D1_L)	A/B
MYC2 (D1_M)	A2/B
MC (P1)	A/Gr
MC (D1)	A/Gr
ACO2 (R2)	A/B
TGA2 (P1)	A/B
TGA2 (R1)	A/B

After the isolation from the gel, the amplicons were fused with the fluorescent proteins using fusion PCR according to Rupar et al. (2015) but with changed temperature and elongation time depending on the melting temperature of the used primers and the length of the amplicon (see Figure 2.2 A for the cloning procedure and Table 2.6 for the list of primer combinations) or Gibson Assembly cloning kit (see NEB for the protocol, Figure 2.2 B for the cloning procedure and Table 2.5 for the list of primers). Briefly, promoter sequences were amplified using A/B primer combinations. B primers contained overlapping sequence, complementary to fluorescent proteins and served as a primer for the second PCR (Table 2.4). Sequence coding for fluorescence protein was amplified using C and D primers (Table 2.4). In the second PCR, the amplified DNA segments were mixed, allowed to anneal and amplified to form a fused construct. Melting temperature of the overlapping sequence (hybridisation fusion zone) was 10 °C higher than the melting temperature of the primer B and it was at least 15 nt long. The amplicons were purified from the gel as described above. The resulting constructs and their lengths are listed in the Results (Table 3.3 and Table 3.4). Promoter::fluorescent protein constructs were afterwards inserted into the pENTR/D TOPO vector (Thermo Fisher Scientific) following the manufacturer's protocol (Figure 2.2 C). In the next step, constructs were recombined from pENTR/D TOPO vector into the binary destination vector pH7WG, pK7WG or pB7WG (VIB) in a process of LR-reaction (Figure 2.2 C).

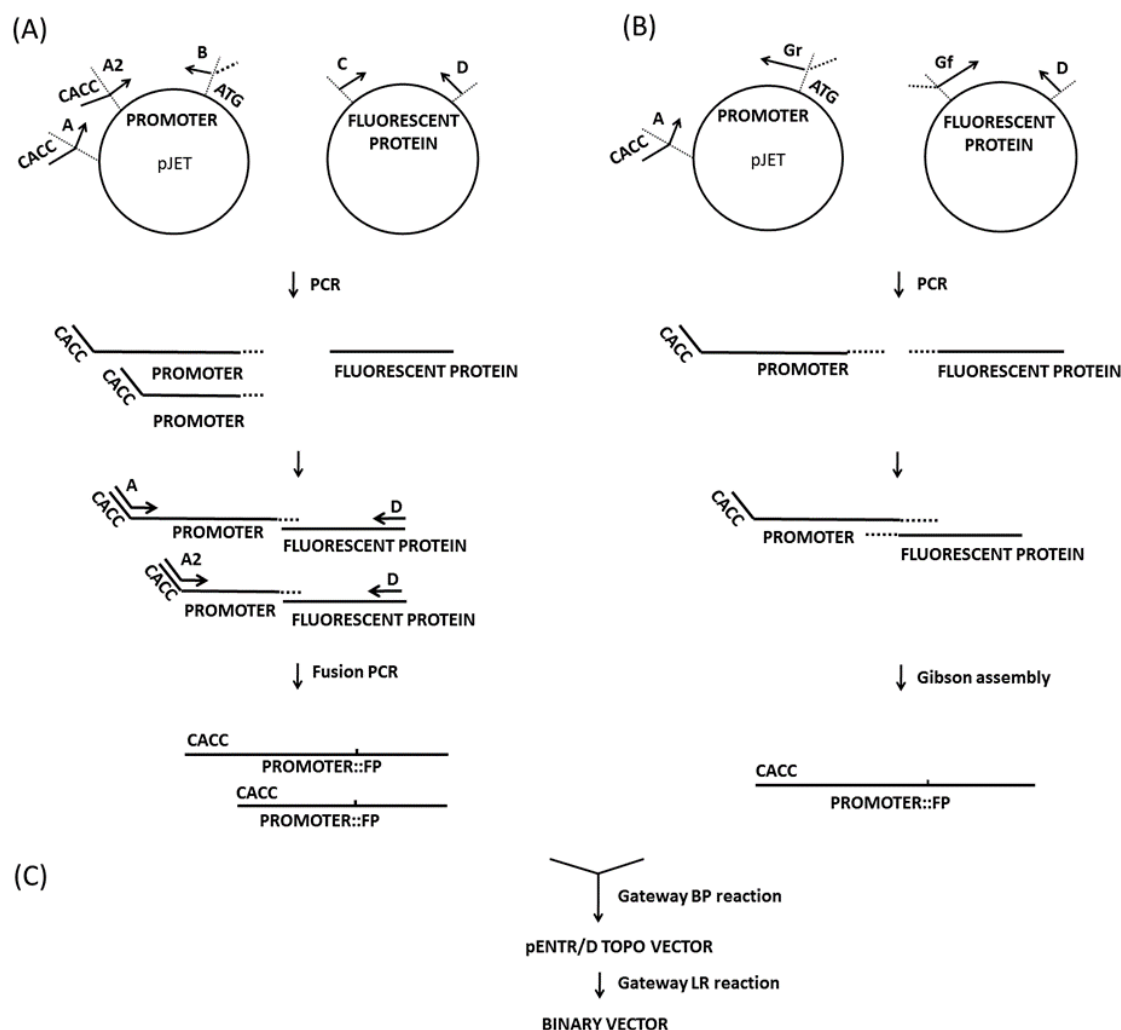


Figure 2.2: Construction of promoter::fluorescent protein constructs. (A) Construction of promoter::fluorescent protein constructs using fusion PCR. The promoter sequence and the coding sequence for the fluorescent protein were amplified from the template plasmids. In the next step, amplicons were fused into a promoter::fluorescent protein construct by fusion PCR. Cycling program for the fusion PCR was explained in Rupar et al., 2015. (B) Construction of promoter::fluorescent protein constructs using the Gibson assembly (NEB). The promoter sequence and the coding sequence for the fluorescent protein were amplified from the template plasmids. In the next step, amplicons were fused into a promoter::fluorescent protein construct by Gibson assembly. (C) Cloning of the promoter::fluorescent protein construct into binary vector. The construct, fused by fusion PCR or Gibson assembly method was introduced into the destination binary vector using Gateway cloning. FP: fluorescent protein.

Table 2.6: Primer combinations for fusion PCR or Gibson assembly. Primers were used to obtain promoter::fluorescent protein constructs. Primer combinations for the construction of PR1b promoter::fluorescent protein constructs are listed in Pezić et al. (2016). * No primers are needed in case of Gibson assembly (constructs with MC promoters).

Construct	Primer
MYC2 (P2_L)::mCherry	A/D
MYC2 (P2_M)::mCherry	A2/D
MYC2 (R2_L)::mCherry	A/D
MYC2 (R2_M)::mCherry	A2/D
MYC2 (R3_L)::mCherry	A/D
MYC2 (R3_M)::mCherry	A2/D
MYC2 (D1_L)::mCherry	A/D
MYC2 (D1_M)::mCherry	A2/D
MC (P3)::YFP*	/
MC (D3)::YFP*	/
ACO2 (R2)::CFP	A/D
ACO2 (R2)::YFP	A/D
TGA2 (P1)::YFP	A/D
TGA2 (P1)::CFP	A/D
TGA2 (R1)::YFP	A/D
TGA2 (R1)::CFP	A/D

Binary vectors pH7WG, pK7WG or pB7WG are suitable for the promoter analysis in plants, as they are free of regulatory elements and they allow for introduction of the desired promoter region and coding sequence. The map of pK7WG vector is presented in Figure 2.3.

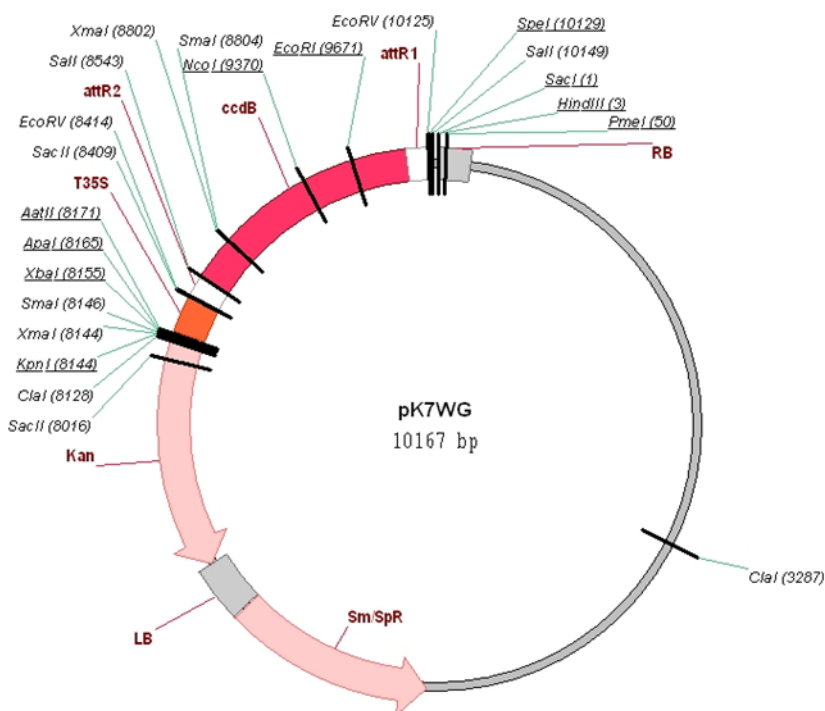


Figure 2.3: Vector map of pK7WG binary vector. pK7WG is free of the promoter region and is thus suitable for promoter analysis. It enables introduction of desired promoter sequence fused to reporter gene using Gateway cloning by homologous recombination through attR1 and attR2 homology regions. It contains spectinomycin selection marker for the selection in *E. coli* and *A. tumefaciens* and kanamycin selection marker for the selection in plants. It contains *ccdB* cassette to disable the growth of *E. coli* transformed with empty vector and left and right T-DNA border (LB, RB) to initiate introduction of the construct between LR and RB into the plant genome. pH7WG and pB7WG binary vectors differ from pK7WG only in regards to plant selection marker. pH7WG contains hygromycin selection marker, while pB7WG contains glufosinate ammonium selection marker.

2.2.5 In vitro DNA assembly cloning reactions

For the optimization of insert cloning in Plant X-tender vectors, different overlap-depending assembly methods were used as described below.

SLiCE extract was prepared as described elsewhere (Messerschmidt et al., 2016) with some modifications. Cells were grown in 100 ml 2 x YT medium in 250 ml baffled flasks to OD600 = 2. SLiCE reactions were performed as described by Zhang (2012) with 50–500 ng of linear vector and an appropriate amount of insert DNA ranging from 1:2 to 1:20 vector to insert molar ratio.

Assembly with NEBuilder HiFi assembly master mix or Gibson assembly master mix was performed according to the manufacturer's recommendations (NEB). Assembly reaction contained 50–500 ng of linear vector, an appropriate amount of insert DNA in a 1:2 to 1:10 vector to insert molar ratio and 2x NEBuilder HiFi or Gibson DNA Assembly Master Mix. Assembly reactions were incubated at 50 °C for 1 h.

Appropriate volumes of the SLiCE, HiFi or Gibson DNA assembled products were transformed into TOP10 *E. coli* (Invitrogen) or NEB5α

(NEB) by electroporation or heat shock according to the manufacturer's protocols. Transformed cells were spread on plates containing appropriate antibiotic, ampicillin (100 µg/ml), spectinomycin (75 µg/ml), kanamycin (50 µg/ml) or rifampicin (20 µg/ml).

2.2.6 In vivo DNA assembly cloning reactions

In vivo DNA assembly cloning was used for the assembly into Level 1 AssemblX vectors and was performed as described by Hochrein et al. (2017). *S. cerevisiae* transformation was performed according to the LiAc/SS carrier DNA/PEG method (Gietz and Schiestl, 2007) using strains YPH500 (ATCC® 76626™) or BY4741 (ATCC® 201388™). The plasmids were isolated from positive colonies using Zymoprep Yeast Miniprep II kit (Zymo Research Corporation) and transformed into electrocompetent ElectroMAX™ DH5α-E™ *E. coli* (Thermo Fisher Scientific).

2.2.7 Construction of Plant X-tender expression vectors

pCambia1300 (Marker Gene Technologies, M1591) was digested with *Bam*HI and *Hind*III (NEB) and purified from the gel. pK7WG2, pB7WG2 and pH7WG2 (Karimi et al., 2002) were digested with *Xba*I and *Sac*I (NEB), allowing the release of T35S-AttR2-*ccdB*-AttR1 cassette from the vector backbone and purified from the gel. The *ccdB* region was amplified from AssemblX pLOA_0-1 Level 0 plasmid (Hochrein et al., 2017) using KG15/KG16 primers, purified from the gel and amplified with KG15/KG18 for pCambia1300 and KG19/KG21 primers for the other three plasmid backbones to add homology regions with flanking restriction enzyme recognition sites (Table 2.7).

Table 2.7: Primers used for the construction of Plant X-tender expression vectors.

Primer	Primer sequence (5' → 3')
KG15_F	CCATGATTACGAATTCGAGCTCGGTACCCGGTAGGGATAACAGGGTAATATCCCTATTGTCCCATTAAATAGGAAAGTCTGGTAACTCAGG
KG16_R	GGGTAATTGAATAACTGCTCCCGTTGTAAAAGTATCCGTAGTGTGACTGAAACCTTAAAGCTTACTGCTGTGTATAAGGG
KG18_R	CGTTGTAAAACGACGGCCAGTGCCGTAGGGATAACAGGGTAATTGAATAACTGCTCCCGTTGTAAAAGT
KG19_F	ATAATTCGCGGTACCCGGGGATCCTAGGGATAACAGGGTAATATCCCTATTGTCCCATTAAATAGGAAAGTCTGGTAACTCAGG
KG21_R	TCTGATCCAAGCTCAAGCTAAGCTGTAGGGATAACAGGGTAATTGAATAACTGCTCCCGTTGTAAAAGT

The gel-isolated I-*Sce*I-A0-*Hind*III-*ccdB*-*Hind*III-B0-I-*Sce*I cassette was assembled into the purified plasmid backbones using different assembly methods and transformed into commercial or homemade One Shot® *ccdB* Survival™ 2 T1R Competent Cells (Thermo Fisher Scientific).

To confirm the functionality of the *ccdB* gene, *ccdB* Survival *E. coli* strain which allows propagation of plasmids containing the *ccdB* gene and *ccdB*-sensitive *E. coli* strain which precludes propagation of plasmids containing the *ccdB* gene were transformed with purified plasmids.

The correct constructions of the Plant X-tender expression vectors were verified by sequencing the homology regions A0 and B0, I-*Sce*I and *Hind*III recognition sites and the *ccdB* gene.

2.2.8 Construction of assembly cassettes and cloning into Plant X-tender expression vectors

pL0A_0-1, pL0A_0-R and pL0A_1-R Level 0 vectors (Hochrein et al, 2017) were linearized with *Hind*III as described in Hochrein et al. (2017). Level 0 units (Table 2.8) were amplified from templates using Phusion® High-Fidelity DNA Polymerase (NEB) and primers with flanking homologous sequences (Table 2.8) according to the manufacturer's protocol. Following agarose gel electrophoresis, Level 0 units were purified from the gel using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel), assembled into Level 0 vectors using NEBuilder HiFi assembly master mix (NEB) and transformed into *E. coli* NEB 5α (NEB).

Table 2.8: Oligonucleotides used for the amplification of Level 0 subunits by PCR. (A) Oligonucleotides used for the amplification of Level 0 subunit for the assembly of expression cassette p35S::H2BRFP_tNOS. (B) Oligonucleotides used for the amplification of Level 0 subunits for the assembly of multigene construct p35S::H2BRFP_tNOS + pNOS::ECFP_t35S. Nucleotides in bold represent overlaps between adjacent parts, e.g. homology regions of destination plasmid (Level 0 AssemblX vector) or sequence of adjacent modules.

(A) 35S::H2BRFP_tNOS

Primer	Primer sequence (5' → 3')	Level 0 unit
KG1_F	CAGGACACTGTATCTGCTACGCTGTTTAT GATTAGCCTTTTCA ATTTCAGAAAGAATGCTAACCC	A0 p35S::H2BRFP_tNOS
KG2_R	AAATGATGAATTGAATTGAAAAGCTA CCCTCACTAAAGGGAAC AAAAGCTGG	p35S::H2BRFP_tNOS AR

(B) Multigene construct / p35S::H2BRFP_tNOS

Primer	Primer sequence (5' → 3')	Level 0 unit
KG3_F	GGACACTGTATCTGCTACGCTGTTTAT GATTAGCCTTTTCAAT TTCAGAAAGAATGCTAACCC	A0 p35S::H2BRFP_tNOS
KG4_R	GCAGCGTTTATTGTGCGAATCTCACTTTA CCCTCACTAAAGGG AACAAAAGCTGG	p35S::H2BRFP_tNOS A1

Multigene construct / pNOS::ECFP_t35S

Primer	Primer sequence (5' → 3')	Level 0 subunits
KG5_F	TTTCGCACAATAAACGCTGCTTCTGTT GAGCGGAGAATTAAGGG AGTCACG	A1 pNOS
KG6_R	CCTTGCTCACCAT GCGAAACGATCCAGATCCGGTGC	pNOS ECFP
KG7_F	TGGATCGTTTCGC ATGGTGAGCAAGGGCGAGGAGC	pNOS ECFP
KG8_R	TCTAGCATGGCCG TTACTTGACAGCTCGTCCATGCCG	ECFP t35S
KG9_F	GCTGTACAAGTAAC GCCCATGCTAGAGTCCGC	ECFP t35S
KG10_R	AAATGATGAATTGAATTGAAAAGCTA AGGTCAGTGGATTTTGG TTTtagg	t35S AR

pL1A-hc / pL1A-lc (A0/AR) Level 1 vectors (Hochrein et al, 2017) were linearized with *Pac*I (NEB). Expression cassettes were released from Level 0 vectors using *Pme*I (NEB), separated by

agarose gel electrophoresis and purified from the gel as described above. Multiple cassettes were assembled into Level 1 vectors using TAR or HiFi. Plasmids were isolated from positive yeast colonies and transformed into electrocompetent ElectroMAX™DH5α-E™ Cells (Thermo Fisher Scientific).

Multigene constructs were released from Level 1 vector with I-SceI (NEB). For inserts with a similar length to the backbone, the plasmid was additionally digested with NheI (NEB) to allow separation of the insert and the backbone by gel electrophoresis. Following isolation from the gel using Wizard® SV Gel and PCR Clean-Up System (Promega), inserts were assembled into *Hind*III linearized Plant X-tender expression vectors by HiFi or SLiCE assembly method. Constructs were transformed into One Shot® TOP10 Chemically Competent *E. coli* (Thermo Fischer Scientific), homemade TOP10 chemically competent *E. coli* or homemade TOP10 electrocompetent *E. coli*. Incorrect assemblies were selected against by the expression of a suicide gene and by antibiotic selection.

2.3 Plant Material

N. benthamiana seeds were soaked in 0.034 % (w/v) gibberellic acid overnight to induce germination. The next day seeds were rinsed three times with sterile water, transferred to soil and grown under controlled environmental conditions (21 ± 2 °C in the light and 18 ± 1 °C in the dark, at a relative humidity of 75 ± 2 %, with 70-90 mmol/m²/s² radiation and a 16-h photoperiod).

Four different potato genotypes (PW363 breeding clone, cv. Rywal, cv. Désirée and NahG-Rywal transgenic line (Baebler et al., 2014)) were used. Potato plants were obtained from the Institute of Plant Breeding and Acclimatisation–National Research Institute, Młochów, Poland (cv. Rywal, cv. NahG-Rywal, PW363 breeding clone) and from the Leibniz Institute of Plant Biochemistry, Halle, Germany (cv. Désirée). Potato plants were grown in stem node tissue culture as previously described (Baebler et al., 2009). Two weeks after node segmentation, they were transferred to soil in a growth chamber and kept under controlled environmental conditions as described for *N. benthamiana*.

2.3.1 Virus inoculation

Green fluorescent protein-tagged PVY N605 infective clone (PVY N605-GFP) was introduced in tobacco plants by the colleague from our research group using biolistic bombardment (Rupar et al., 2015). After several passages of infection, the virus was further multiplied and maintained in tobacco plants, *N. tabacum* L. cv. Xanthi. We ensured that the strength of the inoculum used for the inoculation of potato plants was comparable among all experiments. First, we used frozen PVY N605-GFP infected leaves from the same batch to inoculate tobacco plants in each of the related experiments. After the virus had spread to tobacco systemic leaves, its presence was confirmed under confocal microscope. Next, only the infected areas of the leaves with comparable intensity of GFP fluorescence were used for inoculum preparation. Selected areas of infected tobacco leaves were weighed and ground in 20 mM phosphate buffer supplemented with 0.025 g of diethyldithiocarbamic acid (DIECA) in the ratio 1:4 prior potato inoculation in all experiments. PVY inoculation was performed as described elsewhere (Baebler et al., 2011). In short, the first three lower fully developed leaves of four weeks old plants were dusted with carborundum powder and rubbed with the inoculum

(approximately 60 µl per leaf). After 10 min, the leaves were washed with tap water.

2.3.2 Plant transformation

2.3.2.1 Transient transformation of *Nicotiana benthamiana*

Three to five weeks old *N. benthamiana* plants were used for transient transformation. Constructs were introduced into homemade electrocompetent *Agrobacterium tumefaciens* GV3101 by electroporation (Eppendorf Electroporator 2510) following the manufacturer's procedure at 2000 V and confirmed by colony PCR. The transformed cells were cultured to an OD600 = 0.5, harvested by centrifugation, resuspended in 0.2 mM acetosyringone solution to OD600 = 0.5 and mixed with *A. tumefaciens* transformed with silencing suppressor p19 (kindly provided by Jacek Hennig, Polish Academy of Sciences, Poland) in the ratio 1:1. The mixture was infiltrated into the second fully developed bottom leaf of *N. benthamiana* plants. *A. tumefaciens* GV3101 without binary vector was used as a control.

2.3.2.2 Stable transformation of potato

Stable transformation of plants of cv. Rywal and cv. Désirée with the ACO2::fluorescent protein constructs and MC::fluorescent protein constructs was performed as described by Pezić (2016). Plants carrying TGA2::fluorescent protein constructs were prepared as a part of Master degree thesis within our group (Pezic, 2016).

2.3.3 Hormonal treatment

For sensor testing in transgenic potato plants after stable transformation, three to five weeks old transgenic potato plants were treated with methyl jasmonate (MeJA) (Sigma), aminocyclopropanecarboxylic acid (ACC) (Sigma) or 2,6-dichloroisonicotinic acid (INA) (Sigma). Plants were exposed to MeJA in airtight chambers made of plexiglass for 1 h or sprayed with 300 µM or 1 µM INA (diluted in 1 % ethanol) or 1 mM ACC (diluted in water). One leaf of each treated plant was cut from the plant and sampled for qPCR and RT-qPCR analysis. Another leaf of each treated plant was cut from the plant and analysed under confocal microscope. Samples for qPCR and RT-qPCR were analysed 5 h or 23 h after being exposed to MeJA for 1 h; 1 h or 24 h after INA treatment; and 6 h or 24 h after ACC treatment, while the leaves were analysed under confocal microscope 1 h, 5 h, 6 h, 24 h or 51 h after being exposed to MeJA for 1 h; 2.5 h, 21 h or 24 h after INA treatment; and 6 h, 24 h, 48 h or 55 h after ACC treatment.

In case of transient expression analyses, *N. benthamiana* plants were treated with MeJA, 1 mM ACC, 0.3 mM INA or colorado potato beetle saliva (CPBS) three, four or six days after agroinfiltration with the *A. tumefaciens* containing the construct promoter::fluorescent protein coding sequence. Prior to the application of CPBS, leaves were injured with the tweezers. Plants were analysed immediately, 20 h or 24 h after being exposed to MeJA for 24 h; 48 h after INA treatment; 24 h after ACC treatment; and immediately or 24 h after CPBS treatment under confocal microscope.

As a control, plants were treated with 1 % ethanol, water or were closed in empty airtight chambers for the same time as the treated samples in case of INA, ACC or MeJA treatment, respectively.

2.4 PCR and Sequencing

2.4.1 PCR for the confirmation of transformation

After stable transformation of potato cv. Désirée with the construct MC(D3)::YFP PCR was used to confirm the presence of the construct in the genomic DNA. Detection of the genes coding for fluorescent proteins was performed using KAPA2G Robust HotStart PCR Kit (Kapa Biosystems) according to the manufacturer's protocol using C/D primer pairs from Table 2.4. Undiluted genomic DNA isolated from transgenic potato plants was used as a template.

2.4.2 RT-qPCR and qPCR for sensors testing

After stable transformation of potato cv. Rywal and cv. Désirée with the constructs promoter::fluorescent protein qPCR was used to confirm the presence of the construct in the genomic DNA and RT-qPCR was used for the expression analysis of fluorescent proteins.

RNA was isolated from plant material (one quarter of the leaf) using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions (Supplementary Table 13, RT-qPCR 1, 2 and 4) or using Direct-zol™ RNA MiniPrep Plus (Zymo Research) according to the manufacturer's instructions (Supplementary Table 13, RT-qPCR 3). Samples, analysed by RT-qPCR 1-4 were from the same experiment (see Section 2.3.3).

For RT-qPCR (expression analyses), RNA was treated with the DNase (Qiagen). The quality and quantity of DNase-treated (0.5 µl/DNase per µg RNA) total RNA was determined using agarose gel electrophoresis and Nanodrop and then reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

For qPCR (confirming the presence of the construct promoter::fluorescent protein in the genomic DNA of transgenic potato cultivars), DNA residue in the RNA preparation prior to the DNase treatment was used as a template for qPCR.

For both, qPCR and RT-qPCR, the reactions were performed on a ViiA 7 Real-Time PCR System or The 7900HT Fast Real-Time PCR System (Applied Biosystems) in 384-well plate format using universal cycling conditions. Reactions were performed in a final reaction volume of 5 µL. Each reaction was performed in two replicate wells in two dilutions on the 384-well plate. Primers were designed using Primer Express Software 2 (Applied Biosystems) and synthesized by Integrated DNA Technologies (IDT). SYBR Green I chemistry was used for the detection of fluorescent proteins expression or to detect presence of construct encoding fluorescent proteins in gDNA using primer pair CAGGAGCGCACCATTCTT/GGGTGTGCGCCCTCGAACT. Presence of construct in gDNA or expression of fluorescent proteins was analysed and normalized to expression of cytochrome oxidase (Cox) according to Baebler et al. (2011). For the detection of Cox expression we used TaqMan chemistry as previously described (Baebler et al., 2011). Data were analyzed using the QuantStudio™ Software V1.3 or SDS 2.4 Software. The standard curve method was used for relative gene expression quantification using quantGenius (<http://quantgenius.nib.si>; Baebler et al., 2017).

2.4.3 RT-qPCR for the following of virus spread

Relative PVY RNA abundance was determined in upper non-inoculated leaves of Rywal and NahG-Rywal plants 4 weeks after inoculation with PVY N605-GFP. RNA was isolated from upper non-inoculated

leaves, treated with the DNase and reverse transcribed as described above for RT-qPCR 1, 2 and 4.

The amount of viral RNA was normalized to expression of Cox as described above. For the detection of PVY RNA TaqMan chemistry was used as previously described (Baebler et al., 2011). Relative quantification was performed as described above. Results were obtained from two independent experiments.

2.4.4 Colony PCR and sequencing for the verification of assemblies

Positive assemblies were confirmed by colony PCR using KAPA Taq PCR Kits (Kapa Biosystems) or Maxima Hot Start Green PCR Master Mix (Thermo Fisher Scientific) following the manufacturer's protocols using primers listed in Tables 2.5 and 2.6 or primers specific for pJET or pENTR plasmids in case of promoter::fluorescent constructs verification of the assembly and primers listed in Tables 2.7 and 2.9 in case of Plant X-tender expression cassettes verification of the assembly. For further verification, plasmids were isolated from positive colonies using GeneElute Plasmid Miniprep Kit (Sigma) or NucleoSpin Plasmid Easy Pure (Macherey-Nagel) and analysed by sequencing. DNA concentration and purity were evaluated using NanoDrop ND1000 spectrophotometer (Nanodrop technologies). Plasmid DNA and oligonucleotides were prepared according to service provider requirements (LGC Genomics or GATC service provider). Sequences were analysed with CLC Main Workbench (Qiagen) or AlignX (Lu, 2004). Primers used for the sequencing are listed in Tables 2.5, 2.6 and 2.9.

Table 2.9: List of primers for sequencing and colony PCR.

Primer name	Primer sequence (5' → 3')
M13F	GTAAAACGACGGCCAGT
M13R	CAGGAAACAGCTATGAC
PVIBF	TAGGGATAACAGGGTAATATC
PVIBR8527	AACGGCTCTCTCTTTTATAG
FM176	AGCAGCCGATGTCTGTTGT
FM218	TAGCTCTTGATCCGGCAA
KG022	CACCTTCCTTTTCCACTATCT
FM182	GAAGAAAGCGAAAGGAGC
FM1113	CAAAGACCCCAACGAGAA
KG032	CCTAATGCTTCAACTAACTCC
FM131	AATACGCAAACCGCCTCTC
FM032	GGAGTTAGTTGAAGCATTAGG
KG34	AACCCATCTCATAAATAACG
KG32	GTCCTTGAAGAAGATGGT
CaMV	CCACGTCTTCAAAGCAAGTG
PVIBR	TGTAGGGATAACAGGGTAAT

2.5 Imaging

2.5.1 Macroscopic analyses

2.5.1.1 Dynamics of lesion formation

16 Rywal and 11 NahG-Rywal plants were inoculated as described above. Lesions were counted on each inoculated leaf at 3, 4, 5, 6, 7, 9 and 11 dpi. Statistical analysis was conducted separately per cultivar using R3.4.0 (R Core Team, 2016). Samples (leaves) with more than 50 % missing values maximal number of lesions lower than 4 were filtered out. Cumulative sums of lesion counts in consecutive time points were used to compute the cumulative relative frequency distribution (CRFD) per sample. The filtered data set was imputed using cumulative maximum, and only samples with monotonically non-decreasing functions were kept. Logistic function, cumulative distribution function (CDF) of the logistic distributions, is a solution for a population growth with a carrying capacity model. Nonlinear regression model with the self-starting logistic growth function approach was used to get the function that best describes the data. CDFs were used to predict a response and calculate the probability of lesion forming before or between measured time points. A few discrepancies in the lesion number counted by eye and under confocal microscope (see below) at early time points can be attributed to the lesion size, as some of the lesions were not visible by eye.

2.5.1.2 Dynamics of lesion expansion

To follow the speed of the lesion expansion, leaves from virus inoculated plants from 13 independent experiments for cv. Rywal (more than 100 lesions) and five independent experiments for NahG-Rywal (48 lesions) were continuously monitored using Dino-Lite Edge AM7915MZTL digital microscope (AnMo Electronics Corporation) in different time intervals from immediately after the inoculation on until or only at later time points. Some of the lesions were followed until 42 dpi.

2.5.2 Confocal microscopy

For all confocal imaging Leica TCS LSI confocal microscope with Plan APO 5× objective (Leica Microsystems) or Leica TCS SP5 laser scanning confocal microscope mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems) were used.

2.5.2.1 Settings

Laser lines used for the excitation and emission windows for the detection of particular fluorescent protein, fluorochrome or chlorophyll are specified in Table 2.10.

Table 2.10: Excitation and emission settings.

Following	Excitation	Emission
Enhanced cyan fluorescent protein	405	450 - 530
Monomeric red fluorescent protein	543	570 - 630
Yellow fluorescent protein (YFP)	514	525 - 550
Enhanced green fluorescent protein	488	505 - 530
mCherry	587	570 - 630
Aniline blue fluorochrome	405	460 - 500

Chlorophyll	488	690 - 750
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Fluorescence emissions were collected sequentially through two or three channels. Regions of interest were scanned unidirectionally with frame average at least 2 (Leica TCS LSI confocal microscope; Leica Microsystems) or bidirectionally with the line average 3 (Leica TCS SP5 laser scanning confocal microscope mounted on a Leica DMI 6000 CS inverted microscope; Leica Microsystems) and scan speed 400 Hz. The images were processed and merged using Leica LAS X software (Leica Microsystems). Images are presented as maximum projections from Z-stacks or single sections.

2.5.2.2 Following expression of fluorescent proteins

For sensors testing, the expression of fluorescent protein was followed using confocal microscope before and after the hormonal treatment. Leaves were detached from the plant to image their bottom side or cut into thin slices when imaging cross-sections. All images were acquired using the 5× objective.

For validation of Plant X-tender toolbox, the expression of the fluorescent proteins was followed six days after agroinfiltration using Leica TCS SP5 laser scanning confocal microscope mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems) with an HC PL FLUOTAR 10× objective. Prior to imaging, leaves were detached from the plant to image their bottom side. Three regions of interest per one agroinfiltrated area were analysed. The experiment was repeated two times.

2.5.2.3 Tracking of PVY N605-GFP on detached leaves

To follow the virus spread around the cell death zone and to count the number of lesions with the virus outside cell death zone, one or two Rywal and NahG-Rywal plants were analyzed at eight discrete time points from 3 dpi to 12 dpi, one leaf per plant, one to ten lesions per leaf and two regions of interest per lesion. Leaf was detached from the plant on the particular day post inoculation (dpi) and the upper side of the leaf was analyzed by TCS LSI confocal microscope with Plan APO 5× objective. Lesions were scanned in the channel for the background chloroplast fluorescence detection to estimate the location of the lesion. The edge between the cell death zone and normal tissue was determined using brightfield. We confirmed the virus presence indirectly by the detection of GFP following PVY N605-GFP accumulation. The cell was considered to contain the PVY N605-GFP only when the GFP fluorescence was observed in the cytoplasm. In addition, lambda-scan was performed to differentiate between GFP spectrum and the spectrum of the background signal. The spectrum was attributed to GFP fluorescence by comparing it with publicly available spectra of GFP. The lesions in which PVY was detected outside the death zone were considered as positive lesions. As a negative control, plants were infected with PVY without GFP tag. We merged the results of seven independent experiments to calculate the percentage of positive lesions in each plant/time point as the number of lesions in which the PVY N605-GFP accumulation was detected in at least one cell outside the cell death zone, divided by the number of all analyzed lesions. Z-stack was sized to cover the whole epidermis (approximately 30 µm) and at least 20 µm of the mesophyll.

To follow the virus spread around the lesions in cv. Rywal at higher temperature, PVY N605-GFP inoculated plants were kept at 22 °C until the lesions were formed. The inoculated leaf was detached from cv. Rywal on 4 dpi, cut into pieces and placed on

1.5 % (w/v) agar plate and kept at 22 °C. On four consecutive days (4 dpi, 5 dpi, 6 dpi and 7 dpi) the plate was transferred to 28 °C for 2 hours. The GFP fluorescence intensity of the same area of interest was followed after the temperature shift using TCS LSI macroscope with Plan APO 5× objective. The experiment was repeated twice.

2.5.2.4 Callose staining

The leaves of two cv. Rywal plants were inoculated as described above. Aniline blue fluorochrome solution was prepared according to the provider's instructions (Biosupplies Australia) and infiltrated with a syringe into the second fully developed inoculated leaf from 3 dpi to 7 dpi. The Aniline blue fluorochrome fluorescence was followed inside and outside the cell death zone on detached leaves using TCS LSI Macroscope with Plan APO 5× objective from 3 dpi to 7 dpi, while the GFP was followed at 4 dpi and 6 dpi. The experiment was repeated twice.

Chapter 3

Results

3.1 Construction of Plant X-tender Vector Set

As multigene assembly using AssemblX, a cloning strategy for the assembly of subunits into a multigene structure which exploits overlap-dependent assembly methods in combination with rare-cutting restriction enzymes (Hochrein et al. 2017), is currently feasible only in bacteria (*E. coli*) and yeast (*S. cerevisiae*), we upgraded the AssemblX cloning system for plant multigene expression. The toolkit was expanded by developing the Plant X-tender toolbox, which consists of four Plant X-tender plant expression vectors, *in silico* design tool GenoCAD and protocols for most efficient cloning into the novel vector set.

First, four plant expression vectors compatible with the AssemblX cloning system were generated to facilitate the transfer of multigene constructs into the plant genome. pCAMBIA 1300 and Gateway vectors pK7WG, pH7WG and pB7WG were used as backbones, each with a different marker gene for the selection of transgenic plants to allow maximal flexibility for the user. The cloning regions of these vectors were replaced with a *ccdB* cassette flanked by A0 and B0 homology regions (Hochrein et al., 2017) and restriction enzymes recognition sites (Figure 3.1). Three different overlap-based cloning methods (SLiCE, Gibson and HiFi) were confirmed to be efficient for the construction of AssemblX compatible plant expression vectors. The *ccdB* gene was introduced to disable the growth of *E. coli* cells transformed with the empty vector. The functionality of *ccdB* gene was confirmed, as no colonies grew after the transformation of *ccdB*-sensitive *E. coli* strain with purified plasmids, while transformation of *ccdB* Survival *E. coli* strain was successful.

The nucleotide sequences of constructed Plant X-tender plant expression vectors pCAMBIA_ASX, pK7WG_ASX, pH7WG_ASX and pB7WG_ASX were deposited in GenBank under accession numbers MG561370-MG561373. The Plant X-tender expression vectors and their maps are also available from Addgene (accession numbers 98888-98891).

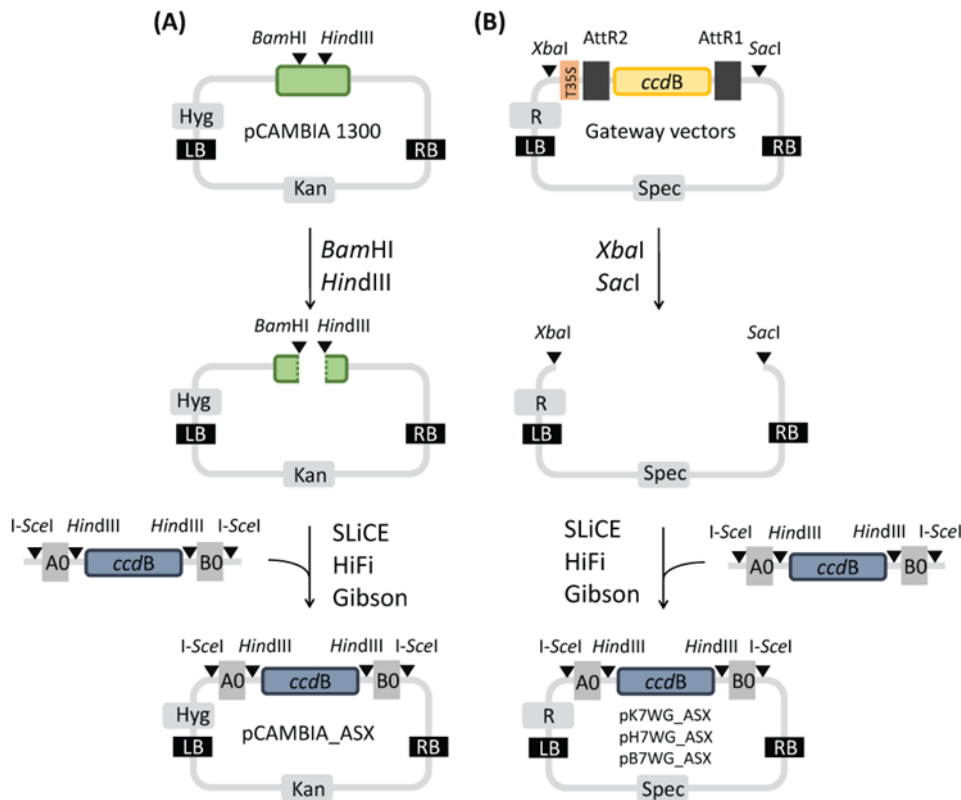


Figure 3.1: Design of Plant X-tender expression vectors. Vector pCambia 1300 (A) or Gateway vectors (pK7WG, pH7WG or pB7WG; B) were used as a backbone. (A) I-SceI-A0-HindIII-ccdB-HindIII-B0-I-SceI cassette was introduced into the MCS region of pCambia1300 by overlap-based cloning method SLiCE after backbone digestion with BamHI and HindIII to obtain pCambia_ASX. (B) T35S-AttR2-ccdB-AttR1 cassette was released from the Gateway plasmid backbone by digestion with XbaI and SacI and replaced with a I-SceI-A0-HindIII-ccdB-HindIII-B0-I-SceI cassette by overlap-based cloning method SLiCE to obtain pK7WG_ASX, pH7WG_ASX or pB7WG_ASX. Two other overlap-based cloning methods (Gibson and HiFi) were also confirmed to be efficient for the construction of AssemblX compatible plant expression vectors. MCS: multiple cloning site, A0/B0: homology regions, Kan: selection marker conferring kanamycin resistance in *E. coli* and *A. tumefaciens*, Spec: selection marker conferring spectinomycin resistance in *E. coli* and *A. tumefaciens*, Hyg: selection marker conferring hygromycin resistance in plants, R: selection marker conferring resistance in plants (kanamycin resistance in pK7WG, hygromycin resistance in pH7WG, herbicide glufosinate-ammonium resistance in pB7WG), LB: left border of T-DNA, RB: right border of T-DNA, ccdB: bacterial suicide gene, HindIII, I-SceI, BamHI, XbaI, SacI: restriction enzyme recognition sites, AttR1/AttR2: Gateway cloning recombination sites, T35S: cauliflower mosaic virus CaMV 35S terminator, SLiCE: Seamless ligation cloning extract cloning method, HiFi: NEBuilder HiFi DNA assembly method, Gibson: Gibson DNA assembly method. Republished from Plant X-tender: An extension of the AssemblX system for the assembly and expression of multigene constructs in plants, Lukan et al., 2018b, PLoS One, 13(1):e0190526, doi: 10.1371/journal.pone.0190526, according to Creative Common Attribution ("CC BY") licence.

3.1.1 Plant X-tender toolbox for the assembly and expression of multigene constructs

To facilitate the use of AssemblX for synthetic biology approaches in plant science, the developed vectors are complemented by the *in silico* design tool GenoCAD and protocols for the most efficient cloning into the novel vector set.

First, the plant grammar implemented in GenoCAD (Coll et al., 2015) was customized by adding sequences of nopaline synthase promoter (pNOS) and cauliflower mosaic virus CaMV 35S terminator (t35S) to the GenoCAD plant library. An additional plant grammar rule was constructed to facilitate virtual assembly of the selected biological parts into the expression cassette. The new rule allows the design of a simple expression cassette segment. The user is guided through the design of an expression cassette that, as a minimum requirement, includes promoter, CDS and terminator. However, it offers flexibility with regard to the CDS, which can represent a plant gene, a fluorescent protein or both, and can be fused to epitope tags and linkers, if needed. The user can also decide to add one or two promoters and terminators. The newly developed grammar is available at Figshare (<https://doi.org/10.6084/m9.figshare.4977464>) and can be imported into the GenoCAD. In the next step, the sequences of the designed expression cassettes were exported from GenoCAD and imported into the AssemblX web tool, in order to combine them in a multigene construct. Thus, the GenoCAD plant grammar complements the AssemblX web tool by supporting the user throughout the design of Level 0 constructs. Moreover, it includes a library of plant genetic parts classified in structural categories which constitute a repository of biological parts sharable with the plant community and easily accessible to all users. The multigene construct, pre-assembled by GenoCAD plant grammar, were virtually assembled in Level 1 AssemblX vectors using the AssemblX web tool. Finally, the plasmid maps of the intended final product were generated by cutting and pasting the A0-B0 flanked assembly module into the final destination backbone, thereby replacing the *HindIII*-*ccdB*-*HindIII* cassette.

The Plant X-tender cloning procedure follows the AssemblX strategy (Figure 3.2). Specific overhang sequences first need to be introduced into subunits (e.g. promoter, CDS, terminator) by PCR using primers overlapping the neighbouring fragment to allow assembly based on overlap-dependent cloning methods and to determine the orientation and order of DNA fragments in AssemblX Level 0 vectors. The as-assembled gene cassettes are combined into a multigene structure in a Level 1 AssemblX vector. In the last step, the construct is transferred into newly developed Plant X-tender expression vectors thus allowing introduction of up to five expression cassettes into a single plasmid. Although it is highly recommended to use the plant grammar implemented in GenoCAD and AssemblX web tool for the design to decrease the risk of introducing errors, one or both design tools could be omitted to apply the cut-and-paste approach.

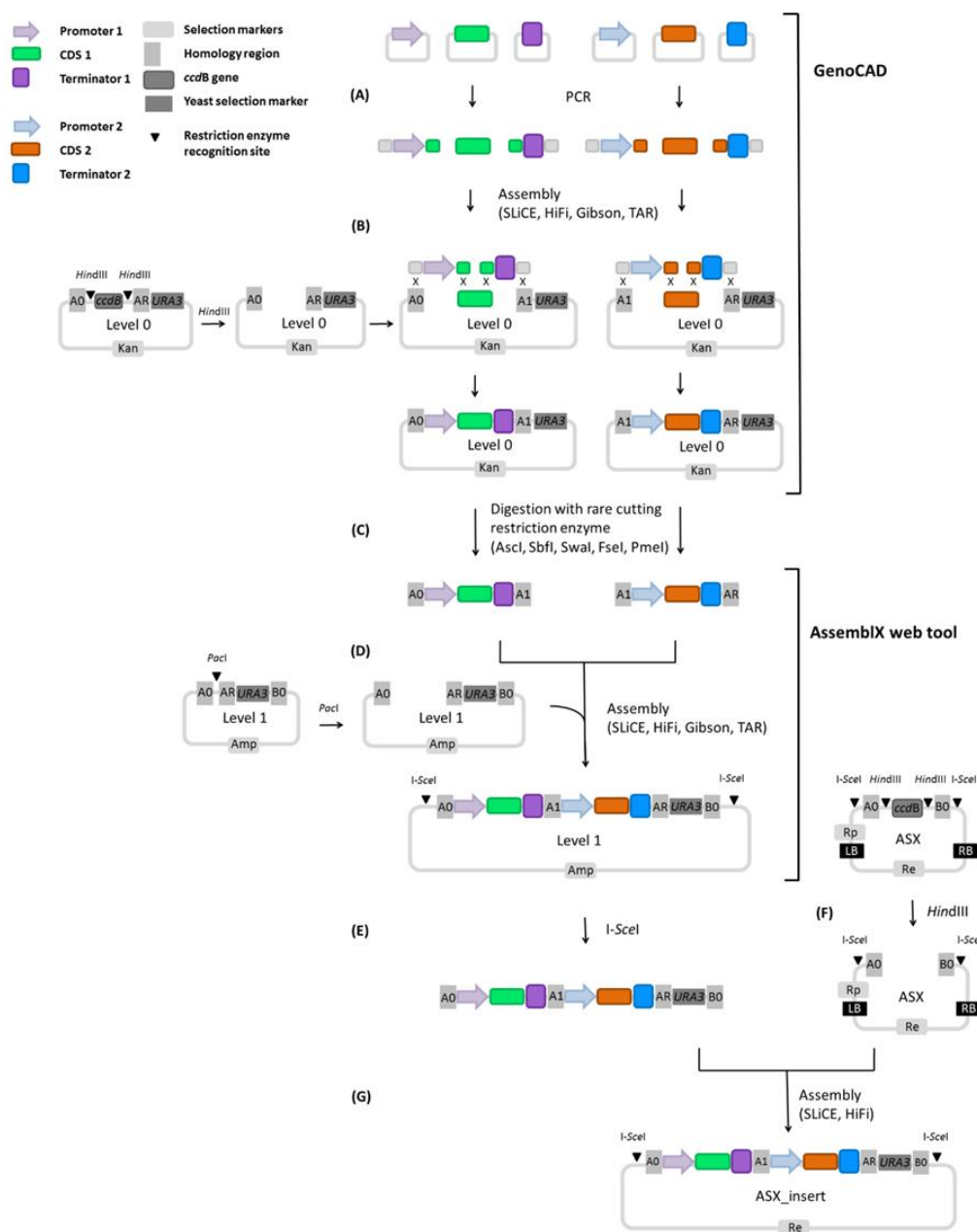


Figure 3.2: Plant X-tender cloning strategy. Diagram showing example of assembly of two expression cassettes into a plant expression vector using Plant X-tender. Definition of parts and design of Level 0 units is done using GenoCAD. Design of multigene cassettes and computation of primers is performed using the AssemblX webtool. (A-D) Assembly of two expression cassettes into a Level 1 vector. (A) PCR amplification of subunits (e.g. promoter, CDS, terminator) using custom-designed primers with appropriate 5' extensions to add overlaps between the individual subunits and chosen Level 0 plasmid. (B) Assembly of subunits into *HindIII* digested Level 0 vectors via overlap-based assembly methods. (C) Assembled cassettes flanked by homology regions are released from the backbone using one of five rare 8-base cutter recognition sites (*AscI*, *SbfI*, *SwaI*, *FsaI*, *PmeI*) flanking the homology regions. (D) Assembly of expression cassettes into *PacI* digested Level 1 vector by the preferred overlap-based assembly method. (E-G) Multigene assembly into Plant X-tender expression

vector. (E) Digestion with I-SceI allows the release of a multigene construct flanked by homology regions A0 and B0 from the Level 1 AssemblX vector. (F) *HindIII* digestion enables the linearization of Plant X-tender expression vector and the release of *ccdB* cassette prior to the assembly. (G) Assembly of a multigene construct and a yeast selection marker (URA3) flanked by homology regions into Plant X-tender expression vector by overlap-based methods exploiting homologous recombination between the homology regions A0 and B0 of the Plant X-tender expression vector and the homology regions A0 and B0 of the insert. A0, A1, AR, B0: homology regions, *HindIII*, I-SceI, *PacI*, *AscI*, *SbfI*, *SwaI*, *FseI*, *PmeI*: restriction enzyme recognition sites, Rp: selection marker conferring resistance in plants, Re: selection marker conferring resistance in *E. coli* and *A. tumefaciens*, Amp: selection marker conferring ampicillin resistance in *E. coli* and *A. tumefaciens*, Kan: selection marker conferring kanamycin resistance in *E. coli* and *A. tumefaciens*, URA3: yeast selection marker, LB: left border of T-DNA, RB: right border of T-DNA, *ccdB*: bacterial suicide gene, SLiCE: Seamless ligation cloning extract cloning method, HiFi: HiFi DNA assembly method, Gibson: Gibson DNA assembly method, TAR: cloning based on transformation-associated recombination, PCR: Polymerase chain reaction, CDS: coding sequence, ASX: Plant X-tender expression vector. Republished from Plant X-tender: An extension of the AssemblX system for the assembly and expression of multigene constructs in plants, Lukan et al., 2018b, PLoS One, 13(1):e0190526, doi: 10.1371/journal.pone.0190526, according to Creative Common Attribution ("CC BY") licence.

3.1.2 Validating the Plant X-tender expression vectors for multigene cloning

The developed Plant X-tender toolbox was tested by cloning the expression cassette p35S::H2BRFP tNOS into Plant X-tender expression vectors. The functionality of the system by transient expression of H2BRFP was further confirmed in *N. benthamiana* (Figure 3.3). This expression cassette, which consists of a histon sequence fused to red fluorescent protein mRFP1, was already confirmed to be functional (Federici et al., 2012), therefore we used it as the proof of concept. The expression cassette was amplified from a template plasmid using primers with appropriate 5' and 3' extensions, giving homology to A0 and AR homology regions and assembled in pL0A_0-R Level 0 vector (Hochrein et al., 2017) by NEBuilder HiFi assembly (Figure 3.3 A, B). It was confirmed that eight out of eight colonies contained the plasmid with DNA insert by colony PCR. Additionally, the correct junction sites in NEBuilder HiFi assembled plasmid were confirmed by sequencing. Subsequently, following the AssemblX procedure, the resulting Level 0 unit was assembled into pL1A-hc / pL1A-lc (A0/AR) Level 1 vector (Hochrein et al., 2017) by TAR and NEBuilder HiFi assembly to compare the efficiency of both (Figure 3.3 C, D). For TAR, all clones analysed by colony PCR were confirmed to contain the correct insert length, while NEBuilder HiFi assembly resulted in less than 60 % of clones with the correct insert length. The correct junction sites of TAR assembled plasmid were confirmed by sequencing.

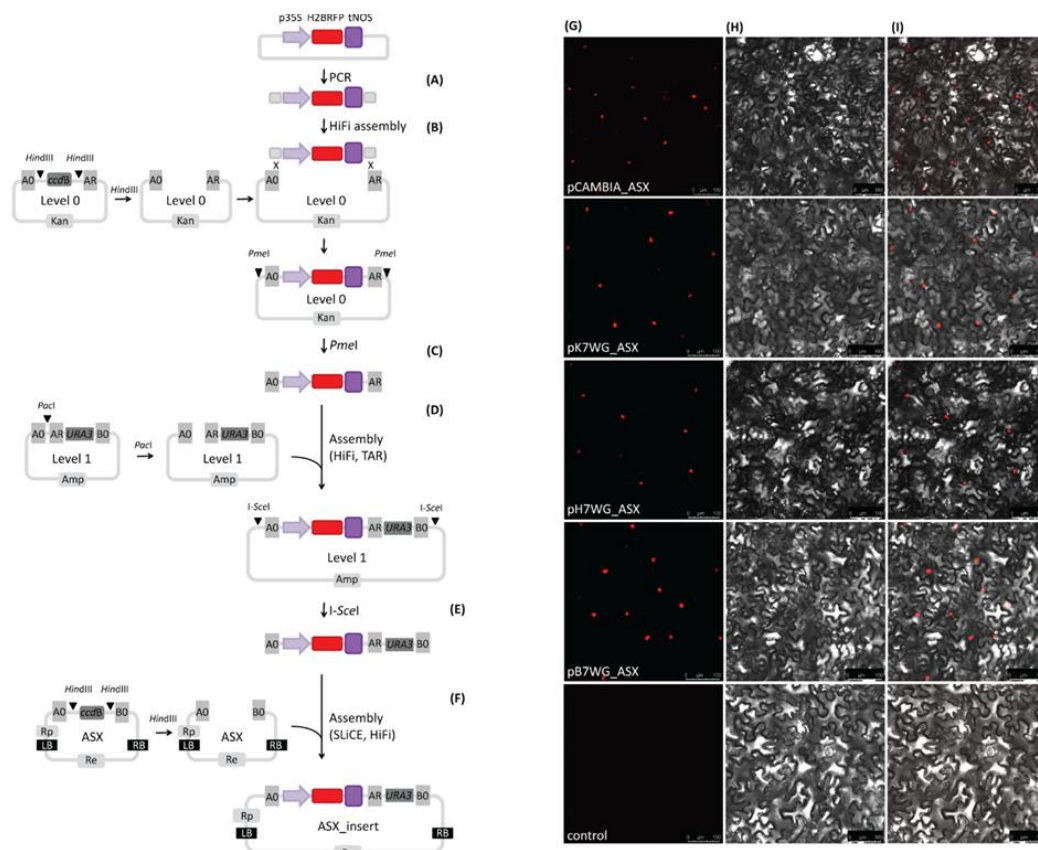


Figure 3.3: Functional evaluation of constructed vectors by cloning expression cassette p35S::H2BRFP_tNOS into Plant X-tender expression vectors. A-F) Scheme of the cloning procedure. (A) Amplification of expression cassette from template plasmid using primers with appropriate 5' and 3' extensions to add A0 and AR homology regions. (B) Expression cassette assembly in *HindIII* restricted pL0A_0-R Level 0 vector by NEBuilder HiFi assembly method. (C) Release of expression cassette with flanking homology regions A0 and AR from Level 0 vector by *PmeI* digestion. (D) Assembly of expression cassette with flanking homology regions A0 and AR into *PacI* digested pL1A-hc / pL1A-lc (A0/AR) Level 1 vector by TAR or NEBuilder HiFi. (E) Release of expression cassette flanked by URA3 yeast selection marker and homology regions A0 and B0 from Level 1 vector by *I-SceI* digestion. (F) Assembly of expression cassette flanked by URA3 yeast selection marker and homology regions A0 and B0 into Plant X-tender expression vectors by SLiCE or NEBuilder HiFi. (G-I) Images of agroinfiltrated *N. benthamiana* leaves obtained by laser scanning confocal microscopy. Leaves were agroinfiltrated with agrobacteria containing pCambia_ASX cassette, pK7WG_ASX cassette, pH7WG_ASX cassette, pB7WG_ASX cassette or empty agrobacteria (top to bottom). (G) Nuclear localisation of RFP. Fluorescence is represented as maximum projections of z-stacks. (H) Bright field. (I) Overlay of G with H. p35S: cauliflower mosaic virus CaMV 35S promoter, H2BRFP: histon sequence fused to red fluorescence protein (mRFP1), tNOS: nopaline synthase terminator, A0, AR, B0: homology regions, Rp: selection marker conferring resistance in plants (hygromycin in the case of pCambia_ASX and pH7WG_ASX, kanamycin in the case of pK7WG_ASX, glufosinate-ammonium in the case of pB7WG_ASX), Re: selection marker conferring resistance in *E. coli* and *A. tumefaciens* (kanamycin in the case of pCambia_ASX, spectinomycin in the case of pK7WG_ASX, pH7WG_ASX and pB7WG_ASX), Amp: selection marker conferring ampicillin resistance in *E. coli* and *A.*

tumefaciens, Kan: selection marker conferring kanamycin resistance in *E. coli* and *A. tumefaciens*, LB: left border of T-DNA, RB: right border of T-DNA, *Hind*III, *I-Sce*I, *Pac*I, *Pme*I: restriction enzyme recognition sites, URA3: yeast selection marker, *ccd*B: bacterial suicide gene, SLiCE: Seamless ligation cloning extract cloning method, HiFi: NEBuilder HiFi DNA assembly method, Gibson: Gibson DNA assembly method, TAR: cloning based on transformation-associated recombination, PCR: Polymerase chain reaction, ASX: Plant X-tender expression vector. Republished from Plant X-tender: An extension of the AssemblX system for the assembly and expression of multigene constructs in plants, Lukan et al., 2018b, PLoS One, 13(1):e0190526, doi: 10.1371/journal.pone.0190526, according to Creative Common Attribution ("CC BY") licence.

In the next step, several cloning and transformation parameters for transferring the assembled expression cassette (Level 1 module) from the AssemblX Level 1 vector to the Plant X-tender expression vector pCambia_ASX were tested (Figure 3.3 E, F). The tested transfer parameters were the cloning method, amount of the plasmid backbone, molar ratio between the plasmid backbone and the insert, transformation method, *E. coli* strain, transformation efficiency of *E. coli* and the volume of cloning mixture used for transformation (Table 3.1 and Table 3.2). Our results proved SLiCE assembly to be more efficient compared to NEBuilder HiFi assembly when 50 ng of the plasmid and a 1:2 molar ratio between the plasmid and the insert were used. A higher number of colonies was obtained when a higher amount of the plasmid was used (150 ng in comparison to 50 ng). Likewise, a higher molar ratio of insert to plasmid (10:1 in comparison to 2:1) resulted in higher transformation efficiency. Our results showed that SLiCE cloning method is applicable even in combination with homemade chemically competent or electrocompetent *E. coli* with lower transformation efficiency in comparison to expensive commercial ones (Table 3.1 and Table 3.2).

Table 3.1: Transformation efficiencies of homemade chemically competent and electrocompetent *E. coli*. Republished from Plant X-tender: An extension of the AssemblX system for the assembly and expression of multigene constructs in plants, Lukan et al., 2018b, PLoS One, 13(1):e0190526, doi: 10.1371/journal.pone.0190526, according to Creative Common Attribution ("CC BY") licence.

Homemade chemically competent and electrocompetent <i>E.</i>	Transformation
homemade TOP10 electrocompetent <i>E. coli</i>	4,22 x 10 ⁷ cfu/pmol
homemade TOP10 chemically competent <i>E. coli</i>	1,87 x 10 ⁵ cfu/pmol
homemade <i>ccd</i> B Survival™ 2 T1 ^R electrocompetent <i>E. coli</i>	1,00 x 10 ⁸ cfu/pmol
homemade <i>ccd</i> B Survival™ 2 T1 ^R chemically competent <i>E.</i>	4,31 x 10 ⁶ cfu/pmol

Table 3.2: Optimization of assembly and transformation methods for the insert cloning into Plant X-tender expression vectors. Expression cassette p35S::H2BRFP tNOS (insert 1) and multigene construct p35S::H2BRFP tNOS + pNOS::CFP t35S (insert 2) were separately assembled into pCAMBIA ASX to determine optimal conditions for insert cloning into the expression vectors. Bottom two rows represent a negative control (the plasmid backbone without the insert). AM: assembly method, SLiCE: Seamless ligation cloning extract cloning method, HiFi: NEBuilder HiFi DNA assembly method, m (ng): amount of pCAMBIA ASX expression vector, MR: molar ratio between the plasmid backbone and the insert, TM: transformation method, *E. coli*: competent *E. coli* used for transformation, HM: homemade TOP10 chemically competent *E. coli*, HM: homemade TOP10 electrocompetent *E. coli*, C: commercial TOP10 chemically competent *E. coli*, T (μl): volume of assembly mixture used for transformation, number of colonies: the number of colonies grown after overnight incubation, cloning efficiency: the ratio between the number of clones with the correct insert length confirmed by colony PCR and the number of colonies subjected to colony PCR. Republished from Plant X-tender: An extension of the AssemblX system for the assembly and expression of multigene constructs in plants, Lukan et al., 2018b, PLoS One, 13(1):e0190526, doi: 10.1371/journal.pone.0190526, according to Creative Common Attribution ("CC BY") licence.

Insert	AM	m (ng)	MR	TM	<i>E. coli</i>	T (μl)	Number of colonies	Cloning efficiency
insert 1	SLiCE	50	1:10	electroporation	HM	2	167	3/6
	SLiCE	50	1:10	heat shock	HM	2	0	0/0
	SLiCE	50	1:10	heat shock	C	2	395	6/6
	SLiCE	50	1:2	electroporation	HM	2	15	5/5
	SLiCE	50	1:2	heat shock	HM	2	0	0/0
	SLiCE	50	1:2	heat shock	C	2	109	6/6
	SLiCE	150	1:2	electroporation	HM	2	107	6/6
	SLiCE	150	1:2	heat shock	HM	2	0	0/0
	SLiCE	150	1:2	heat shock	C	2	132	6/6
	HiFi	50	1:2	electroporation	HM	2	0	0/0
	HiFi	50	1:2	heat shock	C	2	0	0/0
	HiFi	50	1:2	heat shock	C	2	0	0/0
insert 2	SLiCE	50	1:10	electroporation	HM	1,5	41	6/11
	SLiCE	50	1:10	heat shock	HM	2	0	0/0
	SLiCE	50	1:10	heat shock	HM	3	1	0/1
	SLiCE	50	1:10	heat shock	C	2	108	8/10
	SLiCE	150	1:5	electroporation	HM	1,5	0	0/0
	SLiCE	150	1:5	heat shock	HM	2	0	0/0
	SLiCE	150	1:5	heat shock	HM	3	0	0/0
	SLiCE	150	1:5	heat shock	C	2	1	0/1
	SLiCE	150	1:2	electroporation	HM	1,5	0	0/0
	SLiCE	150	1:2	heat shock	HM	2	3	3/3
	SLiCE	150	1:2	heat shock	HM	3	1	1/1
	SLiCE	150	1:2	heat shock	C	2	880	6/10
	HiFi	50	1:5	electroporation	HM	2	0	0/0
	HiFi	50	1:5	heat shock	C	2	8	5/7
negative control	SLiCE	50	/	electroporation	HM	2	0	0/0
	SLiCE	50	/	heat shock	HM	2	0	0/0

The expression cassette p35S::H2BRFP_tNOS was transferred into the pK7WG_ASX, pH7WG_ASX and pB7WG_ASX vectors using the most favourable assembly method determined by the pCAMBIA_ASX transfer, SLiCE (Figure 3.3 C-F). The functionality of the constructs was confirmed by the confocal imaging of H2BRFP upon agroinfiltration of *N. benthamiana* leaves (Figure 3.3 G-I).

Finally, the usability of Plant X-tender for multigene cloning was tested by introducing two expression cassettes, containing two different reporter genes (H2BRFP and ECFP), into Plant X-tender expression vectors. The functionality of the system was confirmed by transient expression of H2BRFP and ECFP in *N. benthamiana* (Figure 3.4). The p35S::H2BRFP_tNOS expression cassette was amplified from the template using primers with appropriate 5' and 3' extensions to add A0 and A1 homology regions and assembled in Level 0 vector pLOA_0-1 (Hochrein et al., 2017) by NEBuilder HiFi assembly. In the case of the pNOS::ECFP_t35S expression cassette, each Level 0 subunit was amplified from separate templates using primers with overlaps between adjacent subunits and destination plasmid. The three subunits were assembled in Level 0 vector pLOA_1-R (Hochrein et al., 2017) by NEBuilder HiFi assembly (Figure 3.4 A, B). GenoCAD and the AssemblX webtool were used to design the construct and the cloning primers. We confirmed that eight out of eight colonies contained the insert for both Level 0 assemblies by colony PCR. The correct junction sites in the assembled plasmids were confirmed by sequencing. Both expression cassettes were subsequently assembled in Level 1 vector pL1A-hc / pL1A-lc (A0/AR) (Hochrein et al., 2017) by TAR and NEBuilder HiFi assembly (Figure 3.4 C, D). According to the colony PCR results, TAR assembly was confirmed to be more efficient in comparison to NEBuilder HiFi assembly in the conditions tested here. For TAR assembly, all clones were confirmed to contain the correct insert length, while NEBuilder HiFi assembly resulted in less than 70 % of clones with the correct insert length in the conditions tested here. The multigene construct consisting of both expression cassettes with flanking homology regions A0/B0 was released from Level 1 vector and cloned into pCAMBIA_ASX vector (Figure 3.4 E, F). Different cloning and transformation methods were evaluated for this step. Lower molar ratio of insert to plasmid was determined to be more efficient. In the tested conditions, NEBuilder HiFi cloning was determined as not applicable in combination with electroporation, while SLiCE could be used in combination with electroporation or heat shock. SLiCE cloning was confirmed to be applicable even in combination with low efficient homemade chemically competent *E. coli* cells (Table 3.1). The functionality of the construct was confirmed by confocal imaging of H2BRFP for the p35S::H2BRFP_tNOS expression cassette and ECFP for the pNOS::ECFP_t35S expression cassette (Figure 3.4 G-J), upon agroinfiltration of *N. benthamiana* leaves.

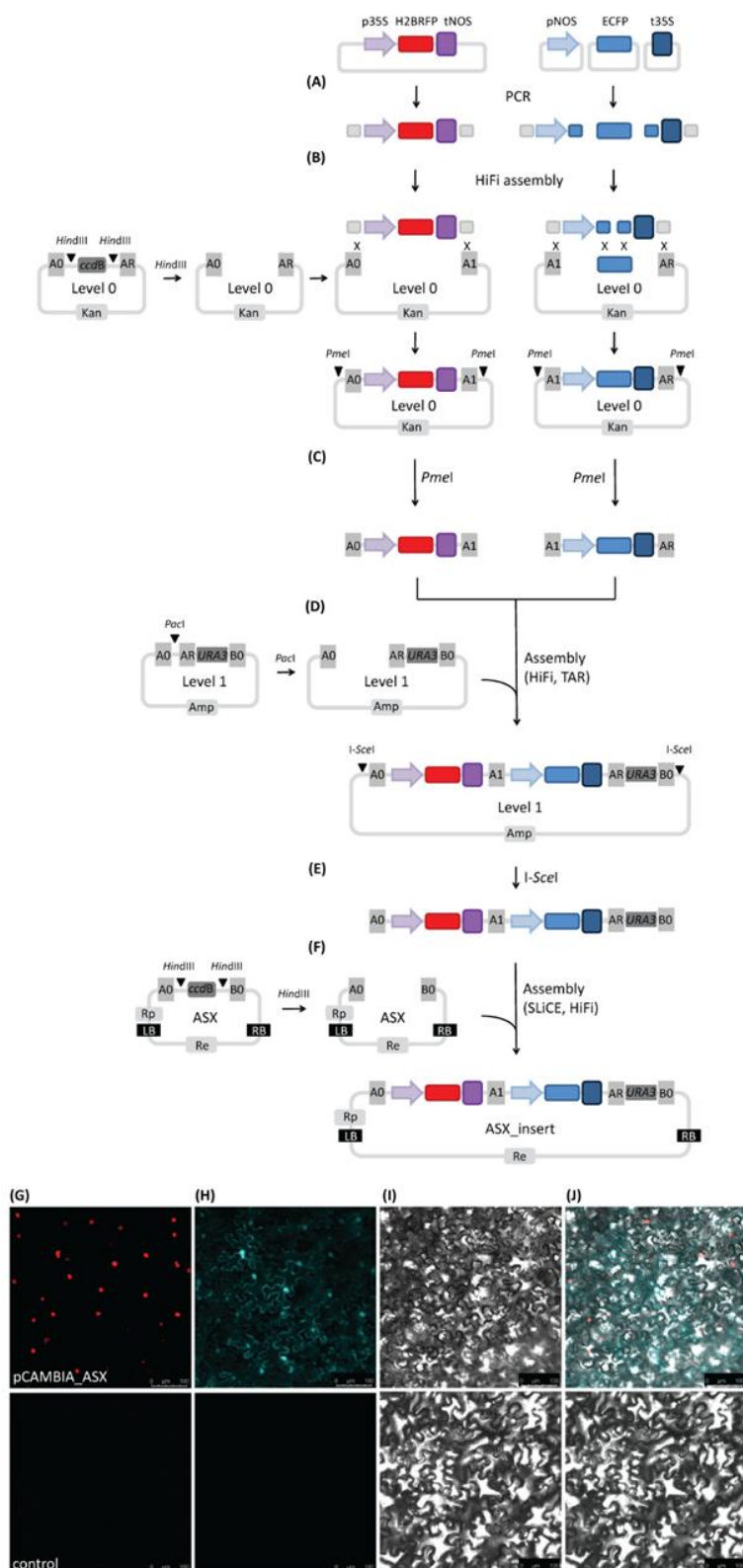


Figure 3.4: Multigene cloning with Plant X-tender expression vectors. Two expression cassettes were cloned into pCAMBIA_{ASX} and introduced into *N. benthamiana*. (A-F) Scheme of cloning procedure. (A) Amplification of expression cassette from template plasmid using primers with appropriate 5' and 3' extension homologies in the case of p35S::H2BRFP tNOS expression cassette. PCR amplification of subunits (pNOS, ECFP, t35S) using custom-

designed primers with appropriate 5' extensions to add overlaps between the individual subunits and chosen Level 0 plasmid in the case of pNOS::ECFP t35S expression cassette. (B) Assembly of subunits into *HindIII* digested Level 0 vectors by NEBuilder HiFi assembly method. (C) Assembled cassettes flanked by homology regions were released from the backbone using *PmeI*. (D) Assembly of expression cassettes into *PacI* digested Level 1 vector by TAR or NEBuilder HiFi. (E) Release of the multigene construct from Level 1 vector using *I-SceI* homing endonuclease, cutting outside the homology regions A0 and B0. (F) Assembly of two expression cassettes and yeast selection marker (URA3) into *HindIII* digested Plant X-tender expression vectors with SLiCE of NEBuilder HiFi. (G-J) Images of agroinfiltrated *N. benthamiana* leaves obtained by laser scanning confocal microscopy. Leaves were agroinfiltrated with *A. tumefaciens* containing pCAMBIA ASX multigene (upper panel) or with empty *A. tumefaciens* (bottom panel). (G) Nuclear localisation of RFP. Fluorescence is represented as a maximum projection of z-stacks. (H) ECFP is localised in the cytoplasm. Fluorescence is represented as maximum projections of z-stacks. (I) Bright field. (J) Overlay of G, H and I. p35S: cauliflower mosaic virus CaMV 35S promoter, H2BRFP: histon sequence fused to red fluorescence protein (mRFP1), tNOS: nopaline synthase terminator, pNOS: nopaline synthase promoter, ECFP: cyan fluorescent protein, t35S: cauliflower mosaic virus CaMV 35S terminator, A0, A1 AR, B0: homology regions, Rp: selection marker conferring hygromycin resistance in plants, Re: selection marker conferring kanamycin resistance in *E. coli* and *A. tumefaciens*, Amp: selection marker conferring ampicillin resistance in *E. coli* and *A. tumefaciens*, Kan: selection marker conferring kanamycin resistance in *E. coli* and *A. tumefaciens*, LB: left border of T-DNA, RB: right border of T-DNA, *HindIII*, *I-SceI*, *PacI*, *AscI*, *SbfI*, *SwaI*, *FseI*, *PmeI*: restriction enzyme recognition sites, URA3: yeast selection marker, *ccdB*: bacterial suicide gene, SLiCE: Seamless ligation cloning extract cloning method, HiFi: NEBuilder HiFi DNA assembly method, Gibson: Gibson DNA assembly method. TAR: cloning based on transformation-associated recombination, PCR: Polymerase chain reaction, ASX: Plant X-tender expression vector. Republished from Plant X-tender: An extension of the AssemblX system for the assembly and expression of multigene constructs in plants, Lukan et al., 2018b, PLoS One, 13(1):e0190526, doi: 10.1371/journal.pone.0190526, according to Creative Commons Attribution ("CC BY") licence.

3.2 Study of Plant Immune Regulatory Networks Through Promoter Analyses

The aim was to elucidate plant immune signalling by developing hormone signalling sensors that were made by fusing selected native promoters with genes encoding fluorescent proteins (Figure 3.5). For this, the selection of appropriate promoters was crucial and performed in the following steps. We focused on hormonal signalling pathways that play dominant roles in plant response to biotic stress, namely those pathways dependent upon SA, JA and ET. To select the candidates, the existing model of Arabidopsis signalling pathways (Miljković, 2012) was improved by retrieving information from the literature (Ramšak, 2015). The model was then inspected to select the candidates. For each signalling pathway, a central transcription factor from a particular pathway and an output of the pathway, regulated directly or indirectly by this transcription factor were selected. In case of ET signalling pathway, for which an output of the signalling pathway is not known, the gene involved in ET synthesis, regulated by a central

transcription factor was selected. To select the best candidate we performed literature search and combined it with experimental data (Baebler et al., 2014, Petek et al., 2014, Stare et al., 2015).

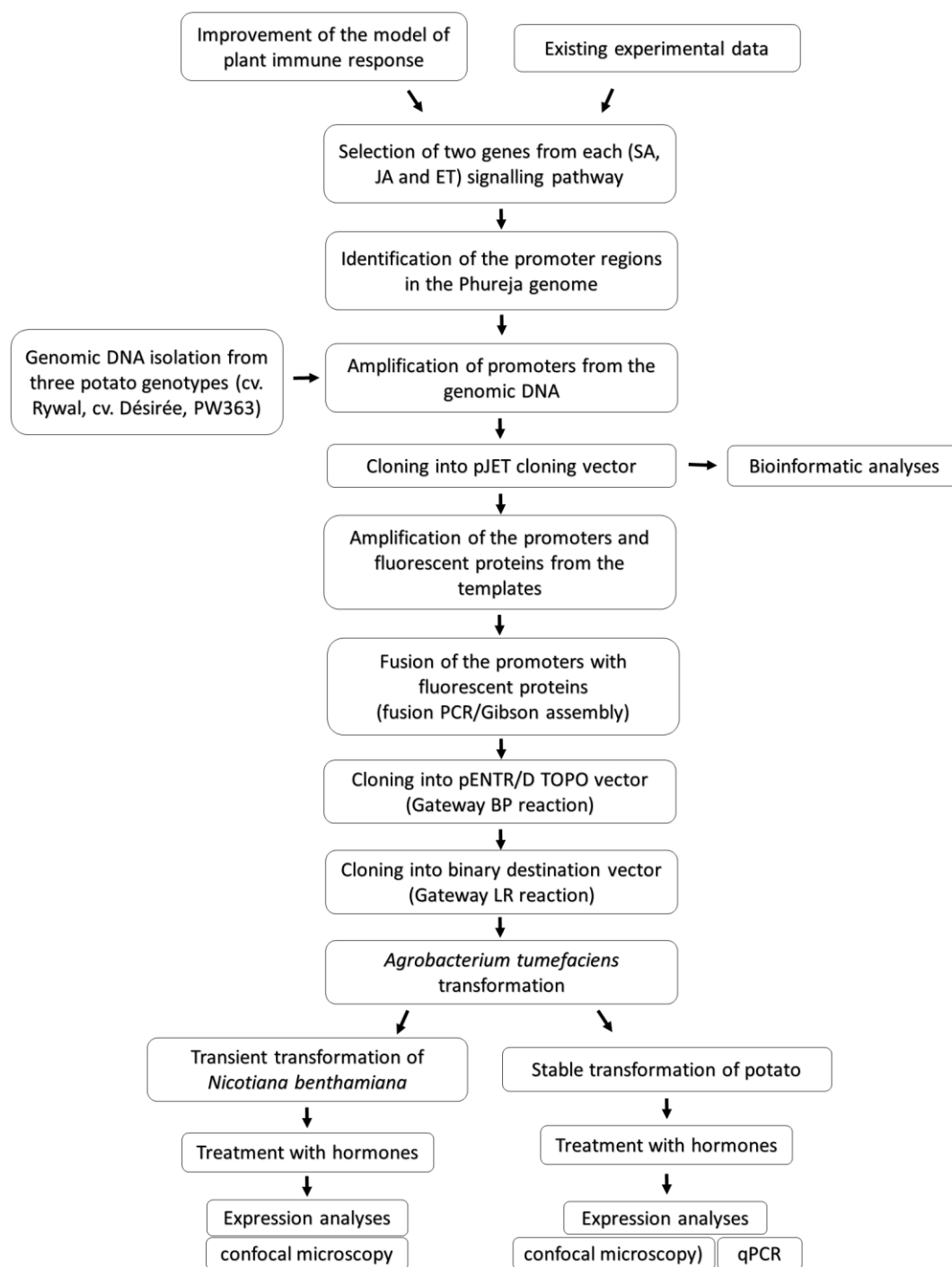


Figure 3.5: Workflow for study of the plant immune regulatory networks through promoter analyses.

As PR1 proteins have long been used as markers of SA-signalling pathway and TGA transcription factors are known to directly regulate their activity through transcriptional regulation (Van Loon, 1997), we focused on those two gene families from SA-signalling pathway. To select the optimal gene candidates from

each family, we searched through the data regarding their expression behaviour after virus inoculation which is known to increase SA levels (Baebler et al., 2014). First, previously published data (Baebler et al., 2014, Petek et al., 2014, Stare et al., 2015) was checked to identify the genes that are responsive to the viral infection in our pathosystem. Moreover, work on the on-going project (J47636) where the colleagues from our working group were studying spatial transcriptomics response to the viral infection has shown that spatiotemporal gene expression behaviour of different genes from SA signalling pathway differs. TGA2 (PGSC0003DMG402023696) and PR1b (PGSC0003DMG400002028) were both responsive to virus infection but had different spatiotemporal gene expression behaviour; PR1b gene featured single expression response wave, while the TGA2 showed double wave of expression response from the point of virus infection, respectively. Therefore, those two genes were selected for further work.

Next, we focused on the genes involved in the JA signalling pathway. It is well known, that MYC2 transcription factor has been required for activation of many JA-responsive genes in *Arabidopsis*, particularly those involved in insect attack (Browse, 2009). Moreover, MYC2 was upregulated in potato leaves after the Colorado potato beetle larvae infestation (Petek et al., 2014). According to these data, MYC2 (PGSC0003DMG400017535) was selected for our experiments. Another gene, known to be upregulated by JA in potato and is presumably acting downstream of MYC2 is cysteine proteinase inhibitor multicystatin (MC). MC (PGSC0003DMG400005950) which was downregulated in potato plants with a silenced JA receptor gene (*coil*), confirming its involvement in JA signalling pathway (Petek et al., 2014), was selected for further work.

Finally, we focused on the genes involved in the ET signalling pathway. Several ERF transcription factors from ET signalling pathway have been linked to biotic stress response. In order to identify the gene from ERF family involved in potato defence response to PVY, we examined a previously published microarray dataset (Baebler et al., 2014). Due to its responsiveness to PVY infection, PGSC0003DMG400010724, which was designated as PTI5, was selected. In contrast to other two signaling pathways, for which proteins regulated by central transcription factors have been known, the protein regulated by PTI5 is not known. However, ERF transcription factors were proposed to also regulate the activity of enzymes involved in ET synthesis (Yang et al., 2015). Therefore, our second candidate gene from ET signalling was from ACO gene family. Expression analysis in tomato showed upregulation of ACO1 following the treatment with ET (Chersicola et al., 2017). The closest potato relative to ACO1 gene from tomato, StACO2 (PGSC0003DMG400013894) was selected for further work.

3.2.1 Promoter sequences of some genes show high nucleotide diversity even within the same cultivar

As high-quality draft potato genome was determined in 2011 by sequencing a unique doubled-monoploid potato clone of *S. tuberosum* group DM (genotype DM, The Potato Genome Sequencing Consortium, 2011), promoter regions were identified based on the location of the selected genes in the DM genome using Solanaceae Genomics Resource and Ensembl Plants portal databases. The regions upstream of the gene were amplified from the genomic DNA of three potato genotypes, cv. Rywal, cv. Désirée and PW363 and sequenced for all selected genes except PTI5. Cloning of PTI5 promoter was performed as a part of another project (N4-0026) by the colleagues from our working group and the regions upstream of the gene were amplified only from the genomic DNA of cv. Rywal. For the promoters of each

gene, the obtained sequences (five for cv. Rywal in the case of PTI5, and three for each genotype in case of the promoters from other genes, except MYC2, for which only two sequences were amplified from cv. Désirée) were compared among each other and to the available model promoter sequence from DM genome. The lengths of promoter sequences from DM were 1513 nt (TGA2), 1619 nt (PR1b), 1357 nt (MYC2), 921 nt (MC), 1519 nt (ACO2) and 976 nt (PTI5). However, the lengths of promoter sequences from different cultivars differed from the lengths in DM due to insertions and deletions.

First, promoters of the TGA2 and PR1b genes from SA signalling pathway were analysed. All three TGA2 promoter sequences from PW363 are identical but differ from the promoter sequences from DM, Rywal and Désirée (Figure 3.6, bottom panel). The relatively low percent identity between DM and PW363 promoter sequences can be attributed to the deletions and insertions within the promoter sequences of the PW363 genotype (Figure 3.6, top panel). On the other hand, all three TGA2 promoter sequences from Rywal and one promoter sequence from Désirée are identical to the DM promoter sequence. However, other two promoter sequences from Désirée differ from the promoter sequence from DM as we determined 99.9 and 98.9 percent identity when we compared them to the promoter sequence from DM (Figure 3.6, bottom panel).

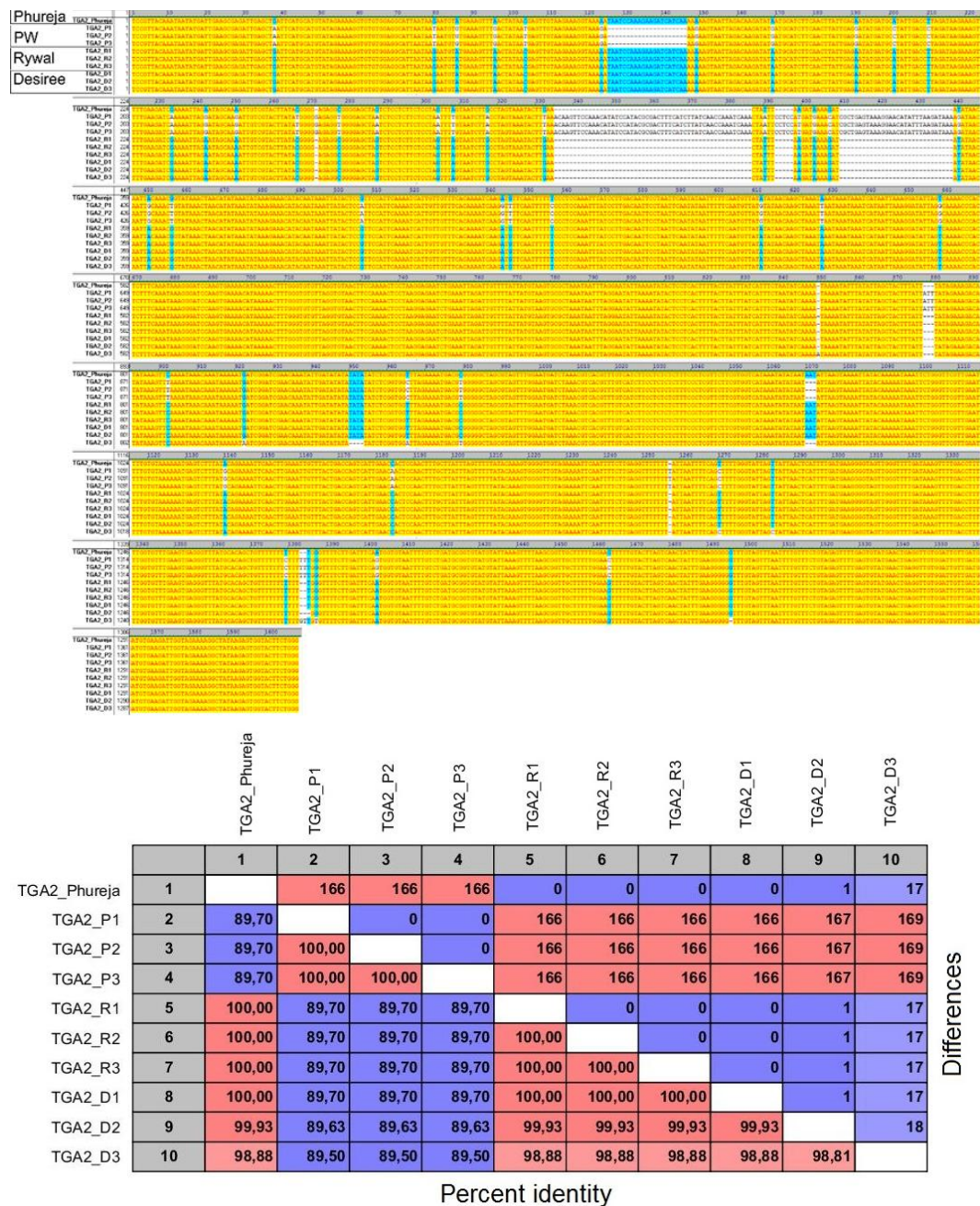


Figure 3.6: Promoter sequences of TGA2 gene in potato genotypes. Top panel: Alignment of TGA2 promoter sequences from different potato genotypes. The first row represents the promoter sequence from DM (Phureja in the Figure), rows 2 - 4 represent promoter sequences from PW363, rows 5 - 7 represent promoter sequences from Rywal, rows 8 - 10 represent promoter sequences from Désirée. Bottom panel: Percent identity (%) and nucleotide differences (number of different nucleotides) between TGA2 promoter sequences from different potato genotypes. The percentage was calculated for the full-length sequences, including insertions and deletions. P: promoter sequence from PW363, R: promoter sequence from cv. Rywal, D: promoter sequence from cv. Désirée, 1 - 3: promoter sequence from colonies 1 - 3.

In comparison to promoter sequences of the TGA2 gene from cv. Rywal and cv. Désirée, promoters of the PR1b gene showed lower percent identity when compared with the promoter sequence from DM and also when compared to each other (Figure 3.7, bottom panel). None of the PR1b promoter sequences from PW363, Rywal and Désirée was identical to the PR1b promoter sequence from DM. Moreover, we did not detect 100% identity between any of the analysed sequences. Even though, PR1b promoter sequences from Désirée showed high percent identity when compared to each other, as only two SNPs were identified (Figure 3.7, top panel). Similarly, PR1b promoter sequences from PW363 showed high percent identity when compared to promoter sequences from cv. Désirée or each other. On the other hand, two PR1b promoter sequences from cv. Rywal feature the lowest percent identity, if compared to the promoter sequences from other genotypes. However, the third PR1b promoter sequence from Rywal resembles promoter sequences from other genotypes rather than other two promoter sequences from Rywal (Figure 3.7, bottom panel).

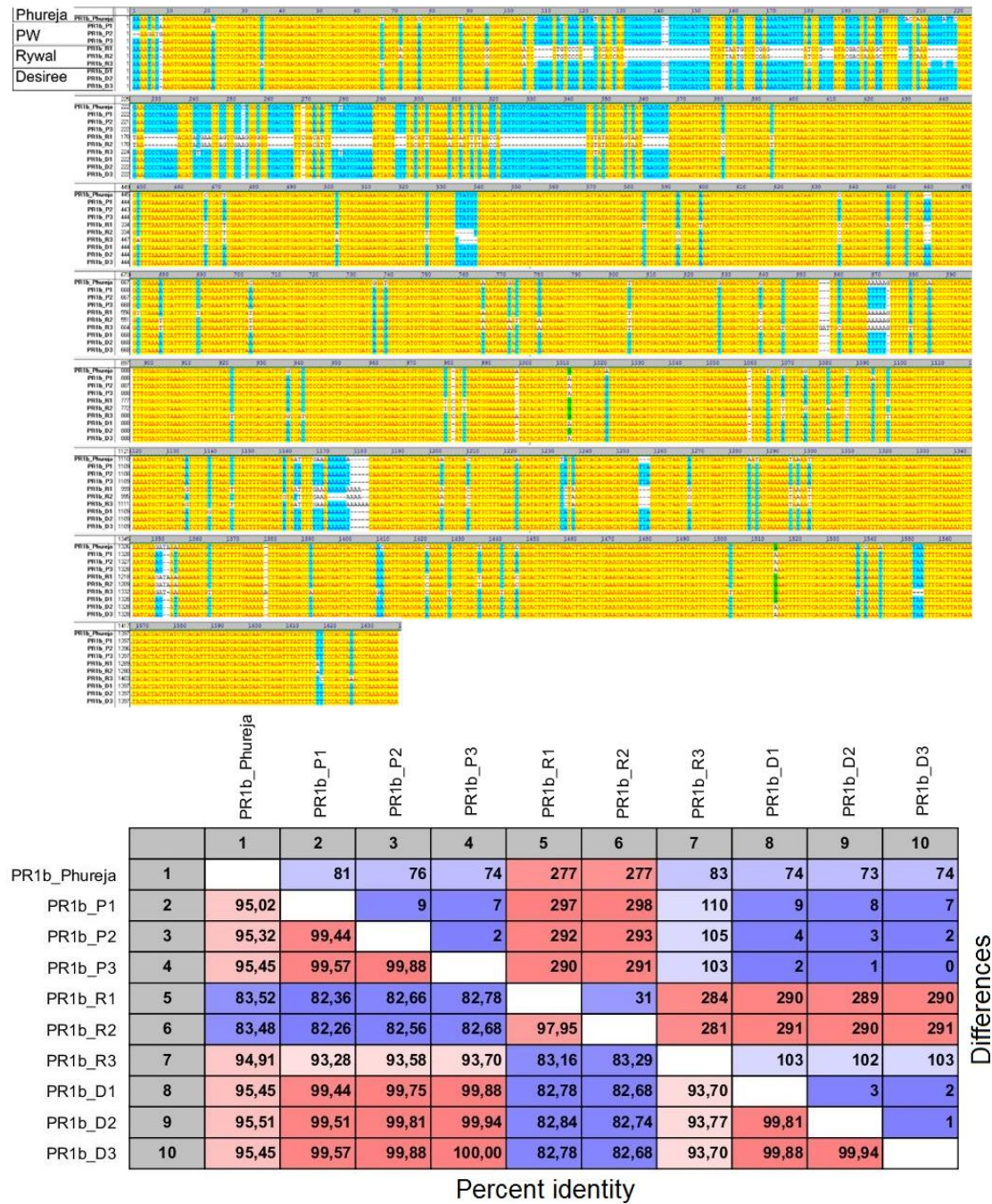


Figure 3.7: Promoter sequences of PR1b gene in potato genotypes. Top panel: Alignment of PR1b promoter sequences from different potato genotypes. The first row represents the promoter sequence from DM, rows 2 - 4 represent promoter sequences from PW363, rows 5 - 7 represent promoter sequences from Rywal, rows 8 - 10 represent promoter sequences from Désirée. Bottom panel: Percent identity (%) and nucleotide differences (number of different nucleotides) between PR1b promoter sequences from different potato genotypes. The percentage was calculated for the full-length sequences, including insertions and deletions. P: promoter sequence from PW363, R: promoter sequence from cv. Rywal, D: promoter sequence from cv. Désirée, 1 - 3: promoter sequence from colonies 1 - 3.

Next, we focused on the promoter sequences of two genes from the JA signalling pathway, MYC2 and MC. The similarity between MYC2 promoters was even lower than in the case of PR1b promoters (Figure 3.8, bottom panel), due to approximately 260 bp long deletion in the middle of the sequence (Figure 3.8, top panel). Interestingly, the deletions were found in the sequences of all three potato genotypes: in one promoter sequence from PW363, in two promoter sequences of Rywal and in both promoter sequences of Désirée, resulting in less than 80 % identity to MYC2 promoter sequence from DM. For the promoter sequences without deletions (two promoter sequences from PW363 and one promoter sequence from Rywal) the identity to MYC2 promoter sequence from DM was more than 90 %.

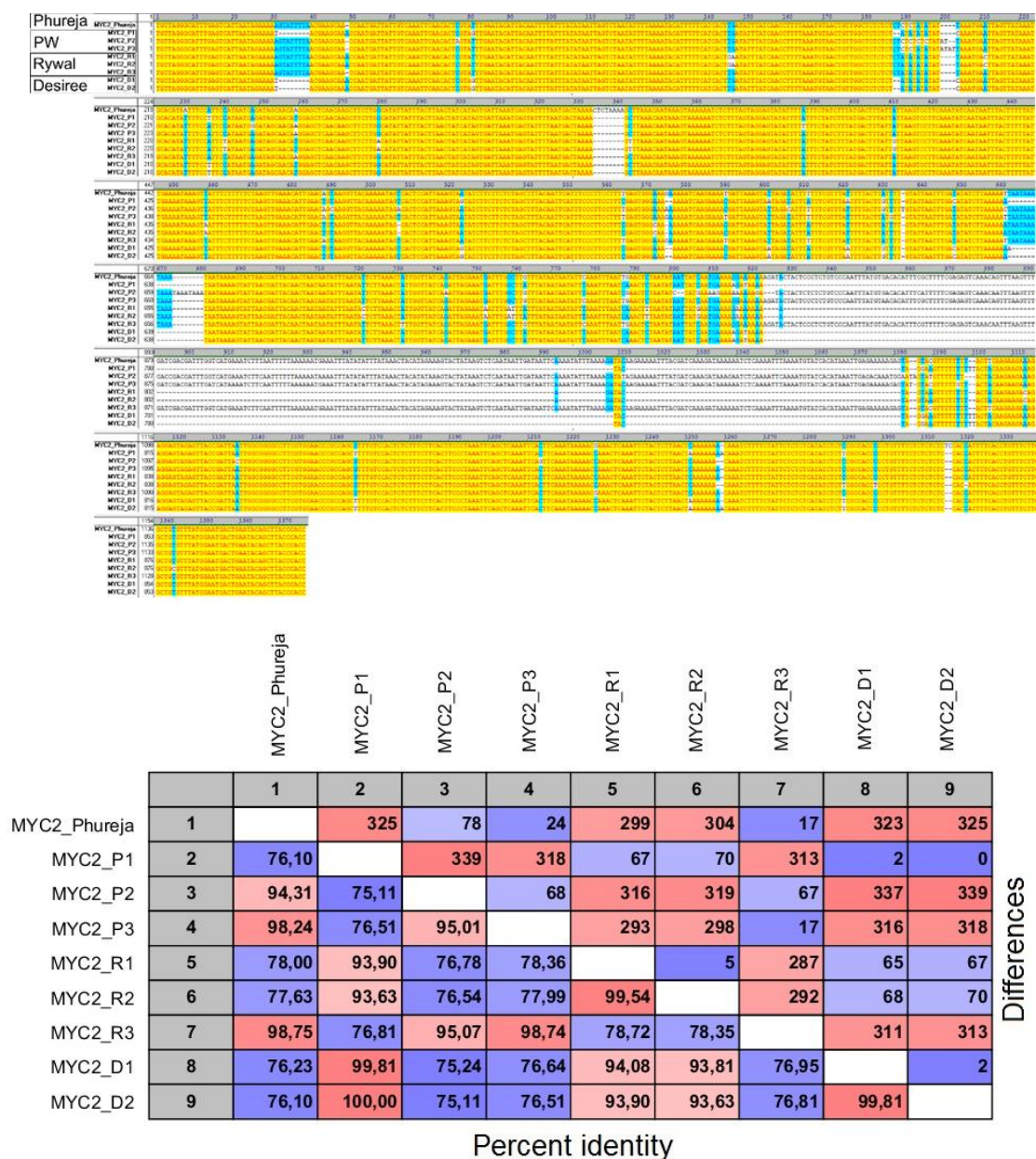


Figure 3.8: Promoter sequences of MYC2 gene in potato genotypes. Top panel: Alignment of MYC2 promoter sequences from different potato genotypes. The first row represents the promoter sequence from DM, rows 2 - 4 represent promoter sequences from PW363, rows 5 - 7 represent promoter sequences from Rywal, rows 8 - 9 represent promoter sequences from Désirée. Bottom panel: Percent identity (%) and nucleotide differences (number of different nucleotides) between MYC2 promoter sequences from different potato genotypes. The percentage was calculated for the full-length sequences, including insertions and deletions. P: promoter sequence from PW363, R: promoter sequence from cv. Rywal, D: promoter sequence from cv. Désirée, 1 - 3: promoter sequence from the colonies 1 - 3.

The similarity between MC promoter sequences was comparable to the similarity between MYC2 promoter sequences (from 74.9 to 99.9 percent identity in case of MC promoters and from 76.1 to 98.8 percent identity in case of MYC2 promoters (Figure 3.9, bottom panel). Interestingly, the region approximately 650 bp upstream of the start codon of the MC gene showed relatively high percent identity when compared among themselves (Figure 3.9, top panel). On the other hand, promoter sequences from Rywal and Désirée upstream of -650 nt showed much lower percent identity (less than 75 %), if compared to the promoter sequence from DM. However, promoter sequences from PW363 resemble the promoter sequence from DM as they showed more than 99 % identity when compared to the promoter sequence from DM.

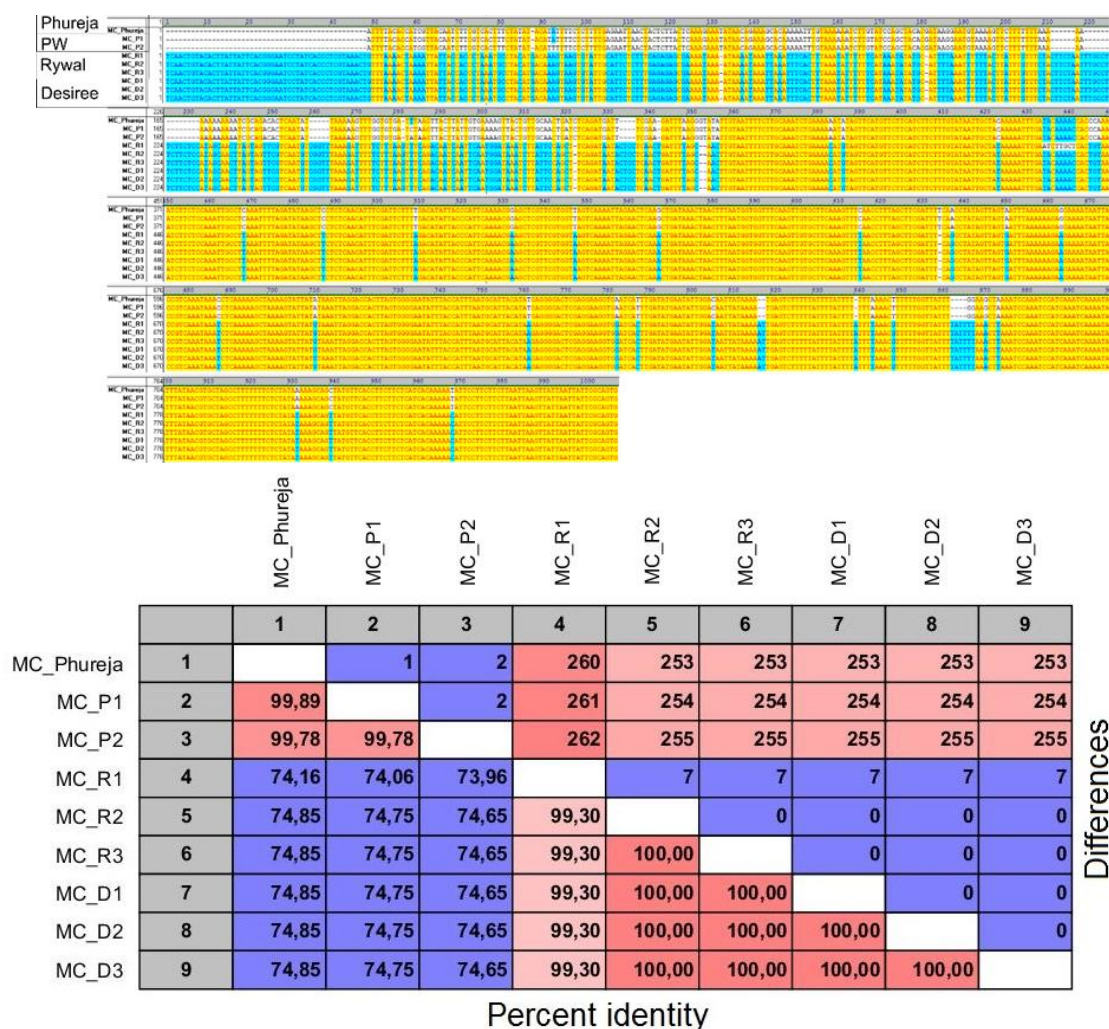


Figure 3.9: Promoter sequences of MC gene in potato genotypes. Top panel: Alignment of MC promoter sequences from different potato genotypes. The first row represents the promoter sequence from DM, rows 2 - 3 represent promoter sequences from PW363, rows 4 - 6 represent promoter sequences from Rywal, rows 7 - 9 represent promoter sequences from Désirée. Bottom panel: Percent identity (%) and nucleotide differences (number of different nucleotides) between MC promoter sequences from different potato genotypes. The percentage was calculated for the full-length sequences, including insertions and deletions. P: promoter sequence from PW363, R: promoter sequence from cv. Rywal, D: promoter sequence from cv. Désirée, 1 - 3: promoter sequence from the colonies 1 - 3.

Finally, we analysed promoter sequences of ACO2 and PTI5 genes involved in ET signalling. All ACO2 promoter sequences showed relatively high (more than 93) percent identity if compared to ACO2 promoter sequence from DM (Figure 3.10, bottom panel). Moreover, the sequence similarity of the ACO2 promoters was relatively high also within the same cultivar and between the cultivars. Instead of long deletions or insertions which were found in the promoters of some other genes, only SNPs and short deletions or insertions were identified in ACO2 promoters (Figure 3.10, top panel), which contributed to such high sequence similarity.

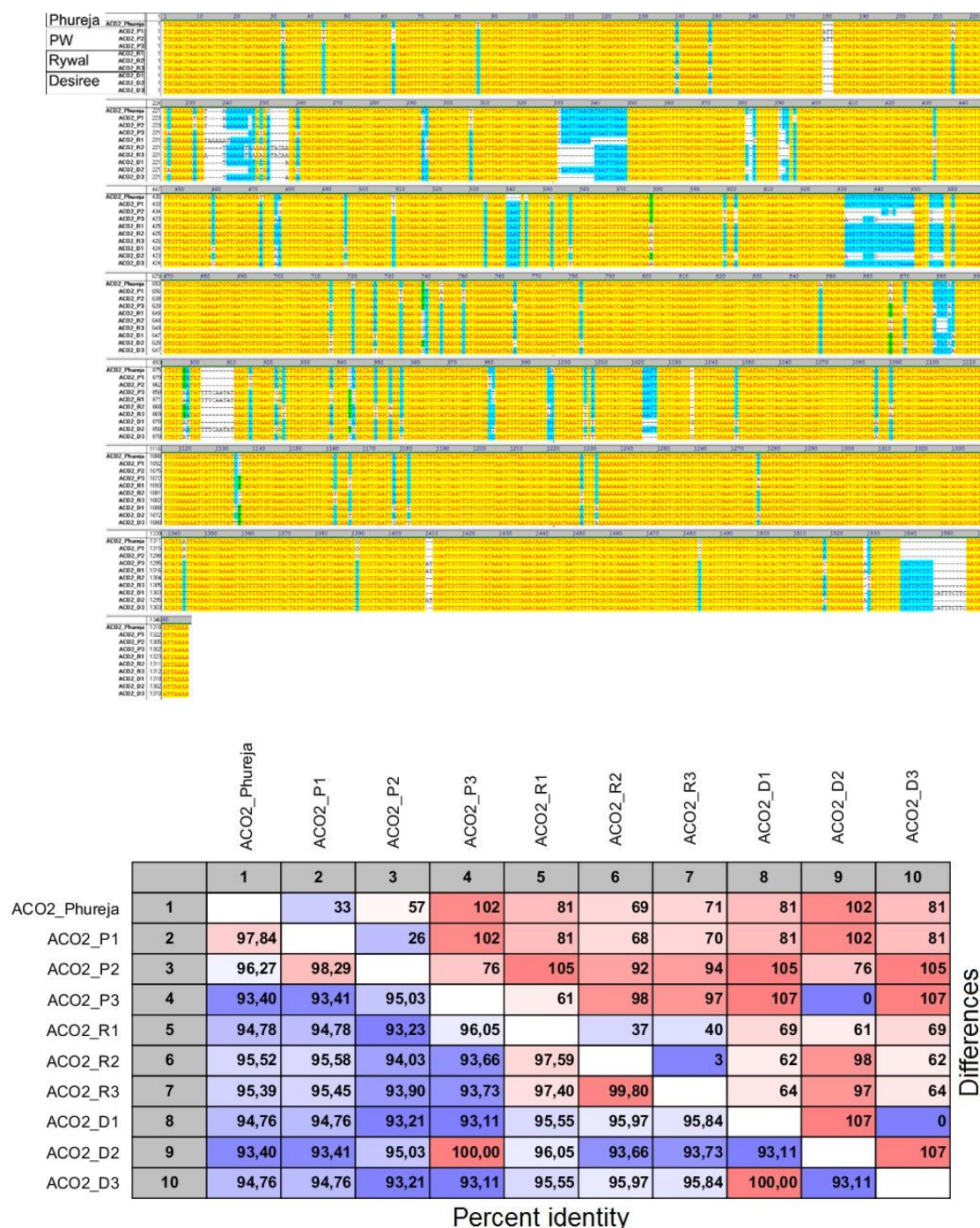


Figure 3.10: Promoter sequences of ACO2 gene in potato genotypes. Top: Alignment of ACO2 promoter sequences from different potato genotypes. The first row represents the promoter sequence from DM, rows 2 – 4 represent promoter sequences from PW363, rows 5 – 7 represent promoter sequences from Rywal, rows 8 – 10 represent promoter sequences from Désirée. Bottom: Percent identity (%) and nucleotide differences (number of different nucleotides) between ACO2 promoter sequences from different potato genotypes. The percentage was calculated for the full-length sequences, including insertions and deletions. P: promoter sequence from PW363, R: promoter sequence from cv. Rywal, D: promoter sequence from cv. Désirée, 1 – 3: promoter sequence from the colony 1 – 3.

Cloning of PTI5 promoter was performed as a part of another project (N4-0026) by the colleagues from NIB and the regions upstream of the gene were amplified only from the genomic DNA of cv. Rywal. Among all the genes, promoter sequences of the PTI5 gene from Rywal showed the lowest percent identity if compared to the PTI5 promoter sequence from DM (less than 70 %;

		PTI5_Phureja	PTI5_R1	PTI5_R2	PTI5_R3	PTI5_R4	PTI5_R5	
		1	2	3	4	5	6	
PTI5_Phureja	1		321	359	324	351	358	Differences
PTI5_R1	2	69,19		73	6	59	72	
PTI5_R2	3	66,23	92,85		75	16	1	
PTI5_R3	4	68,91	99,40	92,65		61	74	
PTI5_R4	5	66,98	94,22	98,41	94,03		15	
PTI5_R5	6	66,32	92,95	99,90	92,75	98,51		
Percent identity								

Figure 3.11, bottom panel). However, the promoter region approximately 600 nt upstream of the start codon of the PTI5 gene showed relatively high similarity to PTI5 promoter sequence from DM, which is evident from

		PTI5_Phureja	PTI5_R1	PTI5_R2	PTI5_R3	PTI5_R4	PTI5_R5	
		1	2	3	4	5	6	
PTI5_Phureja	1		321	359	324	351	358	Differences
PTI5_R1	2	69,19		73	6	59	72	
PTI5_R2	3	66,23	92,85		75	16	1	
PTI5_R3	4	68,91	99,40	92,65		61	74	
PTI5_R4	5	66,98	94,22	98,41	94,03		15	
PTI5_R5	6	66,32	92,95	99,90	92,75	98,51		
Percent identity								

Figure 3.11 (top panel). However, when compared to each other, PTI5 promoter sequences from Rywal showed a relatively high (from 92.7 to 99.9) percent identity.



		PTI5_Phureja	PTI5_R1	PTI5_R2	PTI5_R3	PTI5_R4	PTI5_R5	
		1	2	3	4	5	6	
PTI5_Phureja	1		321	359	324	351	358	Differences
PTI5_R1	2	69,19		73	6	59	72	
PTI5_R2	3	66,23	92,85		75	16	1	
PTI5_R3	4	68,91	99,40	92,65		61	74	
PTI5_R4	5	66,98	94,22	98,41	94,03		15	
PTI5_R5	6	66,32	92,95	99,90	92,75	98,51		
Percent identity								

Figure 3.11: Promoter sequences of PTI5 gene in potato genotypes. Top panel: Alignment of PTI5 promoter sequences from different potato genotypes. The first row represents the promoter sequence from DM, rows 2 - 6 represent promoter sequences from Rywal. Bottom panel: Percent identity (%) and nucleotide differences (number of different nucleotides) between PTI5 promoter sequences from different potato genotypes. The percentage was calculated for the full-length sequences, including insertions and deletions. R: promoter sequence from cv. Rywal, 1 - 5: promoter sequence from the colonies 1 - 5.

3.2.2 Promoter analysis enabled identification of several binding sites for the transcription factors involved in plant immune signalling

The next aim was to determine, if the promoters of the selected genes contain any patterns for binding sites of the transcription factors, known to be involved in plant hormonal signalling. Therefore, we searched for plant cis-regulatory elements and transcription factors specific for known transcription factors binding sites within the promoters of the selected genes using three databases, PlantCARE, TRANSFAC and PLACE. We show the results of the analyses of the promoter sequences of the lengths which were later on used for more detailed investigation (see section 3.3, Table 3.3). These lengths differed from the lengths of the promoter sequences that were used for alignments (see section 3.2.1) in the case of MYC2, MC and PTI5 promoters. Those promoters were truncated from the 5' end as lower number of pathogen responsive cis-regulatory elements were identified in distal regions.

The analysis using PlantCARE and TRANSFAC was performed in all promoter variants of all selected genes. The data can be found in the Appendix (Supplementary Tables 2 and 3 for TGA2, Supplementary Tables 4 and 5 for MYC2, Supplementary Tables 6 and 7 for MC, Supplementary Tables 8 and 9 for ACO2 and Supplementary Tables 10 and 11 for PTI5) or in Pezić et al., 2016 (for PR1b). The candidates for sensor design were further narrowed down based on the pattern of detected binding sites of transcription factors known to be implicated in immune signalling. The ones with the highest number of cis-regulatory elements involved in immune signalling were TGA2, PR1b, ACO2 and PTI5 promoter sequences from Rywal (TGA2(R1), PR1b(R3) and ACO2(R2)), MYC2 and MC promoter sequences from PW363 (MYC2(P2) and MC(P1)) and MC promoter sequence from Désirée (MC(D1)). For the promoters of MC gene, promoter sequences from two different genotypes were selected to

compare their activity based on different pattern of cis-regulatory elements. The selected promoter sequences were further analysed using PLACE database and then, the results were combined from all three databases and the regulatory elements involved in plant immune response within the selected promoter sequences were presented visually in Figures 3.12 - 3.17. Visual presentation of the MC(P1) promoter can be found in the Appendix (Supplementary Figure 1).

As expected, the majority of known regulatory elements involved in defense response identified in the TGA2 promoter were SA-inducible, including TGACG-motif, GT1-motif and several W-boxes (Figure 3.12). Several JA-inducible regulatory elements, including MYC-recognition site and G-box were also identified. On the other hand, no ET-responsive regulatory element was identified within the TGA2 promoter.

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+ TCCGTTACAAA TAATATGATT GAAGCGAGAT TGAGCTGATT CATGCATGTA TAGAAAAGGT GTGTGGAGG
- AGGCAATGTTT ATTATACTAA CTTCGCTCTA ACTCGACTAA GTACGTACAT ATCTTTTCCA CACACCTCC

+ CATTAATAAGA ATGTATGAAA GTTTAACTAT AACGAGTTGT AAGAAAGGTA AAAATAATCC AAAGAAGAT
- GTAATTATTCT TACATACTTT CAAATTGATA TTGCTCAACA TTCTTTCCAT TTTTATTAGG TTTCTTCTA

+ CATCAAAAAA GTAATTAGAC AAGATATAAT GCATCTTCAA CTTATTGAGA ATATGATGCA TAATCAGCT
- GTAGTTTTTTT CATTAACTCTG TTCTATATTA CGTAGAAGTT GAATAACTCT TATACTACGT ATAACTGGA

+ TAGATAAGAAA GTTTTGAAGA TCGAAATTA GAATAGCAAA ATTGTCGTAC TTATACGGGG AGAGGCGGG
- ATCTATCTTTT CAAACTTCT AGCTTTTAAT CTTATCGTTT TAACAGCATG AATATGCCCC TCTCCGCC

+ GAGCTAGTCTC CTCTCTCCT AGTTTAGTAA TCTTGCCCTAG TAAATACTTG AACTATTTAA GATAAAAGA
- CTCGATCAGAG GAGAAGAGGA TCAATCATT AGAAGCGATC ATTTATGAAC TTGATAAATT CTATTTTCT

+ TATATAACAAT TACAAACGGT ATAACTAAC ATATAAATAT AAAGAAACAT ACAATAAATT ATACTCCGT
- ATATATTGTTA ATGTTTGCCA TATTGATTG TATATTTATA TTTCTTTGTA TGTATTTAA TATGAGGCA

+ TCCATTCAAAA TCATTGTTGT TTCACAAAAT CAAGATGTTT AATTTTTTCC CTCCAAATT ATCCTTGAO
- AGGTAAGTTTT AGTAACAACA AAGTGTTTTA GTTCTACAAG TTAAAAAGG GAGGTTTAA TAGGAAGTG

+ AATTCCTAATT CAATATAATT TTTCAATGTT ATATATAACA AGCTAAATA ATAAATAAAT CATAATTAA
- TTAAGGATTAA GTTATATTAA AAGTTACAA TATATATTGT TCGATTATT TATTATTTA GTATTAATT

+ AGGATATTAGT CAAACCCAC TCTTCAAAAT AAAGGATCC AAGTGAAGC ATAAAACTT TGGGTGTGT
- TCCTATAATCA GTTTTGGTG AGAAAGTTTA TTTCCCTAGG TTCACTTTG TATTTTGA ACCCACA

+ TAGGTGTAAT TCCAAACTC CTAAGGAGAA TCTGAAATTA GATTGTTTTA TTATGTAAGT GCGCTAAAT
- ATCCACATTGA AGGTTTGTAG GATTCTCTT AGACTTTAAT CTAACAAAT AATACATTCA CGCGATTGA

+ AATTTAGGAAT ATTAATAAT ACTCCTCACT TTTACTTATT TATCATTTCT AATATCAAAA TAAATATT
- TTAAATCCTTA TAATTTTATA TGAGGAGTGA AAATGAATAA ATAGTAAAGA TTATAGTTTT ATTTTATA

+ TATATTAGCTA CTTTATTTAT AGAAGACAT ATAAAGTTCA AAATAACAA ATAAAAATGA TCGGATCGA
- ATATAATCGAT GAAATAAATA TCTTCTGTA TATTCAAGT TTTATTGTT TATTTTACT AGCCTAGCT

+ ACAAATATTGA TATATATATA TATCTCGTG GTTTAGAAA TGAACGCGGG GCTAGCGTAG TTTGGAATG
- TGTTTTATACT ATATATATA ATAGAGCCAC CAAATCTTTT ACTGGGCCCG CGATCGCATC AAACCTTAC

+ ATCTAAACGTC ACGTTTGTCA TCTTCTCTC TCTTCCCTC GTTCTTCGTG GTCATAAATA TATAAAT
- TAGATTTGAG GCAAAAGT AGAAGGAGAG AGAAGGGAG CAAGAAGCAC CAGTATTAT ATATTATA

+ ATTAAGTAAA TTATACAAA ATAAATTCTG GGTTCGTTGA ATTTTGTGTA AAAATGAGT CTTTATACA
- TAATTCATTTT AATATGTTTT TATTTAAGAC CCAAGCAACT TAAACACAT TTTTACTCA GAAATATGT

+ GAAATTCAC TTGAATTGT TTAATGACCA GTCATTGAAC CACTCCAAT TTGCTTATT AGTTTTAT
- CTTTTAAGTTG AACTTTAACA AATGACTGGT CAGTAACTTG GTGAGGTGA AACGAATAA TCAAAATA

+ ACAAAGTGGGG TTGTAGAAA TTCAATTTTC TTGAGGTTTT TTTATTAATT TTCAGTTTT GGTATTAG
- TGTTTCACCCC AACTCTTTT AGTTAAAG AACTCCAAA AAATAATTAA AAGTCAAAA CCCATAATC

+ TTATTAATCA TTTTATGAA GGGGTAGTTG GGTTTGATA AAGTTTGA CTTTTGGTG TTTGAAGTG
- AATAATTGAGT AAACTACTT CCCCATCAAC CCAAACTAT TTCAAAATG GAAAACCCAC AAACCTTAC

+ AGGGTTTATGC ACAGCTCTT TTTTTTTTG GGTTCCTTG ATTTCAATGG TGTAAATTTG TCTGATCGC
- TCCCAATACG TGTGACAAA AAAAAAAGC CCAAAAGAAC TAAAGTTACC ACATTAAAC AGACTACGC

+ GTGTAATGATT AAAGTTTTAA GCGGTTCCTT TTTGGAAGTT TTTGTACTTA GTCAACTATT TGAAGGGGG
- CACATACATAA TTTCAAAAT CGCCAACGAA AAACCTTCAA AAACATGAAT CAGTGTATA ACTTCCCC

+ GTTTGTAGTTT AATTTTGTCT CTGTAGAGTT TTGAGTGTAT GAACTGAGGT TGTGGATTGT TGAATGTA
- CAAACATCAA TTAACAAA GACATCTCAA AACTCACATA CTTGACTCCA ACACCTAACA ACTACTACT

+ TGTGAAGATTG GTAGAAAAG CTATAAGAGT GGTACTTCTG GGATG
- ACATTTCTAAC CATCTTTTCC GATATTCTCA CCATGAAGAC CCTAC

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Figure 3.12: Visual representation of predicted regulatory elements of TGA2 promoter from *S. tuberosum* cv. Rywal (TGA2(R1), 1513 nt). The predicted regulatory elements potentially involved in defense responses are **CGTCA-motif** (involved in the MeJA-responsiveness), **TC-rich repeats** (involved in defense and stress responsiveness), **W box/ELRECOREPCRPI** (involved in the SA-

responsiveness), **CBNAC binding site** (calmodulin-binding NAC protein), **At4g04450** (WRKY binding site), **WBOXATNPR1** (W-box, recognized by salicylic acid (SA)-induced WRKY DNA binding proteins), **WRKY71OS** (a core of TGAC-containing W-box WRKY binding site within the Pathogenesis-Related Class10 (PR-10) genes), **WBOXNTERF3** (W-box, involved in wounding responsiveness), **MYB1LEPR** (G-box, Tomato Pti4 (ERF) regulates defence-related gene expression via GCC box), **GT1GMSCAM4** (GT-1 motif, involved in pathogen- and salt-induced SCA4 gene expression), **MYCCONSENSUSAT** (MYC recognition site), **TGACG-motif/ASF1MOTIFCAMV** (involved in the JA- and SA-responsiveness), **T/GBOXATPIN2** (T/G-box, involved in JA-responsiveness), **WBOXNTCHN48** (W-box, involved in elicitor-responsive transcription of defense genes), **GT1CONSENSUS** (consensus GT-1 binding site, influences the level of SA-inducible gene expression), **WBOXPCWRKY1** (WB/W box, WRKY proteins binding site in PR1 gene in parsley). The **ATG** indicates the start of the TGA2 coding region. The promoter region is located upstream of the ATG. The regulatory elements were determined by PlantCARE, TRANSFAC and PLACE. The complete list of regulatory elements from the TGA2 promoters from cv. Rywal, Désirée and PW363 with their positions and sequences, predicted with PlantCARE and TRANSFAC can be found in the Appendix (Supplementary Tables 2 and 3).

Similar to the TGA2 promoter, the majority of known regulatory elements involved in defense response identified in the PR1b promoter were SA-responsive (Figure 3.13). In addition to SA-responsive regulatory elements which were also found in the TGA2 promoter, the PR1b promoter contained TCA-element and TGA binding site. Among JA-inducible regulatory elements, MYC-recognition site was identified, while G-box was not identified. In contrast to TGA2 promoter, several ET-responsive regulatory elements were identified, including GCC-box and some other ERF binding sites.

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+ AA AATAGAAGTC AAGAAAAAC TCTCCAATTA CATGATGGAA CAGGAATTCC
- TT TTAICTTicag ttCTTTTTTG AGAGGTTAAT GTACTACCTT GTCCTTAAGG

+ ACGCAGCGGT GACTAGTGGC AGAACCATGA TTTTCAATAA GGGGGTTCAA AATCCGAAGG ATTAAACATA
- TGGCTCGCCA CTGATCACC G TCTTGGTACT AAAAGTTATT CCCCCAAGTT TTAGGCTTCC TAATTTGTAT

+ CGAACTAGCC GAAGGGGGGG TTCGACATCT ATTATATACA TCTAAAAAAT AATTTTAGCC ATGTATATAT
- GCTTGATCGG CTTCGCCCC AAGCTGTAGA TAATATATGT AGATTTTTTA TTAAAAATCGG TACATATATA

+ AGTAATATTT TCCGTCGAAG GGGGTTCCGA TGAACCCCTA AAGACATGTT GGCTCCTCCC TGCCGCTGAC
- TCATTATAAA AGGCAGCTTC CCCCAGCCCT ACTTGGGGAT TTCTGTACAA CCGAGGAGGG ACCGCCACTG

+ CTATTGAAA ACTTTTATCG AAAAATTATA CTTTATATGT AAAATATTAT ATGAAGTACA TTCGTCAGGA
- GATAAACTTT TGAAAAATAGC TTTTAAATAT GAAATATACA TTTTATAATA TACTTCATGT AACAGTCCT

+ ACTACTTTAG GTGTACATAT ATTTATTAAG CATATCAAAT TATTTATCCT CTATTTAATA CTTATTTAAA
- TGATGAAATC CACATGTATA TAAATAATTC GTATAGTTTA ATAAATAGGA GATAAATTAT GAATAAATTT

+ CATGTAACTA TTTATTCGTA AATTCAACTT CAACCCCTTA AAACGATTTA AAAATTAATA ATTCTCATTC
- GTACATTGAT AAATAAGCAT TTAAGTTGAA GTTGGGAATT TTTGCTAAAT TTTTAATTAT TAAGAGTAAG

+ GAAGCTGCCA GGATGTGAGG CAGTTAATAT CTACAGAAAG GACCAAATAT TTTTCTGGT TGTCCATCAC
- CTTCCAGCGT CCTACACTCC GTCATTATA GATGTCTTTC CTGGTTTATA AAAAGCACCA ACAGGTAGTG

+ TTTGTTTATT TTTTGTITTC ATTATATTCA AATTGCTTC AATGGCTAAC AAATTCTCAC TCTTCTCTCT
- AAACAAATAA AAAACAAAAG TAATATAAGT TTAACGAAAG TTACCGATTG TTTAAGAGTG AGAAGAGAGA

+ CGTACAATAA TTTTCTCCCA ATAGATTTAG GGGGGCTTAA TAATATCGAT GGCCTAAAT CATTTTTCCA
- GCATGTTATT AAAAGAGGGT TATCTAAATC CCGCCGAGTT ATTATAGCTA CCGGATTTAA GTAAAAAGGT

+ TGAATATATTT TATAAGTAAA CACTGAATCG CATCTCTCT TGAACAGAGG TTCCATGTTT GAATCCTAAA
- ACTTTATAAA ATATTCATTT GTGACTTAGC GTAGGAGAGA ACTAGTCTCC AAGGTACAAG CTTAGGATTT

+ ATGAAAAATA AAACCTGCTAA ATAGAACTTC CCTTTAAAGG TACTTATGTG ACATAAATCA AATTAATTAG
- TACTTTTATT TTGATT TATCTTGAAG GGAAATTTCC ATGAATACAC TGTATTTAGT TTAATTAATC

+ GACTCCAGCA CACATCCAAG ACATGATTGC ATAAGAAAAA AAGTTTTTTT TCAGACCCCT ATAAATTTGG
- CTGAGGTCGT GTGTAGGTTT TGTACTAAGC TATTCTTTTT TTCAAATAAA AGTCTGGGGA TATTAAAACC

+ AGCCTAAGC CCTTATTTTA GTTGGTTCAC CATTGGTCA TGTCCATGCT TCACGAGGCT GTAGAAATG
- TCGGATTTCC GGAATAAAAT CAACGAAGTG GTAAACAGT ACAGGTACGA AGTGCTCCGA CATCTTGTAC

+ GTTGAGCCCC CATCTAATCG AAAAATAATA TACATCTTTA TGCTTCACGA GGTGTAGAA CATGTGTGAG
- AACTCGGGG GTAGATTACC TTATTTTTAT ATGTAGAAAT ACGAAGTGCT CCAACATCTT GTACACACTC

+ CCCCACTAA TAGAAAAAAT ATACATCTTT TTAATAATTC AAGTCTTCTT ATGTTTTATA GACTTTTTAT
- GGGGTAGATT ATCATTTTTA TATGTAGAAA AATTATTAAG TTCAGAAGAA TACAAAATAT CTGAAAAATA

+ TCCACCCAAA AATGCTTAAT TAGTCTTGT TAACTTTATT TTGATAATAT AATTTTAAA AAAAAAATA
- AGGTGGGTTT TTACGAATTA ATCAGAACAA ATTGAAATAA AACTATTATA TTAAAACTTT TTTTTTTTTT

+ ACAAGAATTA CCTAGATTAA AGTAGACTA TTCTTTAAAG CATATAGTAT TCCCTAAATC ACACGACATG
- TGTCTTAAAT GGATCTAATT TCATACTGAT AAGAAATTTT GTATATCATA AGGGATTTAG TGTGCTGTAC

+ CAAGGTACTA ATCGCATTTG AATTTTCTTT ACATGAAAAA TAAACTCCA CAATGTTTAA ATTTAACAAT
- GTTCCATGAT TAGCTAAAC TTAAGAAA TGTACTTTTT ATTTGAGGT GTTACAAAT TAAATTTGTA

+ CAAAGTTTGA TAAAAATCTA ATCAAATAA AAAAAATGTT CATTTTTGA AAAAACTTAA GGACCAAAAG
- GTTTCAAACT ATTTTATAGT TAGTCTATT TTTTTTACAA GTAAAAACTT TTTTGAATTT CCTGGTTTTT

+ TAATTACTTC TAAGAAATTG AAGGACCAAA ATTGCTCAAC GAAATATACA AGAGACTATT TTGAACCTAC
- ATTAATGAAG ATTCTTTAAC TTCTGGTTT TAACGAGTTG CTTTATATGT TCTCTGATAA AACTTGAATG

+ TATCAAAGAT AAGAGACCAT TTTTATCATT TCCTCTATTA ATTTCTTTG AATTTTCTTC ACACATGCTA
- ATAGTTTCTA TTCTCTGGTA AAAATAGTAA AGGAGATAAT TAAAGCAAC TTAAGAGAAG TGTGTACGAT

+ ATCAAAATTC CACAATTTTT ACTTATAAAT ACACACTTAA TCTCACATT ATAATCACAA TAACCTAGAT
- TAGTTTTAAG GTGTTAAAAA TGAATATTTA TGTGATGAAT AGAGTGATAA TATTAGTGTT ATTGAATCTA

+ TTATTTTCTC TCCACTAAAC CTAAAGCAAA ATG TAC
- AATAAAGAG AGGTGATTTG GATTGCTTT TAC

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Figure 3.13: Visual representation of predicted regulatory elements of PR1b promoter from *S. tuberosum* cv. Rywal (PR1b(R3), 1622 bp). The predicted regulatory elements potentially involved in defense responses are **CGTCA-motif** (involved in the MeJA-responsiveness), **TC-rich repeats** (involved in defense and stress responsiveness), **TCA-element** (involved in salicylic acid responsiveness), **GCC box** (involved in responses to ET), **TGACG-motif** (involved in the MeJA-responsiveness), **TGA1a binding site** (TGA1a transcription factor recognition site), **TGA1A binding site** (TGA1a transcription factor recognition site), **CBNAC binding site** (calmodulin-binding NAC protein), **ERF13/DRF1.3/ESR1 binding site** (ethylene-responsive element binding factor, involved in ethylene and jasmonic acid responsiveness), **ERF1 binding site** (ethylene-responsive element binding factor, involved in ethylene and jasmonic acid responsiveness), **WBOXNTERF3** (W-box, involved in wounding responsiveness), **WBOXATNPR1** (W-box, recognized by salicylic acid (SA)-induced WRKY DNA binding proteins; lowercase), **ASF1MOTIFCAMV** (ASF-1 binding site, involved in salicylic acid and biotic stress responsiveness), **GCCCORE** (core of GCC-box, ethylene-responsive element), **ERELEE4** (ethylene responsive element), **AGCBOXNPGLB** (binding sequence of Arabidopsis AtERFs, conserved in most PR-protein genes, involved in stress responsiveness), **MYCATRD22** (binding site for MYC), **MYCATERD1** (MYC recognition sequence), **WRKY71OS** (a core of TGAC-containing W-box WRKY binding site within the Pathogenesis-Related Class10 (PR-10) genes), **GT1CONSENSUS** (consensus GT-1 binding site, influences the level of SA-inducible gene expression), **MYCCONSENSUAT** (MYC recognition site). The **ATG** indicates the start of the PR1b coding region. The promoter region is located upstream of the ATG. The regulatory elements were determined by PlantCARE, TRANSFAC and PLACE. The complete list of regulatory elements from the PR1b promoter sequences from cv. Rywal, cv. Désirée and PW363 with their positions and sequences, predicted with PlantCARE and TRANSFAC, can be found in Pezić et al., 2016.

In the MYC2 promoter, there were identified regulatory elements involved in all three signalling pathways, including the SA-responsive GT-motif and TCA-element, several ET-responsive ERF transcription factor recognition sites and the MYC2 recognition site involved in the JA-signalling pathway (Figure 3.14). Also identified were several W-boxes and DREB1B recognition site, a motif involved in the oxidative stress response.

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+ TGTTAGGGCA TTTGAGTCAT TAATGAAAA AGTATTTTAA CGAAGCAACC AATGATTATT GTCAAATTCA
- ACAATCCCGT AAACTCAGT ATTATCTTTT TCATAAAATT GCTTCGTGGT TTACTAATAA CAGTTAAGT

+ ACAC TAGTAT TTGAATACAT TACAATTTTA TTTATATAAT TAGTCTAATA TTTTAAAAA CATTTTTCAT
- TGTGATCATA AAC TTATGTA ATGTTAAAAA AAATATATTA ATCAGATTAT AAAATTTTAT GTAAAAAGTA

+ CACTTGATAT TTAICAACTT TTTAAATGTA ACTGTTGGCT CTCTCTCTCT CTCTCTATAT TAAATGAATT
- GTGAAGTATA AATAGTTGGA AAATTTACAT TGACAACCGA GAGAGAGAGA GAGAGATATA ATTTACTTAA

+ AGTTATAAAT GCACATATTT TCCATTCCAT AATGGGATAG CAACAAGGAG CTCAACAAGC TTCTTGGCAT
- TCAATATTTA CGTGTATAAA AGGTAAGGTA TTACCCATATC GTTGTTCCTC GAGTTGTTGG AAGAACCCTA

+ ATTATTTACT TAACTATCAT AGTGATTAAA TGAGTATTTT AATGACTAAA AGCTTAAACA ATAAAGTAAA
- TAATAAATGA ATTGATAGTA TCACTAATTT ACTCATAAAA TTACTGATT TCGAATTTGT TATTTCAATT

+ AAATCTCTTT AGTAGGAGTA TATTTTTTTT CTATCTTTA T GACT TTATTA TTAAGTCCTT CAAATATCAA
- TTTAGAGAAA TCATCCTCAT ATAAAAAAA GATAGAAAATA CTGAAATAAT AATTCAGGAA GTTTATAGTT

+ TAATTTACTT TTTATTGAAA ATAAAGTTAT TTCTTTTTCT AAGTTGAAAC ATTGAACAGC AAGGTTACAA
- ATTAATGAA AAATAACTTT TATTTCAATA AAGAAAAAGA TTCAACTTTG TAACTTGTGG TTCCAATGTT

+ AAATAGACAC TCCATTAAAG TAATATCTTC TTCTCTAGCT TTACAATTCA TTTCCCTTCC TTTTGTGTTG
- TTTATCTGTG AGGTAATTTT ATTATAGAAC AAGAGATCGA AATGTTAAGT AAAGGGAAGG AAAACTCACC

+ GAAAAAATA TCAAGAAAAC GATTAAAGTA TTAGACATTT TTTTCGTTA TTTTACTTGG TTTTGTIATT
- CTTTTTTTTT AGTCTTTTTG CTAATTTTAT AATCTGTAAA AAAAGCAAAT AAAAATGAAC AAAACATAA

+ AATTTGATAT ATCTTAAAAA CTAATAAATA AATAAATAAA TAATAAAAGT ATTAACGATT ACAAATAATA
- TTAAACTATA TAGAATTTTT GATTATTTAT TTATTTATTT ATTATTTTCA TAATTGCTAA TGTGATTAT

+ ATATTTAATA TTCTTAAACT ATTGGTTATC AAATTAGAAA TGATTTGGCT TGTTTATAAT AATATTTAAA
- TATAAATTAT AAGAATTTGA TAACCAATAG TTTAATCTTT ACTAAACCGA ACAAATATTA TTATAAATTT

+ TTTAATCGAA CTCTAATATC TATGAAAAGA AAAAAGAAGA TACTACTCTC TCTGTCCCAA TTTATG TGAC
- AAATTAGCTT GAGATTATAG ATACTTTTCT TTTTTTTTCT ATGATGAGAG AGACAGGGTT AAATACACTG

+ ACATTTCAIT TTTGAGAGT CAAACAATTT AAGT Tggc GAC GATTGG TCATGAAATC TTCAATTTTT
- TGTAAGTAA AAAGCTC TCA CT TTGTTAAA TTCAAAGTGG CTGCTAAA CC ACTTACTTTAG AAGTTAAAAA

+ TAAAAATAAA ATTTATATAT TTATAAATA CATATAAAGT ACTATAAATC TCAATAATTG AT AATTCAAA
- ATTTTATTTT TAAATATATA AATATTTGAT GTATATTTCA TGATATTTAG AGTTATTAAC TATTAAGTTT

+ ATATTTAAAA GATATAGAAA AAATTTATGA TCAAGATAA AGAATCTCAA AATTCAAAAT GTATCACATA
- TATAAATTTT CTATATCTTT TTTAATAACT AGTTCTATT TCTTAGAGTT TTAAGTTTAA CATAGTGTAT

+ AATTGAGACA AATGSAATAG TATTTTTT TAG TACAAGA AGAAAGAGGA GTAGAGTTAC CGATTACTTG
- TTAACTCTGT TTACTTTATC ATACAAAAAA ATGATGTTCT TCTTTCTCT CATCTCAATG GCTAATGAAC

+ GCGGGGCCCT CGTGAACCC GCCAGCGTTT GTCCACTCTC TTTCTTCACT TCCCTAAAT CAGCTCAAAT
- CGCCCCGGA GCACCTTGGG CGGTGCGAAA CAGGTGAGAG AAAGAAGTGA AGGGATTTAA GTGAGTTTA

+ TCAGTTCAAA TAAAAACCAA ACTCAAATTC TACTTTAATC TCAAAAAACA AATCTTTTCT ATCTCTCTC
- AGTCAAGTTT ATTTTGGTT TGAGTTTAAAG ATGAGAATTG AGTTTTTTGT TTAGAAAAGA TAAGAGAGAG

+ ATAICTCTCC GCCACCCCTT GTTTTCTCTC TCTCCACCAT TTTCACTGT TTTTGTCTGT GTTTATGGAA
- TATACAGAGG CGGTGGGGGA CAAAAGAGAG AGAGGTGGTA AACTGGACA AAGAACGACA CAAATACCTT

+ TGACTGAATA CAGCTTACCC ACC
- ACTGACTTAT GTCGAATGGG TGG

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Figure 3.14: Visual representation of predicted regulatory elements of MYC2 promoter from *S. tuberosum* PW363 (MYC2(P2), 1353 bp). The predicted regulatory elements potentially involved in defense responses are **TCA-element** (involved in salicylic acid responsiveness), Box-W1/W box/ELRECOREPCR1 (involved in the SA-responsiveness; lowercase), **TC-rich repeats** (involved in defense and stress responsiveness), CBNAC binding site (calmodulin-binding NAC protein binding site), **GT-1 recognition site** (involved in SA-inducible gene expression, GT-1 binds to upstream PR-1a

promoter region), **WRKY48 recognition site** (involved in stress and pathogen response), ERF039 recognition site (contains GCC-box pathogenesis-related promoter element), **DREB1B recognition site** (involved in oxidative stress and salicylic acid responsiveness), **ERF9 recognition site** (contains GCC-box pathogenesis-related promoter element), **GT1CONSENSUS** (consensus GT-1 binding site, influences the level of SA-inducible gene expression), GT1GMSCAM4 (GT-1 motif, involved in pathogen- and salt-induced SCaM-4 gene expression), **WRKY71OS** (a core of TGAC-containing W-box WRKY binding site within the Pathogenesis-Related Class10 (PR-10) genes), **WBOXNTERF3** (W-box, involved in wounding responsiveness), **MYCCONSUSAT** (MYC recognition site), WBOXATNPR1 (W-box, recognized by salicylic acid (SA)-induced WRKY DNA binding proteins), *WBOXPCWRKY1* (WB/W box, WRKY proteins binding site in PR1 gene in parsley; italics), **ERELEE4** (ethylene responsive element), **SEBFCONSSTPR10A** (binding site of the potato silencing element binding factor (SEBP) gene found in promoter of PR-10a gene). The **ATG** indicates the start of the PR1b coding region. The promoter region is located upstream of the ATG. The crucial regulatory elements were determined by PlantCARE, TRANSFAC and PLACE. The complete list of regulatory elements from the PR1b promoter sequences from cv. Rywal, cv. Désirée and PW363 with their positions and sequence, predicted with PlantCARE and TRANSFAC, can be found in the Appendix (Supplementary Figures 4 and 5).

The majority of the identified regulatory elements in the MC promoter were involved in JA signalling, including MYC-recognition site, TGACG-motif and G-box (Figure 3.15). We also identified several regulatory elements from SA-signalling, including TGA1A binding site and several WRKY binding sites. No ET-responsive elements were identified. Interestingly, regulatory elements involved in Ca²⁺ and oxygen responsiveness within the MC promoter sequence were identified.

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+ TCAACTGTAC ACTTATTATT CACGGGAATC CTATCACCTT CGTAACTGT TTAAGACTAA AATTATTACC
- AGTTGACATG TGAATAATAA GTGCCCTTAG GATAGTGGGA GCATTGACA AATTTTGATT TTAATAATGG

+ ACCCTAATG CCTACATGAC AAGTAAAGTG TATTGCATTC TCCTTGAGAG AGCGAAAAA AAATAAATA
- TGGGATTAC GGAATGACTG TTTTCATTCAC ATAACGTAAG AGAACTCTC TCGCTTTTAT TTTATTTTAT

+ AATTAAATTT CACTTTAAAT ATTTTTCAT AAATATATGC ATTTTAAAT TTTTACTTAT TTGTTTCTAC
- TTAATTTAAA GTGAATTTA TAAAAAAGTA TTTATATACG TAAATTTAA AAAATGAATA AACAAAGATG

+ TTCTAATTTG CCTTCTTCTC CATAGCAACT TAGTAGTTCG TCAATGTCGG TTTAAAGATT TTTCTTTAAG
- AAGATTAAC GGAAGAAGAG GTATCGTTGA ATCATCAACAGTACAGCC AATTTTCTAA AAAAGAAATTC

+ TTTTCAGACAA TCAATTCAAT GGATAAATCT TATTCAGCT TCAGATAATA CTCCTCAACT GATTTTAGCA
- AAAGTCTGTT AGTTAAGTTA CCTATTTAGA ATAAGGTCGA AGTCTATTAT GAGGAGTTGA CTAAATCGT

+ ACTTGTAATT TTCTTGCAA TCTGAAACA TGTAGTTCAT GTTCTTCATC TTCCTTTGAT AATTGCTATA
- TGAACATTAA AAGAACGTTT AGACTTTTGT ACATCAAGTA CAAGAAGTAG AAGAAACATA TTAACGATAT

+ AAAATTTGAT ACAAACCAC TGTAAATCTT CTCCAAATTG CCTTAAATTT TAGATATAAG CTCTCTCAA
- TTTTAACTA TGTTTTGGTG ACATTTAGAA GAGGTTTAA GGAATTTAAA ATCTATATTC GAAGAGATT

+ CATTTCGATT CTTTAGACAT ATTACCCATT CAAAACGAAG CTCGTTCTGT GTAAGTCAA ATTAGACTC
- GTAAAGCTAA GAAATCTGTA TAATGGGTAA GTTTTGCTTC GAGCAAGCAC CATTCAGTCT TAATCTTGAG

+ AAATGATAA ACTAACTTTA ATGGTGGTTT CAAGTTGTGC TTCAAAGTTT TAACCTTTAG CTTCGATTTT
- TTTGACTATT TGATTGAAT TACCACCAA GTTCAACAG AGTTTCAA ATTGGAATC GAAGCTAAAG

+ AGCTATAGT TAGTGATTTA AAAAGAAGA AATAAATTAC CGTCAATAA ATCTCAAAA GCTAAAAGTA
- TCGGATATCA ATCACTAAAT TTTTCTTCT TTTTAAATG CGAGTTTATT TAGAGTTTTT CGATTTTCAT

+ TTATGTAAT TAGGACCACT TAGTGGGGAA TATTACCAT TTAATGCATT ACATAAGAGG GGAATCGAGG
- AATACATTTA ATCCTGGTGA ATCACCCCTT ATAAATGGTA AATTACGTAA TGTATTCTCC CCGAGCTCC

+ AAGTGGATC ACTTGATATG AATATTGGAT AATTATAAAA ATTGAGTCTT TTTATTTTAT TTTATTTTAA
- TTCCACCTAG TGAATATATC TTATAACCTA TTAATATTTT TAATCTCAGAA AAATAAATA AAATAAATT

+ ACGTTTTTGG TTATTTTATT TTAACCTTAA ATCCAAATCC ATCAAAATCAA AATATTTATA ACGTGCTAGC
- TGCBAAAACC AATAAATAA AATTGAAT TAGTTTAGG TAGTTTAGT TTATAAATA TGCACGATCG

+ CTTTTTTTCT CTATATAAAG CAGTTATGTT CACCTTCTTC TCATCACAAA AACATTCCCT CTCTTAAAT
- GAAAAAAAGA GATATATTTT GTCAATACAA GTGGAAGAAG AGTAGTGT TTGTAAGGAA GAAGAATTAA

+ AAGTATTAA TTATTCGCAG TGATC
- TTCAATAATT AATAAGCGTC ACTAC

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Figure 3.15: Visual representation of predicted regulatory elements of MC promoter from *S. tuberosum* cv. Rywal (MC(D1), 1002 bp). The predicted regulatory elements involved in biotic stress responses are **CGTCA-motif** (involved in the MeJA-responsiveness), **TC-rich repeats** (involved in defense and stress responsiveness), **TGACG-motif** (involved in the MeJA-responsiveness), **G-box** (involved in JA-responsiveness), **TGA1A** binding site (TGA1A transcription factor recognition site), **WRKY** (WRKY binding site), **DREB1B** (involved in tobacco streak virus infection and PR gene expression), **AT4G12670** (involved in SA and JA responsiveness), **MYCCONSUAT** (MYC recognition site), **GT1CONSENSUS** (consensus GT-1 binding site, influences the level of SA-inducible gene expression), **WRKY71OS** (a core of TGAC-containing W-box WRKY binding site within the Pathogenesis-Related Class10 (PR-10) genes), **WBOXATNPR1** (W-box, recognized by salicylic acid (SA)-induced WRKY DNA binding proteins; lowercase), **ACGTATERD1** (involved in Ca²⁺ responsiveness), **ASF1MOTIFCAMV** (ASF-1 binding site, involved in salicylic acid and biotic stress responsiveness), **GT1GMSCAM4** (involved in pathogen responsiveness), **CURECORECR** (involved in oxygen response), **TCA1MOTIF** (involved in SA responsiveness), **T/GBOXATPIN2** (involved in JA responsiveness), **MYB1LEPR** (G-box, Tomato Pti4(ERF) regulates defence-related gene expression via GCC box), **ABRERATCAL**

(involved in Ca²⁺ responsiveness), **WBOXNTERF3/WBOXHVIS01** (W-box), **WBBOXPCWRKY1** (WB/W box, WRKY proteins binding site in PR1 gene in parsley). The **ATG** indicates the start of MC coding region. The promoter region is located upstream of the ATG. The regulatory elements were determined by PlantCARE, TRANSFAC and PLACE. The complete list of MC promoter domains from cv. Rywal, Désirée and PW363 with their positions and sequence, predicted with PlantCARE and TRANSFAC, are specified in Tables 6 and 7.

In the ACO2 promoter, GT1-motif, TCA-element and many W-boxes from the SA signalling pathway, a MYC-recognition site and a G-box involved in JA signalling and several ET-responsive ERF recognition sites were identified (Figure 3.16).

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+ TGCAACTAAC ATACTTAgacTAATAAAAT ATAAATGGCT TTTCTCACTT GTTGAAGCC ATATAAGTTT
- ACGTTGATTG TATGAATACT GATTATTTTA TATTTACCGA AAAGAGTGAA CAAACTTCGG TATATTCAAA

+ TTCTTCAATC TATATCGGTG TAAGTTCTTG GTCAAAGATT GCATATTCTA GATATATGCT TTATAATTAA
- AAGAGTTAG ATATAGCCAC ATTCAAGAAceagtCTAA CGTATAAGAT CTATATACGA AATATTAATT

+ GAAAAAAAAG GAAATTAGTT AAGATAAATT TCCACAATTA ATTATACAAA AGTTAGGTTG ATGATGAAAT
- CTTTTTTTTT CTTTAATCAA TTCTATTTAA AGGTGTTAAT TAATATGTTT TCAATCCAAC TACTACTTTA

+ GGAtgacACA GCAAAAAAAA ATAAAAATAA AAAATACAAC AACTATGATA TGTTCAAAAAT TCAAATATTT
- CCTACTGTGT CGTTTTTTTT TATTTTTIAT TTTTATGTTG TTGATACTAT ACAAGTTTTA AGTTTATAAA

+ AGTCAAGAAT AGTTACTTAG AATTTAATTG GATTAAATGA AATAATTGAA GTAACACAGG TTCTTTTtg
- TeagtCTTA TCAATGAATC TTAAATTAAC CTAATTTACT TTATTAACTT CATTGTGTCC AAGAAAATAC

+ acTTTAAAG TGTCTTTTTT TTTGTAATTA CAATTAGTTA AATTACCATG AGAATACTAA GTCTATTATC
- TGAAATTTTC ACAGAAAAAAA AAACATTAAT GTTAATCAAT TTAATGGTAC TCTTATGATT CAGATAAATAG

+ TTTCTTGTTA ATATATATAA GTTCAATATA ATTCCGACTT TCTGTATCAA CCATTTTCTA TAAATCCAT
- AAAGAACAAT TATATATATT CAAGTTATAT TAAGCTGGAA AGACATAGTT GGTAAAAGAT ATTTAAGGTA

+ TAACAATCAA ATTCTTTTTT TTGGAATCAA TTTTGTITAT CATTCTATTT CATATTTTTT TAATAAAGAT
- ATTGTTAGTT TAAGAAAAAAA AACCTTAGTT AAAACAAAATA GTAAGATAAA GTATAAAAAA ATTATTCTA

+ ATTTTATTAT AATAGTCAA TAATATTCAA ATAAACGTTA TCGTTATAAG GTCTTGCTA TATTAAAAA
- TAAAAATAA TTATeagtTT ATTATAAGTT TATTTGCAAT AGCAATATTC CAGAACAGAT ATAATTTTTT

+ TTTCCATGGG GCAAAATGTG CAGCCCTAAA AATGTGAATT GTGTGGCAAC TTCTAAATAG TACCCTTTG
- AAAGGTACCC CGTTTTACAC GTCGGGATT TACACTTAA CACACCGTTG AAGATTTATC ATGGGAAAAe

+ TCAAACTGAA CCAAAACAGTT TATAAACACA TGAAAAAAAT AAATTAACAA AAAGTTTTAG TCAAGAtgac
- agtCTGACTT GGTGTGTCAA ATATTGTCT ACTTTTTTTA TTTAATTGTT TTTCAAATe-agtCTACTG

+ CAAAATTTAG CCACTAGAAA AATGGGTCCA AATTCTAACT GTCCCAACTC TAATGGGTAG AGTAGAAGAT
- GTTTTAAATC GGTGATCTTT TTACCCAGGT TTAAGATTGA CAGGGTTGAG ATTACCCATC TCATCTTCTT

+ TATGAAAGTT TACTAGGCTG TCATTTTCTA TCGATATAAA TATTAAGTAA TTTTATTCAT TCGACATTTA
- ATACTTTCAA ATGATCCGAc agtAAAAAGAT AGCTATATTT ATAATTCATT AAAATAAGTA AGCTGTAAAT

+ GATAGTGGCA AAGCTAGAAT GTCATTATGA ATTegacGA ATTTTATAAT TTAGTTTAAA TTTTATATTT
- CTAACACCGT TTCGATCTTA cagtAATACT TAAACTGGCT TAAATATTA AATCAAATTT AAAATATAAAA

+ ATCTTAAAAA TTCATTGAGC GTATTTTAAA AACTTAATAA CTTAATAAAC TAAGATCTTT CCTTTAGTTA
- TAGAATTTTT AAGTAACTCG CATAAAATTT TTGAATTAT GAATTATTG ATTCTAGAAA GGAAATCAAT

+ GGAGAATTGA TCAAAAGGTA ACATATATAA TCCGAAAAAT CATTTTATC CTATATTGTG AAATATAATT
- CCTCTAACT AGTTTCCAT TGTATATATT AGGCTTTTTA GTAAAAATAG GATATAACAC TTTATATTAA

+ TTTGATGAA CGTGATTCAA TTAATCCCT CTTACCTAG CTTTCCCTT AAGCTTAAGC AGATGAAATA
- AAAGCTACTT GCCTAAGTT AATTTAGGGA GAAATGATC GAAAGGGAAA TTCGAATTCG TCTACTTTAT

+ AAATTTTCAA AAAAAATTAT GCGATATTCA TTATCTATAT TGAAGTTGAA CTAAATATAA ATTCATCCAG
- TTTAAAAAGTT TTTTATAA CGCTATAAGT AATAGATATA ACTTCACTT GATTTATATT TAAGTAGGTC

+ AAAAAATTCAT ATTAAAAATA AAGTGATCCC CAACAGAGCG ATTACATATT TAGAACCCAA AATTATTTTA
- TTTTAAAGTA TAATTTTTAT TTCACTAGGG GTTGTCTCGC TAATGTATAA ATCTTGGGTT TTAATAAAAAT

+ TTTCTACTAT TCAATTATTA AATACTCCCC TACTAGCTAT ATATGGATTT CTTTCCCTAT AAATACCCAA
- AAAGATGATA AGTTAATAAT TTAAGAGGGG ATGATCGATA TATACCTAAA GAAAGGGATA TTTATGGGTT

+ ACAAAGCCCTC AATCTTTTAC ACACAAAAAAA CTCACCTTCA ATATCTTCCA TCTTTTTATT CCACACACTA
- TGTTTCGGAG TTAGAAAAATG TGTGTTTTT GAGTGGAAGT TATAGAAGGT AGAAAAATAA GGTGTGTGAT

+ TTTACTCAA ATAGAAAAAAA ATATTCTT CATTTCCTC AAGAAATTAA AAATG
- AAATGAGTTT TATCTTTTT TATAAAGAA GGTAAGAAG TTCTTTAATT TTTAC

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Figure 3.16: Visual representation of predicted regulatory elements of the ACO2 promoter from *S. tuberosum* cv. Rywal (ACO2(R2), 1521 bp). The predicted regulatory elements potentially involved in defense responses are **TC-rich repeats** (involved in

defense and stress responsiveness), **Box-W1/W box/ELRECOREPCR1** (involved in the SA-responsiveness), **EIRE** (elicitor-responsive element), **TCA-element** (involved in salicylic acid responsiveness), **WUN-motif** (wound-responsive element), **WRKY** (WRKY binding site), **GT-1 binding site** (negative regulator of PR-1a gene expression), **MYB1LEPR** (G-box, Tomato Pti4(ERF) regulates defence-related gene expression via GCC box), **WBOXHVIS01** (W-box element), **WRKY710S** (a core of TGAC-containing W-box WRKY binding site within the Pathogenesis-Related Class10 (PR-10) genes), **WBOXNTERF3** (W-box, involved in wounding responsiveness), **MYCCONSENSUSAT** (MYC recognition site), **GT1CONSENSUS** (consensus GT-1 binding site, influences the level of SA-inducible gene expression), **WBOXPCWRKY1** (WB/W box, WRKY proteins binding site in PR1 gene in parsley), **WBOXATNPR1** (W-box, recognized by salicylic acid (SA)-induced WRKY DNA binding proteins), **ERELEE4** (ethylene responsive element), **MYCATERD1** (MYC recognition sequence), **MYCATERD22** (MYC recognition sequence), **T/GBOXATPIN2** (T/G-box, involved in JA-responsiveness). The **ATG** indicates the start of the ACO2 coding region. The promoter region is located upstream of the ATG. The crucial regulatory elements were determined by PlantCARE, TRANSFAC and PLACE. The complete list of regulatory elements from the ACO2 promoter sequences from cv. Rywal, cv. Désirée and PW363 with their positions and sequence, predicted with PlantCARE and TRANSFAC, can be found in the Appendix (Supplementary Tables 8 and 9).

Analysis of the PTI5 promoter revealed the presence of several ERF recognition sites, involved in the ET-signalling pathway (Figure 3.17). Identification of the TGA1a recognition site and several W-boxes within the PTI5 promoter sequence suggested the involvement of TGA and WRKY transcription factors in the regulation of PTI5 transcription. SA-responsive TGACG-motif, GT1-motif, TCA-element and JA-responsive MYC-recognition site were also identified in the PTI5 promoter. In addition, a regulatory element involved in the oxygen response was identified.

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+ GGTCATCAC GACGATTTCAGTCAATAGTACAAAATTT TCTTTCTTT CAACGTCA TA TGTTAAGATG
- CCAAGTAGTG CTGCTAAGT GCACGTTATC ATGTTTAA AGAAAAGAA GTTCCAGTAT AC AATTCTAC

+ TTCTTAGATT AACTGCATAA TATTTCTACT CAACAGAACA AAGTGTTTTA CTCTCACCT TCTCTCACAT
- AAGAACTCAA TTGACGTATT ATAAAGATGA GTTGTCTTGT TTCACAAAAT GAAGAGTGGA AGAGAGTGTA

+ AAAGTTATTA TTCTACTAA ATATATAAAA AAAAAAATT GGACATTCTT TACATATAAT AGTGGACAAA
- TTTCAATAAT AAAGATGATT TATATATTTT TTTTTTTTAA CCTGTAAGGA ATGTATATTA TCACCTGTTT

+ ATTAATGTTA AAAAAAATG CACCAATATT GCAACATGA GGTACTATTA GTGCTCCAIG TGAAAAGTCA
- TAATTACAAT TTTTTTTTAC GTGGTTATAA CGTTTGTACT CAGTATAAT CACGAGGTAC ACTTTT cagt

+ AGGATAACTT TGAATCTTAA CCCAAGACG AGAGACTATT TTGGGCCCTT TCTCGAA TGA CTACAACTAA
- TCCTATTGAA ACTTAGAATT GGGTTTCTGC TCTCTGATAA AACCCGGAAA AGAGCTTACT GATGTTGATT

+ GCATATTTTT GTTCTTTTGA AGATTAGCAT GCACATAAAA TTATAATCAA GGGCAAAGTT AAAGTGTGAG
- CGTATAAAA CAAGAAACT TCTAATCGTA CGTGATTTT AATATTAGTT CCCGTTTCAA TTTCA CAGT C

+ TTACAAATTC AACTCAATTT AATAACTATG ATCCGAATCT AGCATAAAAT AAATGTATTT CAAACCTCGT
- AATGTTTAAG TTGAGTTAAA TTATTGATAC TAGGCTTAGA TCGTATTTA TTTACATAAA GTTTGGAGCA

+ ATCATTTTGA GCTATATGTA ACTGAAACG GCGT TTTTAC CACTAATAIC CACCTCAAAA AAGCGCTCTT
- TAGTAAACT CGATATACAT TGACTTTTGC CCGCAAAGTG GTGATTATAG GTGGAGTTT TTCCGCAGAA

+ CCACGGGCAA CAACTTTTCT TTAATTATTC TAAAGATGGT CAAATTACGT CCAATCTTTT TTTAATAAAA
- GGTGCCCGTT GTTGAAAGGA AATTAATAAG ATTCTAC ca gtt TAATGCA GGTTAGAAAA AAAATTATTT

+ AAACATCAAT ACTCTGGAAA ATATTGGTTG Acagt tCTCT TAAATAAAAC CAAGTA TACA TCATTGTACA
- TTTGTAGTTA TGAGACCTTT TATAACCAAC TGTCAGAGA ATTTATTTT GTTCATATGT AGTAAGATGT

+ TGTACACAAT TAACTAACT TGAATATCA ACGCAACAA GAGGTAAITA ATTAAACCAT GTCATATCCC
- AATGTGTTA ATTGATTGTA ACTTTATAGT TGCGTTTGT CTCCATTAAAT TAATTTGGTA CAGTATAGGG

+ ATCAAGACAC CAACCATATA ATATATATTG TATAAATGA AAAAGACTTG CACAATTTT CACTTGTTTC
- TAGTTCTGTG GTTGGTATAT TATATATAAC ATATTTAACT TTTTCTGAAC GTGTTAAAAA GTGAACAAAG

+ CTCATACTTT CCTCTTTCTC CACGAGATAA ATAAATTC AAAAAATCCC ACAATTAAC GATTACTTTT
- GAGTATGAAA CGAGAAAGAG GTGCTCTATT TATTTTAACT ATTTTATAGG TGTTAATTG CTAATGAAAA

+ CTAGTATCCC TTTATGTAAA ATACCTCATA AACTAAACA TTTTCCCCA CAAATTAACC AAAATCCCTT
- GATCATAGG AAAATACATT TATGGAGTAT TTTGATTTGT AAAAGGGGT GTTTAATTGG TTTTAGGGAA

+ CTAATTATAG CT ATG
- GATTAATATC GA TAC

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Figure 3.17: Visual representation of predicted regulatory elements of PTI5 promoter from *S. tuberosum* cv. Rywal (PTI5(R1), 992 bp). The crucial regulatory elements that potentially regulate defense responses are **CGTCA-motif** (involved in jasmonic acid responsiveness), **TGACG-motif/ASF1MOTIFCAMV** (involved in jasmonic acid and salicylic acid responsiveness), **TC-rich repeats** (involved in defence and stress responsiveness), **ERE/ERELEE4** (ethylene-responsive element), **Box-W1/W box** (fungal elicitor response element), **TCA-element** (involved in salicylic acid responsiveness), **Opaque-2 recognition site** (TGACG-sequence-specific binding site, TGA1A binding site), **RITA-1 recognition site** (TGA1A recognition site), **HBP-1a recognition site** (TGA1A recognition site), **TGA1a recognition site** (TGA1 recognition site), **GT-1 recognition site** (Ca^{2+} -dependent CaM-binding protein recognition site), **ERF069 recognition site** (contains GCC-box pathogenesis-related promoter element), **AT1G28160 recognition site** (ethylene response factor recognition site), **At1g68550 recognition site** (ethylene response factor recognition site), **WRKY48 recognition site** (recognition site of stress and pathogen induced WRKY48, recognizes the TTGACC W-box), **TGA1a recognition site** (TGA1a recognition site), **CACGTGMOTIF** (involved in defense-

related gene expression), MYCCONSENSUSAT (MYC recognition site), CURECORECR (involved in oxygen response), GT1CONSENSUS (involved in salicylic acid responsiveness), WRKY71OS (a core of TGAC-containing W-box, binding site of WRKY71), WBOXNTERF3 (W-box element, involved in the activation of ERF3 gene by wounding), WBOXATNPRI (recognized by SA-induced WRKY; lowercase), ***ELRECOREPCR1/WBBOXPCWRKY1*** (W-box, elicitor responsive element). The **ATG** indicates the start of the PTI5 coding region. The crucial regulatory elements were determined by PlantCARE, TRANSFAC and PLACE. The complete list of regulatory elements from the PTI5 promoter sequences from cv. Rywal, cv. Désirée and PW363 with their positions and sequences, predicted with PlantCARE and TRANSFAC, can be found in the Appendix (Supplementary Tables 10 and 11).

3.3 Design of Plant Immune Response Sensors

Study of binding sites for the transcription factors involved in immune signalling enabled identification of the promoters which could be later on used as sensors of plant immune response. These are TGA2(R1), PR1b(R3), MYC2(P2), MC(D1), ACO(R2) and PTI5(R1), since regulatory elements involved in plant signalling pathways were identified in all of them (see Section 3.2.2). For this purpose, we focused on the design of fluorescent transcriptional biosensor, which would be activated through transcription. This was achieved through the fusion of the promoter regions of the selected genes (Figure 1.10, Figure 2.2, Table 3.3) with fluorescent reporters. The fusion of PTI5 promoter with fluorescent protein was performed as a part of another project (N4-0026) by the colleagues from our working group and is not explained in this doctoral dissertation.

Table 3.3: Lengths of the amplified promoter regions. Note that the lengths of the promoter regions differ from the theoretical length (see Figure 2.1) due to the deletions or insertions in some promoters. Lengths of the PR1b promoters are listed in Pezić et al., 2016.

Promoter	Primer	Length of the amplicon
MYC2 (P2)	A/B	1357
MYC2 (P2)	A2/B	1083
MYC2 (R2)	A/B	1357
MYC2 (R2)	A2/B	1083
MYC2 (R3)	A/B	833
MYC2 (R3)	A2/B	559
MYC2 (D1)	A/B	833
MYC2 (D1)	A2/B	559
MC (P1)	A/Gr	921
MC (D1)	A/Gr	1002
ACO2 (R2)	A/B	1521
TGA2 (P1)	A/B	1583
TGA2 (R1)	A/B	1513

In order to be able to follow the activity of different promoters simultaneously, three different fluorescent proteins with distinct emission spectra, YFP, CFP and mCherry were selected. Several promoter::fluorescent protein constructs with different combinations of promoters and fluorescent proteins

(Table 3.4) were prepared. We tested two different assembly protocols for the fusion of promoters with fluorescent proteins, fusion PCR (Rupar et al., 2015) and Gibson Assembly (NEB) to determine, if both are suitable for the construction of biosensors. Results confirmed that both assembly protocols are suitable for the construction of fluorescent transcriptional biosensor (see Section 3.3.1.2).

Table 3.4: Constructs promoter::fluorescent protein obtained by fusion PCR or Gibson assembly. /: no primers were needed due to the protocol used (Gibson assembly). Length of the PR1b promoter::fluorescent protein constructs are listed in Pezić et al., 2016.

Construct	Primer combination	Length of the amplicon	Assembly protocol
MYC2 (P2_L)::mCherry	A/D	2068	fusion PCR
MYC2 (P2_S)::mCherry	A2/D	1544	fusion PCR
MYC2 (R2_L)::mCherry	A/D	1794	fusion PCR
MYC2 (R2_S)::mCherry	A2/D	1270	fusion PCR
MYC2 (R3_L)::mCherry	A/D	2068	fusion PCR
MYC2 (R3_S)::mCherry	A2/D	1544	fusion PCR
MYC2 (D1_L)::mCherry	A/D	1794	fusion PCR
MYC2 (D1_S)::mCherry	A2/D	1270	fusion PCR
MC (P3)::YFP	/	1641	Gibson
MC (D3)::YFP	/	1722	Gibson
ACO2 (R2)::CFP	A/D	2241	fusion PCR
ACO2 (R2)::YFP	A/D	2241	fusion PCR
TGA2 (P1)::YFP	A/D	2303	fusion PCR
TGA2 (P1)::CFP	A/D	2303	fusion PCR
TGA2 (R1)::YFP	A/D	2233	fusion PCR
TGA2 (R1)::CFP	A/D	2233	fusion PCR

To enable transient transformation of *N. benthamiana* or stable transformation of potato with several different constructs at the same time, constructs were cloned into three different binary vectors with the same backbone (pK7WG, pH7WG and pB7WG) but different plant selection markers (kanamycin, hygromycin, glufosinate ammonium) using Gateway cloning. All available combinations are listed in Table 3.5.

Table 3.5: Combinations of promoter::fluorescent protein constructs. Binary vector: construct was cloned into one of three destination binary vectors, GV3101: constructs were transformed into *A. tumefaciens* GV3101, LBA4404: constructs were transformed into *A. tumefaciens* LBA4404, ✓: constructs were transformed into GV3101 or LBA4404 *A. tumefaciens* strain, *results were obtained as a part of Master degree thesis and are presented in Pezić, 2016.

Construct	Destination binary vector	<i>A. tumefaciens</i>	
		GV3101	LBA4404
MYC2 (P2_S)::mCherry	pH7WG	✓	
MYC2 (R2_L)::mCherry	pH7WG	✓	
MYC2 (R2_S)::mCherry	pH7WG	✓	
MYC2 (R3_L)::mCherry	pH7WG	✓	
MYC2 (R3_S)::mCherry	pH7WG	✓	
MYC2 (D1_L)::mCherry	pH7WG	✓	✓
MYC2 (D1_S)::mCherry	pH7WG	✓	
MC (P3)::YFP	pB7WG	✓	
MC (D3)::YFP	pB7WG	✓	✓
ACO2 (R2)::CFP	pK7WG	✓	✓
ACO2 (R2)::YFP	pK7WG	✓	✓
PR1b (R3)::mCherry *	pH7WG	✓	✓
PR1b (D1)::YFP *	pH7WG	✓	✓
TGA2 (R1)::CFP *	pK7WG	✓	✓
TGA2 (P1)::CFP *	pK7WG	✓	✓
TGA2 (R1)::YFP *	pK7WG	✓	✓
TGA2 (P1)::YFP *	pK7WG	✓	✓

3.3.1 Analysis of sensors responsiveness

3.3.1.1 The most promising promoters were selected by transient transformation of *Nicotiana benthamiana*

Some of the developed constructs were tested through transient expression analysis to identify the most promising combinations of promoters and fluorescent proteins following agroinfiltration of *N. benthamiana* (Table 3.6). The ones performing best were in next stage used for stable transformation of potato.

Table 3.6: The list of selected promoter::fluorescent protein gene constructs for the screening by transient transformation of *N. benthamiana*. * Transient transformation of *N. benthamiana* with PR1b and TGA promoters was performed as a part of a Masters degree thesis. Results are presented in Pezić, 2016.

Construct	Binary vector
MYC2 (R2_L)::mCherry	pH7WG
MYC2 (R3_L)::mCherry	pH7WG
MYC2 (D1_L)::mCherry	pH7WG
MC (P3)::YFP	pB7WG
MC (D3)::YFP	pB7WG
PR1b (D1)::YFP *	pH7WG
TGA2 (R1)::YFP *	pK7WG

Promoter activity was determined by the detection of fluorescence under confocal microscope after induction with different elicitors. Elicitors were selected according to the signalling pathway in which the genes of selected promoters are involved. Therefore, transgenic plants harboring constructs containing MC and MYC2 promoters with the volatile derivative of JA, methyl jasmonate (MeJA). In addition, wounding in combination with CPBS was used as an inducer of JA signalling pathway. The results, obtained after treating transgenic plants harbouring constructs containing PR1b promoters with the SA functional analogue INA are presented in Pezić, 2016.

First, two MYC2 promoters from cv. Rywal (R2, 1093 bp and R3, 1347 bp) and MYC2 promoter from cv. Désirée (D1, 1073 bp) were analysed 6 days after transient transformation of *N. benthamiana*. When following the activity of the promoter prior the induction, expression of mCherry could be detected. Next, the transiently transformed plants were exposed to MeJA for 24 hours and the promoter activity was followed 24 hours post treatment, at which point the activation of the promoter was not detected. Similarly, a drop of CPBS on the wounded leaf did not trigger the expression of the fluorescent protein under MYC2 promoter one hour post treatment.

Next, MC promoters were analysed. *N. benthamiana* plants were transiently transformed with MC(P1)::YFP construct (MC promoter from PW363) and MC(D3)::YFP construct (MC promoter from cv. Désirée) and treated with MeJA 4 days post agroinfiltration. No activity of MC(P1) promoter was detected prior to activation (Figure 3.18 left top), while the analysis under confocal microscope immediately or 20 hours after being exposed to MeJA for 24 hours resulted in YFP expression (Figure 3.18 right top). On the other hand, strong expression of YFP was detected already prior to the induction of MC(D3) promoter (Figure 3.18 left bottom). In addition, YFP expression was also detected following MeJA treatment (Figure 3.18 right bottom). Strong expression of YFP was observed in the mesophyll and lower epidermis in the leaves of plants, transformed with MC(D3)::YFP, in all conditions. However, further image analysis, including quantification, will be needed to determine if the fluorescence intensity is significantly changed after treatment and thus MC(P1) and MC(D3) promoters could be used as sensors of plant immune response.

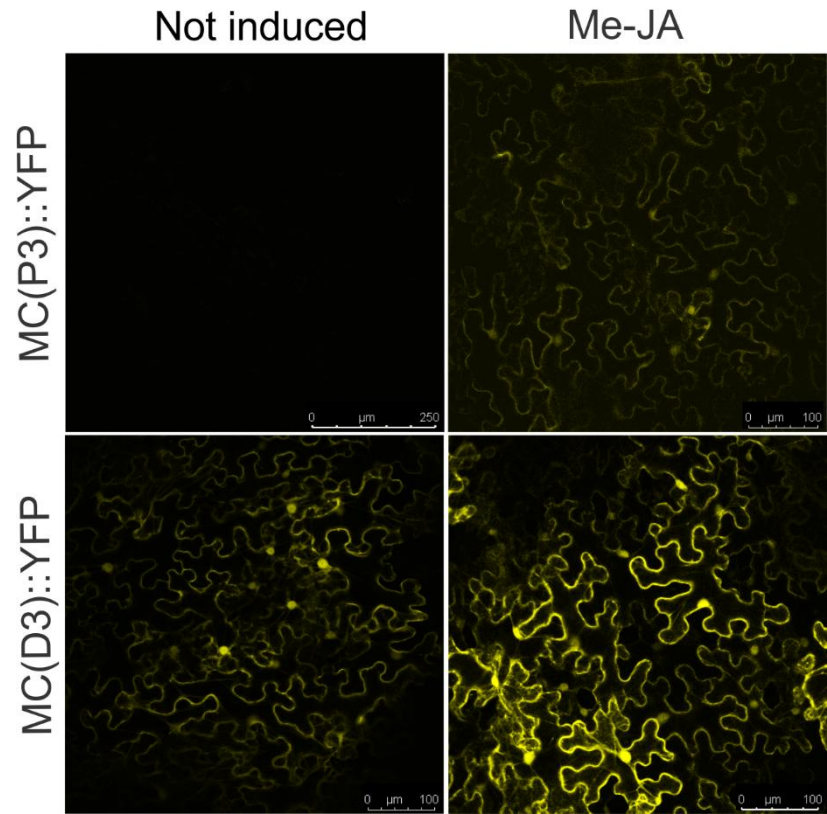


Figure 3.18: Expression analysis of multicystatin (MC) promoters. *N. benthamiana* plants were agroinfiltrated with MC(P3)::YFP and MC(D3)::YFP constructs. Expression of YFP was followed 24 h after plants were being exposed to MeJA for 24 hours (right column) or prior to the treatment (left column) in plant agroinfiltration with MC(P3)::YFP (top row) or MC(D3)::YFP (bottom row). P3: MC promoter from PW363, D3: MC promoter from Désirée.

Results obtained with PR1b and TGA2 promoters are presented in Pezić (2016) and are discussed in the Discussion Section.

3.3.1.2 Stable transformation of potato with designed sensors did not necessarily lead to detection of fluorescent signal

According to the results from transient expression analysis, MYC promoters were excluded from further analysis and two different potato cultivars (Rywal and Désirée) were transformed with constructs containing MC, ACO2, TGA2 and PR1b promoters. Transgenic plants were generated by stable transformation with several constructs containing MC, ACO2 and TGA2 promoters, while no transgenic line with any of the PR1b promoter constructs could be generated. All stable transformations, which resulted in the production of transgenic potato plants are listed in Table 3.7.

Table 3.7: Stably transformed potato plants (cv. Rywal and cv. Désirée) after transformation with the selected promoter::fluorescent protein constructs. ✓ denotes that transgenic plants were generated for the specific construct-cultivar combination. *Stable transformation of cv. Rywal and cv. Désirée with the constructs containing TGA2 promoters was

performed as a part of Master degree thesis. Results are presented in Pezić, 2016.

Construct	Binary	Rywal	Désirée
MC (P3) ::YFP	pB7WG		
MC (D3) ::YFP	pB7WG		✓
ACO2 (R2) ::CFP	pK7WG	✓	✓
ACO2 (R2) ::YFP	pK7WG	✓	
PR1b (R3) ::mCherry	pH7WG		
PR1b (D1) ::YFP	pH7WG		
TGA2 (R1) ::CFP	pK7WG		✓
TGA2 (P1) ::CFP	pK7WG		
TGA2 (R1) ::YFP	pK7WG		
TGA2 (P1) ::YFP *	pK7WG	✓	✓

First, the generated transgenic potato plants were analysed for the presence of the promoter::fluorescent protein construct in the genomic DNA. 2 lines per construct were tested in the case of transgenic plants containing TGA2 and ACO2 promoter constructs and 13 lines in the case of transgenic plants containing MC promoter constructs. Construct was detected in the genomic DNA in at least one of the analysed transformants for all tested combinations. Results are shown in Table 3.8.

Table 3.8: Confirmation of the promoter::fluorescent protein construct DNA presence in the transgenic potato plants. Presence of the gene coding for the fluorescent protein in the gDNA was detected by PCR (constructs marked with *) or qPCR. Construct, line number, and the presence of the DNA product are shown. ✓ denotes that the construct was present in the gDNA, ✗ denotes that the construct was not present in the gDNA.

Construct	Genotype	Line number	Construct in
pK7WG_ACO2 (R2)::CFP	Rywal	1	✓
pK7WG_ACO2 (R2)::CFP	Rywal	2	✓
pK7WG_ACO2 (R2)::CFP	Désirée	2	✓
pK7WG_ACO2 (R2)::CFP	Désirée	3	✓
pK7WG_ACO2 (R2)::YFP	Rywal	1	✗
pK7WG_ACO2 (R2)::YFP	Rywal	2	✓
pK7WG_TGA2 (P1)::YFP	Rywal	3	✓
pK7WG_TGA2 (P1)::YFP	Rywal	5	✗
pK7WG_TGA2 (P1)::YFP	Désirée	1	✓
pK7WG_TGA2 (P1)::YFP	Désirée	3	✓
pK7WG_TGA2 (R1)::CFP	Désirée	4	✓
pK7WG_TGA2 (R1)::CFP	Désirée	5	✓
pB7WG_MC (D3)::YFP*	Désirée	1	✓
pB7WG_MC (D3)::YFP*	Désirée	2	✓
pB7WG_MC (D3)::YFP*	Désirée	3	✓
pB7WG_MC (D3)::YFP*	Désirée	4	✗
pB7WG_MC (D3)::YFP*	Désirée	5	✓
pB7WG_MC (D3)::YFP*	Désirée	6	✓
pB7WG_MC (D3)::YFP*	Désirée	7	✓
pB7WG_MC (D3)::YFP*	Désirée	8	✓
pB7WG_MC (D3)::YFP*	Désirée	9	✓
pB7WG_MC (D3)::YFP*	Désirée	10	✓
pB7WG_MC (D3)::YFP*	Désirée	11	✓
pB7WG_MC (D3)::YFP	Désirée	12	✗
pB7WG_MC (D3)::YFP	Désirée	13	✓

Table 3.9: Selected transgenic lines and treatment conditions for the analysis of sensors responsiveness with confocal microscopy. Transgenic line construct, genotype, transgenic line number, chemical used for the treatment and its concentration, treatment time and analysis method (confocal imaging of bottom leaf surface or leaf cross-section) are presented. ✓ denotes which method was used for the analysis of the particular transgenic plant.

Construct	Genotype	Line number	Treatment	Treatment time	Confocal (bottom leaf surface)	Confocal (leaf cross-section)
TGA	Désirée	1/3/4/5/7	0.3 mM	1h/24h	✓	
TGA	Désirée	1/2/6/8	0.3 mM	1h/24h	✓	
TGA	Rywal	4/5/6/7	0.3 mM	2.5h/21h	✓	✓
ACO	Rywal	3/8/9	1 mM ACC	6h/48h	✓	
ACO	Désirée	6/7/10	1 mM ACC	6h/24h	✓	
ACO	Rywal	2/7	1 mM ACC	55h	✓	
MC	Désirée	14	MeJA	6h/51h	✓	
MC	Désirée	12/15/16/17/1	MeJA	1h	✓	
MC	Désirée	13	MeJA	5h/24h	✓	

Next, the promoter activities were tested through expression analyses using confocal microscopy. Selected transgenic lines and treatment conditions are listed in Table 3.9.

Elicitors for the induction of promoters were selected according to the signalling pathway in which the genes of selected promoters are involved. Therefore, transgenic plants were treated with the constructs containing TGA promoters from SA signalling pathway with INA, transgenic plants with the constructs containing ACO promoters were treated with ethylene precursor 1-Aminocyclopropanecarboxylic acid (ACC) and transgenic plants with the construct containing MC promoter were treated with MeJA. The 0.3 mM INA was selected as the treatment with this concentration resulted in higher PR1b gene expression if compared to 1 mM INA in the transient expression screening (Pezic, 2016). ACC concentration was selected according to our previous experiments with other ET-responsive genes (unpublished).

Treatment time was selected according to the available microarray expression data for the selected genes or the genes from the same gene family obtained after the treatments of potato plants with the above-mentioned elicitors (Wiesel et al., 2015). TGA gene showed the highest expression when the plants were analysed 1 hour after SA treatment, while MC and ACO genes showed higher expression after the plants were exposed to MeJA and ACC for 6 hours.

YFP expression was detected in some of the transgenic plants transformed with the MC(D3)::YFP construct (lines 13, 14 and 18; Figure 3.19). As expected from transient expression screening, in those lines, the fluorescence was also observed prior to the induction with MeJA. However, in more than half of the analysed transgenic lines no YFP fluorescence was detected prior or after the treatment. For one of those (line 12) we confirmed that MC(D3)::YFP construct was not inserted in the genomic DNA (Table 3.8). As other transgenic lines were not tested for the presence of the construct in the genomic DNA, the absence of fluorescence cannot be attributed to low transformation efficiency.

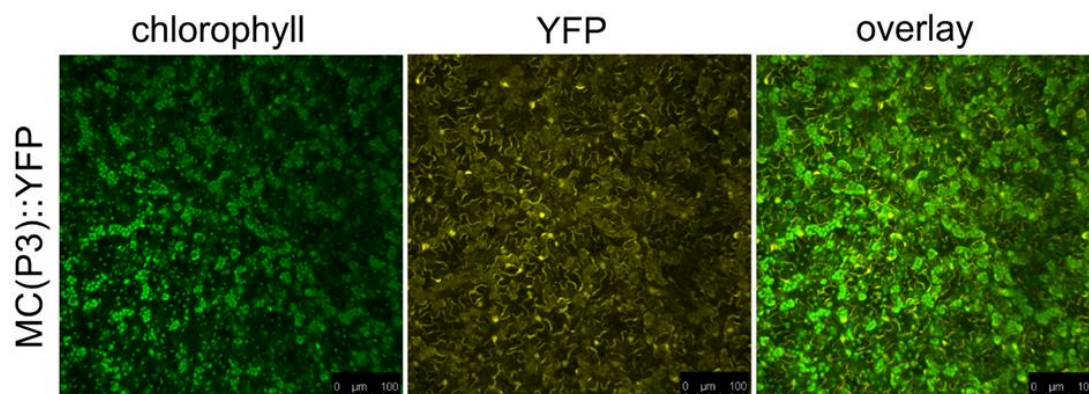


Figure 3.19: YFP fluorescence in plants of cv. Rywal containing the MC(P3)::YFP construct (transgenic line 14). The bottom surface of the leaf was analysed 51 hours after the MeJA treatment. From left to right: chlorophyll fluorescence (green with this filter set), YFP fluorescence in the epidermal cells (yellow with this filter set), overlay of chlorophyll and YFP fluorescence. Scale bars 100 μm .

On the other hand, we did not detect the fluorescence after INA treatment in any of the analysed transgenic plants, transformed with ACO2 or TGA2 promoter constructs when imaging the bottom leaf surface, which is in accordance with the results obtained by transient expression screening. However, as some promoters could be selectively induced only in the particular cell type, we also analysed cross sections of the leaves from transgenic Rywal plants transformed with the TGA(P1)::YFP construct treated with INA to get better insight into the fluorescence in different cell types (Figure 3.20). Even though we observed fluorescence in the mesophyll cells, we attributed this to the autofluorescence as it was also detected in the non-transgenic Rywal plants. On the other hand, we detected YFP fluorescence in the epidermal cells, which we did not detect in the nontransgenic Rywal plants (Figure 3.20). Further analyses will be needed to exclude the possibility that this fluorescence is the result of unspecific promoter induction.

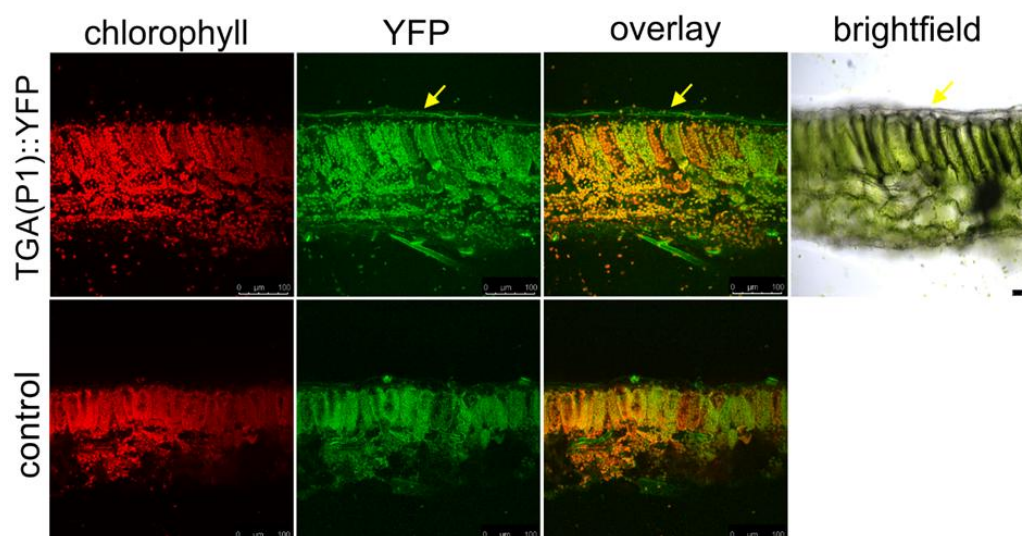


Figure 3.20: YFP fluorescence in plants of cv. Rywal containing the TGA(P1)::YFP construct (transgenic line 5). Upper row: cross sections of the leaves 2.5 hours after INA treatment. Bottom row: non-transformed potato plant. From left to right: chlorophyll fluorescence (false-coloured red), YFP fluorescence (false-coloured green), overlay of chlorophyll and YFP fluorescence (scale bars 100 μm), brightfield (scale bar 50 μm). Yellow arrow represents YFP fluorescence in the upper epidermis. Note that chlorophyll is false-coloured red and YFP is false-coloured green so the overlap can be clearly represented as yellow.

Next, promoter activities were tested through expression analyses using RT-qPCR. Selected transgenic lines and treatment conditions are listed in Table 3.10.

Table 3.10: Selected transgenic lines and treatment conditions for the analysis of sensors responsiveness with RT-qPCR. Transgenic line construct, genotype, transgenic line number, chemical used for the treatment and its concentration and treatment time are presented.

Construct	Genotype	Line number	Treatment	Treatment
TGA	Désirée	4/5/6/9	0.3 mM INA	1h/24h
TGA	Désirée	1/3/7/8/9/10	0.3 mM INA	1h/24h
TGA	Rywal	3/5	0.3 mM INA	1h/24h
ACO	Rywal	1/2/4/10	1 mM ACC	6h/24h
ACO	Désirée	2/3/5/8	1 mM ACC	6h/24h
ACO	Rywal	1/2/4/5/6/7/8/9/10	1 mM ACC	6h/24h
MC (D3)::YFP	Désirée	12/13/14	MeJA	5h/23h
MC (D3)::YFP	Désirée	15/16/17/18	MeJA	5h

Promoter activities are presented as the ratio between the relative copy numbers of the treated sample and the relative copy numbers of the control (Table 3.11). Only the results for the transgenic lines, previously analysed for the presence of the construct in the genomic DNA, are shown. Results for the other transgenic lines are available in the Appendix (Supplementary Table 12). Relative copy numbers are available in the Appendix (Supplementary Table 13).

Table 3.11: Promoter activities, tested in promoter::fluorescent protein transgenic lines using RT-qPCR. Ratios between the relative copy numbers of the treated sample (T) and control (C) are presented. Construct, genotype, transgenic line number, treatment, T/C ratio and the presence of gDNA are shown. LOD: expression was under the limit of detection, ✓: the construct was detected in the genomic DNA, x: the construct was not detected in genomic DNA. Samples marked with * were selected for further analysis.

Construct	Genotype	Line	Time	Treatment	Ratio (T/C)	gDNA
TGA (P1)::YFP	Rywal	3	1 h	INA	LOD	✓
			1 h	control		
			24 h	INA	LOD	
			24 h	control		
		5	1 h	INA	LOD	✓
			1 h	control		
			24 h	INA	LOD	
			24 h	control		
TGA (R1)::CFP	Désirée	4*	1 h	INA	1.83	✓
			1 h	control		
			24 h	INA	0.67	
			24 h	control		
		5	1 h	INA	1.28	✓
			1 h	control		
			24 h	INA	0.76	
			24 h	control		
TGA (P1)::YFP	Désirée	1*	1 h	INA	1.52	✓
			1 h	control		
			24 h	INA	LOD	
			24 h	control		
		3	1 h	INA	LOD	✓
			1 h	control		
			24 h	INA	LOD	
			24 h	control		

Continued

Construct	Genotype	Line	Time	Treatment	Ratio (T/C)	gDNA
ACO (R2)::CFP	Rywal	1*	6 h	ACC	2.88	✓
			6 h	control		
			24 h	ACC	1.20	
		2	24 h	control		✓
			6 h	ACC	0.35	
			6 h	control		
ACO (R2)::CFP	Désirée	2*	24 h	ACC	LOD	✓
			24 h	control		
			6 h	ACC	2.25	
		3	6 h	control	1	✓
			6 h	ACC	1.79	
			24 h	ACC	1.71	
ACO (R2)::YFP	Rywal	1	24 h	control		✗
			6 h	ACC	0.43	
			6 h	control		
		2	24 h	ACC	0.72	✓
			24 h	control		
			6 h	ACC	1.26	
MC (D1)::YFP	Désirée	12	6 h	ACC	1.59	✗
			24 h	control		
			5 h	MeJA	LOD	
		13	5 h	control	LOD	✓
			5 h	MeJA	1.15	
			23 h	MeJA	1.73	

The most promising transgenic lines (the ones with the highest ratio between the relative copy numbers of treated sample and control, namely TGA(R1)::CFP_Désirée_line4, TGA(P1)::YFP_Désirée_line1, ACO(R2)::CFP_Rywal_line1 and ACO(R2)::CFP_Désirée_line2) were selected for further analysis (Table 3.11, samples marked with *). Repeated experiments with increased number of analysed samples (three replicates) showed no significant difference between the expression in the controls and in the treated samples (Figure 3.21).

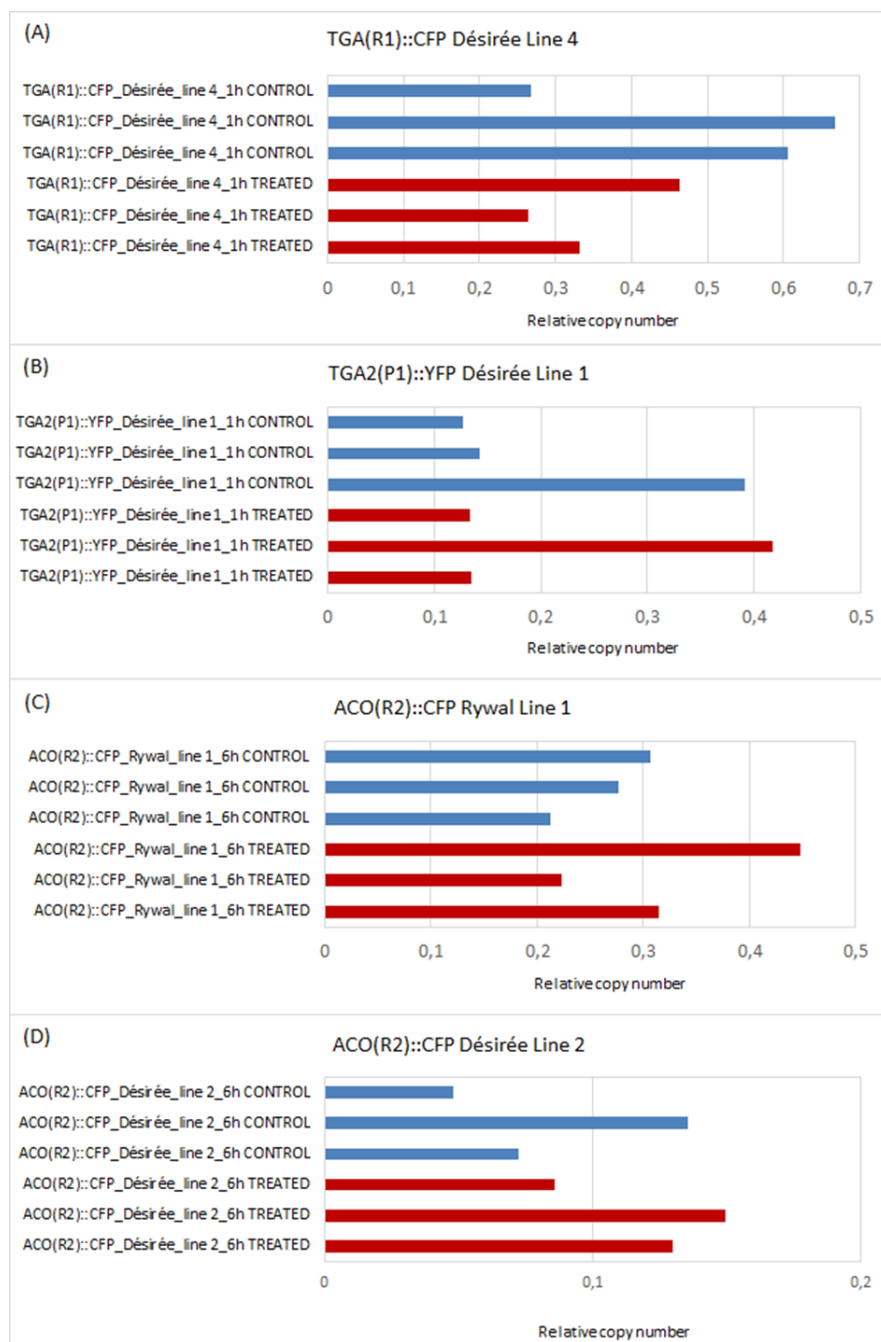


Figure 3.21: Promoter activities, tested in selected promoter::fluorescent protein transgenic lines using RT-qPCR. Selected transgenic plants were plants of cv. Désirée transformed with the TGA(R1)::CFP construct, transgenic line 4 (A), plants of cv. Désirée transformed with the TGA2(P1)::YFP construct, transgenic line 1 (B), plants of cv. Rywal transformed with the ACO(R2)::CFP construct, transgenic line 1 (C) and plants of cv. Désirée transformed with the ACO(R2)::CFP construct, transgenic line 2 (D). Relative copy numbers of treated samples and controls are presented.

The results show that the transgene expression levels vary between different transgenic lines generated within the same stable transformation (Table 3.11). For example, the expression level of YFP under ACO(R2) promoter was three times higher in the plant of line 2, if compared to the plant of line 1. Moreover,

the results show high variability in the expression levels between different plants of the same transgenic line (Figure 3.21).

3.4 Dynamics of Events is Crucial for Effectiveness in Hypersensitive Response-Conferred Resistance

3.4.1 PVY is multiplying outside the necrotic lesions in hypersensitive response-conferred resistance

In PVY-infected Rywal, the virus is arrested in the inoculated leaves and could not spread systemically. This was determined for Wilga (Baebler et al., 2014) and N605 (Table 3.12) PVY strains by the absence of the symptoms and viral RNA in systemic leaves.

Table 3.12: Relative PVY RNA abundance in the systemic leaves in Rywal and NahG-Rywal plants after PVY inoculation. Relative PVY RNA abundance was determined in upper non-inoculated leaves 4 weeks after inoculation with PVY N605-GFP. Results were obtained from two independent experiments. Relative abundance of PVY RNA was followed using RT-qPCR.

Experiment	Genotype	Plant No.	Leaf No.	Relative viral RNA
Exp 1	Rywal	P1	L1	Under LOD
		P2	L1	Under LOD
Exp 2		P3	L1	Under LOD
			L2	Under LOD
		P4	L1	Under LOD
			L2	Under LOD
Exp 1	NahG-Rywal	P1	L1	3.8
		P2	L1	9.3
Exp 2		P3	L1	1.0
			L2	1.8
		P4	L1	5.6
			L2	1.7

To study the link between resistance and cell death in *Ny-1* resistance-gene-mediated response, potato plants of cv. Rywal were infected with a GFP-tagged PVY (PVY N605-GFP). In this infective clone, GFP was inserted between NIb and CP coding regions and was flanked by protease recognition sites, allowing excision from the polyprotein and release into the cytoplasm after accumulation (Rupar et al., 2015). In order to investigate the possible spread of the virus around the lesion, the area around the cell death zone was scanned in PVY N605-GFP inoculated leaf at 7 dpi using confocal microscope. Individual cells or small clusters of cells expressing GFP were observed at the margins of some of the analyzed lesions (Figure 3.22 A, B). In plants infected with PVY N605 without GFP tag, GFP fluorescence was not observed, excluding the possibility that the fluorescence could be attributed to the background signal (Figure 3.22 C).

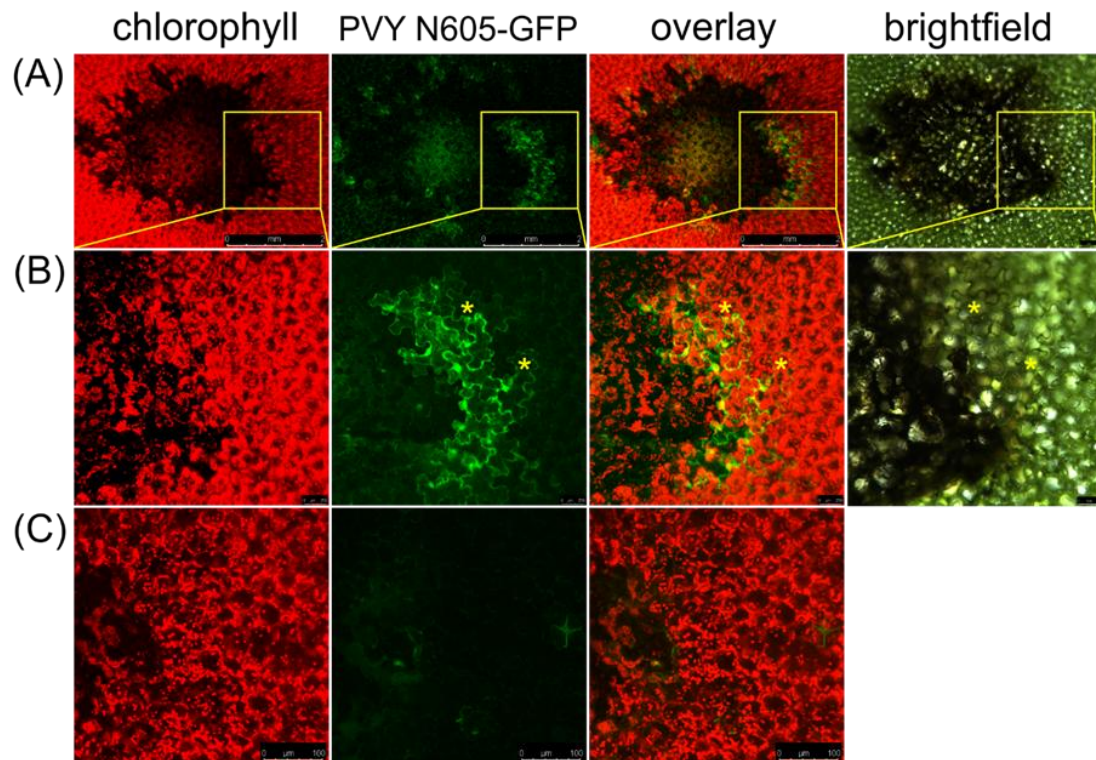


Figure 3.22: PVY is located outside the cell death zone in potato cv. Rywal. Lesions were scanned by laser scanning confocal microscopy. (A) PVY N605-GFP accumulation as observed 7 dpi. From left to right: chlorophyll fluorescence (false-coloured red), PVY N605-GFP accumulation (green), overlay of chlorophyll fluorescence and PVY N605-GFP accumulation (scale bars 2 mm), brightfield (scale bar 500 μ m). (B) High-magnification of the boxed areas in A (scale bars 250 μ m for confocal microscopy and 500 μ m for brightfield). Figure C shows a lesion after inoculation with PVY without GFP tag as a control (scale bars 100 μ m). Asterisks indicate two representative infected cells located outside the cell death zone. Chlorophyll and GFP fluorescence are presented as maximum projections of z-stacks. Published in Lukan et al. (2018a). Republished from Cell death is not sufficient for the restriction of potato virus Y spread in hypersensitive response-conferred resistance in potato, Lukan et al., 2018a, *Frontiers in Plant Science*, 9, 168. doi: 10.3389/fpls.2018.00168, according to Creative Common Attribution ("CC BY") licence.

The occurrence of PVY N605-GFP infected cells outside the cell death zone in cv. Rywal was further investigated at different time points after inoculation. PVY N605-GFP accumulation was detected outside some of the lesions at all analyzed time points (Appendix, Supplementary Table 14 A).

However, the number of cells in which the PVY N605-GFP accumulation was observed was similar at early and late time points (from 3 to 11 dpi) (Figure 3.23; Appendix, Supplementary Table 14 A). The virus accumulation could not be detected before visible lesion formation using our infectious clone.

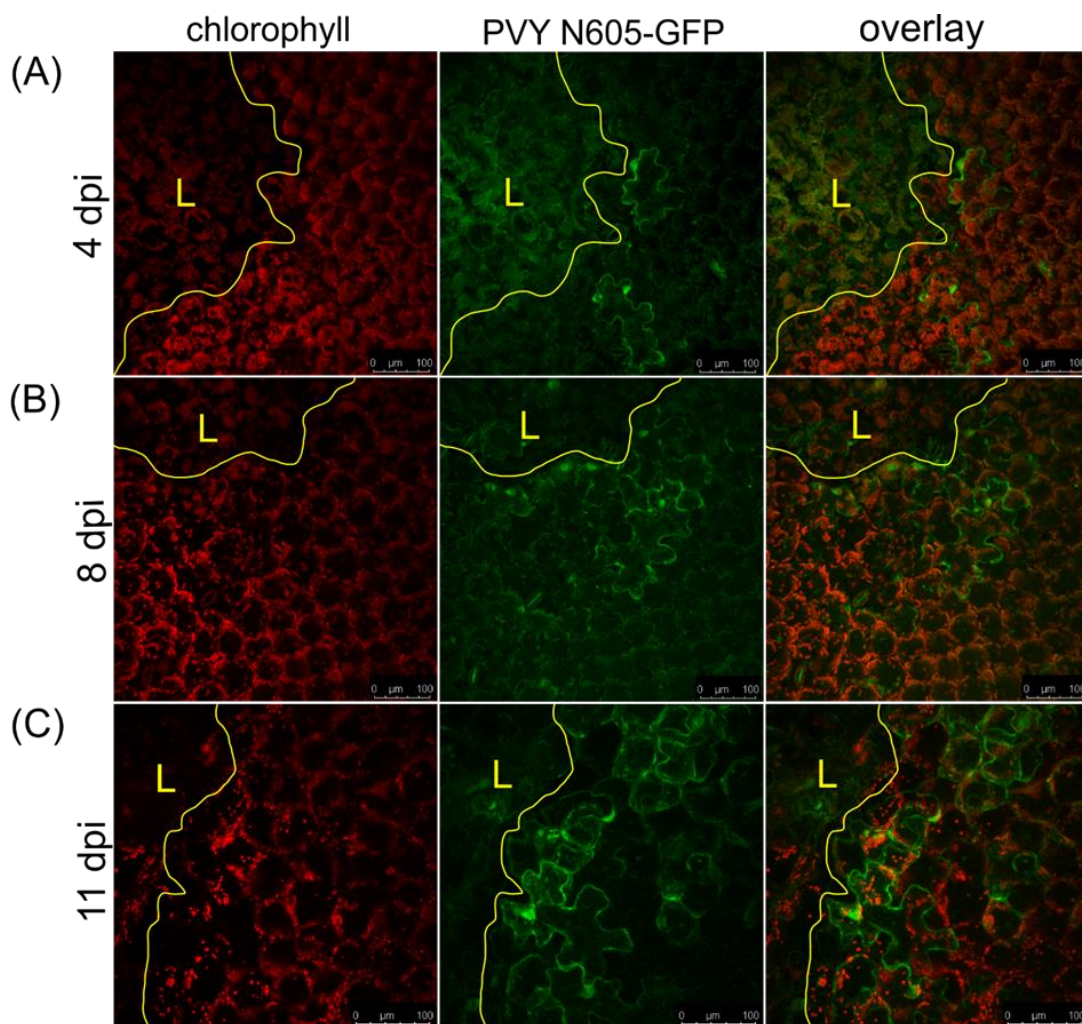


Figure 3.23: PVY was detected outside the cell death zone in cv. Rywal at different time points following the infection. Lesions at 4 dpi (A), 8 dpi (B) and 11 dpi (C) are shown. From left to right: chlorophyll fluorescence (red), PVY N605-GFP accumulation (green), overlay of chlorophyll fluorescence and PVY N605-GFP accumulation. PVY N605-GFP accumulation was detected outside some of the lesions at all analyzed time points after inoculation (Appendix, Supplementary Table 14 A). The lesion is marked by the yellow L and the edge of the cell death zone is marked by the yellow line. Scale bars are 100 μm . Republished from Cell death is not sufficient for the restriction of potato virus Y spread in hypersensitive response-conferred resistance in potato, Lukan et al., 2018a, *Frontiers in Plant Science*, 9, 168. doi: 10.3389/fpls.2018.00168, according to Creative Common Attribution ("CC BY") licence.

3.4.2 Virus spread is continuous in SA depleted plants

NahG-Rywal transgenic plants are unable to restrict the virus close to the site of inoculation, which leads to the systemic spread of the virus (Baebler et al., 2014). Therefore, they were investigated in a similar setup to study the dynamics of virus spread around the lesions in the non-resistant host. The PVY N605-GFP accumulation was followed in the cells around the cell death zone from 3 to 12 dpi by confocal microscopy. As expected, the PVY N605-GFP accumulation was observed outside some of the lesions for all analyzed time points from 4 dpi on (Figure 3.24; Appendix, Supplementary Table 14 A). PVY N605-GFP accumulation could not be detected at the time of the first lesion occurrence (3 dpi) or before lesion formation. The number of the epidermal cells around the cell death zone in which the PVY N605-GFP accumulation was observed, was increasing from 4 to 11 dpi. Beside the epidermal cells, also the number of the GFP-containing mesophyll cells was increasing from 4 to 11 dpi, confirming the concentric spread of the virus, as already observed by Rupar et al. (2015).

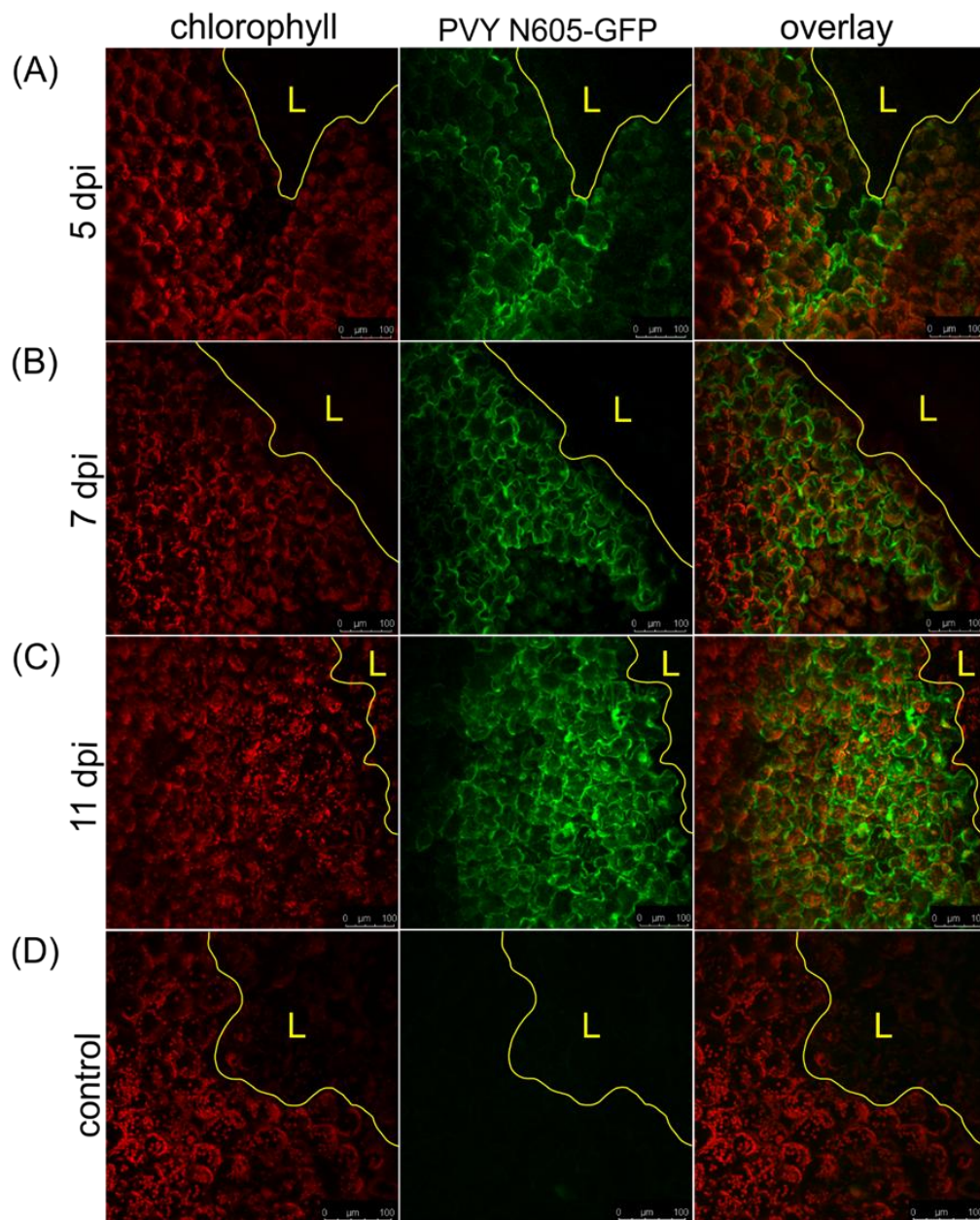


Figure 3.24: PVY spread around the cell death zone in NahG-Rywal transgenic plants. Lesions at 5 dpi (A), 7 dpi (B) and 11 dpi (C) are shown. Figure D shows a lesion, which developed after the plants were inoculated with PVY without GFP tag. From left to right: chlorophyll fluorescence (false-coloured red), PVY N605-GFP accumulation (green), overlay of chlorophyll fluorescence and PVY N605-GFP accumulation. The number of the cells around the cell death zone in which PVY N605-GFP accumulation was detected was higher at later time points after inoculation. The lesion is marked by the yellow L and the edge of the cell death zone is marked by the yellow line. Scale bars are 100 μm . Republished from Cell death is not sufficient for the restriction of potato virus Y spread in hypersensitive response-conferred resistance in potato, Lukan et al., 2018a, *Frontiers in Plant Science*, 9, 168.

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3.4.3 Virus is outside the cell death zone in all lesion developmental stages in hypersensitive response-conferred resistance

The percentage of lesions with detected PVY N605-GFP accumulation outside the cell death zone (positive lesions) was determined for both genotypes at different time points following inoculation (Figure 3.25). PVY N605-GFP accumulation was detected outside the cell death zone for some of the lesions at all time points after inoculation. In cv. Rywal, the percentage of positive lesions was higher at earlier time points (3 dpi and 4 dpi). In NahG-Rywal, the percentage of positive lesions increased over time, reaching more than 80 % at 11 dpi.

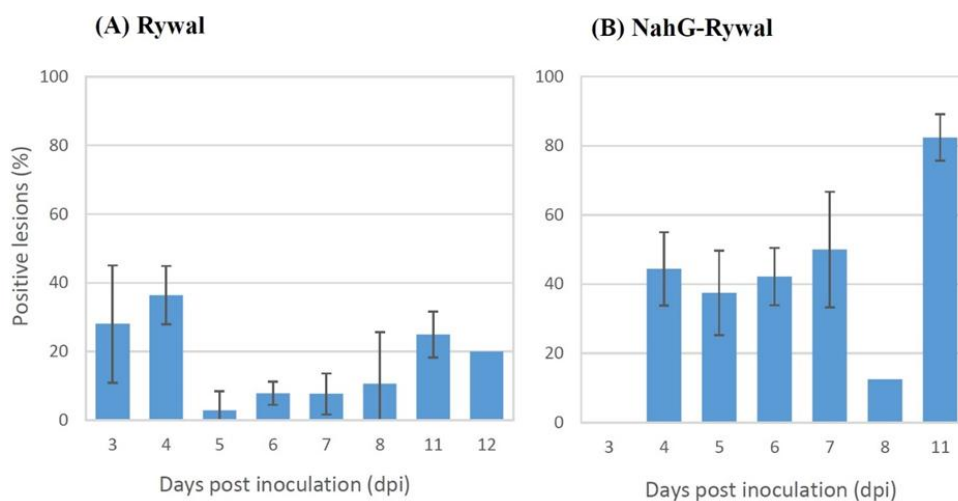


Figure 3.25: Percentage of lesions with detected PVY outside the cell death zone. Rywal and NahG-Rywal plants were inoculated with PVY N605-GFP to follow PVY N605-GFP accumulation in the cells around the cell death zone on different days post inoculation (dpi). The percentage of positive lesions was calculated as the number of lesions with PVY N605-GFP accumulation detected in at least one cell outside the cell death zone, divided by the number of all analyzed lesions. Error bars represent the standard error of repeated measurements ($n = 2-7$). Data of individual experiments are available in Appendix, Supplementary Table 14 A. Republished from Cell death is not sufficient for the restriction of potato virus Y spread in hypersensitive response-conferred resistance in potato, Lukan et al., 2018a, *Frontiers in Plant Science*, 9, 168. doi: 10.3389/fpls.2018.00168, according to Creative Commons Attribution ("CC BY") licence.

In order to exclude the possibility that the lesions analyzed at later time points after inoculation are newly formed lesions, while the virus is successfully enclosed in older lesions, dynamics of lesions development was studied. Statistical analysis was performed by the colleague from our working group as a part of the project J4-7636. Results showed that the probability that the lesion is formed after 7 dpi is less than 2 % for both genotypes (Figure 3.26, Table 3.13). On the other hand, experimental results showed that the virus was present outside the cell death zone in all lesion developmental stages and the percentage of positive lesions at 8, 11 and 12 dpi was at least 10 %. As the percentage of positive lesions observed under confocal microscope after 7 dpi was at least 5-fold higher than the probability that the lesion is formed after 7 dpi, we can

conclude that the virus is present outside the cell death zone in all developmental stages of lesions.

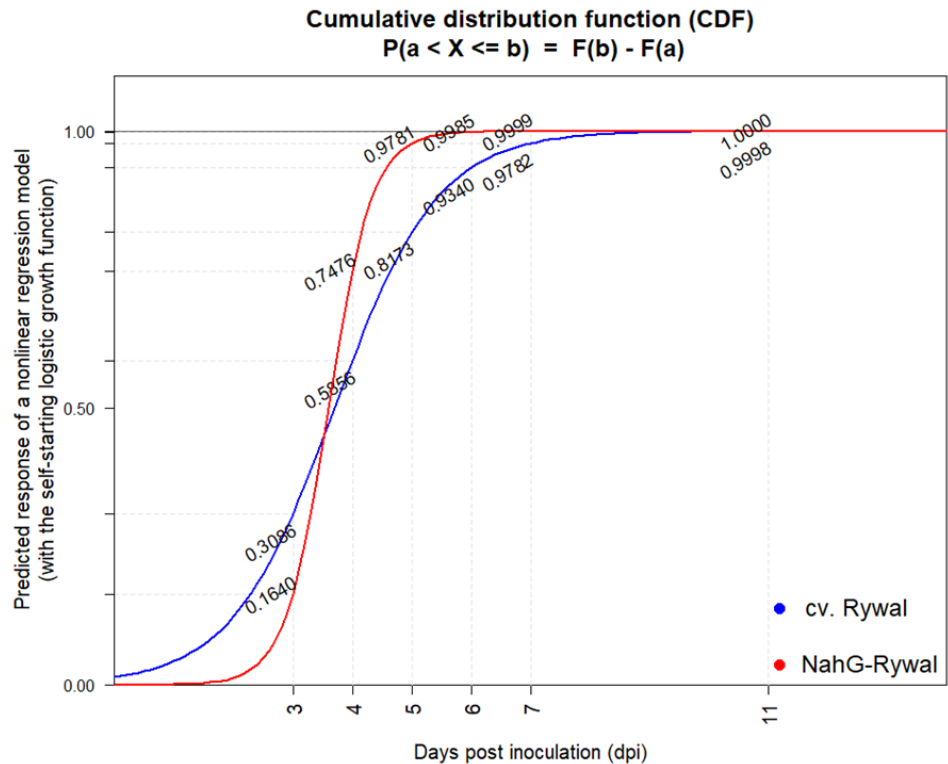


Figure 3.26: Cumulative probability distribution functions of lesion appearance after PVY inoculation. Lesions were counted at 3, 4, 5, 6, 7, 9 and 11 dpi on all the three inoculated leaves (Appendix, Supplementary Image 1) after PVY N605-GFP inoculation in potato cv. Rywal and NahG-Rywal. To determine the probability that the observed lesion is formed until the particular dpi, cumulative probability distribution function was determined for Rywal (blue) and NahG-Rywal (red). X-axis: dpi, y-axis: probability that the randomly observed lesion is formed until the particular dpi. Republished from Cell death is not sufficient for the restriction of potato virus Y spread in hypersensitive response-conferred resistance in potato, Lukan et al., 2018a, Frontiers in Plant Science, 9, 168. doi: 10.3389/fpls.2018.00168, according to Creative Common Attribution ("CC BY") licence.

Table 3.13: The probability that the observed lesion was formed until the particular day post inoculation. The probability was calculated after PVY N605-GFP inoculation of cv. Rywal (A) and NahG-Rywal (B) plants by nonlinear regression model with logistic function. Republished from Cell death is not sufficient for the restriction of potato virus Y spread in hypersensitive response-conferred resistance in potato, Lukan et al., 2018a, Frontiers in Plant Science, 9, 168. doi: 10.3389/fpls.2018.00168, according to Creative Common Attribution ("CC BY") licence.

(A) cv. Rywal

dpi	Probability (%)
3	30.86
4	58.56
5	81.73

(B) NahG-Rywal

dpi	Probability (%)
3	16.40
4	74.76
5	97.81

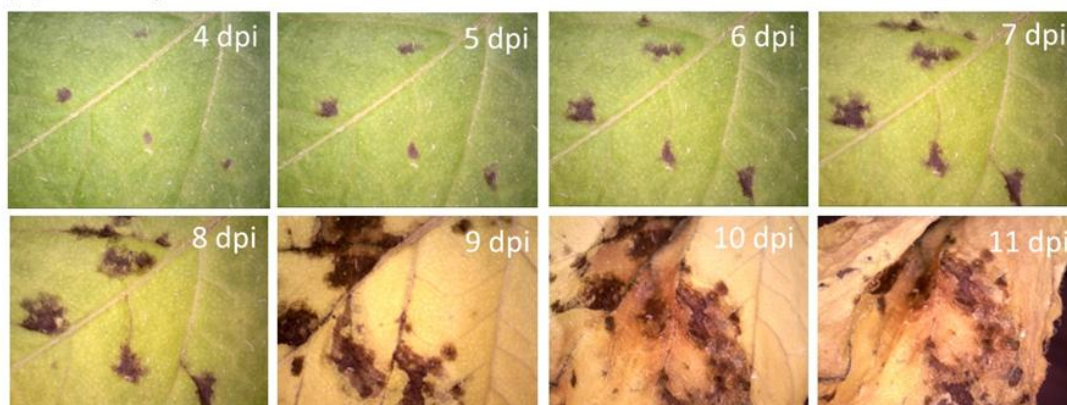
6	93.40
7	97.82
11	99.98

6	99.85
7	99.99
11	100.00

3.4.4 Cell death zone is continuously expanding in hypersensitive response-conferred resistance

In order to determine the dynamics of lesion expansion, lesions were followed over the longer time period by digital microscope until the lesions expanded into large necrotic lesions covering the majority of the leaf (NahG-Rywal) or the senescence occurred (cv. Rywal). In NahG-Rywal, unlimited expansion of all analyzed lesions was observed (Figure 3.27 A). Although the lesion expansion was hardly visible by eye in cv. Rywal at a later dpi, using digital microscopy it was possible to determine that the majority of analyzed lesions continuously expanded even at later time points (Figure 3.27 B). However, compared to NahG-Rywal, the expansion was much slower. On the other hand, some of the analyzed lesions became fully developed already at early time points after inoculation in cv. Rywal.

(A) NahG-Rywal



(B) Rywal

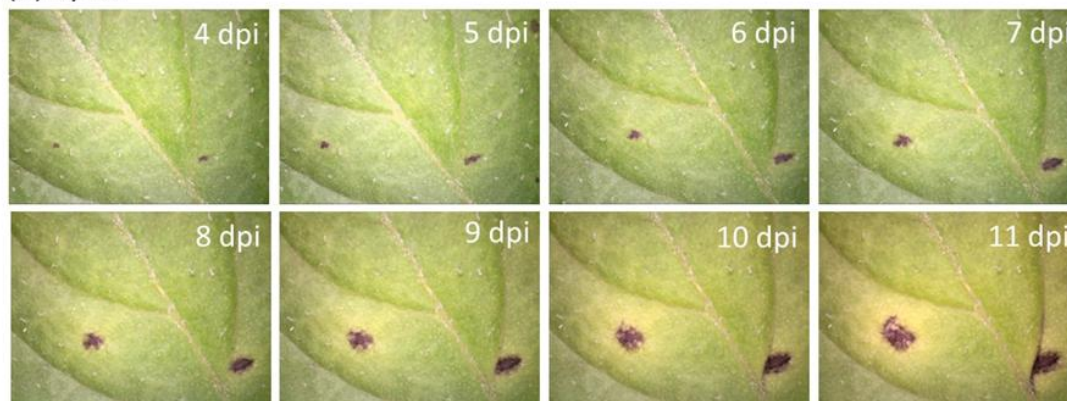


Figure 3.27: Dynamics of lesion expansion in NahG-Rywal (A) and cv. Rywal (B) 4 to 11 days after PVY N605-GFP inoculation (dpi). Lesions expansion was followed by DinoLite digital microscope in 5 (NahG-Rywal) and 13 (Rywal) independent experiments. Images

presented in (A) and (B) were taken at the same magnification and working distance.

3.4.5 Virus-infected cells at the periphery of the necrotic lesion are able to reinitiate infection

The *Ny-1* gene mediated HR-conferred resistance is temperature dependent, as the higher temperature (28 °C) leads to the abortion of resistance manifested as a lack of lesion formation and systemic spread of the virus (Szajko et al., 2008). To test whether the GFP-containing cells at the periphery of the necrotic lesions contain the PVY N605-GFP, which is able to reinitiate infection, this feature was exploited in our system. The temperature shift facilitated the spread of PVY N605-GFP, initiating from the infected cells at the cell death zone margin, and resulted in the development of secondary infection foci, as the number of GFP-containing cells around the cell death zone was higher at later time points after inoculation (Figure 3.28). We confirmed that the cells at the periphery of the cell death zone are alive and contain the PVY N605-GFP, which is able to reinitiate infection.

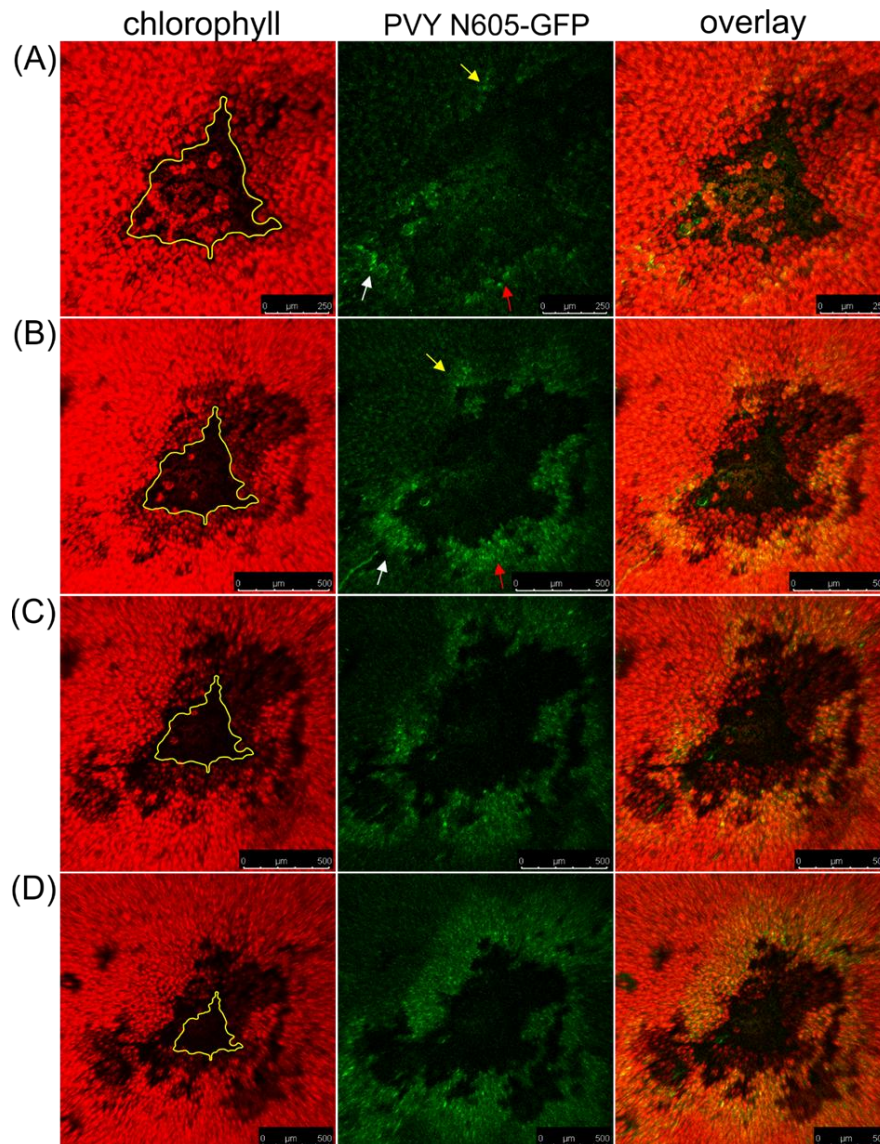


Figure 3.28: The spread of the PVY following the temperature shift in cv. Rywal. Plants were kept at 22 °C to allow lesion formation. At 4 dpi, the inoculated leaf was detached, cut into pieces, placed on 1.5 % (w/v) agar plate and kept at 22 °C. At 4 dpi (A), 5 dpi (B), 6 dpi (C) and 7 dpi (D), the plate was transferred to 28 °C for 2 hours for confocal microscopy imaging. The higher temperature was used as a condition under which multiplication and spread of the virus are not inhibited by *Ny-1* gene, while the lower temperature as a means to visualize the lesion expansion due to *Ny-1* gene activity. From left to right: chlorophyll fluorescence (false-coloured red), PVY N605-GFP accumulation (green), overlay of chlorophyll fluorescence and PVY N605-GFP accumulation. The number of GFP-containing cells around the cell death zone was higher at later time points after inoculation confirming the spread of the PVY N605-GFP. The size of the lesion on 4 dpi is indicated by the yellow line. Arrows indicate the development of secondary infection foci. Scale bars are 250 μm (A) or 500 μm (B-D). Republished from Cell death is not sufficient for the restriction of potato virus Y spread in hypersensitive response-conferred resistance in potato, Lukan et al., 2018a,

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3.4.6 Virus can be found outside the formed "callose ring"

To study the pattern of callose deposition in potato-PVY interaction, callose deposits were followed at several time points following the virus inoculation (Figure 3.29). In Rywal, the callose was accumulated inside the cell death zone at 3 dpi (Figure 3.29 A). At 4 dpi, callose deposits were detected at the margin between the cell death zone and the tissue outside the cell death zone forming a continuous ring around the cell death zone. The amount of the callose inside the cell death zone decreased in time. Moreover, the intensity of the staining at the margin of the cell death zone decreased after 5 dpi. The callose was rarely detected outside the cell death zone at all time points.

In NahG-Rywal, the callose was accumulated inside the cell death zone at 3 dpi (Figure 3.29 B). In contrast to Rywal, a continuous callose ring around the cell death zone was rarely detected. At 4 dpi callose was detected in some of the cells on the outside edge of the cell death zone. At 5 dpi, callose was detected in the cells even further from the cell death zone. The intensity of the staining decreased in time after 5 dpi.

The features described above were determined for the majority of the analysed lesions. However, as there were different stages of lesions present at the particular dpi, different types of staining were observed at each dpi. Moreover, there was no staining in some of the analysed lesions at all time points for both genotypes.

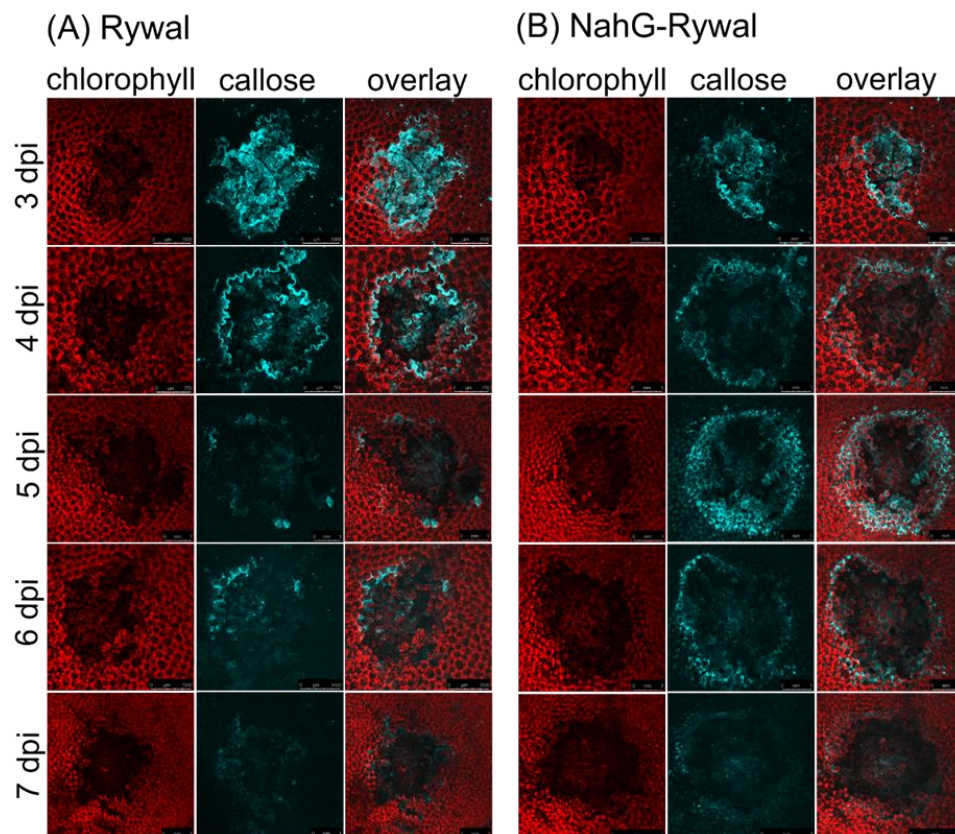


Figure 3.29: Callose deposition in potato-PVY interaction. Callose deposition was followed in cv. Rywal (A) and cv. NahG-Rywal (B) from 3 to 7 days post inoculation with the PVY N605-GFP (dpi). From left to right: chlorophyll fluorescence (false-coloured red), callose deposits (blue), overlay of chlorophyll fluorescence and callose deposits. Scale bars are 750 μ m.

We next investigated if the callose deposition is crucial for the virus restriction at the periphery of the necrotic lesions in cv. Rywal. The PVY N605-GFP accumulation was detected inside (Figure 3.30, white arrow) but in some cells also outside the callose ring (Figure 3.30, yellow arrow). This indicates that the callose is either not blocking the spread of the virus to cells adjacent of necrotic lesions or callose deposition at the levels below our detection limit is already sufficient for its function.

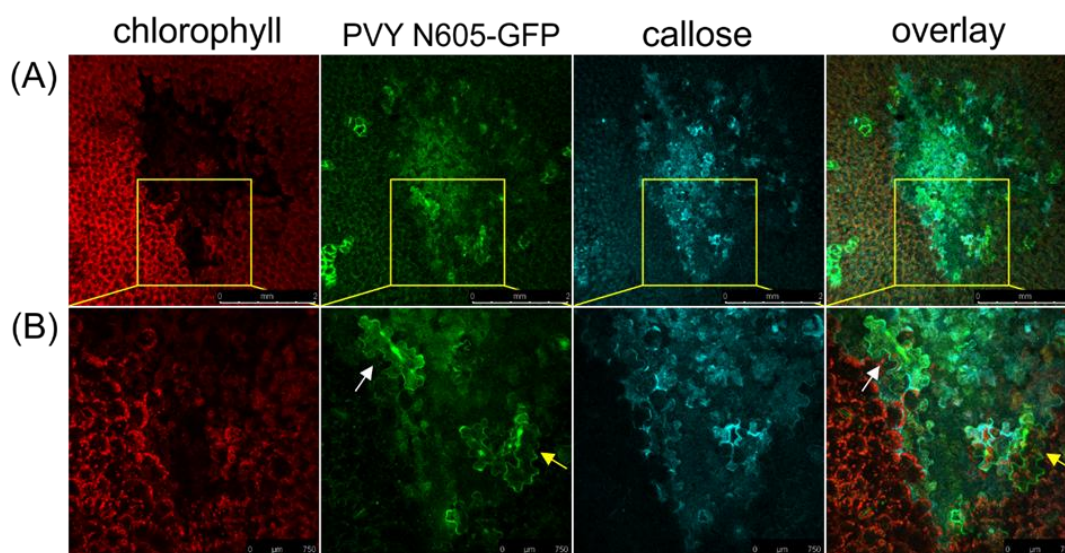


Figure 3.30: Virus escape is not blocked by callose deposition in cv. Rywal. (A) PVY was detected outside the callose deposits 6 dpi. (B) High-magnification of the boxed areas in A (scale bars are 750 μ m). From left to right: chlorophyll fluorescence (false-coloured red), PVY N605-GFP accumulation (green), callose (blue), overlay of all three channels (scale bars are 2 mm). The infected cell inside the callose ring is shown by the white arrow, while the infected cell outside the callose ring is shown by the yellow arrow. Republished from Cell death is not sufficient for the restriction of potato virus Y spread in hypersensitive response-conferred resistance in potato, Lukan et al., 2018a, *Frontiers in Plant Science*, 9, 168. doi: 10.3389/fpls.2018.00168, according to Creative Common Attribution ("CC BY") licence.

Chapter 4

Discussion

4.1 Plant X-tender Pave the Way for Increasingly Complex Approaches for Large-Scale Engineering in Plant Synthetic Biology

When designing biosensors, we came across a major drawback of available cloning methods for plant synthetic biology. The basic design of plant expression vectors rarely permits the cloning and transfer of more than a single expression cassette into plant cells (Dafny-Yelin and Tzfira, 2007). Therefore, there is a growing need for simple but flexible cloning strategies for multigene cloning (Liu and Stewart, 2015). This encouraged us to develop Plant X-tender, a set of tools that complements the AssemblX toolkit (Hochrein et al., 2017) and thus enables easier development of plant synthetic biology applications.

If the activity of different promoters needs to be followed simultaneously, several promoter::fluorescent protein constructs with different combinations of promoters and fluorescent proteins first need to be introduced into the plant genome. In our previous design, each promoter::fluorescent protein construct was introduced into a separate binary vector. In such a set-up, only one sensor could be introduced into a plant genome at the time. This could pose a problem, especially in the case of time-consuming stable transformation of potato. However, Plant X-tender will support the development of multigene fluorescent transcriptional biosensor which will enable the introduction of several promoter::fluorescent protein expression cassettes into the plant genome at the same time.

When designing Plant X-tender expression vectors, we focused on providing full flexibility regarding the design of the expression cassettes. The selection of appropriate regulatory elements is crucial since the repetitive use of the same promoter for expression of multiple genes was shown to be associated with transgene silencing (Matzke & Mosher, 2014; Peremarti et al., 2010; Schubert et al., 2004). In addition, in the AssemblX system, the cloning steps for each assembly reaction are designed in a user-oriented workflow, allowing the user to freely choose the final orientation of modules and thus avoid gene silencing driven by inverted repeats (Ghareeb et al., 2016; Matzke et al., 2002; Mette et al., 2000; Schubert et al., 2004). Moreover, this planning strategy allows the use of linkers between expression cassettes in order to improve expression, folding and/or stability of the proteins when expressing recombinant fusion proteins (Chen et al., 2013). Although a few plant expression plasmids for multigene cloning have already been developed, they are rarely fully flexible regarding regulatory elements, orientation of expression cassettes and addition of linkers between them, if

required. With these problems in mind, Plant X-tender expression vectors are available as empty backbones (Figure 3.1).

One major advantage of the Plant X-tender vector system presented here is the flexibility in the choice of a selection marker. Since there are reports on interactions between selection markers and gelling agents (Maheswaran et al., 1992), as well as connections between selection markers and regenerability (Schöpke et al., 1996), our set of Plant X-tender expression vectors, including three expression vectors with the same backbone (pK7WG ASX, pH7WG ASX and pB7WG ASX) but different selection markers, enables the selection of the most appropriate selection marker matching the target tissue as well as transformation media (Figure 3.1). According to our experience, the backbone can affect the expression pattern as well as transformation efficiency. Therefore, we constructed an additional Plant X-tender expression vector, pCambia ASX, with the intention of providing flexibility in terms of backbones (Figure 3.1).

Another advantage of the novel vector set is the inclusion of the *ccdB* gene (Figure 3.1), which precludes growth of non-recombinant clones (Bernard and Couturier, 1992) resulting in higher cloning efficiency. In addition Plant X-tender enables scar-free assembly, which is especially important since the introduced sequences can affect transgene function (Lampropoulos et al., 2013). However, this last property is already available also in recently developed COLORFUL-circuit vectors (Ghareeb et al., 2016).

Although restriction enzyme-based methods derived from the Golden Gate strategy share a number of characteristics that encourage their adoption by the scientific community, they have one major disadvantage in common. All parts are required to be devoid of any restriction sites used in the assembly. This could pose a problem, as Ghareeb et al. (2016) noticed a high occurrence of restriction enzyme recognition sites used in Golden Gate technology in several plant genomes. To overcome this downside of restriction enzyme-based methods, Ghareeb et al. (2016) exploited the use of a newly discovered rare-cutting restriction enzyme. Despite the utility and advantages of the system, annealing of short sticky ends may have limited affinity and specificity when assembling multiple DNA parts in one pot (reviewed in Chao et al., 2015). Since the cloning approach presented here is based on overlap-based cloning methods which utilize longer overlapping homology regions, the affinity and specificity issue is overcome. Moreover, since our cloning approach is based on rare-cutting restriction enzymes in combination with overlap-based methods, it is sequence-independent and thus avoids elimination of unwanted restriction sites. However, the issue of introducing sequence errors and time-consuming parts domestication could nowadays be avoided by DNA synthesis.

Another advantage of the AssemblX-based approach presented here lies in the flexibility to select the most appropriate method among several overlap-based assembly methods according to the user requirements (Figure 3.2). To determine the most efficient assembly method and cloning conditions, we evaluated different overlap-based assembly methods for cloning in various conditions. Cloning efficiency of 100 % in the case of Level 0 assembly by HiFi for all expression cassettes could be attributed to optimized homology regions present in Level 0 vectors and *ccdB* counterselection. In contrast, using TAR assembly method for Level 1 assembly resulted in higher cloning efficiency, if compared to the HiFi assembly method (data not shown). Our results are consistent with the results of de Kok et al. (2014), who obtained higher cloning efficiency by TAR assembly in comparison to a highly promoted commercial assembly kit (Gibson assembly). In the

last step of the cloning strategy followed here, we assembled the constructs into Plant X-tender expression vectors. Results from the assembly of p35S::H2BRFP_tNOS expression cassette (Table 3.2) are in agreement with the results from Zhang et al. (2012), who claimed that increasing the amount of the insert at the same vector amount as well as increasing the amount of the vector and the insert yield higher cloning efficiencies. In contrast, results obtained by the assembly of p35S::H2BRFP_tNOS + pNOS::ECFP_t35S multigene construct (Table 3.2) speak in favour of lower molar ratio. The transformation of highly efficient chemically competent *E. coli* with a SLiCE assembly mixture containing 150 ng of plasmid and a 2-fold molar excess of insert resulted in a higher number of colonies in comparison to higher molar ratio (1:5) of vector and insert (Table 3.2). Our results from the multigene assembly are consistent with the results from Okegawa and Motohashi (2015) and Motohashi (2015), who observed a significant decrease of cloning efficiency by increasing the molar ratio of the vector to insert. High cloning efficiency could be attributed to optimized homology regions (Hochrein et al., 2017) introduced in the Plant X-tender expression vectors and to *ccdB* counterselection. Although SLiCE has originally been used in combination with commercially available highly competent *E. coli* cells (Zhang et al., 2012), our results showed that SLiCE is applicable even in combination with low efficient homemade chemically competent or electrocompetent *E. coli* (Table 3.1), which is in agreement with the published data from Motohashi (2015) and Messerschmidt et al. (2016). However, in the case of more demanding cloning settings or low plasmid concentrations, highly competent *E. coli* cells are recommended.

4.2 In silico and Expression Promoter Analyses Enabled Identification of the Candidate Genes for Sensors Development

Recognition of the pathogen is followed by the synthesis of plant hormones in the host plant, which play crucial roles in mediating downstream transcriptional reprogramming resulting in effective defense responses (Birkenbihl et al., 2017). Therefore, the promoters of the genes from plant hormonal signalling pathways are particularly useful to study characteristics of signalling in plant-pathogen interaction.

Nucleotide alignments of the promoter sequences from selected genes involved in plant hormonal signalling showed high variability between and within the genotypes. Results show that promoter sequences of the same gene can differ between the genotypes. Moreover, the majority of the genes had different promoter sequences even within the same genotype, e.g. all three TGA2 promoters from Désirée differ in several SNPs (Figure 3.6), two PR1b promoters from Rywal differ from the third one in longer deletions (Figure 3.7).

On the other hand, only a few identical promoters were identified in different potato genotypes, e.g. TGA2 promoters from Rywal and Désirée (Figures 3.6), MYC2 promoters from PW363 and Désirée (Figure 3.8), ACO2 promoters from PW363 and Désirée (Figure 3.10) and within the same genotype. Among all analysed genes, only in the cases of TGA2 promoters and MC all three promoters were identical within the same genotype (TGA promoters from PW363 and Rywal and MC promoters from Désirée, Figures 3.6, 3.9). However, as only three promoter sequences were analysed per genotype, it is possible that even TGA2 and MC promoters differ within the same cultivar.

The next step was *in silico* analysis of promoter sequences, which resulted in the identification of several regulatory elements that are known to be highly represented in the promoters of pathogen-inducible genes (Hernandez-Garcia and Finer, 2014). Interestingly, regulatory elements from all three plant hormonal signalling pathways were identified in the promoters of all selected genes, suggesting that the crosstalk between signalling pathways is regulated through transcription (Table 4.1).

Table 4.1: Regulatory elements involved in hormonal signalling (JA, SA and ET signalling pathways) in the promoters of selected genes (TGA2, PR1b, MYC2, PTI5, ACO2 and MC), summarized from Figures 3.12-3.17.

Hormonal signalling pathway	Regulatory element	TGA2 (R1)	PR1b (R3)	MYC2 (P2)	PTI5 (R1)	ACO2 (R2)	MC (D1)
JA	CGTCA-motif	✓	✓		✓		✓
	T/GBOXATPIN2	✓				✓	✓
	MYCATERD22 (MYC recognition sequence)					✓	
	MYCATERD1 (MYC recognition sequence)		✓			✓	
	MYCCONSUSAT (MYC recognition site)	✓	✓	✓	✓	✓	✓
	AT4G12670						✓
SA	Box-W1/W box/ELRECOREPCR1/WBBOXPCWRKY1	✓		✓	✓	✓	
	WBOXATNPR1	✓	✓	✓	✓	✓	✓
	TGACG-motif/ASF1MOTIFCAMV	✓	✓		✓		✓
	GT1CONSUSAT	✓	✓	✓	✓	✓	✓
	GT-1 recognition site			✓			
	DREB1B recognition site			✓			✓
	ASF1MOTIFCAMV		✓				✓
	TCA-element		✓	✓	✓	✓	
	TGA1a binding site		✓				
	TGA1A binding site		✓				✓
	Opaque-2 (TGA1A) recognition site, TGACG-sequence-specific binding site				✓		
	RITA-1 (TGA1A) recognition site				✓		
	HBP-1a (TGA1A) recognition site				✓		
	TGA1A recognition site				✓		
	TGA1a recognition site				✓		
	SEBFCNSSTPR10A (silencing element binding factor (SEBP) gene found in promoter of PR-10a gene)			✓			
	GT-1 binding site (negative regulator of PR-1a gene expression)					✓	
	At4g04450 (WRKY binding site)	✓					
	WRKY71OS (WRKY binding site within the Pathogenesis-Related Class10 (PR-10) genes)	✓	✓	✓	✓	✓	✓
	WBBOXPCWRKY1 (WRKY proteins binding site in PR1 gene in parsley)	✓		✓		✓	✓
	WRKY48 recognition site (involved in stress and pathogen response)			✓			
	WRKY binding site					✓	✓
	WRKY48 recognition site (stress and pathogen induced WRKY48, recognizes the TTGACC W-box)				✓		
	WBOXHVIS01 (W-box element)					✓	✓
	AT4G12670						✓
	TCA1MOTIF						✓

Continued

Hormonal signalling pathway	Regulatory element	TGA2 (R1)	PR1b (R3)	MYC2 (P2)	PTI5 (R1)	ACO2 (R2)	MC (D1)
ET	ERF13/DRF1.3/ESR1 binding site		✓				
	ERF1 binding site		✓				
	GCCCORE		✓				
	ERE/ERELEE4		✓	✓	✓	✓	
	AT1G28160 recognition site				✓		
	At1g68550 recognition site				✓		
	MYB1LEPR (Tomato Pti4 (ERF) regulates defence-related gene expression)	✓					✓
	ERF039 recognition site (contains GCC-box pathogenesis-related promoter element)			✓			
	ERF9 recognition site (GCC-box pathogenesis-related promoter element)			✓			
	ERF069 recognition site (GCC-box pathogenesis-related promoter element)				✓		
	MYB1LEPR (G-box, Tomato Pti4(ERF) regulates defence-related gene expression via GCC box)					✓	
	GCC box (pathogen response)		✓				

W-boxes, known also as WRKY-binding sites, were identified in promoters of all analysed genes, but were more abundant in ACO2 promoter (Table 4.1). Interestingly, we identified the binding site of WRKY71OS, which was before identified in the promoters of the pathogenesis-related Class10 (PR-10) genes in parsley (Eulgem et al., 1999) in PR1b(R3) promoter, but also in all other analysed promoters (Table 4.1).

GCC box, which is another pathogen-inducible regulatory element, was identified in the promoters of several ET-responsive genes (Phukan et al., 2017). For example, it was suggested that the activation of GCC box in promoters of tobacco PR genes directs PR gene expression after ET induction (Ohme-Takagi and Shinshi, 1995). This is in consistency with our results, as we showed that the PR1b(R2) promoter is induced after ET treatment (Pezic, 2016). Moreover, we showed that GCC-box is highly represented in the promoters of PTI5 gene (Table 4.1). It is well known that ERF transcription factors regulate the molecular response to pathogen attack by binding to the GCC box (Müller and Munné-Bosch, 2015). Therefore, the presence of GCC boxes within the promoter of PTI5 transcription factor suggests regulation of PTI5 by other ERF transcription factors through transcription (Table 4.1). This is in agreement with the results from Oñate-Sánchez et al. (2007), who claimed that AtERF14 plays a unique role in plant defence response regulating the expression of other ERF genes. According to McGrath et al. (2005), AtERF2 is also a positive regulator of JA-responsive defence genes. This is in consistency with our results as GCC-box elements was identified in the promoters of MYC2 and MC genes (Table 4.1).

TCA element is another regulatory element that is abundant in the promoters of pathogen-inducible genes. This regulatory element was identified only in the PR1b(R3) promoter (Table 4.1). As TCA element was involved in the expression of PR-2d gene after TMV infection or SA-induction (Shah and Klessing, 1996), it could also play a role in the expression of PR1b gene after PVY infection or INA-induction.

Some increase in the fluorescence intensity was observed in the MC promoter from PW363 after induction with MeJA (Figure 3.18), which could be mediated by the G-box regulatory element present in the MC(P1) promoter (Table 4.1). Our results are in agreement with the results from Kim et al., who proposed the involvement of G-box in the expression of JA-inducible proteinase inhibitor in tobacco (Kim et al., 1992). However, G-box was also identified in the MC promoter from Désirée, which is not MeJA-inducible (Figure 3.18). Moreover, G-box was identified in the TGA2(R1) promoter, which did not show any activity after induction with MeJA (Pezic et al., 2016).

Yang et al. identified MYC binding site in the tobacco PR1 gene promoters (Yang et al., 1997). In accordance with the results from Yang et al., our results confirm the presence of MYC recognition site in the PR1b promoter also in potato (cv. Rywal, Table 4.1). These results are suggesting the crosstalk between SA (PR1b) and JA (MYC2) signalling pathway. However, expression driven by PR1b promoter was not activated after MeJA treatment in our experiments (Pezic, 2016).

Activator sequence-1 (as-1) is a SA-responsive element bound by some members of TGA transcription factors, isolated from tobacco and Arabidopsis (Yang et al., 1997). The regulatory element as-1-like is found in the promoters of several PR1 genes and is bound by the members of TGA transcription factors (Kim et al., 2002; Zhou et al., 2000, Zhang et al., 1999). It is composed of two TGA boxes (TGACG) and a linker (reviewed in Alves et al., 2013). TGA boxes, well documented as TGA-binding sites which are the centres of palindromic sequence, were found in promoters of tobacco PR1a and AtPR1, which are known as markers for the late NPR1-dependent genes (Krawczyk et al., 2002; Butterbrodt et al., 2006; Thibaud-Nissen et al., 2006). Several studies demonstrated the binding of TGA to the TGA-boxes within PR1 promoter in vivo after SA induction in Arabidopsis and tobacco (Lam et al., 1995; Després et al., 2000; Krawczyk et al., 2002; Johnson et al., 2003; Thibaud-Nissen et al., 2006). However, not all of them are supporting the idea of independence between two adjacent TGA-boxes. For example, Lam et al. (1995) detected very low binding affinities of AtTGA2 to a probe with a single TGACG-motif. Similarly, tobacco TGA2.1 requires a two-palindrome sequence for the binding (Krawczyk et al., 2002). On the other hand, some of them are supporting the idea of independence between two adjacent TGA-boxes. For example, AtTGA2 can independently bind the negative (LS5) and positive (LS7) SA-inducible regulatory element (ACGTCA). Both elements are complementary to the TGA-box (Després et al., 2000). Similarly, tobacco TGA2.2 and TGA1a, which show higher similarity to AtTGA2 in comparison to TGA2.1, are able to bind to a single palindrome (Krawczyk et al., 2002).

In our study, analysis of the promoter region of PR1b gene resulted in the identification of the TGA box, while as-1 sequence was not present (Table 4.1). To explain the discrepancies between the results of different studies, Lam et al. (1995) suggested that each member of the TGA factor family may be differentially regulated and show different DNA-binding characteristics. Moreover, binding of TGA2 to the as-1-like element is responsible for the SA-induced transcription of early NPR1-dependent genes (Qin et al., 1994; Blanco et al., 2009), which are activated 30-120 minutes after SA treatment, with the peak of expression at around four hours (Zhang and Singh, 1994). As PR1 genes were determined as late induced by SA, first detectable after three to five hours (Qin et al., 1994; Niggeweg et al., 2000b; Kliebenstein et al., 2006) with the peak of expression after 24 or more hours (Ward et al., 1991; Qin et al., 1994; Spoel et al., 2003; Uquillas et al., 2004), the presence of as-1 sequence in the promoters of

some PR1 genes promoters might not be crucial for the activity. This hypothesis is in agreement with the results, obtained within our working group, as PR1b(D3) promoter lacking the as-1 sequence was activated after INA treatment (Pezic, 2016). In addition, a PR1 promoter derivate lacking the as-1-like element responds to INA in an NPR1-dependent manner also in Arabidopsis (Pape et al., 2010).

In silico promoter analyses that were performed are sufficient for the identification of the most promising promoter candidates, but do not guarantee that the promoters will be functional *in vivo*. Therefore, in the next step, the candidate promoters were analysed through *in vivo* analyses to confirm or disprove their functionality. Many different *in vivo* validation systems for the promoter analysis exist, however, the generated results are largely determined by the expression system used for functional analysis. For example, transient expression analysis is relatively simple and rapid, if compared to stable transformation, but does not always provide the correct information on promoter function (Hernandez-Garcia and Finer, 2014).

One drawback of the use of transient transformation for promoter study is the potential absence of transcription factors required to interact with cis-elements, which can modify the expected expression patterns in heterologous systems (Hernandez-Garcia and Finer, 2014). For example, some transcription factors, which are crucial for the promoter responsiveness to hormonal induction in potato might be absent in *N. benthamiana* (Table 4.1). Moreover, further analysis, such as the study of promoter activity after PVY infection in HR could not be performed on *N. benthamiana* as PVY does not induce HR in this host (Goodin et al., 2008). In addition, promoters removed from their native context and fused to new genes frequently display different expression profiles (Hernandez-Garcia and Finer, 2014).

Therefore, the candidate promoters were functionally characterized in potato following stable transformation. The transformation efficiency (introduction of the construct into the potato genome) was relatively high for some constructs (more than 80 % in the case of MC(D1) promoter; Table 3.8). However, it was not possible to detect expression in some transgenic lines even though the presence of the promoter::fluorescent protein construct in the genomic DNA was confirmed (Table 3.11).

The absence of the signal could be attributed to several different post-transcriptional phenomena. The first one following the transcription is RNA silencing. Even though the promoters are activated and the mRNA coding for the fluorescent protein is generated, translation into fluorescent protein could be prevented by RNA silencing. In case of successful protein translation, fluorescent proteins could be subjected to protein degradation. Fluorescent proteins are free from any signal peptide and are thus expressed in the cytoplasm, where they are exposed to degradation. In addition, the absence of the signal could also be explained by fluorescence quenching. For example, De Jonge et al. (2017) observed a decrease in the GFP, RFP and VENUS-derived fluorescence in plant tissue after SA treatment. The loss of the signal was not dependent on the fluorescent protein abundance, suggesting the interference between SA and fluorescence (De Jonge et al., 2017). On the other hand, some of the promoters might be functional, but too weak to produce fluorescence which would exceed the detected autofluorescence (Rugbjerg et al., 2015). The absence of the signal could also be attributed to the position effect as random integration of promoter constructs in plant genome may result in large variability in expression (Hernandez-Garcia and Finer, 2014).

If we ignore all technical problems in confocal imaging, the absence of the fluorescent signal in transformed lines can indicate that the selected promoter is not functional. Variations in promoters (either insertions/deletions or single nucleotide polymorphisms) can affect promoter activity (see Section 1.1.4.1). As the analysed promoters are highly variable, the promoter, selected for expression analysis might be defective in some essential elements (see Section 3.2.1). Moreover, potential missing enhancers located in the distal promoter region due to the truncation of the promoter at the 5' end during cloning might affect promoter activity (Lee and Young, 2000).

However, using confocal microscopy, it was possible to identify a few promoters, which could potentially be used as sensors of plant immune response, namely MC(P1), MC(D3) (Figures 3.18, 3.19) and PR1b(D1) (Pezic, 2016).

Expression of YFP was observed 24 and 48 hours after the activation of PR1b(D1) promoter with 1 mM ACC and 0.3 mM INA, respectively, while the treatment with MeJA did not result in YFP expression. Similarly, treatment of TGA2(R1)::YFP transgenic plants with 0.3 mM INA resulted in YFP expression, but was weaker if compared to the fluorescence obtained after induction of PR1b(D1) promoter. In addition, treatment with 1 % ethanol (control) also resulted in YFP fluorescence, albeit weaker. However, further analyses will be needed to exclude the possibility that this fluorescence is the result of unspecific promoter induction. Moreover, we identified wounding responsive regulatory element WBOXNTERF3 in the TGA2(R1) promoter (Figure 3.12), allowing the possibility that the fluorescence is the result of the injury. However, in plants, transformed with *A. tumefaciens* with no binary plasmid, YFP expression was not detected.

No wounding regulatory elements were identified in the MC promoters, suggesting that the fluorescence is not the result of the mechanical treatment (Table 4.2). However, further quantification analysis will be needed to determine if the fluorescence intensity is significantly changed after treatment and thus the selected promoters could be used as sensors of plant immune response. Moreover, elicitors for SA, JA and ET signalling pathways (INA, MeJA and ACC) have not been tested for the induction of each of the selected promoters to identify potential crosstalks, as is was already performed for PR1b and TGA2 promoters (Pezic, 2016). Induction of PR1b(D3) promoter with INA and ACC resulted in promoter activation, while MeJA did not activate the promoter, suggesting the presence of crosstalk between SA and ET signalling pathway (Pezic, 2016).

We did not detect any activity of MC(P1) promoter prior to activation with MeJA, while expression of YFP was detected already prior to the induction of MC(D3) promoter (Figure 3.18). The differential activity of MC(D3) promoter compared to MC(D1) and MC(P1) promoter can be explained by different regulatory elements present in those promoters (Figure 3.15 and Supplementary Figure 1). For example, two 5'UTR Py-rich stretch motifs, known as cis-acting regulatory element conferring high transcription levels were found in MC(D1) promoter, while only one 5'UTR Py-rich stretch motif was identified in MC(P1) promoter (Appendix, Supplementary Table 6). We conclude that sequence diversity can lead to differences in functionality.

Table 4.2: Regulatory elements involved in stress response in the promoters of selected genes (TGA2, PR1b, MYC2, PTI5, ACO2 and MC), summarized from Figures 3.12-3.17.

Responsiveness	Regulatory element	TGA2 (R1)	PR1b (R3)	MYC2 (P2)	PTI5 (R1)	ACO2 (R2)	MC (D1)
defense and stress responsiveness	TC-rich repeats	✓	✓	✓	✓	✓	✓
calmodulin-binding NAC protein	CBNAC binding site	✓	✓	✓			
wounding responsiveness	WBOXNTERF3	✓	✓	✓	✓	✓	
pathogen- and salt-induced SCA4 gene expression	GT1GMSCAM4	✓		✓			✓
elicitor-responsive transcription of defense genes	WBOXNTCHN48	✓					
involved in stress responsiveness	AGCBOXNPGLB		✓				
dependent CaM-binding protein recognition site	GT-1 recognition site				✓		
defense-related gene expression	CACGTGMOTIF				✓		
involved in oxygen response	CURECORECR				✓		✓
elicitor-responsive element	EIRE					✓	
wound-responsive element	WUN-motif					✓	
involved in Ca ²⁺ responsiveness	ACGTATERD1						✓
involved in Ca ²⁺ responsiveness	ABRERATCAL						✓

On the other hand, we did not identify any promoters, which could potentially be used as sensors of plant immune response based on their gene expression by RT-qPCR. Even though the results of preliminary testing were promising for several promoter::fluorescent protein constructs (Table 3.11), repeated experiments with the increased number of analysed samples showed no significant difference between the expression in the controls and in the treated samples (Figure 3.21). These results are not surprising, as high variability of transgene expression in independent transgenic lines is frequently observed in plants (Nagaya et al., 2005).

To increase robustness of analysis, nucleus localization signal (NLS) will be added to fluorescent proteins to enable transport of the protein into the nucleus. The signal in the nucleus is concentrated in the small area and not scattered through the whole cytoplasm, which results in higher fluorescence intensity and enables easier quantification (Ghareeb et al., 2016). Therefore, this approach will enable quantification of the fluorescence intensity prior and following the induction of MC(P1) promoter to determine if MC(P1) promoter could be used as a sensor of plant immune response. In addition, it will help to exclude the possibility that the fluorescence is the result of unspecific induction of TGA2 promoters by increasing fluorescence intensity. Moreover, measuring fluorescence which is concentrated in the nucleus will enable detection of small changes in expression after induction and could potentially allow identification of promoters, which were now determined as unresponsive to the hormonal

treatment. This will be especially crucial in case of promoters with low expression.

Moreover, for proper analysis of promoters in stably transformed plants, a number of transgenic plants should be generated and screened for gene expression, which makes screening through stable transformation limited for high-throughput promoter analysis (Hernandez-Garcia and Finer, 2014). Since screening various promoters using transient transformation of potato is more problematic compared to some other species such as *Arabidopsis* due to low transformation efficiency (low number of cells are transformed by agroinfiltration) and high signal-to-noise ratio (high autofluorescence in potato) we will enable some additional screening options. A high-throughput screening system with promoters fused to GUS will be developed and their response to different elicitors will be monitored in a leaf disc system. Once the most promising promoter sequences will be selected, we will continue with stable transformation and quantification based on fluorescent proteins using newly generated transgenic plants.

Taken altogether we can conclude that transcriptional regulation in potato shows additional complexity also through promoter variability. Potato is highly heterozygous autotetraploid ($2n = 4x = 48$). The presence of the paralogous sequences and repetitive transposable elements makes its genome highly complicated (The potato genome sequencing consortium, 2011). As potato has four sets of chromosomes, presence of up to four different promoters per loci is possible. Perhaps high diversity in promoters enables specificity of transcriptional response in response to different environmental and developmental cues. We have also detected high variability of promoters between potato cultivars. Variabilities in the promoters could be responsible for different phenotypical and molecular responses to PVY infection in different sensitive potato genotypes (Baebler et al., 2011).

As this type of sensors enable measuring of the signal in real time in living plant cells, the developed sensors will be used in the studies which require detailed spatiotemporal resolution, such as multi-way interactions. In addition, development of sensors will enable detailed site-specific identification of the cells with interesting expression profile on the single cell level prior to tissue dissection.

4.3 Restriction of Potato Virus Y Spread in Ny-1-Gene-Mediated Hypersensitive Response-Conferred Resistance is Dynamic Process Not Conferred by Cell Death or Callose Deposition

Traditionally, HR-associated cell death was envisioned as crucial for disease resistance in host-pathogen interaction, which results in the restriction of pathogen growth and spread. This notion was first challenged in 1972, when Király et al. suggested, that it is not the necrosis of host tissues which inhibits or prevents the pathogen from further growth, but before necrosis, unknown mechanisms inhibit or even kill the pathogen. The necrosis observed in HR was suggested to be the result of the death or inhibition of the growth of the pathogen, rather than a direct defense mechanism (Király et al., 1972). Later on, however, it became clear that the relative importance of cell death in this form of disease resistance may vary depending on the specific pathosystem (Yu et al., 1998).

In plant-virus pathosystems, the cell death was shown to be uncoupled from resistance in the HR in different host-virus interactions including viruses from several genera, such as *Potexvirus* (PLAMV and PVX), *Caulimovirus* (CaMV), *Tombusvirus* (tomato bushy stunt virus (TBSV)), *Cucumovirus* (CMV), *Carmovirus* (TCV) and *Tobamoviruses* (TMV and tomato mosaic virus (ToMV)) (Ando et al., 2014; Cawly et al., 2005; Chu et al., 2000; Hafez et al., 2012; Ishibashi et al., 2007; Komatsu et al., 2010; Chandra-Shekara et al., 2004; Liu et al., 2009). Our study is the first showing similar results also for the interaction of a member of another virus family, *Potyviridae*, with its host, in an agronomically important pathosystem (Figure 3.22). However, besides our study, only studies investigating tobacco-TMV interaction were focused on the precise localization of the virus in relation to the cell death zone. Electron microscopy examination of lesions formed in *Nicotiana glutinosa* after infection with TMV confirmed the presence of the virus just outside the edge of the lesion (Da Graça and Martin, 1976; Milne, 1966), as confirmed also by ELISA (Antoniw and White, 1986). Results of two confocal microscopy studies confirmed the presence of isolated cells or small clusters of cells infected with TMV-GFP (Murphy et al., 2002; Wright et al., 2000) or TMV-GUS (Arce-Johnson et al., 1995) at the edge of necrotic lesions. Our results are in agreement with the results studying tobacco-TMV interaction as we have visualized a similar pattern of virus escape. As in the case of the TMV-tobacco pathosystem (Wright et al., 2000), we also show that a virus outside the cell death zone is alive and able to reinitiate infection from the cells surrounding the lesion (Figure 3.28). In previous studies, the temperature shift assay was also exploited to determine the time needed for complete elimination of the TMV in HR interaction with the host. In resistant tobacco plants at least 12 days at 22 °C were needed to avoid any virus spread after the temperature shift (reviewed in Weststeijn, 1981). These results suggest the presence of the viable virus outside the lesion also in late time points after inoculation, which is in accordance with our results showing that the virus is located outside the lesion in all lesion developmental stages (Figure 3.26, Table 3.13).

The production of ROS after pathogen attack has been proposed to be involved in the resistance mediated by HR (reviewed in Zurbriggen et al., 2010). The transcriptomic analysis of potato cv. Rywal after PVY infection showed strong upregulation of redox-related genes from 1 dpi, suggesting that a burst of ROS may have an important role in the defense response also in our pathosystem (Baebler et al., 2014). Among those, RBOHD (Sotub06g025550) showed different spatiotemporal expression profiles in PVY-infected resistant and susceptible genotypes, suggesting its involvement in the restriction of virus spread. As RBOH gene family has also been proposed to be involved in the production of ROS during the defense responses in several other pathosystems (Torres and Dangl, 2005), we prepared transgenic plants of cv. Rywal with silenced RBOHD gene to test its involvement in virus restriction in potato-PVY pathosystem. In the transgenic plants PVY spread systemically, which was manifested as lesions in upper, noninoculated leaves. We further confirmed that the virus spread is no longer restricted in RBOHD-silenced transgenic plants by detecting viral RNA in upper non-inoculated leaves by RT-qPCR. Our results show that functional RBOHD is required for the Ny-1 resistance-gene-mediated resistance to PVY. Results were obtained as a part of a Master degree thesis within our group (Dušak, in process).

Moreover, we elucidated some additional characteristics of HR-conferred resistance using our pathosystem. To determine whether the cells that harbour viable viral elements are in time also

engulfed by cell death zone, the dynamics of lesions expansion was followed over the longer time period, until the leaf was completely senescent. Results show that, in this pathosystem, the lesions are first visible 3 dpi and while some lesions maintain their size after 5 dpi, the expansion of the majority of lesions could be observed even after 10 dpi (Figure 3.27 B). In N-gene resistance-gene-mediated response against TMV infection, Wright and coworkers were visually studying the potential expansion of lesion in later stages but were not able to detect it and thus concluded that an alternative mechanism exists that selectively eliminates the remaining infected cells (Wright et al., 2000). In the Ny-1 resistance-gene-mediated responses the infected cells observed outside the cell death zone can be eventually subjected to cell death. In the SA-depleted plants, both the virus and the cell death zone are spreading faster than in cv. Rywal (Figure 3.27 A, B). Therefore HR-conferred resistance should be regarded as a process where the dynamics of events is crucial for effectiveness.

One of the common mechanisms that control the viral movement by preventing cell-to-cell spread of the virus is plasmodesmata closure, which is often regulated by callose deposition (Lee and Lu, 2011). For example, in plants infected with soybean mosaic virus (SMV), callose deposition was extensive within necrotic viral lesions, but could also be detected outside the lesion, suggesting that callose deposits at plasmodesmata could be an early defence strategy limiting the spread of the virus (Li et al., 2012). Callose deposition has also been reported at plasmodesmata in plants infected with many other viruses, including TMV and PVY (reviewed in Zavaliev et al., 2011). However, these studies did not include precise localization of the infected cells in relation to the callose deposits. Our results show the presence of extensive callose deposition on the margin of the cell death zone, engulfing the majority of infected cells and the outside tissue, but the virus was present also outside the callose deposits (Figure 3.30), suggesting that the callose deposition is not sufficient for virus restriction. However, we cannot exclude the possibility that the callose is contributing to the resistance in HR in potato, albeit at a concentration below the limit of our detection method. Physical blockage of plasmodesmata was also not detected in resistant tobacco-TMV interaction, where results were obtained by studying symplastic continuity with micro-injected dyes, although the involvement of callose deposits in the plasmodesmata closure was not investigated (Wright et al., 2000).

Chapter 5

Conclusions

As hypothesised, *in silico* analysis of the promoters of selected genes from hormonal signalling pathways resulted in the identification of several binding sites specific for the transcription factors involved in immune signalling. The activity of the promoter variants with the most promising combination of regulatory elements was analysed in transient and stable expression set-up after promoters were fused with reporter genes coding for fluorescent proteins. The results showed that sequence diversity leads to functional diversity. This enabled identification of the best candidates which could be later on used as sensors of plant immune response.

Plant X-tender, a toolbox for cloning and delivery of multiple DNA fragments into the plant cells was developed. Validation of the toolbox by cloning two cassettes into the newly developed vectors and following their functionality in a transient expression setup confirmed the hypothesis that multigene constructs can be delivered into plant cells in a streamlined and highly efficient way. The developed toolbox paves the way for more complex and increasingly flexible approaches for large-scale engineering and will support faster introduction of synthetic biology into the plant science.

In the third part of my dissertation, the relationship between cell death and restriction of PVY spread in the *Ny-1* genetic background of potato was investigated. The results show that the virus is multiplying in the immediate vicinity of the cell death zone at all time points post inoculation, yet no virus spread to the neighbouring tissues was observed. Although the possibility cannot be excluded that cell death contributes to limitation of viral spread and/or multiplication, cell death is not sufficient to completely prevent PVY from spreading to cells adjacent to necrotic lesions. This supports the hypothesis that the HR-associated cell death is a separate phenomenon from the resistance mechanism(s) at work in the potato-PVY pathosystem. It is suggested that this might be a general feature of HR against viruses as our results are in agreement with the results obtained with the TMV-tobacco pathosystem.

The obtained data which suggest the involvement of RBOHD in virus restriction could be further used for the improvement of the model of plant immune response. Moreover, the developed experimental approaches and knowledge could be transferable to other pathosystems and the technology developed also to other applications in biotechnology.

Appendix A

Supplementary Material

A.1 Supplementary Material for Section 3.1

Republished from Plant X-tender: An extension of the AssemblX system for the assembly and expression of multigene constructs in plants, Lukan et al., 2018b, PLoS One, 13(1):e0190526, doi: 10.1371/journal.pone.0190526, according to Creative Commons Attribution ("CC BY") licence.

Supplementary Table 1: Primers for sequencing and colony PCR.

	Backbone	Insert	Primers for sequencing	Primers for colony PCR	Length of the amplicon
Construction of Plant X-tender expression vectors	pCAMBIA_ASX	homology regions A0 and B0, I-SceI and HindIII recognition sites and <i>ccdB</i> gene	M13F, M13R	M13F/M13R	896 bp
	pK7WG_ASX	homology regions A0 and B0, I-SceI and HindIII recognition sites and <i>ccdB</i> gene	PVIBF, PVIBR8527	PVIBF/PVIBR	830 bp
	pH7WG_ASX	homology regions A0 and B0, I-SceI and HindIII recognition sites and <i>ccdB</i> gene	PVIBF, PVIBR8527	PVIBF/PVIBR	830 bp
	pB7WG_ASX	homology regions A0 and B0, I-SceI and HindIII recognition sites and <i>ccdB</i> gene	PVIBF, PVIBR8527	PVIBF/PVIBR	830 bp
Level 0 assembly	pLOA_0-R	p35S::H2BRFP_tNOS	FM176, FM218	FM176/FM218	3133 bp
	pLOA_0-1	p35S::H2BRFP_tNOS	FM176, FM218	FM176/FM218	2888 bp
	pLOA_1-R	pNOS::ECFP_t35S	FM176, FM218	FM176/FM218	2038 bp
Level 1 assembly	pL1A-hc / pL1A-lc (A0/AR)	p35S::H2BRFP_tNOS	FM182, FM131	KG022/FM182	773 bp
	pL1A-hc / pL1A-lc (A0/AR)	p35S::H2BRFP_tNOS + pNOS::ECFP_t35S	FM182, FM131, KG032	KG022/FM182, FM1113/FM032	773 bp, 702 bp
Plant X-tender expression vectors assembly and <i>A. tumefaciens</i> transformation	pCAMBIA_ASX	p35S::H2BRFP_tNOS	M13F, M13R	M13R/FM032	2854 bp
	pCAMBIA_ASX	p35S::H2BRFP_tNOS + pNOS::ECFP_t35S	M13F, M13R, KG032	M13R/KG022	716 bp
	pK7WG_ASX	p35S::H2BRFP_tNOS	PVIBF, PVIBR8527	PVIBF/FM032	2807 bp
	pH7WG_ASX	p35S::H2BRFP_tNOS	PVIBF, PVIBR8527	PVIBF/FM032	2807 bp
	pB7WG_ASX	p35S::H2BRFP_tNOS	PVIBF, PVIBR8527	PVIBF/FM032	2807 bp

A.2 Supplementary Material for Sections 3.2 and 3.3

Supplementary Table 2: Cis-regulatory elements in promoter sequences of TGA2 promoters from different potato cultivars, identified by PlantCARE. 1583 bp long promoter sequences from PW636 (TGA2(P1), TGA2(P2), TGA2(P3)), 1513 nt long sequences from Rywal (TGA2(R1), TGA2(R2), TGA2(R3)) and Désirée (TGA2(D1): 1513 nt, TGA2(D2): 1512 nt, TGA2(D3): 1508 nt) were analysed using PlantCARE web service. Promoter TGA2(R1), used for a more detailed investigation, is grey shaded, while known defence-related regulatory elements are green shaded. The position, the strand and the sequence of each cis-regulatory element is specified. If more than three repeats of the particular regulatory element were present in the sequence, only the number of these elements is stated (with no position information).

Name of regulatory element	Sequence of regulatory element	PW363			Rywal			Désirée		
		TGA2 (P1)	TGA2 (P2)	TGA2 (P3)	TGA2 (R1)	TGA2 (R2)	TGA2 (R3)	TGA2 (D1)	TGA2 (D2)	TGA2 (D3)
RY-element	CATGCATG	42(+)	42(+)	42(+)	42(+)	42(+)	42(+)	42(+)	42(+)	42(+)
Box 4	ATTAAT	72(+)	72(+)	72(+)	72(+)	72(+)	72(+)	72(+)	72(+)	72(+)
TCT-motif	TCTTAC	110(-)	110(-)	110(-)	110(-)	110(-)	110(-)	110(-)	110(-)	110(-)
AAGAA-motif	gGTAAAGAAA	118(+)	118(+)	118(+)						
AAGAA-motif	GAAAGAA	124(+)	124(+)	124(+)						
Box-W1	TTGACC	183(+)	183(+)	183(+)	204(+)	204(+)	204(+)	204(+)	204(+)	204(+)
SUTR Py-rich	TTTCTTCTCT	191(-)	191(-)	191(-)	212(-)	212(-)	212(-)	212(-)	212(-)	212(-)
F-box	CTATTCTCATT				237(-)	237(-)	237(-)	237(-)	237(-)	237(-)
TCT-motif	TCTTAC	292(+)	292(+)	292(+)						
CTAG-motif	ACTAGCAGAA				295(-)	295(-)	295(-)	295(-)	295(-)	295(-)
ATC-motif	AGTAATCT				307(+)	307(+)	307(+)	307(+)	307(+)	307(+)
circadian	CAAAGATATC	322(+)	322(+)	322(+)						
TATCCAT/C-motif	TATCCAT	328(+)	328(+)	328(+)						
circadian	CAANNNNATC	353(+)	353(+)	353(+)						
AE-box	AGAAACAT	461(+)	461(+)	461(+)	1416(-)	1416(-)	1416(-)	1416(-)	1415(-)	1411(-)
circadian	CAANNNNATC	542(+)	542(+)	542(+)	475(+)	475(+)	475(+)	475(+)	475(+)	475(+)
Box 4	ATTAAT	605(-)	605(-)	605(-)	1165(+)	1165(+)	1165(+)	1165(+)	1165(+)	1159(+)
Box I	TTTCAA	651(+)	651(+)	651(+)	584(+)	584(+)	584(+)	584(+)	584(+)	584(+)
chs-Unit 1 m1	ACCTACCACAC	692(-)	692(-)	692(-)	625(-)	625(-)	625(-)	625(-)	625(-)	625(-)
ATCT-motif	AATCTAATCT	733(-)	733(-)	733(-)	666(-)	666(-)	666(-)	666(-)	666(-)	666(-)
ATCT-motif	AATCTGATCG									831(+)
Unnamed 6	taTAAATATct	831(-)	831(-)	831(-)	764(-)	764(-)	764(-)	764(-)	764(-)	765(-)
ARE	TGGTTT				870(+)	870(+)	870(+)	870(+)	870(+)	870(+)
Unnamed 6	taTAAATATct	926(+)	926(+)	926(+)	856(+)	856(+)	856(+)	856(+)	856(+)	853(+)
box 5	AGCCACC	938(-)	938(-)	938(-)						
G-box	TGACGTGG	952(+)	952(+)	952(+)						879(+)
CGTCA-motif	CGTCA	952(-)	952(-)	952(-)	882(-)	882(-)	882(-)	882(-)	882(-)	879(-)
G-box	CACGTC	953(-)	953(-)	953(-)						880(-)
GC-motif	CCCCCG				887(-)	887(-)	887(-)	887(-)	887(-)	
TGACG-motif	TGACG	952(+)	952(+)	952(+)	882(+)	882(+)	882(+)	882(+)	882(+)	879(+)
Unnamed 1	CGTGG	955(+)	955(+)	955(+)	958(+)	958(+)	958(+)	958(+)	958(+)	955(+)
CGTCA-motif	CGTCA	988(+)	988(+)	988(+)	918(+)	918(+)	918(+)	918(+)	918(+)	915(+)
TGACG-motif	TGACG	988(-)	988(-)	988(-)	918(-)	918(-)	918(-)	918(-)	918(-)	915(-)
G-Box	CACGTT	991(+)	991(+)	991(+)	921(+)	921(+)	921(+)	921(+)	921(+)	918(+)
Unnamed 1	CGTGG	1028(+)	1028(+)	1028(+)						882(+)
chs-CMA1a	TTACTTAA	1049(-)	1049(-)	1049(-)	982(-)	982(-)	982(-)	982(-)	982(-)	976(-)
TC-rich repeats	ATTTTCTTCA	1114(-)	1114(-)	1114(-)						
HSE	AGAAAATTCG	1117(+)	1117(+)	1117(+)	1050(+)	1050(+)	1050(+)	1050(+)	1050(+)	1044(+)
HSE	AAAAAATTTTC	1131(-)	1131(-)	1131(-)	1064(-)	1064(-)	1064(-)	1064(-)	1064(-)	1058(-)
TC-rich repeats	ATTTTCTTCA	1200(-)	1200(-)	1200(-)	1133(-)	1133(-)	1133(-)	1133(-)	1133(-)	1127(-)
HSE	AGAAAATTCG	1203(+)	1203(+)	1203(+)	1136(+)	1136(+)	1136(+)	1136(+)	1136(+)	1130(+)
TC-rich repeats	ATTTTCTTCA	1213(+)	1213(+)	1213(+)	1146(+)	1146(+)	1146(+)	1146(+)	1146(+)	1140(+)
Box 4	ATTAAT	1233(+)	1233(+)	1233(+)						
AC-I	CCCACCTACC	1282(-)	1282(-)	1282(-)	1214(-)	1214(-)	1214(-)	1214(-)	1214(-)	1208(-)
LAMP-element	TCTTATCA	1295(-)	1295(-)	1295(-)	1227(-)	1227(-)	1227(-)	1227(-)	1227(-)	1221(-)
AT-rich element	ATAGAAATCAA				1299(-)	1299(-)	1299(-)	1299(-)	1298(-)	1295(-)
Box-W1/W box	TTGACC	1305(+)	1305(+)	1305(+)	1237(+)	1237(+)	1237(+)	1237(+)	1237(+)	1231(+)
Sp1	CC(G/A)CCC	1466(-)	1466(-)	1466(-)	1396(-)	1396(-)	1396(-)	1396(-)	1395(-)	
AE-box	AGAAACAT	1486(-)	1486(-)	1486(-)	394(+)	394(+)	394(+)	394(+)	394(+)	394(+)
GT1-motif	AATCCACA	1522(-)	1522(-)	1522(-)	1452(-)	1452(-)	1452(-)	1452(-)	1451(-)	1447(-)
as-2-box	GATAatGATG	1533(+)	1533(+)	1533(+)	1463(+)	1463(+)	1463(+)	1463(+)	1462(+)	1458(+)
O2-site	GATGATGTGG	1536(+)	1536(+)	1536(+)	1466(+)	1466(+)	1466(+)	1466(+)	1465(+)	1461(+)
W box	TTGACC	183(+)	183(+)	183(+)	204(+)	204(+)	204(+)	204(+)	204(+)	204(+)
Skn-1 motif	GTCAAT	4	4	4	4	4	4	4	4	4
Unnamed 4	CTCC	12	12	12	11	11	11	11	11	11
CAAT-box		29	29	29	28	28	28	28	28	28
TATA-box		89	89	89	97	97	97	97	97	93

Transcription factor	Sequence of regulatory element	PW363				Rywal			Désirée	
		TGA2 (P1)	TGA2 (P2)	TGA2 (P3)	TGA2 (R1)	TGA2 (R2)	TGA2 (R3)	TGA2 (D1)	TGA2 (D2)	TGA2 (D3)
PBF	aAAAGG	55 (+)	55 (+)	55 (+)	55 (+)	55 (+)	55 (+)	55 (+)	55 (+)	55 (+)
PBF	aAAAGG	55 (-)	55 (-)	55 (-)	55 (-)	55 (-)	55 (-)	55 (-)	55 (-)	55 (-)
BPC1	AGAAAg	113 (+)	113 (+)	113 (+)	113 (+)	113 (+)	113 (+)	113 (+)	113 (+)	113 (+)
BPC1	AGAAAg	123 (+)	123 (+)	123 (+)						
BPC1	AGAAAg	196 (+)	196 (+)	196 (+)	217 (+)	217 (+)	217 (+)	217 (+)	217 (+)	217 (+)
GT-1	GTAATAa	301 (+)	301 (+)	301 (+)	321 (+)	321 (+)	321 (+)	321 (+)	321 (+)	321 (+)
At4g36620	atCAACCA	351 (+)	351 (+)	351 (+)						
RIN	cataTTTAAga	404 (+)	404 (+)	404 (+)						
Myb-15	TAACAA	422 (+)	422 (+)	422 (+)	355 (+)	355 (+)	355 (+)	355 (+)	355 (+)	355 (+)
AZF3	aaattATACTc	474 (-)	474 (-)	474 (-)	407 (-)	407 (-)	407 (-)	407 (-)	407 (-)	407 (-)
At3g51080	ttTTTCC	531 (-)	531 (-)	531 (-)	465 (-)	465 (-)	465 (-)	465 (-)	465 (-)	465 (-)
Myb-15	TAACAA	594 (+)	594 (+)	594 (+)	527 (+)	527 (+)	527 (+)	527 (+)	527 (+)	527 (+)
ASR-1	ACCCA	643 (+)	643 (+)	643 (+)	576 (+)	576 (+)	576 (+)	576 (+)	576 (+)	576 (+)
ASR-1	TGGGT	689 (-)	689 (-)	689 (-)	622 (-)	622 (-)	622 (-)	622 (-)	622 (-)	622 (-)
HSFA2	CCAAAA	710 (+)	710 (+)	710 (+)	643 (+)	643 (+)	643 (+)	643 (+)	643 (+)	643 (+)
AZF3	aaaatATACTc	782 (+)	782 (-)	782 (-)	715 (-)	715 (-)	715 (-)	715 (-)	715 (-)	715 (-)
BPC1	AGAAAg	862 (+)	862 (+)	862 (+)	792 (+)	792 (+)	792 (+)	792 (+)	792 (+)	793 (+)
OCSBF-1	ACGTG	954 (-)	954 (-)	954 (-)						881 (-)
ABI5	ACGTGg	954 (+)	954 (+)	954 (+)						881 (+)
OCSBF-1	CACGT	991 (+)	991 (+)	991 (+)	921 (+)	921 (+)	921 (+)	921 (+)	921 (+)	918 (+)
ASR-1	TGGGT	1077 (-)	1077 (-)	1077 (-)	1220 (-)	1220 (-)	1220 (-)	1220 (-)	1220 (-)	1004 (-)
CBNAC	tTGCTT	1169 (+)	1169 (+)	1169 (+)	1102 (+)	1102 (+)	1102 (+)	1102 (+)	1102 (+)	1096 (+)
HSFA2	tTTTGG	1246 (-)	1246 (-)	1246 (-)	1361 (-)	1361 (-)	1361 (-)	1361 (-)	1360 (-)	1172 (-)
ASR-1	TGGGT	1249 (-)	1249 (-)	1249 (-)	1010 (-)	1010 (-)	1010 (-)	1010 (-)	1010 (-)	1175 (-)
C1	ggggTAGTTgg	1280 (-)	1280 (-)	1280 (-)	1212 (-)	1212 (-)	1212 (-)	1212 (-)	1212 (-)	1206 (-)
ASR-1	TGGGT	1288 (-)	1288 (-)	1288 (-)	1290 (-)	1290 (-)	1290 (-)	1290 (-)	1289 (-)	1214 (-)
PBF	CCTTTt	1309 (+)	1309 (+)	1309 (+)	1241 (+)	1241 (+)	1241 (+)	1241 (+)	1241 (+)	1235 (+)
PBF	CCTTTt	1309 (-)	1309 (-)	1309 (-)	1241 (-)	1241 (-)	1241 (-)	1241 (-)	1241 (-)	1235 (-)
HSFA2	tTTTGG	1312 (-)	1312 (-)	1312 (-)	1287 (-)	1287 (-)	1287 (-)	1287 (-)	1286 (-)	1238 (-)
FLC	tttTTCTcttttgggtt	1348 (-)	1348 (-)	1348 (-)						
AGL20	ttttctttTTTGGg	1349 (+)	1349 (+)	1349 (+)	1169 (+)	1169 (+)	1169 (+)	1169 (+)	1169 (+)	
PI	tTTCTcttttggg	1350 (-)	1350 (-)	1350 (-)						
AGL15	TTTCTcttttgggtt	1351 (+)	1351 (+)	1351 (+)	1171 (+)	1171 (+)	1171 (+)	1171 (+)	1171 (+)	
AGL9	ttcttTTTTTg	1352 (+)	1352 (+)	1352 (+)						
HSFA2	tTTTGG	1357 (-)	1357 (-)	1357 (-)	1178 (-)	1178 (-)	1178 (-)	1178 (-)	1178 (-)	
ASR-1	TGGGT	1360 (-)	1360 (-)	1360 (-)	1181 (-)	1181 (-)	1181 (-)	1181 (-)	1181 (-)	
CBNAC	tTGCTT	1426 (+)	1426 (+)	1426 (+)	1356 (+)	1356 (+)	1356 (+)	1356 (+)	1355 (+)	1352 (+)
HSFA2	tTTTGG	1431 (-)	1431 (-)	1431 (-)	1244 (-)	1244 (-)	1244 (-)	1244 (-)	1244 (-)	1357 (-)
HSFA4A	aCTATT	1456 (+)	1456 (+)	1456 (+)	1386 (+)	1386 (+)	1386 (+)	1386 (+)	1385 (+)	1382 (+)
ATHSFA1D	TGTAGT	1474 (-)	1474 (-)	1474 (-)	1404 (-)	1404 (-)	1404 (-)	140		

Supplementary Table 4: Cis-regulatory elements in promoter sequences of MYC2 promoters from different potato cultivars, identified by PlantCARE. Promoter sequences from PW636 (MYC2(P1): 1073 nt, MYC2(P2): 1353 nt, MYC2(P3): 1352 nt), Rywal (MYC2(R1): 1095 nt, MYC2(R2): 1093 nt, MYC2(R3): 1347 nt) and Désirée (MYC2(D1): 1073 nt and MYC2(D2): 1073 nt) were analysed using PlantCARE web service. Promoters, used for a more detailed investigation, are grey shaded, while known defence-related regulatory elements are green shaded. The position, the strand and the sequence of each cis-regulatory element is specified. If more than three repeats of the particular regulatory element were present in the sequence, only the number of these elements is stated (with no position information).

Name of regulatory element	Sequence of regulatory element	MYC2 (P1)	PW363 MYC2 (P2)	MYC2 (P3)	MYC2 (R1)	Rywal MYC2 (R2)	MYC2 (R3)	Désirée MYC2 (D1)	MYC2 (D2)
GCN4 motif	TGAGTCA	13(+)	13(+)	13(+)	13(+)	13(+)	13(+)	13(+)	13(+)
HSE	AGAAAATTCG	25(+)						25(+)	25(+)
AT-rich	TAAAATACT		31(-)	31(-)	31(-)	31(-)	31(-)		
ARE	TGGTTT	39(-)			47(-)	47(-)		39(-)	39(-)
chs-CMA2a	TCACCTGA	133(+)	140(+)	140(+)			140(+)	133(+)	133(+)
MBS	TAACTG	162(+)	169(+)	169(+)	170(+)	170(+)	169(+)	162(+)	162(+)
TATC-box	TATCCCA		243(-)						
chs-CMA1a	TTACTTAA	275(+)	286(+)	288(+)	285(+)	285(+)	284(+)	275(+)	275(+)
AT-rich	TAAAATACT	302(-)	313(-)	315(-)	312(-)	312(-)	311(-)	302(-)	302(-)
ARE	TGGTTT	322(-)	1204(-)	1202(-)	945(-)	945(-)		923(-)	922(-)
HSE	AAAAAATTTTC	337(+)	348(+)	350(+)	347(+)	347(+)	346(+)	337(+)	337(+)
3-AF1 binding	AAGAGATATTT	338(-)	349(-)	351(-)	348(-)	348(-)	347(-)	338(-)	338(-)
AT-rich	ATAGAAATCAA	363(-)		376(-)	373(-)	373(-)	372(-)	363(-)	363(-)
TCA-element	GAGAAGAATA	505(-)	516(-)	518(-)	514(-)	514(-)	514(-)	505(-)	505(-)
P-box	GCCTTTTGAGT		548(+)	550(+)			546(+)		
P-box	CCTTTTG		549(+)	551(+)			547(+)		
circadian	CAANNNNATC				560(+)	560(+)			
AAGAA-motif	gGTAAAGAAA			561(+)			557(+)		
AAGAA-motif	GAAAGAA			563(+)			559(+)		
ACE	AAAACGTTTA	563(+)	576(+)		573(+)	573(+)		563(+)	563(+)
TC-rich repeats	ATTTCTTCA			573(-)			569(-)		
3-AF1 binding	AAGAGATATTT							577(+)	577(+)
Unnamed 6	taTAAATATct	580(-)	925(-)	923(-)	590(-)	590(-)	919(-)	580(-)	580(-)
AT1-motif	ATTAATTTTACA	614(+)						614(+)	614(+)
OBP-1 site	TACACTTTTGG						704(+)		
AT-rich	TAAAATACT	775(+)						776(+)	775(+)
GCN4 motif	TGAGTCA		746(-)	739(-)			735(-)		
HSE	AAAAAATTTTC	786(-)	594(-)	596(-)			592(-)	787(-)	786(-)
GCN4 motif	TGAGTCA		837(-)				831(-)		
Unnamed 1	CGTGG	849(+)	1131(+)	1127(+)	872(+)	872(+)	1124(+)	850(+)	849(+)
Box-W1/W box	TTGACC		875(+)						
MBS	CGGTCA		876(-)						
GAG-motif	AGAGAGT	873(-)	815(-)		896(-)	896(-)		874(-)	873(-)
HD-Zip 1/HD-	CAAT(A/T)ATTG		962(-)	960(-)			956(-)		
Sp1	CC(G/A)CCC	992(+)	1272(+)	1271(+)	1014(+)		1266(+)	992(+)	992(+)
HSE	AGAAAATTCG	998(+)		1017(+)			1013(+)		
TC-rich repeats	ATTTCTTCA		1074(+)						
AC-I	(T/C)(T/C)(C/T)ACC(T/C)AC	1063(+)	1343(+)	1342(+)	1085(+)	1083(+)	1337(+)	1063(+)	1063(+)
GAG-motif	AGAGAGT		1155(-)	1153(-)			1148(-)		
Skn-1 motif	GTCAT	4	5	4	5	5	5	4	4
5'UTR Py-rich	TTTCTTCTCT	2	4	2	2	2	2	2	2
Box 4	ATTAAT	4	3	5	4	4	5	4	4
Unnamed 4	CTCC	5	6	7	6	6	8	5	5
TATA-box		73	95	96	78	78	96	73	73
CAAT-box		28	38	41	28	28	41	28	28

Supplementary Table 5: Transcription factor binding sites in promoter sequences of MYC2 promoters from different potato cultivars, identified by TRANSFAC. TRANSFAC database was used to analyse promoter sequences from PW636 (MYC2(P1): 1073 nt, MYC2(P2): 1353 nt, MYC2(P3): 1352 nt), Rywal (MYC2(R1): 1095 nt, MYC2(R2): 1093 nt, MYC2(R3): 1347 nt) and Désirée (MYC2(D1): 1073 nt and MYC2(D2): 1073 nt) to predict transcription factor binding sites (regulatory elements). Promoters used for more detailed investigation are grey shaded, while some of the known defence related regulatory elements are green shaded. The position, the strand and the sequence of each transcription factor binding site is specified in the table as well as the transcription factors, which bind to these transcription factor binding sites.

Transcription factor	Regulatory element	PW				Rywal		Désirée	
		MYC2 (P1)	MYC2 (P2)	MYC2 (P3)	MYC2 (R1)	MYC2 (R2)	MYC2 (R3)	MYC2 (D1)	MYC2 (D2)
TRB2	tTAGGGca	3 (-)	3 (-)	3 (-)	3 (-)	3 (-)	3 (-)	3 (-)	3 (-)
CBNAC	AAGCAa	35 (-)	43 (-)	43 (-)	43 (-)	43 (-)	43 (-)	35 (-)	35 (-)
ATHB-6	ccAATGAtt	42 (+)	49 (+)	49 (+)	50 (+)	50 (+)	49 (+)	42 (+)	42 (+)
PBF	CCTTTt	151 (-)	158 (-)	158 (-)	159 (-)	159 (-)	158 (-)	151 (-)	151 (-)
PBF	CCTTTt	151 (+)	158 (+)	158 (+)	159 (+)	159 (+)	158 (+)	151 (+)	151 (+)
MYB3R5	taaatgtaaCTGTTg	156 (+)	163 (+)	163 (+)	164 (+)	164 (+)	163 (+)	156 (+)	156 (+)
MYB3R4	aaatgtaaCTGTTgg	157 (+)	164 (+)	164 (+)	165 (+)	165 (+)	164 (+)	157 (+)	157 (+)
MYB3R1	aaatgtaaCTGTTgg	157 (+)	164 (+)	164 (+)	165 (+)	165 (+)	164 (+)	157 (+)	157 (+)
BPC1	ctgttgctctcTCTCtctctc		172 (-)						
BPC6	tgttgctctcTCTCtctctc		173 (+)	173 (+)					
BPC1	gttgctctctcTCTCtctctc		174 (-)	174 (-)					
BPC6	tgttgctctcTCTCtctctc		175 (+)	175 (+)					
BPC1	tggctctctcTCTCtctctc		176 (-)	176 (-)					
BPC6	ggctctctcTCTCtctctc		177 (+)	177 (+)					
BPC1	gctctctctcTCTCtctctc		178 (-)	178 (-)					
BPC6	ctctctctcTCTCtctctc		179 (+)	179 (+)					
BPC1	tctctctctcTCTCtctctc		180 (-)						
BPC6	ctctctctcTCTCtctctc		181 (+)	181 (+)					
BPC6	ctctctctcTCTCtctctc		183 (+)						
GT-1	taTTTAC	272 (-)	283 (-)	285 (-)	282 (-)	282 (-)	281 (-)	272 (-)	272 (-)
ARF16	ttaaTgacTAAAGcttaaa	307 (-)	318 (-)	320 (-)			316 (-)	307 (-)	307 (-)
AT4G12670	tAAAGGcttaacaataaagtaaaaa	316 (+)	327 (+)	329 (+)			325 (+)	316 (+)	316 (+)
TRP2	aaagcttAAACaataaagta	318 (+)	329 (+)	331 (+)	328 (+)	328 (+)	327 (+)	318 (+)	318 (+)
TRP1	aaagctTAAACaataaagtaaaaa	318 (+)	329 (+)	331 (+)	328 (+)	328 (+)	327 (+)	318 (+)	318 (+)
SRS7	aaAGCTTaaacaataaagtaaaaa	318 (+)	329 (+)	331 (+)			327 (+)	318 (+)	318 (+)
DOF4.3	AAAAATctcttt	339 (-)	350 (-)	352 (-)	349 (-)	349 (-)	348 (-)	339 (+)	339 (-)
DOF4.3	aaaatcTCTTT	339 (+)	350 (+)	352 (+)	349 (+)	349 (+)	348 (+)	339 (-)	339 (+)
AZF3	gAGTATatttt	355 (+)	366 (+)	368 (+)	365 (+)	365 (+)	364 (+)	355 (+)	355 (+)
Myb-15	tTGTTA				397 (-)	397 (-)			
ATHB-6	tcAATAAtt	406 (+)	417 (+)	419 (+)	416 (+)	416 (+)	415 (+)	406 (+)	406 (+)
ATHB1	tcAATAAttta	406 (+)	417 (+)	419 (+)	416 (+)	416 (+)	415 (+)	406 (+)	406 (+)
NAC078	tttTATTGaaaataaag	419 (-)	430 (-)	432 (-)	429 (-)	429 (-)	428 (-)	419 (-)	419 (-)
SQUA	acAAAAAatag	476 (-)	487 (-)	489 (-)	486 (-)	486 (-)	485 (-)	476 (-)	476 (-)
REM16	ccttcctttgagtgGGAaAa		545 (+)	547 (+)			543 (+)		
DEL2	cttccttttgAGTGGgaaaa		546 (+)	548 (+)			544 (+)		
PBF	CCTTTt		549 (-)	551 (-)			547 (-)		
PBF	CCTTTt		549 (+)	551 (+)			547 (+)		
DEL1	tttgagTGGGAaaaaaaatc		552 (-)	554 (-)			550 (-)		
HSFA4A	AATAGt	481 (-)		494 (-)			490 (-)	481 (-)	481 (-)
At3g51080	GGAAaAa	549 (+)	560 (+)					549 (+)	549 (+)
MYB3R1	aaAACGAttaaagta	563 (-)	576 (-)		573 (-)	573 (-)		563 (-)	563 (-)
MYB3R4	aaAACGAttaaagta	563 (-)	576 (-)		573 (-)	573 (-)		563 (-)	563 (-)
MYB3R5	aAACGAttaaagtaa	564 (-)	577 (-)		574 (-)	574 (-)		564 (-)	564 (-)
AT4G27900	ttagacatttttTCGTTtatt		591 (-)	593 (-)	588 (-)	588 (-)	589 (-)		
GT-1	cttaaaaAAATA							629 (-)	
TRP1	atatctTAAAAactaataaataa		639 (+)						
MYB3R4	ttAACGAttacaact	649 (-)	682 (-)	675 (-)	670 (-)	670 (-)	671 (-)	650 (-)	649 (-)
MYB3R1	ttAACGAttacaact	649 (-)	682 (-)	675 (-)	670 (-)	670 (-)	671 (-)	650 (-)	649 (-)
MYB3R5	tAACGAttacaacta	650 (-)	683 (-)	676 (-)	671 (-)	671 (-)	672 (-)	651 (-)	650 (-)
HMG-1	gattACAAC	654 (-)	687 (-)	680 (-)	675 (-)	675 (-)	676 (-)	655 (-)	654 (-)
HSFA4A	aCTATT	685 (+)	718 (+)	711 (+)	706 (+)	706 (+)		686 (+)	685 (+)
HSFA2	tTTTGG						709 (-)		
NAC92	tttggCTTGTTt		744 (-)	737 (-)			733 (-)		
GT-1	TTTTTttttact	791 (+)					790 (+)	792 (+)	791 (+)
PBF	aAAAGG			794 (+)			790 (-)		
PBF	aAAAGG			794 (-)			794 (+)		
At3g51080	GGAAaAa			798 (+)			799 (-)		
ARF16	atttcgtTTTTGgagagtaaa						837 (+)		
CBF3	tttgaTCGACgatt						868 (+)		
MADS-A	ttttaaAAAATgaaat						901 (+)		
ATHSFA1D	aCTACA	800 (+)		935 (+)	823 (+)	823 (+)	931 (+)	801 (+)	800 (+)
AZF3	aaaagATACTa		805 (-)	803 (-)	799 (-)	799 (-)			
BPC1	AGAAAg	809 (+)		942 (+)			938 (+)	810 (+)	809 (+)

AT1G53910	ccgattaattgCGGGGcctt	828 (+)			851 (+)	851 (+)		829 (+)	828 (+)
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Transcription factor	Regulatory element	MYC2 (P1)	MYC2 (P2)	MYC2 (P3)	MYC2 (R1)	MYC2 (R2)	MYC2 (R3)	MYC2 (D1)	MYC2 (D2)
RAP2.1	ttggCGGGGcctt	836 (+)			859 (+)	859 (+)		837 (+)	836 (+)
RAP2-10	tggCGGGGcc	837 (-)			860 (-)	860 (-)		838 (-)	837 (-)
RAP2-6	tggCGGGGcc	837 (-)			860 (-)	860 (-)		838 (-)	837 (-)
RRTF1	tggCGGGGcc	837 (-)			860 (-)	860 (-)		838 (-)	837 (-)
E2L	tGGCGGg	837 (+)			860 (+)	860 (+)		838 (+)	837 (+)
LBD2	TGGCGggccttcgt	837 (-)			860 (-)	860 (-)		838 (-)	837 (-)
MADS-A	atttcATTTTcgaga		843 (-)						
LBD2	gcggggccttCGTGG	839 (+)			862 (+)	862 (+)		840 (+)	839 (+)
LOB	gccttcgtggaaccGCCAgc	844 (+)			867 (+)	867 (+)		845 (+)	844 (+)
LBD2	CTTCGtggaaccgc	846 (-)			869 (-)	869 (-)		847 (-)	846 (-)
LBD23	tTCGTGgaaccgcc	847 (-)			870 (-)	870 (-)		848 (-)	847 (-)
LBD2	tcgtggaaccGCCA	848 (+)			871 (+)	871 (+)		849 (+)	848 (+)
TCP14	gtggaaCCCGCagcttttg	850 (-)			873 (-)	873 (-)		851 (-)	850 (-)
TCP7	GTGGAaccgc	850 (+)			873 (+)	873 (+)		851 (+)	850 (+)
TCP16	GTGGAaccgc	850 (-)			873 (-)	873 (-)		851 (-)	850 (-)
E2L	cCCGCCa	856 (-)			879 (-)	879 (-)		857 (-)	856 (-)
AT4G27900	aatttaagtttgaCCGACgatt		866 (-)						
CBF3	tttgaTCGACgatt			872 (+)					
WRKY48	gtTTGACcga		873 (-)						
CBF3	tttgaCCGACgatt		874 (+)						
ERF039	tgaCCGACga		876 (-)						
AT1G77200	tgaCCGACgatttg		876 (+)						
BPC1	cTTTCT	878 (-)						879 (-)	878 (-)
HSFA1E	cCGACG		879 (+)						
DREB1B	CCGAC		879 (+)						
MADS-A	ttttaaAAAATgaaat			905 (+)					
MYB24	aCCAAActc	924 (-)						925 (-)	924 (-)
ATHSFA1D	aCTACA		937 (+)	959 (+)					
ATHB-6	tcAATAAtt		961 (+)	959 (+)			955 (+)		
ATHB5	tcAATAAttga		961 (+)	959 (+)			955 (+)		
ATHB1	tcAATAAttga		961 (+)	960 (-)			955 (+)		
ATHB-5	caATAAttg		962 (-)	961 (-)			956 (-)		
ATHB-6	aaTAATTga		963 (-)				957 (-)		
ARR1	aaaGAATCtc		1019 (+)						
WRKY	TGACTgaatac	1051 (-)		1063 (+)				1051 (-)	1051 (-)
bHLH28	gtatGTACGttt			1065 (+)					
SPL5	atGTACGtt			1066 (+)			1060 (+)		
ARF16	tgtacgtTTTTGttactacaa			1066 (+)					
SPL4	tGTACGttt			1066 (+)			1061 (+)		
SPL12	tGTACGtt						1061 (+)		
HSFA4A	AATAGt		1066 (-)						
ASR-1	ACCCA	1067 (+)		1075 (-)				1067 (+)	1067 (+)
Myb-15	tTGTTA								
AZF3	tAGTATgtttt		1068 (+)	1080 (+)					
ATHSFA1D	aCTACA		1082 (+)	1089 (+)					
BPC1	AGAAAg		1091 (+)				1084 (+)		
bZIP53	accgattACTTGgcg		1109 (+)						
bZIP44	accgattACTTGgcg		1109 (+)	1108 (+)					
AT1G53910	ccgattacttgCGGGGcctt		1110 (+)	1116 (+)			1103 (+)		
RAP2.1	ttggCGGGGcctt		1118 (+)	1117 (-)			1111 (+)		
RAP2-10	tggCGGGGcc		1119 (-)	1117 (-)			1112 (-)		
RAP2-6	tggCGGGGcc		1119 (-)	1117 (-)			1112 (-)		
RRTF1	tggCGGGGcc		1119 (-)	1117 (+)			1112 (-)		
E2L	tGGCGGg		1119 (+)	1117 (-)			1112 (+)		
LBD2	TGGCGggccttcgt		1119 (-)	1119 (+)			1112 (-)		
LBD2	gcggggccttCGTGG		1121 (+)	1124 (+)			1114 (+)		
LOB	gccttcgtggaaccGCCAgc		1126 (+)	1126 (-)			1119 (+)		
LBD2	CTTCGtggaaccgc		1128 (-)	1127 (-)			1121 (-)		
LBD23	tTCGTGgaaccgcc		1129 (-)	1128 (+)			1122 (-)		
LBD2	tcgtggaaccGCCA		1130 (+)	1130 (-)			1123 (+)		
TCP16	GTGGAaccgc		1132 (-)	1130 (+)			1125 (-)		
TCP7	GTGGAaccgc		1132 (+)	1130 (-)			1125 (+)		
TCP14	gtggaaCCCGCagcgtttg		1132 (-)	1136 (-)			1125 (-)		
E2L	cCCGCCa		1138 (-)	1140 (+)			1131 (-)		
At5g54070	cCAGCG		1142 (+)	1158 (-)	883 (+)	883 (+)	1135 (+)		
BPC1	ctTTTCT		1160 (-)	1204 (-)	901 (-)	901 (-)	1153 (-)		
MYB24	aCCAAActc		1206 (-)	1262 (-)	947 (-)	947 (-)			
RAP2.1	atctCTCCGccac		1263 (-)	1262 (-)	1005 (-)		1257 (-)		
AT1G53910	atctctCCGCacccccctgtt		1263 (-)	1262 (-)	1005 (-)		1257 (-)		
AT1G28160	atctctcGCCAacc		1263 (-)	1262 (+)	1005 (-)		1257 (-)		
At2g33710	atctctcGCCAacc		1263 (+)	1262 (+)	1005 (+)		1257 (+)		
ERF73	atctctcGCCAaccctgtt		1263 (+)	1262 (-)	1005 (+)		1257 (+)		
RAP2.6	atctctcGCCAacc		1263 (-)	1264 (+)	1005 (-)		1257 (-)		
RAP2-10	ctCTCCGcca		1265 (+)	1264 (+)	1007 (+)	1009 (+)	1259 (+)		
RRTF1	ctCTCCGcca		1265 (+)	1265 (+)	1007 (+)		1259 (+)		
ERF4	tctcCGCCAaccctt		1266 (+)	1265 (+)	1008 (+)		1260 (+)		
ERF9	tctcCGCCAaccctgttttc		1266 (-)	1265 (-)	1008 (-)		1260 (-)		
AT1G53910	tctccGCCAaccctgttttc		1266 (-)	1330 (-)	1008 (-)		1260 (-)		
WRKY	TGACTgaatac		1331 (-)	1346 (+)	1073 (-)	1071 (-)	1325 (-)		

ASR-1 **ACCCA** 1347 (+) 1089 (+) 1087 (+) 1341 (+)

Supplementary Table 6: Cis-regulatory elements in promoter sequences of MC promoters from different potato cultivars, identified by PlantCARE. 921 nt long promoter sequences from PW636 (MC(P1) and MC(P2)), 1002 nt long promoter sequences from Rywal (MC(R1), MC(R2), MC(R3)) and 1002 nt long promoter sequences from Désirée (MC(D1), MC(D2) and MC(D3)) were analysed using PlantCARE web service. Promoters, used for a more detailed investigation, are grey shaded, while known defence-related regulatory elements are green shaded. The position, the strand and the sequence of each cis-regulatory element is specified. If more than three repeats of the particular regulatory element were present in the sequence, only the number of these elements is stated (with no position information).

Name of regulatory element	Sequence of regulatory element	PW363		Rywal			Désirée		
		MC (P1)	MC (P2)	MC (R1)	MC (R2)	MC (R3)	MC (D1)	MC (D2)	MC (D3)
CGTCA-motif	CGTCA	25(+)	25(+)	249(+)	249(+)	249(+)	249(+)	249(+)	249(+)
TGACG-motif	TGACG	25(-)	25(-)	249(-)	249(-)	249(-)	249(-)	249(-)	249(-)
GATA-motif	GATAGGA			29(-)	29(-)	29(-)	29(-)	29(-)	29(-)
5UTR Pv-rich stretch	TTTCTTCTCT		43(+)	653(-)	653(-)	653(-)	653(-)	653(-)	653(-)
Box II	TGGTAATAA			63(-)	63(-)	63(-)	63(-)	63(-)	63(-)
TCT-motif	TCITAC	69(+)	69(+)						
Skn-1 motif	GTCAT			86(-)	86(-)	86(-)	86(-)	86(-)	86(-)
CATT-motif	GCAATC			105(+)	105(+)	105(+)	105(+)	105(+)	105(+)
HSE	AAAAAATTTC			185(-)	185(-)	185(-)	185(-)	185(-)	185(-)
AE-box	AGAAACAA			201(-)	201(-)	201(-)	201(-)	201(-)	201(-)
MBS	CAACTG	240(+)	240(+)	2(+)	2(+)	2(+)	2(+)	2(+)	2(+)
TATCCAT/C-motif	TATCCAT			299(-)	299(-)	299(-)	299(-)	299(-)	299(-)
TC-rich repeats	ATTTTCTTCA	314(+)	314(+)	389(+)	389(+)	389(+)	389(+)	389(+)	389(+)
MBS	CAACTG			336(+)	336(+)	336(+)	336(+)	336(+)	336(+)
Sp1	CC(G/A)CCC	362(+)	362(+)	69(+)	69(+)	69(+)	69(+)	69(+)	69(+)
TC-rich repeats	ATTTTCTTCA	371(+)	371(+)	446(+)	446(+)	446(+)	446(+)	446(+)	446(+)
Unnamed 4	CTCC	376(+)	376(+)	228(+)	228(+)	228(+)	228(+)	228(+)	228(+)
ARE	TGGTTT	395(-)	359(-)	585(+)	585(+)	585(+)	585(+)	585(+)	585(+)
Unnamed 4	CTCC	406(-)	406(-)	331(+)	331(+)	331(+)	331(+)	331(+)	331(+)
circadian	CAANNNNATC	422(-)	422(-)	674(+)	674(+)	674(+)	674(+)	674(+)	674(+)
Unnamed 4	CTCC	452(-)	452(-)	451(+)	451(+)	451(+)	451(+)	451(+)	451(+)
Unnamed 1/3	CGTGG	462(+)	462(+)	537(+)	537(+)	537(+)	537(+)	537(+)	537(+)
ARE	TGGTTT	510(+)	510(+)	434(-)	434(-)	434(-)	434(-)	434(-)	434(-)
CGTCA-motif	CGTCA	597(-)	597(-)	671(+)	671(+)	671(+)	671(+)	671(+)	671(+)
TGACG-motif	TGACG	597(-)	597(-)	671(-)	671(-)	671(-)	671(-)	671(-)	671(-)
GT1-motif	GGTTAA			610(-)	610(-)	610(-)	610(-)	610(-)	610(-)
TC-rich repeats	ATTTTCTTCA			655(-)	655(-)	655(-)	655(-)	655(-)	655(-)
TCCACCT-motif	TCCACCT			772(-)	772(-)	772(-)	772(-)	772(-)	772(-)
chs-CMA2a	TCACTTGA			779(+)	779(+)	779(+)	779(+)	779(+)	779(+)
circadian	CAANNNNATC	793(+)	793(+)	874(+)	874(+)	874(+)	874(+)	874(+)	874(+)
Unnamed 6	taTAAATAtct	809(-)	809(-)	890(-)	890(-)	890(-)	890(-)	890(-)	890(-)
G-Box	CACGTT	819(-)	819(-)	900(-)	900(-)	900(-)	900(-)	900(-)	900(-)
5UTR Pv-rich stretch	TTTCTTCTCT	832(+)	832(+)	913(+)	913(+)	913(+)	913(+)	913(+)	913(+)
ACE	AAAACGTTTA			838(-)	838(-)	838(-)	838(-)	838(-)	838(-)
Box 4	ATTAAT	905(-)	905(-)	986(-)	986(-)	986(-)	986(-)	986(-)	986(-)
MBS	TAACTG			931(-)	931(-)	931(-)	931(-)	931(-)	931(-)
CAAT-box		21	21	21	21	21	21	21	21
TATA-box		52	54	49	50	50	50	50	50

Supplementary Table 7: Transcription factor binding sites in promoter sequences of MC promoters from different potato cultivars, identified by TRANSFAC. TRANSFAC database was used to analyse promoter sequences from PW636 (MC(P1) and MC(P2)), 1002 nt long promoter sequences from Rywal (MC(R1), MC(R2), MC(R3)) and 1002 nt long promoter sequences from Désirée (MC(D1), MC(D2) and MC(D3)) to predict transcription factor binding sites (regulatory elements). Promoters, used for a more detailed investigation, are grey shaded, while some of the known defence related regulatory elements are green shaded. The position, the strand and the sequence of each transcription factor binding site is specified in the table as well as the transcription factors, which bind to these transcription factor binding sites.

Transcription factor	Regulatory element	PW363		Rywal			Désirée		
		MC (P1)	MC (P2)	MC (R1)	MC (R2)	MC (R3)	MC (D1)	MC (D2)	MC (D3)
MYB3R1	ttacaccaTCGTTac	4 (+)	4 (+)						
ZML2	caCCATCgttacaatcttc	7 (+)	7 (+)						
AT3G25990	caccatCGTTAcaatcttcgt	7 (+)	7 (+)						
GATA15	cATCGTTacaatcttcgtc	10 (+)	10 (+)						
ANAC094	cacttattattcACGGGga			10 (-)	10 (-)	10 (-)	10 (-)	10 (-)	10 (-)
GT-1	taTTCAC			17 (-)	17 (-)	17 (-)	17 (-)	17 (-)	17 (-)
DEL1	tattcaCGGGAatcctatcac			17 (-)	17 (-)	17 (-)	17 (-)	17 (-)	17 (-)
TGA1A	tTCGTCa	23 (+)	23 (+)						
BPC1	cTTTCT	30 (-)	30 (-)						
AT3G25990	ttttgagaatTAACtactctt	52 (-)	52 (-)						
DDF2	taaattgcCTACAtga			75 (-)	75 (-)	75 (-)	75 (-)	75 (-)	75 (-)
WRKY	TGACAaagtaa			87 (+)	87 (+)	87 (+)	87 (+)	87 (+)	87 (+)
BPC1	AGAAAg	90 (+)	90 (+)						
DOF4.3	AAAAGttcttt	145 (-)	145 (-)						
DOF4.3	aaaagtTCTTT	145 (+)	145 (+)						
DOF4.3	AAAGTtctttt	146 (-)	146 (-)						
DOF4.3	aaagttCTTTT	146 (+)	146 (+)						
GT-1	TCTTTttttaaa	151 (+)	151 (+)						
GT-1	tttaaaaAAAAA	157 (-)	157 (-)						
MYB24	aagTTGGt	195 (+)	195 (+)						
MYB3R5	gaaaagttaCTGTTg	225 (+)	225 (+)						
MYB3R1	aaaagttaCTGTTgc	226 (+)	226 (+)						
MYB3R4	aaaagttaCTGTTgc	226 (+)	226 (+)						
DOF4.3	AAAGTtactgt	227 (-)	227 (-)						
DOF4.3	aaagttACTGT	227 (+)	227 (+)						
LBD2	GTTTCgtcaatgtcgg			246 (-)	246 (-)	246 (-)	246 (-)	246 (-)	246 (-)
TGA1A	tTCGTCa			247 (+)	247 (+)	247 (+)	247 (+)	247 (+)	247 (+)
LBD2	tcgtcaatgtCGGTT			248 (+)	248 (+)	248 (+)	248 (+)	248 (+)	248 (+)
AT1G77200	gtcaatGTCGGttt			250 (-)	250 (-)	250 (-)	250 (-)	250 (-)	250 (-)
DDF2	caaTGTCGgtttaaa			252 (+)	252 (+)	252 (+)	252 (+)	252 (+)	252 (+)
CBF3	caatGTCGgtttaa			252 (-)	252 (-)	252 (-)	252 (-)	252 (-)	252 (-)
ARF8	aaTGTCGgt			253 (+)	253 (+)	253 (+)	253 (+)	253 (+)	253 (+)
RAP2.1	atgtCGGTTtaaa			254 (+)	254 (+)	254 (+)	254 (+)	254 (+)	254 (+)
PHYPADRAFT 64121	atgTCGGTtaaa			254 (+)	254 (+)	254 (+)	254 (+)	254 (+)	254 (+)
ARF1	atGTCGgt			254 (-)	254 (-)	254 (-)	254 (-)	254 (-)	254 (-)
ERF039	atGTCGgtt			254 (+)	254 (+)	254 (+)	254 (+)	254 (+)	254 (+)
DRE1C	ATGTCggt			254 (+)	254 (+)	254 (+)	254 (+)	254 (+)	254 (+)
DREB1B	GTCGG			256 (+)	256 (+)	256 (+)	256 (+)	256 (+)	256 (+)
ZML1	tcattGTTTcatct	312 (+)	312 (+)						
GATA15	cATGTTTctcatctcttt	313 (+)	313 (+)	388 (+)	388 (+)	388 (+)	388 (+)	388 (+)	388 (+)
ZML2	caTGTTCttcatctcttt	313 (+)	313 (+)	388 (+)	388 (+)	388 (+)	388 (+)	388 (+)	388 (+)
ASR-1	ACCCA	364 (+)	364 (+)						
HSFA2	CCAAa	366 (+)	366 (+)						
GATA16	catgtatTTCATgttcttcatctt			379 (+)	379 (+)	379 (+)	379 (+)	379 (+)	379 (+)
ATHSFA1D	TGTAGt			381 (-)	381 (-)	381 (-)	381 (-)	381 (-)	381 (-)
ZML1	tcattGTTTcatct			387 (+)	387 (+)	387 (+)	387 (+)	387 (+)	387 (+)
RIN	caaaTTTTAga	389 (+)	389 (+)						
AT3G25990	aaccacTGTAAtcttctcca			435 (+)	435 (+)	435 (+)	435 (+)	435 (+)	435 (+)
ANAC042	attaccattcaaaACGGAG	436 (-)	436 (-)						
ASR-1	ACCCA	439 (+)	439 (+)	514 (+)	514 (+)	514 (+)	514 (+)	514 (+)	514 (+)
MYB3R1	aaAACGGagctcggt	447 (-)	447 (-)						
MYB3R4	aaAACGGagctcggt	447 (-)	447 (-)						
MYB3R5	aAACGGagctcggt	448 (-)	448 (-)						
CAMTA1	gaagCTCGTtcg			527 (+)	527 (+)	527 (+)	527 (+)	527 (+)	527 (+)
WRKY43	(t/ala)GCAAAat	468 (+)	468 (+)	543 (+)	543 (+)	543 (+)	543 (+)	543 (+)	543 (+)
DOF4.3	AAACTaacttt	494 (-)	494 (-)	569 (-)	569 (-)	569 (-)	569 (-)	569 (-)	569 (-)
DOF4.3	aaactaACTTT	494 (+)	494 (+)	569 (+)	569 (-)	569 (-)	569 (-)	569 (-)	569 (-)
AT3G25990	aaactaactTTAATGgtggt	494 (-)	494 (-)	569 (-)	569 (-)	569 (-)	569 (-)	569 (-)	569 (-)
HMG-1	GTTGTgctt	519 (+)	519 (+)	594 (+)	594 (+)	594 (+)	594 (+)	594 (+)	594 (+)
AT3G25990	acctatAGTTAgtaatttaa	558 (+)	558 (+)						
MYB3R5	aagtttaaCCTTTa			605 (+)	605 (+)	605 (+)	605 (+)	605 (+)	605 (+)
MYB3R1	agtttttaaCCTTTa			606 (+)	606 (+)	606 (+)	606 (+)	606 (+)	606 (+)
MYB3R4	agtttttaaCCTTTa			606 (+)	606 (+)	606 (+)	606 (+)	606 (+)	606 (+)

Continued

Transcription factor	Regulatory element	MC (P1)	MC (P2)	MC (P3)	MC (R1)	MC (R2)	MC (R3)	MC (D1)	MC (D2)
C1	cctaTAGTTag	559 (-)	559 (-)	633 (-)	633 (-)	633 (-)	633 (-)	633 (-)	633 (-)
AT4G27900	a(g/a)gaaaataattaCCGTCAAat	583 (-)	583 (-)	657 (-)	657 (-)	657 (-)	657 (-)	657 (-)	657 (-)
DEL1	(g/a)gaaaataatTACCGtcaaat	584 (+)	584 (+)	658 (+)	658 (+)	658 (+)	658 (+)	658 (+)	658 (+)
MYB3R5	aaataattaCCGTCa	587 (+)	587 (+)	661 (+)	661 (+)	661 (+)	661 (+)	661 (+)	661 (+)
MYB3R1	aataattaCCGTCAa	588 (+)	588 (+)	662 (+)	662 (+)	662 (+)	662 (+)	662 (+)	662 (+)
MYB3R4	aataattaCCGTCAa	588 (+)	588 (+)	662 (+)	662 (+)	662 (+)	662 (+)	662 (+)	662 (+)
AT1G77200	ttaCCGTCAaataa	593 (+)	593 (+)	667 (+)	667 (+)	667 (+)	667 (+)	667 (+)	667 (+)
ANAC042	tACCGTcaaatataaacctcaa	594 (+)	594 (+)						
ANAC094	tACCGTcaaatataaacctc	594 (+)	594 (+)						
TCP16	attaggACCAC	635 (+)	635 (+)	709 (+)	709 (+)	709 (+)	709 (+)	709 (+)	709 (+)
TCP24	attaGGACCact	635 (+)	635 (+)	709 (+)	709 (+)	709 (+)	709 (+)	709 (+)	709 (+)
AT4G27900	tegegaatatttaCCATTtaat	650 (-)	650 (-)	724 (-)	724 (-)	724 (-)	724 (-)	724 (-)	724 (-)
MYB3R5	gaataatttaCCATTt	654 (+)	654 (+)	728 (+)	728 (+)	728 (+)	728 (+)	728 (+)	728 (+)
GT-1	taTTTAC	657 (-)	657 (-)	731 (-)	731 (-)	731 (-)	731 (-)	731 (-)	731 (-)
EDT1	catTTAATgc	664 (+)	664 (+)	738 (+)	738 (+)	738 (+)	738 (+)	738 (+)	738 (+)
DOF4.3	AATGAgctcttt	735 (-)	735 (-)						
DOF4.3	aatgagTCITT	735 (+)	735 (+)						
GT-1	TATTTttttaaa	752 (+)	752 (+)						
AGL9	aacttTTTTGg	763 (+)	763 (+)						
LBD2	tcgaggaaggTGGAT			765 (+)	765 (+)	765 (+)	765 (+)	765 (+)	765 (+)
MYB3R5	ttatttttaaACGTTt			832 (+)	832 (+)	832 (+)	832 (+)	832 (+)	832 (+)
MYB3R1	tatttttaaACGTTt			833 (+)	833 (+)	833 (+)	833 (+)	833 (+)	833 (+)
MYB3R4	tatttttaaACGTTt			833 (+)	833 (+)	833 (+)	833 (+)	833 (+)	833 (+)
HSFA2	tTTTGG	768 (-)	768 (-)	845 (-)	845 (-)	845 (-)	845 (-)	845 (-)	845 (-)
TRP1	taaaactTAAATccaaatccatca			862 (+)	862 (+)	862 (+)	862 (+)	862 (+)	862 (+)
AT4G12670	tTAAATccaaatccatcaaatcaaaat			867 (+)	867 (+)	867 (+)	867 (+)	867 (+)	867 (+)
SRS7	aaATCCAaatccatcaaatcaaaa	788 (+)	788 (+)	869 (+)	869 (+)	869 (+)	869 (+)	869 (+)	869 (+)
OCSBF-1	ACGTG	820 (-)	820 (-)	901 (-)	901 (-)	901 (-)	901 (-)	901 (-)	901 (-)
PBF	CCTTTt	829 (-)	829 (-)	910 (-)	910 (-)	910 (-)	910 (-)	910 (-)	910 (-)
PBF	CCTTTt	829 (+)	829 (+)	910 (+)	910 (+)	910 (+)	910 (+)	910 (+)	910 (+)
DOF4.3	AAAGCagctat	846 (-)	846 (-)						
DOF4.3	aaagcaGCTAT	846 (+)	846 (+)						
MYBA51	agcaGTTATgt			929 (-)	929 (-)	929 (-)	929 (-)	929 (-)	929 (-)

Supplementary Table 8: Cis-regulatory elements in promoter sequences of ACO2 promoters from different potato cultivars, identified by PlantCARE. Promoter sequences from PW636 (ACO2(P1): 1523 nt, ACO2(P2): 1506 nt and ACO2(P3): 1515 nt), promoter sequences from Rywal (ACO2(R1): 1536 nt, ACO2(R2): 1521 nt, ACO2(R3): 1522 nt) and promoter sequences from Désirée (ACO2(D1): 1529 nt, ACO2(D2): 1515 nt and ACO2(D3): 1529 nt) were analysed using PlantCARE web service. Promoter, used for a more detailed investigation, is grey shaded, while known defence related regulatory elements are green shaded. The position, the strand and the sequence of each cis-regulatory element is specified. If more than three repeats of the particular regulatory element were present in the sequence, only the number of these elements is stated (with no position information).

Name of regulatory element	Sequence of regulatory element	PW363				Rywal			Désirée	
		ACO2 (P1)	ACO2 (P2)	ACO2 (P3)	ACO2 (R1)	ACO2 (R2)	ACO2 (R3)	ACO2 (D1)	ACO2 (D2)	ACO2 (D3)
Box 4	ATTAAT	31(+)	31(+)					177(+)		177(+)
TC-rich repeats	ATTTCTTCA	67(+)	67(+)	68(+)	68(+)	68(+)	68(+)	68(+)	68(+)	68(+)
Box-W1/W	TTGACC	99(-)	99(-)	100(-)	100(-)	100(-)	100(-)	100(-)	100(-)	100(-)
WUN-motif	TCATTACGAA			134(+)			134(+)		134(+)	
TCA-element	CCATCITTTT			142(-)			142(-)		142(-)	
Box 4	ATTAAT	179(+)	179(+)	177(+)	177(+)	177(+)	177(+)		177(+)	
AT1-motif	ATTAATTTTACA	179(+)	179(+)	177(+)	177(+)	177(+)	177(+)	177(+)		177(+)
Unnamed 2	AACCTAACCT	192(-)	192(-)	190(-)	190(-)	190(-)	190(-)	190(-)	190(-)	190(-)
MRE	AACCTAA	195(-)	195(-)	193(-)	193(-)	193(-)	193(-)	193(-)	193(-)	193(-)
O2-site	GATGACATGA	205(+)	205(+)	203(+)	203(+)	203(+)	203(+)	203(+)	203(+)	203(+)
GCN4 motif	TGTGTCA	216(-)	216(-)	214(-)	214(-)	214(-)	214(-)	214(-)	214(-)	214(-)
TC-rich repeats	ATTTCTTCA	324(-)	324(-)	315(-)					315(-)	
EIRE	TTCGACC				452(+)	452(+)	453(+)			
ACE	AAAACGTTTA	600(-)	600(-)	589(-)	592(-)	592(-)	593(-)	591(-)	589(-)	591(-)
CG-motif	CCATGGGG	642(+)			634(+)	634(+)	635(+)	633(+)		633(+)
P-box	CCITTTG	702(+)	685(+)	674(+)	694(+)	694(+)	695(+)	693(+)	674(+)	693(+)
ACE	AAAACGTTTA	720(-)	703(-)							
TC-rich repeats	ATTTCTTCA	739(-)	722(-)	711(-)	731(-)	731(-)	732(-)	730(-)	711(-)	730(-)
CAT-box	GCCACT	788(+)	771(+)	759(+)	780(+)	780(+)	781(+)	779(+)	759(+)	779(+)
MBS	TAACGT	814(+)	797(+)	785(+)	806(+)	806(+)	807(+)	805(+)	785(+)	805(+)
TCA-element	GAGAAGAATA	841(+)	824(+)	812(+)	833(+)	833(+)	834(+)	832(+)	812(+)	832(+)
chs-CMA1a	TTACTTAA	894(-)	877(-)			883(-)	884(-)	885(-)		885(-)
3-AF3 binding	CACTATCTAAC					907(-)	908(-)			
CAT-box	GCCACT					914(-)	915(-)			
GATA-motif/-	GATAGGG	922(+)	905(+)	902(+)	923(+)			913(+)	902(+)	913(+)
Box-W1/W	TTGACC					943(+)	944(+)	945(+)		945(+)
WUN-motif	TCATTACGAA	943(+)	926(+)	923(+)	944(+)	932(+)	933(+)	934(+)	923(+)	934(+)
MBS	CGGTCA					944(-)	945(-)	946(-)		946(-)
ATCT-motif	AATCTGATCG	951(+)	934(+)							
ARE	TGGTTT	973(+)	956(+)	953(+)	974(+)			964(+)	953(+)	964(+)
Unnamed 4	CTCC	1062(-)	1045(-)	1042(-)	1063(-)	1051(-)	1052(-)		1042(-)	
ATCC-motif	CAATCCTC	1063(-)	1046(-)	1043(-)	1064(-)			1051(-)	1043(-)	1051(-)
P-box	CCITTTG	1073(-)	1056(-)	1053(-)	1074(-)	1062(-)	1063(-)	1061(-)	1053(-)	1061(-)
LTR	CCGAAA	1093(-)	1076(-)	1073(+)	1094(+)	1082(+)	1083(+)	1081(+)	1073(+)	1081(+)
G-Box	CACGTT	1140(-)	1123(-)			1129(-)	1130(-)	1128(-)		1128(-)
circadian	CAANNNNATC	1149(+)	1132(+)	1129(+)	1150(+)	1138(+)	1139(+)	1133(-)	1129(+)	1133(-)
ERE	ATTTCAAA	1205(+)	1188(+)	1185(+)	1206(+)				1185(+)	
Box I	TTTCAAA	1206(+)	1189(+)	1186(+)	1207(+)	1195(+)	1196(+)		1186(+)	
TC-rich repeats	ATTTCTTCA			1228(-)	1249(-)	890(+)	891(+)		1228(-)	
GA-motif	ATAGATAA	1232(-)	1215(-)	1212(-)	1233(-)	1221(-)	1222(-)	1220(-)	1212(-)	1220(-)
GARE-motif	TCTGTTG	1302(-)	1285(-)	1282(-)	1303(-)	1291(-)	1292(-)	1290(-)	1282(-)	1290(-)
AAGAA-motif	gGTAAAGAAA	1389(-)	1372(-)	1371(-)	1392(-)	1378(-)	1379(-)	1377(-)	1371(-)	1377(-)
AAGAA-motif	GAAAGAA	1390(-)	1373(-)	1372(-)	1393(-)	1379(-)	1380(-)	1378(-)	1372(-)	1378(-)
TCA-element	CCATCITTTT	1459(+)	1442(+)	1441(+)	1462(+)	1448(+)	1449(+)	1447(+)	1441(+)	1447(+)
Unnamed 4	CTCC	1456(+)	1439(+)	1346(+)	1367(+)	1355(+)	1356(+)	1354(+)	1346(+)	1354(+)
3-AF3 binding	AAGAGATATT					1489(-)	1490(-)			
HSE	A(A/G)AAAAATT(T)/(C/G)	4	3	2	3	2	3	4	2	4
Skn-1 motif	GTCAT	6	6	6	6	6	6	6	6	6
CAAT-box		31	31	34	30	28	28	32	34	32
TATA-box		94	89	100	97	98	98	97	100	97

Supplementary Table 9: Transcription factor binding sites in promoter sequences of ACO2 promoters from different potato cultivars, identified by TRANSFAC. TRANSFAC database was used to analyse promoter sequences from PW636 (ACO2(P1): 1523 nt, ACO2(P2): 1506 nt and ACO2(P3): 1515 nt), promoter sequences from Rywal (ACO2(R1): 1536 nt, ACO2(R2): 1521 nt, ACO2(R3): 1522 nt) and promoter sequences from Désirée (ACO2(D1): 1529 nt, ACO2(D2): 1515 nt and ACO2(D3): 1529 nt) to predict transcription factor binding sites (regulatory elements). Promoter, used for a more detailed investigation, is grey shaded, while some of the known defence related regulatory elements are green shaded. The position, the strand and the sequence of each transcription factor binding site is specified in the table as well as the transcription factors, which bind to these transcription factor binding sites.

Transcription factor	Transcription factor binding site	PW			Rywal			Désirée		
		ACO2 (P1)	ACO2 (P2)	ACO2 (P3)	ACO2 (R1)	ACO2 (R2)	ACO2 (R3)	ACO2 (D1)	ACO2 (D2)	ACO2 (D3)
Dof3	tgGCTTTtttc	36 (-)	36 (-)	36 (-)	36 (-)	36 (-)	36 (-)	36 (-)	36 (-)	36 (-)
MADS-A	acgaaaAAAATggaaa			139 (+)					139 (+)	
PBF	aAAAGG	145 (+)	145 (+)		146 (+)	146 (+)	146 (+)	146 (+)		146 (+)
PBF	aAAAGG	145 (-)	145 (-)		146 (-)	146 (-)	146 (-)	146 (-)		146 (-)
Opaque-2	GATGAaatga	205 (+)	205 (+)	203 (+)	203 (+)	203 (+)	203 (+)	203 (+)	203 (+)	203 (+)
HMG-1	aaatACAC	239 (-)	239 (-)		242 (-)	242 (-)	242 (-)			
WRKY	atatttAGTCA	272 (+)	272 (+)	263 (+)	275 (+)	275 (+)	275 (+)	271 (+)	263 (+)	271 (+)
HSFA4A	AATAGt	285 (-)	285 (-)	276 (-)	288 (-)	288 (-)	288 (-)	284 (-)	276 (-)	284 (-)
BPC1	cTTTCT	428 (-)	429 (-)	418 (-)	420 (-)	420 (-)	420 (-)	419 (-)	418 (-)	419 (-)
SBF-1	ttcttgTTAATata	430 (+)	431 (+)	420 (+)	422 (+)	422 (+)	422 (+)	421 (+)	420 (+)	421 (+)
Mvb-15	tTGTTA	433 (-)	434 (-)	423 (-)	425 (-)	425 (-)	425 (-)	424 (-)	423 (-)	424 (-)
BPC1	cTTTCT	466 (-)	467 (-)	456 (-)	458 (-)	458 (-)	458 (-)	457 (-)	456 (-)	457 (-)
At4g36620	atcAACCA	474 (+)	475 (+)	464 (+)	466 (+)	466 (+)	466 (+)	465 (+)	464 (+)	465 (+)
Mvb-15	TAACAa	499 (+)	500 (+)	489 (+)	491 (+)	491 (+)	491 (+)	490 (+)	489 (+)	490 (+)
HSFA2	tTTTGG	517 (-)	518 (-)	507 (-)	509 (-)	509 (-)	509 (-)	508 (-)	507 (-)	508 (-)
GT-1	TATTTtttaaat	551 (+)	552 (+)	541 (+)	543 (+)	543 (+)	543 (+)	542 (+)	541 (+)	542 (+)
SBF-1	taataagTTAATAat		579 (+)	568 (+)					568 (+)	
HSFA4A	AATAGt	579 (-)	580 (-)	569 (-)	571 (-)	571 (-)	571 (-)	570 (-)	569 (-)	570 (-)
HSFA4A	AATAGt	694 (-)	677 (-)	666 (-)	686 (-)	686 (-)	686 (-)	685 (-)	666 (-)	685 (-)
SED	gtacCCTTTt	698 (+)	681 (+)		690 (+)	690 (+)	690 (+)			
PBF	CCTTTt	702 (-)	685 (-)	674 (-)	694 (-)	694 (-)	694 (-)	693 (-)	674 (-)	693 (-)
PBF	CCTTTt	702 (+)	685 (+)	674 (+)	694 (+)	694 (+)	694 (+)	693 (+)	674 (+)	693 (+)
GT-1	tttaaaaACATA	727 (-)	710 (-)							
Mvb-15	TAACAa	753 (+)	736 (+)	724 (+)	745 (+)	745 (+)	745 (+)	744 (+)	724 (+)	744 (+)
HSFA2	CCAAaAa	778 (+)	761 (+)	749 (+)	770 (+)	770 (+)	770 (+)	769 (+)	749 (+)	769 (+)
ASR-1	TGGGT	801 (-)	784 (-)	772 (-)	793 (-)	793 (-)	793 (-)	792 (-)	772 (-)	792 (-)
ASR-1	TGGGT	832 (-)	815 (-)	803 (-)	824 (-)	824 (-)	824 (-)	823 (-)	803 (-)	823 (-)
ANTL	attCGACatt	910 (+)	893 (+)			899 (+)	899 (+)	901 (+)		901 (+)
ARR10	cgAATCT							921 (-)		921 (-)
PBF	aAAAGG	1074 (-)	1057 (-)	1054 (-)	1075 (-)	1063 (+)	1063 (+)	1062 (+)	1054 (-)	1062 (+)
PBF	aAAAGG	1074 (+)	1057 (+)	1054 (+)	1075 (+)	1063 (-)	1063 (-)	1062 (-)	1054 (+)	1062 (-)
OCSBF-1	ACGTG	1141 (-)	1124 (-)			1130 (-)	1130 (-)	1129 (-)		1129 (-)
GT-1	tttaaaaAAAAA							1194 (-)		1194 (-)
ASR-1	ACCCA	1326 (+)	1309 (+)	1306 (+)	1327 (+)	1315 (+)	1315 (+)	1314 (+)	1306 (+)	1314 (+)
HSFA2	CCAAaAa	1328 (+)	1311 (+)	1308 (+)	1329 (+)	1317 (+)	1317 (+)	1316 (+)	1308 (+)	1316 (+)
HSFA4A	aCTATT	1347 (+)	1330 (+)	1327 (+)	1348 (+)	1336 (+)	1336 (+)	1335 (+)	1327 (+)	1335 (+)
AGL3	ctagctataTATGGattt	1374 (-)	1357 (-)			1363 (-)	1363 (-)	1362 (-)		1362 (-)
AGL3	ctagctataTATGGattt	1374 (-)	1357 (-)			1363 (-)	1363 (-)	1362 (-)		1362 (-)
AGL3	ctagctataTATGGattt	1374 (-)	1357 (-)			1363 (-)	1363 (-)	1362 (-)		1362 (-)
RIN	CTATAtatgg	1378 (-)	1361 (-)			1367 (-)	1367 (-)	1366 (-)		1366 (-)
ASR-1	ACCCA	1406 (+)	1389 (+)	1388 (+)	1409 (+)	1395 (+)	1395 (+)	1394 (+)	1388 (+)	1394 (+)
HSFA4A	aCTATT	1478 (+)	1461 (+)	1460 (+)	1481 (+)	1467 (+)	1467 (+)	1466 (+)	1460 (+)	1466 (+)
GT-1	taTTTAC	1480 (-)	1463 (-)	1462 (-)	1483 (-)	1469 (-)	1469 (-)	1468 (-)	1462 (-)	1468 (-)
FLC	atttactcaaatAGAAaAa	1481 (+)	1464 (+)			1470 (+)	1470 (+)	1469 (+)		1469 (+)
AGL15	ttactcaaAATAGaaa	1483 (+)	1466 (+)			1472 (+)	1472 (+)	1471 (+)		1471 (+)

Supplementary Table 10: Cis-regulatory elements in promoter sequences of PTI5 promoters from different potato cultivars, identified by PlantCARE. Promoter sequences from Rywal (PTI5(R1): 992 nt, PTI5(R2): 1008 nt, PTI5(R3): 991 nt, PTI5(R4): 1008 nt and PTI5(R5): 1008 nt were analysed using the PlantCARE web service. Promoter, used for a more detailed investigation, is grey shaded, while known defence related regulatory elements are green shaded. The position, the strand and the sequence of each cis-regulatory element is specified. If more than three repeats of the particular regulatory element were present in the sequence, only the number of these elements is stated (with no position information).

Name of regulatory element	Sequence of regulatory element	Rywal				
		PTI5 (R1)	PTI5 (R2)	PTI5 (R3)	PTI5 (R4)	PTI5 (R5)
G-Box	CACGAC	8(+)	8(+)	8(+)	8(+)	8(+)
G-Box	CACGTGAAA	16(-)	16(-)	16(-)	16(-)	16(-)
G-Box/ABRE	CACGTG	19(+)	19(+)	19(+)	19(+)	19(+)
5UTR Py-rich stretch	TTTCTTCTCT	39(+)	39(+)	39(+)	39(+)	39(+)
AAGAA-motif	GAAAGAA	45(-)		45(-)	45(-)	
CGTCA-motif	CGTCA	54(+)	54(+)	54(+)	54(+)	54(+)
TGACG-motif	TGACG	54(-)	54(-)	54(-)	54(-)	54(-)
Skn-1 motif	GTCAT	55(+)	55(+)	55(+)	55(+)	55(+)
MBS	TAACTG	80(+)		80(+)	80(+)	
GARE-motif	TCTGTTG	101(-)	101(-)	101(-)	101(-)	101(-)
TC-rich sequence	ATTTTCTCCA	203(-)	206(-)	202(-)	206(-)	206(-)
Box 4	ATTAAT	211(+)	214(+)	210(+)	214(+)	214(+)
Unnamed 4	CTCC	264(+)		263(+)		
G-Box	CACATGG	266(-)		265(-)		
circadian	CAAAGATATC	283(-)		282(-)		
Unnamed 11	TCCACATAGA		294(-)		294(-)	294(-)
GT1-motif	GGTTAA	297(-)	326(-)	296(-)	326(-)	326(-)
GA-motif	AAAGATGA		333(+)		333(+)	333(+)
Skn-1 motif	GTCAT	337(-)		336(-)	366(-)	336(-)
TGA-element	AACGAC		365(+)			
MBS	TAACTG	418(-)	447(-)	417(-)	447(-)	447(-)
ERE	ATTTCAAA	477(+)	506(+)	476(+)	506(+)	506(+)
Box 1	TTTCAAA	478(+)	507(+)	477(+)	507(+)	507(+)
AT1-motif	ATTAATTTTACA			492(-)		
MBS	TAACTG	509(+)	538(+)	508(+)	538(+)	538(+)
TCCACCT-motif	TCCACCT	539(+)		538(+)		
TGA-element	AACGAC		546(+)		546(+)	546(+)
Unnamed 1	CGTGG	561(-)	577(-)	560(-)	577(-)	577(-)
SARE	TTGACCATCTT	593(-)	609(-)		609(-)	609(-)
Box-W1/W box	TTGACC	598(-)	614(-)		614(-)	614(-)
TCA-element	CCATCTTTTT	612(+)		611(+)	628(+)	
ARE	TGGTTT	677(-)	693(-)	676(-)	693(-)	693(-)
Unnamed 13	TCCAAGTATA	679(+)	695(+)	678(+)	695(+)	695(+)
Box 4	ATTAAT	747(-)	763(-)	746(-)	763(-)	763(-)
ARE	TGGTTT	754(-)	770(-)	753(-)	770(-)	770(-)
O2-site	GATGACATGG	757(-)	773(-)	756(-)	773(-)	773(-)
Skn-1 motif	GTCAT	761(+)	777(+)	760(+)	777(+)	777(+)
TATC-box	TATCCCA	765(+)	781(+)	764(+)	781(+)	781(+)
box E	ACCCATCAAG	767(+)	783(+)	766(+)	783(+)	783(+)
Unnamed 4	CTCC	858(+)	874(+)	857(+)	874(+)	874(+)
Unnamed 1	CGTGG	860(-)	876(-)	859(-)	876(-)	876(-)
ACE	AAAACGTTTA	945(-)	961(-)	944(-)	961(-)	961(-)
TC-rich sequence	ATTTTCTCCA	950(+)	966(+)	949(+)	966(+)	966(+)
L-box	AAATTAACCAAC	962(+)		961(+)	978(+)	
GT1-motif	GGTTAAT	964(-)		963(-)	980(-)	
GT1-motif	GGTTAA	965(-)		964(-)	981(-)	
CAAT-box		21	20	21	21	20
TATA-box		47	49	44	48	48

Supplementary Table 11: Transcription factor binding sites in promoter sequences of PTI5 promoters from different potato cultivars, identified by TRANSFAC. TRANSFAC database was used to analyse promoter sequences from Rywal (PTI5(R1): 992 nt, PTI5(R2): 1008 nt, PTI5(R3): 991 nt, PTI5(R4): 1008 nt and PTI5(R5): 1008 nt) to predict transcription factor binding sites (regulatory elements). Promoter, used for a more detailed investigation, is grey shaded, while some of the known defence-related regulatory elements are green shaded. The position, the strand and the sequence of each transcription factor binding site is specified in the table as well as the transcription factors, which bind to these transcription factor binding sites.

Transcription factor	Regulatory element	Rywal				
		PTI5 (R1)	PTI5 (R2)	PTI5 (R3)	PTI5 (R4)	PTI5 (R5)
ANAC042	atcagcagcatttcACGTGc	6 (-)	6 (-)	6 (-)	6 (-)	6 (-)
ANAC094	tCACGAcgatttcacgtg	7 (+)	7 (+)	7 (+)	7 (+)	7 (+)
ANAC094	cagcagcatttcACGTGc	8 (-)	8 (-)	8 (-)	8 (-)	8 (-)
Opaque-2	gatttcACGTGca	14 (+)	14 (+)	14 (+)	14 (+)	14 (+)
FAR1	atttcACGTGcaata	15 (+)	15 (+)	15 (+)	15 (+)	15 (+)
BIM1	tttCACGTGc	16 (-)	16 (-)	16 (-)	16 (-)	16 (-)
PHYPADRAFT 72483	ttcACGTGca	17 (-)	17 (-)	17 (-)	17 (-)	17 (-)
BIM3	ttcACGTGca	17 (-)	17 (-)	17 (-)	17 (-)	17 (-)
BIM2	ttcACGTGca	17 (-)	17 (-)	17 (-)	17 (-)	17 (-)
BEE2	ttcACGTGca	17 (-)	17 (-)	17 (-)	17 (-)	17 (-)
Opaque-2	ttcACGTGcaata	17 (-)	17 (-)	17 (-)	17 (-)	17 (-)
PHYPADRAFT 72483	ttCACGTGca	17 (+)	17 (+)	17 (+)	17 (+)	17 (+)
BIM3	ttCACGTGca	17 (+)	17 (+)	17 (+)	17 (+)	17 (+)
BIM2	ttCACGTGca	17 (+)	17 (+)	17 (+)	17 (+)	17 (+)
BEE2	ttCACGTGca	17 (+)	17 (+)	17 (+)	17 (+)	17 (+)
BIM1	ttCACGTGca	18 (+)	18 (+)	18 (+)	18 (+)	18 (+)
RITA-1	tCACGTg	18 (+)	18 (+)	18 (+)	18 (+)	18 (+)
OCSBF-1	CACGT	19 (+)	19 (+)	19 (+)	19 (+)	19 (+)
OCSBF-1	ACGTG	20 (-)	20 (-)	20 (-)	20 (-)	20 (-)
HSFA4A	AATAGt	26 (-)	26 (-)	26 (-)	26 (-)	26 (-)
DOF4.3	aaatttTCTTT	35 (+)	35 (-)	35 (+)	35 (-)	35 (-)
DOF4.3	AAATTTcttt	35 (-)	35 (+)	35 (-)	35 (+)	35 (+)
GBF6	ttcAACGTcatatgt	49 (+)	49 (+)	49 (+)	49 (+)	49 (+)
bZIP44	ttcAACGTcatatgt	49 (-)	49 (-)	49 (-)	49 (-)	49 (-)
bZIP53	ttcAACGTcatatgt	49 (-)	49 (-)	49 (-)	49 (-)	49 (-)
HBP-1a	tcaaCGTCat	50 (+)	50 (+)	50 (+)	50 (+)	50 (+)
At5g54070	cCAACG		50 (+)			50 (+)
Opaque-2	tcaACGTcatatg	50 (-)	50 (-)	50 (-)	50 (-)	50 (-)
TGA1A	aACGTCa	52 (+)	52 (+)	52 (+)	52 (+)	52 (+)
GT-1	tGTTAAgatgtt	61 (-)	61 (-)	61 (-)	61 (-)	61 (-)
SPL15	attcCTTACatata	185 (-)	188 (-)	184 (-)	188 (-)	188 (-)
bHLH28	ttcCTTACatata	186 (-)	189 (-)	185 (-)	189 (-)	189 (-)
SPL5	ttcCTTACatata	186 (-)	189 (-)	185 (-)	189 (-)	189 (-)
SPL3	tCCCTTAc	187 (+)	190 (+)	186 (+)	190 (+)	190 (+)
HSFA4A	AATAGt	198 (-)	201 (-)	197 (-)	201 (-)	201 (-)
EDT1	aaaTTAATgt	209 (+)	212 (+)	208 (+)	212 (+)	212 (+)
GT-1	gttaaaaaAAAA	217 (-)		216 (-)		
HSFA4A	aCTATT	254 (+)	283 (+)	253 (+)	283 (+)	283 (+)
ASR-1	ACCCA	300 (+)	329 (+)	299 (+)	329 (+)	329 (+)
SRS7	ataactTTAAAtcttaacccaaa		313 (+)		313 (+)	313 (+)
TRP1	ataactTTAAAtcttaacccaaa		313 (+)		313 (+)	313 (+)
TRP2	taactttAAATCttaaccca		314 (+)		314 (+)	314 (+)
HSFA4A	aCTATT	315 (+)	344 (+)	314 (+)	344 (+)	344 (+)
HSFA2	tTTTGG	319 (-)	348 (-)		348 (-)	348 (-)
PBF	CCITTt	326 (+)	355 (+)	325 (+)	355 (-)	355 (-)
PBF	CCITTt	326 (-)	355 (-)	325 (-)	355 (+)	355 (+)
HMG-1	gactACAAC	339 (-)	368 (-)	338 (-)	368 (-)	368 (-)
ATHSFA1D	aCTACA	340 (+)	369 (+)	339 (+)	369 (+)	369 (+)
LEC2	agCATGCacat	376 (+)	405 (+)	375 (+)	405 (+)	405 (+)
AT4G27900	aatcAAGGGcaaaagttaaagtg	395 (+)	424 (+)	394 (+)	424 (+)	424 (+)
AT1G77200	taaagtGTCAGtta	410 (-)	439 (-)	409 (-)	439 (-)	439 (-)
ARR1	tccGAATCta	452 (+)	481 (+)	451 (+)	481 (+)	481 (+)
ARR10	cgAATCT	454 (-)	483 (-)	453 (-)	483 (-)	483 (-)
ERF11	aaaaCGGCGt	515 (-)		514 (-)		
MYB3R4	aaAACGGCgttttca	515 (-)		514 (-)		
MYB3R1	aaAACGGCgttttca	515 (-)		514 (-)		
MYB3R5	aaAACGGCgttttca	516 (-)		515 (-)		
ERF069	aaCGGCGt	516 (-)		515 (-)		
AT1G28160	aaCGGCGgttttca	516 (+)		515 (+)		
At1g68550	aaCGGCGt	516 (+)		515 (+)		
CAMTA1	aaacGGCGTttt	516 (+)		515 (+)		
bZIP44	atcCACTCaaaaaaa	538 (-)		537 (-)		
CBF3	tgaaaACGACgttt		542 (+)		542 (+)	542 (+)
bZIP48	ttCACCTcaaaaaa	539 (+)		538 (+)		
CAMTA1	aaaaGGCGTctt	549 (+)	565 (+)	548 (+)	565 (+)	565 (+)
PBF	aAAAGG	549 (-)	565 (-)	548 (-)	565 (+)	565 (-)
PBF	aAAAGG	549 (+)	565 (+)	548 (+)	565 (-)	565 (+)

Continued

Transcription factor	Regulatory element	PTI5 (R1)	PTI5 (R2)	PTI5 (R3)	PTI5 (R4)	PTI5 (R5)
LBD2	AGGCGtcttccacgg	552 (-)	568 (-)	551 (-)	568 (-)	568 (-)
LBD23	ggcgtcttcCACGGg	553 (+)	569 (+)	552 (+)	569 (+)	569 (+)
REM16	tTTTCacctcaaaaaaggcgt		553 (-)		553 (-)	553 (-)
LBD2	gcgtcttccaCGGGC	554 (+)	570 (+)	553 (+)	570 (+)	570 (+)
FAR1	cttccACGGGcaaca	558 (+)	574 (+)	557 (+)	574 (+)	574 (+)
WRKY48	atgGTCAAat	596 (+)	612 (+)		612 (+)	612 (+)
Opaque-2	attACGTCcaatc	604 (-)		603 (-)	620 (-)	
NAC6	TTACGt	605 (-)	621 (-)	604 (-)	621 (-)	621 (-)
ARF16	tttttaataAAAAacatcaa	619 (-)		618 (-)	635 (-)	
PBF	CCTTTt		631 (+)			631 (+)
PBF	CCTTTt		631 (-)			631 (-)
TGA1A	tACATCa	687 (+)		686 (+)	703 (+)	
AT3G25990	ggtacacaatTAACtaactt			700 (-)		
MYB3R4	tcAACGCaacaaga	728 (-)	744 (-)	727 (-)	744 (-)	744 (-)
MYB3R1	tcAACGCaacaaga	728 (-)	744 (-)	727 (-)	744 (-)	744 (-)
AT3G25990	aacaagAGGTAattaattaaa	736 (+)	752 (+)	735 (+)	752 (+)	752 (+)
MYB61	gacACCAAccatata	776 (+)	792 (+)	775 (+)	792 (+)	792 (+)
TGA1a	aaaAAGACttgcacaatttttc	810 (+)	826 (+)	809 (+)	826 (+)	826 (+)
BPC1	ctTTCT	854 (-)		853 (-)	870 (-)	
MYB3R4	taAACGAttactttt	896 (-)		895 (-)	912 (-)	
MYB3R1	taAACGAttactttt	896 (-)		895 (-)	912 (-)	
MYB3R5	aAACGAttacttttc	897 (-)		896 (-)	913 (-)	
At3g60580	gtATCCC	914 (+)		913 (+)	930 (+)	
HSFA2	CCAAaA	969 (+)	985 (+)	968 (+)	985 (+)	985 (+)
At4g36620	atcAACCA		980 (+)			980 (+)

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> MC_P1, 922nt

+ ATTTTACACC ATCGTTACAA TCTTCCGTCAC TTTCTATATA CATATTTTCT CTTTGTAGAA TTAACACTCT
- TAAATGTGG TAGCAATGTT AGAATCAGTG AAAGATATAT GTATAAAAGA GAAACTCTT AATTGATGAG

+ TTACTCAAGG AAATATAACA GAAAGCACAA AAATTTGTAA AAAATCTTGT ATCCAGCTAC ACGATAAGGA
- AATGAGTTTC TTTATATTGT CTTTCGTGTT TTTAAACATT TTTTGAACA TAGGTGCAIG TGCTATTCTT

+ ATGTAAAGT TCTTTTTTTA AAAAAAAAAA AAATCGCAGA CACTCAATAT TAAAAAGTTT GGTGTGATCT
- TACATTTTCA AGAAAAAAT TTTTTTTTTT TTTAGCGTCT GTGAGTTATA ATTTTTCAAA CCACACTAGA

+ AAGTTACTTA TTGTGAAAG TTACTGTTGC AACTGATCTC AGATGATTTC GAAGATTAA GGGTATATTG
- TTCAATGAAT AACACTTTTC AATGACAAGC TTGACTAGAG TCTACTAAAG CTTCTAAAT CCCATATAAC

+ TAAATTTCTT GCAAACTGA AAAAATATAG TTCATGTTCT TCTTCTCT TGTATAATTG CTACAAAAAT
- ATTAAAAGAA CGTTTAGACT TTTTATATC AAGTACAAGA AGTAGAAGAA ACATATTAA GATGTTTTTA

+ TTGATACAAA ACCACCCAAA ATCTTCTCC AAATGCCTCA AATTTTAGAT ATAAGCTCCT CTCACATTT
- AACTATGTTT TGGTGGGTTT TAGAAGAGGT TTAACGGAGT TTAATACTA TATTCGAGGA GAGTTGTAAA

+ CGATTCTTtgagGATATTAC CCATTCAAAA CGGAGCTCGT TCGTGGTTAG TCAAAATTAG AACTCAAGCT
- GCTAAGAAA CTGTATATG GTAAGTTTT GCCTCGAGCA AGCACCANTCAGTTTAATC TTGAGTTGGA

+ GATAAACTAA CTTTAATGTT GGTTCAGT TGTGCTTCAA AGTTTCAACC TTTAGCTTGG ATTTTCAACC
- CTATTTTGATT GAAATTACCA CCAAAGTTCA ACACGAAGTT TCAAAGTTGG AAATCGAAGC TAAAAGTGG

+ TATAGTTAGT AATTTAAAA AGAGGAAAT AATTACCGTC AAATAAACCT CAAAAAGCTA AAGTATTAT
- ATATCAATCA TTAATTTTT TCTCCTTTA TTAATGGagttTATTGGA GTTTTCGAT TTTTATAATA

+ ATAAATTAGG ACCACTTAGT GGGGAATATT TACCATTAA TGCAATCAT ATGAGGGGAC TCGAGGAAGG
- TATTTAATCC TGGTGAATCA CCCCTTATA ATGGTAAAT ACGTAATCTA TACTCCCTG AGCTCCCTCC

+ TGAATCATTT GATATGAATA TTGGACAATT ATAAATGAG TCTTTTATT TTAATTTTT AAAACTTTTT
- ACTTACTAAA CTATACTTAT AACCTGTTAA TATTTTACTC AGAAAAATAA AATAAAAAAA TTTTGAAAAA

+ TGGTTATTGG GAAGCTAAAA TCCAAATCCA TCAAATCAA ATATTIATAA CGTGCTAGCC TTTTCTCTC
- ACCAATAAAC CTTGATTTT AGGTTTAGGT AGTTTAGTTT TATAAATATT GCACGATCGG AAAAAAAGAG

+ TATAAAAGC AGCTATGTT ACCTTCTTCT CATCACAAA ATATTCCTTC TTCTTAATTA AGTTATTAA
- ATATTTTTCG TCGATACAG TGAAGAAGA GTAGTGTTT TATAAGGAAG AAGAATTAA TCAATAATTA

+ TATTCGAGT GATG
- ATAAGCGTCA CTAC

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Supplementary Figure 1: Visual representation of predicted regulatory elements of MC promoter from *S. tuberosum* cv. Rywal (MC(P1), 922 bp). The predicted regulatory elements involved in biotic stress responses are **CGTCA-motif** (involved in the MeJA-responsiveness), **TC-rich repeats** (involved in defense and stress responsiveness), **TGACG-motif** (involved in the MeJA-responsiveness), **TGA1A binding site** (TGA1A transcription factor recognition site), **WBOXNTERF3/WBOXHVIS01** (W-box), **WBOXATNPR1** (W-box, recognized by salicylic acid (SA)-induced WRKY DNA binding proteins; lowercase), **ASF1MOTIFCAMV** (ASF-1 binding site, involved in salicylic acid and biotic stress responsiveness), **WRKY71QS** (a core of TGAC-containing W-box WRKY binding site within the Pathogenesis-Related Class10 (PR-10) genes), **GT1CONSENSUS** (consensus GT-1 binding site, influences the level of SA-inducible gene expression), **MYCCONSENUAT** (MYC recognition site), **G-box** (involved in defense responsiveness), **ACGTATERD1** (involved in Ca²⁺ responsiveness), **GT1GMSCAM4** (involved in pathogen responsiveness), **TCA1MOTIF** (involved in SA responsiveness), **T/GBOXATPIN2** (involved in JA responsiveness), **MYB1LEPR** (G-box, Tomato Pti4(ERF) regulates defence-related gene expression via GCC box), **ABRERATCAL** (involved in Ca²⁺ responsiveness), **WBOXPCWRKY1** (WB/W box, WRKY proteins binding site in PR1 gene in parsley). The **ATG** indicates the start of MC coding region. The promoter region is located upstream of the ATG. The crucial regulatory elements were determined by PlantCARE, TRANSFAC and PLACE. The complete list of MC promoter domains from cv. Rywal, Désirée and PW636 with their positions and sequence, predicted with PlantCARE and TRANSFAC, are specified in the Supplementary Tables 6 and 7.

Supplementary Table 12: Promoter activities, tested in all obtained promoter::fluorescent protein transgenic lines using RT-qPCR. Ratio between the relative copy numbers of treated sample and relative copy numbers of the control are presented. Genotype: genotype of the plant used for stable transformation with the construct promoter::fluorescent protein, Ratio(T/C): ratio between the relative copy numbers of treated sample and relative copy numbers of the control, LOD: expression was under the limit of detection, /: no result available due to failed quality control of the target, ✓: the construct was detected in the genomic DNA, x: the construct was not detected in genomic DNA.

Construct	Genotype	Line	Time	Treatment	Ratio(T/C)	gDNA
TGA(P1)::YFP	Rywal	3	1 h	INA	LOD	✓
			1 h	control	LOD	
			24 h	INA	LOD	
		5	24 h	control	LOD	x
			1 h	INA	LOD	
			1 h	control	LOD	
TGA(R1)::CFP	Désirée	4	1 h	INA	1,83	✓
			1 h	control	0,67	
			24 h	INA	0,67	
		5	1 h	INA	1,28	✓
			1 h	control	0,76	
			24 h	INA	0,76	
		6	24 h	control	0,87	n.a.
			1 h	INA	0,87	
			1 h	control	1,86	
		9	24 h	INA	LOD	n.a.
			1 h	control	LOD	
			24 h	INA	LOD	
TGA(P1)::YFP	Désirée	1	1 h	INA	1,52	✓
			1 h	control	LOD	
			24 h	INA	LOD	
		3	24 h	control	LOD	✓
			1 h	INA	LOD	
			1 h	control	LOD	
		7	1 h	INA	/	n.a.
			1 h	control	1,21	
			24 h	INA	1,21	
		8	24 h	control	2,55	n.a.
			1 h	INA	/	
			1 h	control	/	
		9	24 h	INA	2,13	n.a.
			1 h	control	0,71	
			1 h	INA	0,71	
		10	24 h	control	/	n.a.
			1 h	INA	/	
			1 h	control	0,96	

Continued

Construct	Genotype	Line	Time	Treatment	Ratio(T/C)	gDNA
ACO(R2)::CFP	Rywal	1	6 h	ACC	2,88	✓
			6 h	control		
			24 h	ACC	1,20	
			24 h	control		
		2	6 h	ACC	0,35	✓
			6 h	control		
			24 h	ACC	LOD	
			24 h	control		
		4	6 h	ACC	0,98	n.a.
			6 h	control		
			24 h	ACC	/	
			24 h	control		
ACO(R2)::CFP	Désirée	10	6 h	ACC	0,27	n.a.
			6 h	control		
			24 h	ACC	1,96	
			24 h	control		
		2	6 h	ACC	2,25	✓
			6 h	control		
			24 h	ACC	1,00	
			24 h	control		
		3	6 h	ACC	1,79	✓
			6 h	control		
			24 h	ACC	1,71	
			24 h	control		
		5	6 h	ACC	/	n.a.
			6 h	control		
			24 h	ACC	LOD	
			24 h	control		
		8	6 h	ACC	0,46	n.a.
			6 h	control		
			24 h	ACC	/	
			24 h	control		

Continued

Construct	Genotype	Line	Time	Treatment	Ratio(T/C)	gDNA
ACO(R2)::YFP	Rywal	1	6 h	ACC	0,43	✕
			6 h	control		
			24 h	ACC	0,72	
			24 h	control		
		2	6 h	ACC	1,26	✓
			6 h	control		
			24 h	ACC	1,59	
			24 h	control		
		3	6 h	ACC	0,99	n.a.
			6 h	control		
		4	6 h	ACC	0,08	n.a.
			6 h	control		
			24 h	ACC	0,10	
			24 h	control		
		5	6 h	ACC	0,70	n.a.
			6 h	control		
			24 h	ACC	1,06	
			24 h	control		
		6	6 h	ACC	0,66	n.a.
			6 h	control		
			24 h	ACC	1,00	
			24 h	control		
		7	6 h	ACC	1,16	n.a.
			6 h	control		
			24 h	ACC	1,22	
			24 h	control		
		8	6 h	ACC	0,87	n.a.
			6 h	control		
			24 h	ACC	0,67	
			24 h	control		
		9	6 h	ACC	1,4	n.a.
			6 h	control		
			24 h	ACC	1,55	
			24 h	control		
		10	6 h	ACC	1,05	n.a.
			6 h	control		
			24 h	ACC	0,26	
			24 h	control		
MC (D1)::YFP	Désirée	12	5 h	MeJA	LOD	✕
			5 h	control		
			23 h	MeJA	LOD	
			23 h	control		
		13	5 h	MeJA	1,15	✓
			5 h	control		
			23 h	MeJA	1,73	
			23 h	control		
		14	5 h	MeJA	3,91	n.a.
			5 h	control		
			23 h	MeJA	0,15	
			23 h	control		
		15	5 h	MeJA	1,48	n.a.
		15	5 h	control		n.a.
		16	5 h	MeJA	1,49	n.a.
		16	5 h	control		n.a.
		17	5 h	MeJA	0,18	n.a.
		17	5 h	control		n.a.
		18	5 h	MeJA	2,88	n.a.
		18	5 h	control		n.a.

Supplementary Table 13: Relative copy numbers of treated samples and relative copy numbers of the controls and the ratio between the relative copy numbers of the treated sample and the relative copy numbers of the control (Ratio (T/C)) are presented. Copy numbers were normalized (see Methods section). LOD: expression was under the limit of detection, LOQ: expression was under the quantification limit, QC target failed: quality control of the target failed, /: no result available due to failed quality control of the target.

RT-qPCR 1

Sample	Relative copy number	Relative copy number (T/C)
TGA(R1)::CFP_Désirée_line 4_1h_T	0,1883	1,8270
TGA(R1)::CFP_Désirée_line 4_1h_C	0,1031	
TGA(R1)::CFP_Désirée_line 4_24h_T	0,1128	0,6683
TGA(R1)::CFP_Désirée_line 4_24h_C	0,1687	
TGA(R1)::CFP_Désirée_line 5_1h_T	0,1515	1,2795
TGA(R1)::CFP_Désirée_line 5_1h_C	0,1184	
TGA(R1)::CFP_Désirée_line 5_24h_T	0,1590	0,7572
TGA(R1)::CFP_Désirée_line 5_24h_C	0,2099	
TGA(P1)::YFP_Désirée_line 1_1h_T	0,1435	1,5190
TGA(P1)::YFP_Désirée_line 1_1h_C	0,0945	
TGA(P1)::YFP_Désirée_line 1_24h_T	0,0415	/
TGA(P1)::YFP_Désirée_line 1_24h_C	Under LOQ	
TGA(P1)::YFP_Désirée_line 3_1h_T	Under LOD	/
TGA(P1)::YFP_Désirée_line 3_1h_C	Under LOD	
TGA(P1)::YFP_Désirée_line 3_24h_T	Under LOD	/
TGA(P1)::YFP_Désirée_line 3_24h_C	Under LOD	
TGA(P1)::YFP_Rywal_line 3_1h_T	Under LOD	/
TGA(P1)::YFP_Rywal_line 3_1h_C	Under LOD	
TGA(P1)::YFP_Rywal_line 3_24h_T	Under LOD	/
TGA(P1)::YFP_Rywal_line 3_24h_C	Under LOD	
TGA(P1)::YFP_Rywal_line 5_1h_T	Under LOD	/
TGA(P1)::YFP_Rywal_line 5_1h_C	Under LOD	
TGA(P1)::YFP_Rywal_line 5_24h_T	Under LOD	/
TGA(P1)::YFP_Rywal_line 5_24h_C	Under LOD	
ACO(R2)::CFP_Rywal_line 1_6h_T	0,3916	2,8754
ACO(R2)::CFP_Rywal_line 1_6h_C	0,1362	
ACO(R2)::CFP_Rywal_line 1_24h_T	0,2638	1,1994
ACO(R2)::CFP_Rywal_line 1_24h_C	0,2199	
ACO(R2)::CFP_Rywal_line 2_6h_T	0,0730	0,3508
ACO(R2)::CFP_Rywal_line 2_6h_C	0,2080	
ACO(R2)::CFP_Rywal_line 2_24h_T	Under LOD	/
ACO(R2)::CFP_Rywal_line 2_24h_C	0,4540	
ACO(R2)::CFP_Désirée_line 2_6h_T	0,2266	2,2509
ACO(R2)::CFP_Désirée_line 2_6h_C	0,1007	
ACO(R2)::CFP_Désirée_line 2_24h_T	0,1853	0,9964
ACO(R2)::CFP_Désirée_line 2_24h_C	0,1860	
ACO(R2)::CFP_Désirée_line 3_6h_T	0,1222	1,7862
ACO(R2)::CFP_Désirée_line 3_6h_C	0,0684	
ACO(R2)::CFP_Désirée_line 3_24h_T	0,2113	1,7088
ACO(R2)::CFP_Désirée_line 3_24h_C	0,1237	
ACO(R2)::YFP_Rywal_line 1_6h_T	0,0780	0,4332
ACO(R2)::YFP_Rywal_line 1_6h_C	0,1800	
ACO(R2)::YFP_Rywal_line 1_24h_T	0,0770	0,7178
ACO(R2)::YFP_Rywal_line 1_24h_C	0,1073	
ACO(R2)::YFP_Rywal_line 2_6h_T	0,8845	1,2643
ACO(R2)::YFP_Rywal_line 2_6h_C	0,6996	
ACO(R2)::YFP_Rywal_line 2_24h_T	0,9815	1,5922
ACO(R2)::YFP_Rywal_line 2_24h_C	0,6164	

Continued

Sample	Relative copy number	Relative copy number (T/C)
MC(D1)::YFP_Désirée_line 12_5h_T	Under LOD	/
MC(D1)::YFP_Désirée_line 12_5h_C	Under LOD	
MC(D1)::YFP_Désirée_line 12_23h_T	Under LOD	/
MC(D1)::YFP_Désirée_line 12_23h_C	Under LOD	
MC(D1)::YFP_Désirée_line 13_5h_T	3,7272	1,1478
MC(D1)::YFP_Désirée_line 13_5h_C	3,2473	
MC(D1)::YFP_Désirée_line 13_23h_T	1,8137	1,7723
MC(D1)::YFP_Désirée_line 13_23h_C	1,0234	

RT-qPCR 2

Sample	Relative copy number	Relative copy number (T/C)
ACO(R2)::YFP_Rywal_line 3_6h_T	0,7848	0,9909
ACO(R2)::YFP_Rywal_line 3_6h_C	0,7920	
ACO(R2)::YFP_Rywal_line 5_6h_T	2,2903	0,7006
ACO(R2)::YFP_Rywal_line 5_6h_C	3,2690	
ACO(R2)::YFP_Rywal_line 5_24h_T	2,9535	1,0624
ACO(R2)::YFP_Rywal_line 5_24h_C	2,7799	
MC(D1)::YFP_Désirée_line 14_5h_T	11,9100	3,9131
MC(D1)::YFP_Désirée_line 14_5h_C	3,0436	
MC(D1)::YFP_Désirée_line 14_23h_T	1,9625	0,1522
MC(D1)::YFP_Désirée_line 14_23h_C	12,8923	
TGA(R1)::CFP_Désirée_line 6_1h_T	0,1294	0,8672
TGA(R1)::CFP_Désirée_line 6_1h_C	0,1493	
TGA(R1)::CFP_Désirée_line 6_24h_T	0,1991	1,8585
TGA(R1)::CFP_Désirée_line 6_24h_C	0,1071	
TGA(P1)::YFP_Désirée_line 7_1h_T	QC target failed	/
TGA(P1)::YFP_Désirée_line 7_1h_C	0,0080	
TGA(P1)::YFP_Désirée_line 7_24h_T	0,0079	1,2141
TGA(P1)::YFP_Désirée_line 7_24h_C	0,0065	
TGA(R1)::CFP_Désirée_line 9_1h_T	Under LOD	/
TGA(R1)::CFP_Désirée_line 9_1h_C	Under LOQ	
TGA(P1)::YFP_Désirée_line 8_1h_T	0,0456	2,5546
TGA(P1)::YFP_Désirée_line 8_1h_C	0,0178	
TGA(P1)::YFP_Désirée_line 9_1h_T	0,0307	2,1330
TGA(P1)::YFP_Désirée_line 9_1h_C	0,0144	
TGA(P1)::YFP_Désirée_line 10_1h_T	0,0231	/
TGA(P1)::YFP_Désirée_line 10_1h_C	QC target failed	
TGA(P1)::YFP_Désirée_line 8_24h_T	QC target failed	/
TGA(P1)::YFP_Désirée_line 8_24h_C	QC target failed	
TGA(R1)::CFP_Désirée_line 9_24h_T	Under LOD	/
TGA(R1)::CFP_Désirée_line 9_24h_C	Under LOD	
TGA(P1)::YFP_Désirée_line 9_24h_T	0,0489	0,7104
TGA(P1)::YFP_Désirée_line 9_24h_C	0,0688	
TGA(P1)::YFP_Désirée_line 10_24h_T	0,0411	0,9565
TGA(P1)::YFP_Désirée_line 10_24h_C	0,0429	
ACO(R2)::CFP_Rywal_line 4_6h_T	0,2940	0,9776
ACO(R2)::CFP_Rywal_line 4_6h_C	0,3007	
ACO(R2)::CFP_Rywal_line 10_6h_T	0,1375	0,2679
ACO(R2)::CFP_Rywal_line 10_6h_C	0,5132	
ACO(R2)::CFP_Désirée_line 5_6h_T	QC target failed	/
ACO(R2)::CFP_Désirée_line 5_6h_C	0,1680	
ACO(R2)::CFP_Désirée_line 8_6h_T	0,4687	0,4598
ACO(R2)::CFP_Désirée_line 8_6h_C	1,0193	
ACO(R2)::YFP_Rywal_line 4_6h_T	0,0229	0,0833
ACO(R2)::YFP_Rywal_line 4_6h_C	0,2746	
ACO(R2)::YFP_Rywal_line 6_6h_T	0,7854	0,6567
ACO(R2)::YFP_Rywal_line 6_6h_C	1,1959	

RT-qPCR 3

Sample	Relative copy number	Relative copy number (T/C)
ACO(R2)::YFP_Rywal_line 7_6h_T	1,2264	1,1579
ACO(R2)::YFP_Rywal_line 7_6h_T	1,0591	
ACO(R2)::YFP_Rywal_line 8_6h_T	1,1860	0,8663
ACO(R2)::YFP_Rywal_line 8_6h_C	1,3690	
ACO(R2)::YFP_Rywal_line 9_6h_T	3,5969	1,4024
ACO(R2)::YFP_Rywal_line 9_6h_C	2,5649	
ACO(R2)::YFP_Rywal_line 10_6h_T	0,4313	1,0549
ACO(R2)::YFP_Rywal_line 10_6h_C	0,4088	
MC(D1)::YFP_Désirée_line 15_5h_T	5,0120	1,4843
MC(D1)::YFP_Désirée_line 15_5h_C	3,3767	
MC(D1)::YFP_Désirée_line 16_5h_T	16,1757	1,4851
MC(D1)::YFP_Désirée_line 16_5h_C	10,8921	
MC(D1)::YFP_Désirée_line 17_5h_T	0,5625	0,1845
MC(D1)::YFP_Désirée_line 17_5h_C	3,0494	
MC(D1)::YFP_Désirée_line 18_5h_T	16,9547	2,8762
MC(D1)::YFP_Désirée_line 18_5h_C	5,8948	
ACO(R2)::CFP_Rywal_line 4_24h_T	1,8056	/
ACO(R2)::CFP_Rywal_line 4_24h_C	QC target failed	
ACO(R2)::CFP_Rywal_line 10_24h_T	2,4462	1,9582
ACO(R2)::CFP_Rywal_line 10_24h_C	1,2492	
ACO(R2)::CFP_Désirée_line 5_24h_T	0,6699	/
ACO(R2)::CFP_Désirée_line 5_24h_C	QC target failed	
ACO(R2)::CFP_Désirée_line 8_24h_T	QC target failed	/
ACO(R2)::CFP_Désirée_line 8_24h_C	4,0291	
ACO(R2)::YFP_Rywal_line 6_24h_T	3,1139	0,9961
ACO(R2)::YFP_Rywal_line 6_24h_C	3,1260	
ACO(R2)::YFP_Rywal_line 7_24h_T	2,7393	1,2186
ACO(R2)::YFP_Rywal_line 7_24h_C	2,2479	
ACO(R2)::YFP_Rywal_line 8_24h_T	1,1962	0,6660
ACO(R2)::YFP_Rywal_line 8_24h_C	1,7963	
ACO(R2)::YFP_Rywal_line 9_24h_T	6,2757	1,5466
ACO(R2)::YFP_Rywal_line 9_24h_C	4,0577	
ACO(R2)::YFP_Rywal_line 10_24h_T	0,4384	0,2626
ACO(R2)::YFP_Rywal_line 10_24h_C	1,6695	

RT-qPCR 4

Sample	Relative copy number	Relative copy
TGA(R1)::CFP_Désirée_line 4_1h_T	0,3321	0,6876
TGA(R1)::CFP_Désirée_line 4_1h_T	0,2633	
TGA(R1)::CFP_Désirée_line 4_1h_T	0,4636	
TGA(R1)::CFP_Désirée_line 4_1h_C	0,6055	
TGA(R1)::CFP_Désirée_line 4_1h_C	0,6678	
TGA(R1)::CFP_Désirée_line 4_1h_C	0,2669	
TGA(P1)::YFP_Désirée_line 1_1h_T	0,1351	0,8818
TGA(P1)::YFP_Désirée_line 1_1h_T	0,4180	
TGA(P1)::YFP_Désirée_line 1_1h_T	0,1327	
TGA(P1)::YFP_Désirée_line 1_1h_C	0,3913	
TGA(P1)::YFP_Désirée_line 1_1h_C	0,1426	
TGA(P1)::YFP_Désirée_line 1_1h_C	0,1272	
ACO(R2)::CFP_Rywal_line 1_6h_T	0,3148	1,2402
ACO(R2)::CFP_Rywal_line 1_6h_T	0,2234	
ACO(R2)::CFP_Rywal_line 1_6h_T	0,4484	
ACO(R2)::CFP_Rywal_line 1_6h_C	0,2127	
ACO(R2)::CFP_Rywal_line 1_6h_C	0,2767	
ACO(R2)::CFP_Rywal_line 1_6h_C	0,3060	
ACO(R2)::CFP_Désirée_line 2_6h_T	0,1299	1,4264
ACO(R2)::CFP_Désirée_line 2_6h_T	0,1493	
ACO(R2)::CFP_Désirée_line 2_6h_T	0,0857	
ACO(R2)::CFP_Désirée_line 2_6h_C	0,0721	
ACO(R2)::CFP_Désirée_line 2_6h_C	0,1356	
ACO(R2)::CFP_Désirée_line 2_6h_C	0,0481	

A.3 Supplementary Material for Section 3.4

Republished from Cell death is not sufficient for the restriction of potato virus Y spread in hypersensitive response-conferred resistance in potato, Lukan et al., 2018a, Frontiers in Plant Science, 9, 168. doi: 10.3389/fpls.2018.00168, according to Creative Common Attribution ("CC BY") licence.

Supplementary Image 1: Number of lesions developed on potato leaves after inoculation with PVY N605-GFP. Lesions were counted in 16 cv. Rywal and 11 NahG-Rywal plants on all three inoculated leaves from 3 dpi to 7 dpi and on 11 dpi to study the dynamics of lesions formation. (A) Numbers of lesions observed on each plant leaf at each timepoint is shown. X: detached leaf for confocal microscopy, F: the leaf fell off the plant, /: lesions not counted. Some of the leaves (marked with green in the tables) from this experiment were detached from the plants and used to follow virus spread around the lesions (see exp2 in Supplementary Table 1). (B) Numbers of lesions for each plant leaf from 3 to 11 dpi for selected plants for both genotypes. (C) Standardized imputed cumulative sums of lesions on each leaf from 3 to 11 dpi for selected plants (see Methods for data filtering details/description).

(A)

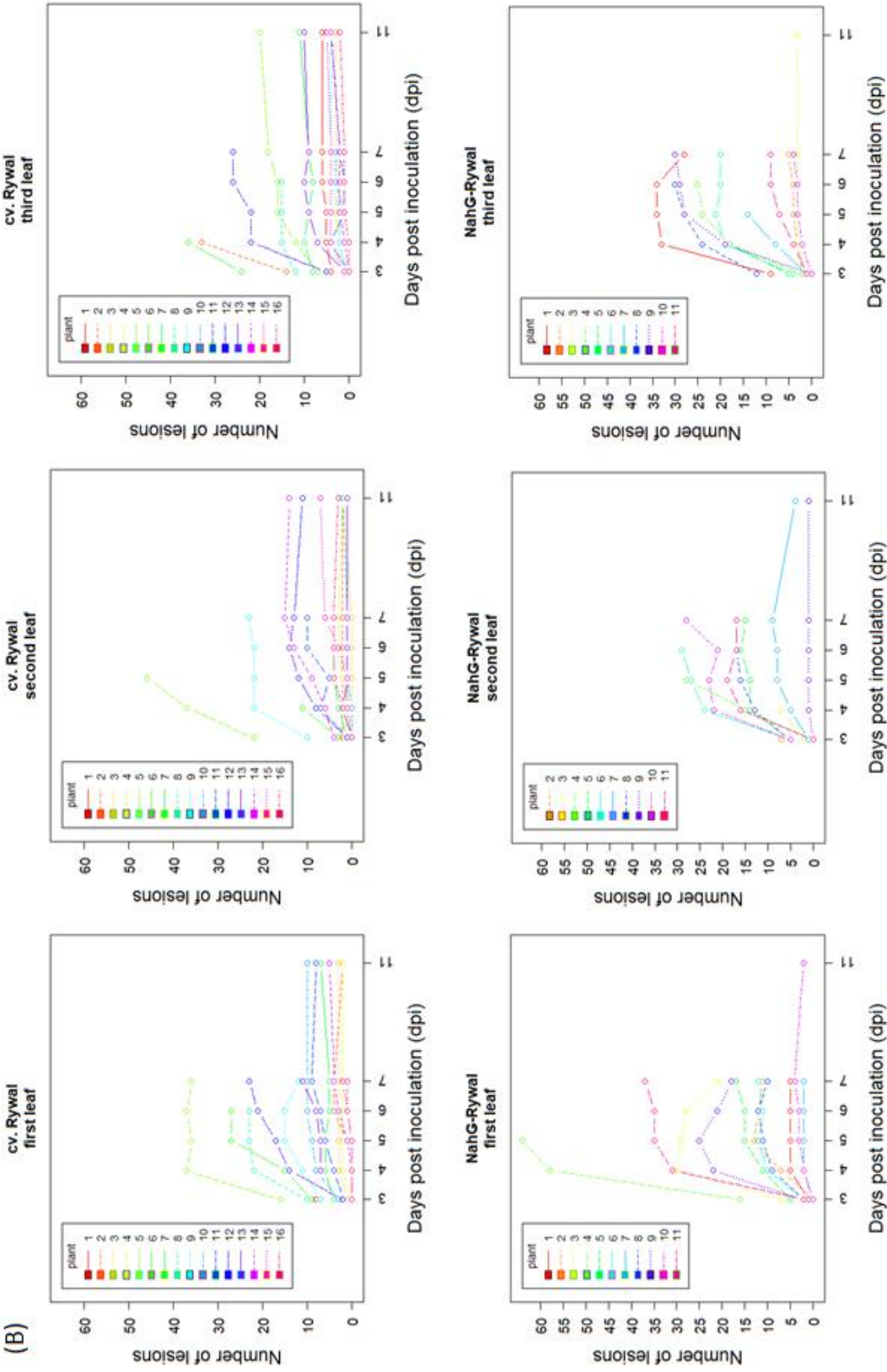
cv. Rywal

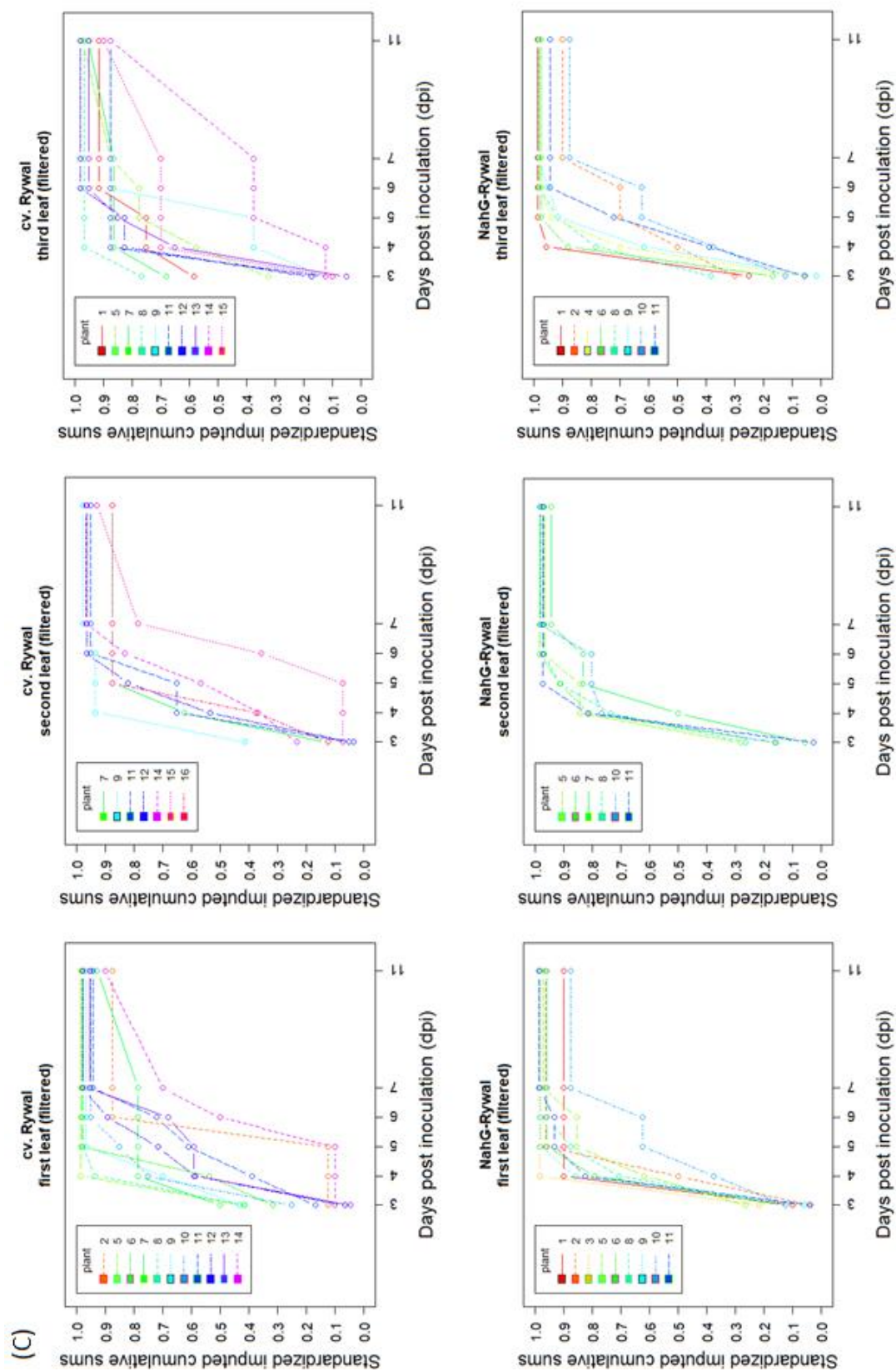
plant no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16																																
dpi/leaf no	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3																								
3dpi	8	2	4	0	2	14	1	0	1	1	16	22	7	9	3	24	4	1	8	10	0	12	7	10	1	3	0	0	2	1	0	2	1	5	/	0	1	0	4	0	0	1	0	0	0			
4dpi	x	x	5	0	2	33	3	0	2	2	1	2	37	37	12	15	11	36	6	3	10	22	1	15	11	22	2	8	0	0	4	7	4	14	8	22	7	2	7	0	6	1	0	1	4	0	2	0
5dpi	x	x	5	1	2	x	3	0	3	2	2	3	36	46	16	27	x	x	6	4	9	23	1	15	15	22	2	9	1	1	6	5	1	17	12	22	7	1	9	1	9	2	0	1	4	0	4	1
6dpi	x	x	6	4	2	x	3	0	2	2	3	x	37	x	16	27	x	x	5	4	8	23	1	15	15	22	4	10	1	3	7	10	1	21	14	26	8	1	10	3	13	2	1	3	4	1	4	1
7dpi	x	x	6	4	2	x	3	0	3	2	3	x	36	x	18	F	x	x	5	9	F	1	x	12	23	4	10	1	3	9	10	2	23	13	26	1	1	19	4	15	2	2	6	4	1	4	1	
11dpi	x	x	6	2	2	x	3	2	3	2	2	x	F	x	20	F	x	x	7	x	11	F	2	x	F	x	4	10	1	x	8	F	4	F	11	F	F	1	10	5	14	4	F	7	5	F	3	2

NahG-Rywal

plant no	1	2	3	4	5	6	7	8	9	10	11	
dpi/leaf no	1	2	3	1	2	3	1	2	3	1	2	3
3dpi	1	F	9	0	7	2	1	16	0	2	5	0
4dpi	5	F	33	7	x	3	30	7	3	58	15	18
5dpi	5	F	34	13	x	4	29	x	3	64	28	24
6dpi	5	F	34	12	x	4	28	x	3	x	25	16
7dpi	5	F	28	11	x	5	21	x	3	x	F	17
11dpi	F	F	F	x	/	F	x	3	x	x	x	x

(B)





Supplementary Table 14: Lesions with the PVY N605-GFP accumulation detected outside the cell death zone. (A) Number of lesions with

the PVY N605-GFP accumulation detected outside the cell death zone after inoculation. Results from seven independent experiments (Exp 1-7) are presented for cv. Rywal and NahG-Rywal. At each time point (3-12 dpi) one or two plants (A, B) were analysed (one leaf per plant). The number of positive lesions (number of lesions with the PVY N605-GFP accumulation detected outside the cell death zone) on the particular day post inoculation / the number of all analysed lesions are shown. - denotes that a plant was not analysed at a particular timepoint. (B) Number of the GFP-containing cells around the positive lesions in cv. Rywal. n.c. denotes that GFP-containing cells around the particular positive lesions were not counted. Republished from Cell death is not sufficient for the restriction of potato virus Y spread in hypersensitive response-conferred resistance in potato, Lukan et al., 2018a, *Frontiers in Plant Science*, 9, 168. doi: 10.3389/fpls.2018.00168, according to Creative Common Attribution ("CC BY") licence.

(A)**cv. Rywal**

Exp	Plant	Number of positive lesions at given time point							
		3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	8 dpi	11 dpi	12 dpi
Exp1	A	-	3/5	2/6	-	3/8	3/10	-	2/10
Exp2	A	3/4	4/9	0/3	1/4	0/10	-	1/5	-
	B	-	2/10	0/6	1/7	0/2	-	1/3	-
Exp3	A	2/10	-	-	-	-	-	-	-
	B	4/7	-	-	-	-	-	-	-
Exp4	A	-	-	0/24	1/15	1/20	0/18	-	-
	B	-	-	0/20	0/12	-	-	-	-
Exp5	A	-	-	1/11	3/29	3/14	-	-	-
Exp6	A	0/10	4/9	3/10	2/9	0/11	-	-	-
Exp7	A	-	-	-	-	-	-	-	-

NahG-Rywal

Exp	Plant	Number of positive lesions at given time point							
		3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	8 dpi	11 dpi	12 dpi
Exp1	A	-	-	6/6	-	5/5	-	-	-
Exp2	A	0/9	4/6	2/7	3/6	3/7	-	4/5	-
	B	-	1/2	3/4	1/4	2/2	-	2/2	-
Exp3	A	-	-	-	-	-	-	-	-
	B	-	-	-	-	-	-	-	-
Exp4	A	-	-	3/17	4/15	2/14	2/16	-	-
	B	-	-	1/11	-	-	-	-	-
Exp5	A	-	-	4/9	4/10	-	-	-	-
Exp6	A	0/10	4/10	5/10	8/10	6/8	-	-	-
Exp7	A	-	-	-	-	-	-	8/10	-

Continued

(B)**cv. Rywal**

Exp	Plant	Number of GFP-containing cells around positive lesions							
		3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	8 dpi	11 dpi	12 dpi
Exp1	A		3, 3, 2	2, 3	-	1, 4, >5	3, >5, 2	-	>5, 3
Exp2	A	>5, 1, 3	2, 2, 2, 2	0	2	0	-	>5	-
	B		n.c., 2	0	1	0	-	>5	-
Exp3	A	5, 2	-	-	-	-	-	-	-
	B	>5, >5, 1, 4	-	-	-	-	-	-	-
Exp4	A	-	-	0	1	>5	0	-	-
	B	-	-	0	0	-	-	-	-
Exp5	A	-	-	1	3, 1, 5	4, 1, >5	-	-	-
Exp6	A	0	1, 1, 1, 3	4, 2, 2	3, 1	0	-	-	-
Exp7	A	-	-	-	-	-	-	-	-

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Publications Related to the Thesis

Journal Articles

- Lukan, T., Baebler, Š., Pompe-Novak, M., Guček, K., Zagorščak, M., Coll, A., & Gruden, K. (2018a). Cell death is not sufficient for the restriction of potato virus Y spread in hypersensitive response-conferred resistance in potato. *Frontiers in Plant Science*. 9, 168. doi: 10.3389/fpls.2018.00168
- Lukan, T., Machens, F., Coll, A., Baebler, Š., Messerschmidt, K., & Gruden, K. (2018b). Plant X-tender: An extension of the AssemblX system for the assembly and expression of multigene constructs in plants. *PLoS ONE*, 13(1):e0190526. doi: 10.1371/journal.pone.0190526

Biography

Tjaša Lukan was receiving scholarship for gifted pupils from 2004 till 2013, graduated from high school with honours (2008) and received a recognition award for outstanding study achievements in the 1st, 2nd and 3rd year of her undergraduate study of Biochemistry. Tjaša finished her studies in 2013 (Biochemistry, Department of Chemistry and Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia) with the diploma thesis entitled »Opredelitev in uporaba monoklonskih protiteles IgM proti zaporedju EFTYQLTSSPTC, izbranem na osnovi modela bilitranslokaze« (supervisor: Prof. Dr. Vladka Čurin Šerbec, co-supervisor: Dr. Uroš Rajčević, Blood Transfusion Centre of Slovenia, University of Ljubljana, Slovenia). In the context of her diploma thesis, she published one scientific publications entitled »New monoclonal antibodies against bilitranslocase as a diagnostic tool in determining the progress of clear cell renal cell carcinoma« as a shared first author. From 2013 she is working as a young researcher at the National Institute of Biology, Department of Biotechnology and Systems Biology under the supervision of Prof. Dr. Kristina Gruden. In the same year she enrolled in the third-level study program Nanosciences and Nanotechnologies at the Jožef Stefan International Postgraduate School, Ljubljana, Slovenia. She is working on the topics related to investigation of interactions between potato virus Y (PVY) and its hosts, different types of potato cultivars. Her research is focused on identifying key components of the plant defense signalling network in order to provide insights into potato responses to PVY infection. In 2016, she visited the Institute of Biochemistry and Biology (Supervisor: Dr. Katrin Messerschmidt, Group Cell2Fab, University of Potsdam, Germany), where she spent a month, designing the system for the assembly and expression of multigene constructs in plants. Throughout her study Tjaša attended several scientific meetings to extend the knowledge of her research topics. She actively participated in several workshops related to different microscopy techniques and bioinformatic analyses of biological systems and conferences covering the topics within the area of plant science, mainly plant-pathogen interactions. Moreover, Tjaša actively participated in events aimed at educating the public, such as Fascination of Plants Day and Researchers' Night and was involved as a working mentor for the master theses of three undergraduate students.