Dissection of Innate Immunity in Tomato and Tolerance to Bacterial Wilt in Solanaceae species

Anastasia Naumenko

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Plant Pathology, Physiology and Weed Science Department

Boris A. Vinatzer

John M. McDowell

Richard E. Veilleux

Bingyu Zhao

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ABSTRACT

Unlike mammals, plants do not have specific immune cells. However, plants can still recognize pathogens and defend themselves. They do that by recognizing microbial-associated molecular patterns (MAMPs) and secreted pathogen proteins, called effectors. MAMP-triggered immunity (MTI) relies on recognition of MAMPs by leucine-rich repeats (LRRs) pattern-recognition receptors (PRRs). The best-studied LRR PRR is Flagellin-Sensitive 2 (Fls2), the receptor of a 22-amino acid long epitope of bacterial flagellin, called flg22. In this project, alleles of *FLS2* of different tomato cultivars were sequenced and compared to each other to get insight into natural selection acting on *FLS2* and to identify residues important for ligand binding. This information may be used in the future to engineer Fls2 for improved ability to recognize flagellin. MTI can be suppressed by effectors secreted by bacteria into plant cells through the type III secretion system. On the other hand, plants are equipped with repertoires of resistance proteins, which can recognize some pathogen effectors. If a pathogen carries an effector that is recognized, effector-triggered immunity (ETI) is activated and the plant is resistant. Here, eggplant breeding lines were screened for their ability to activate ETI upon recognition of effectors of the soil borne pathogen *Ralstonia solanacearum*, a causative agent of bacterial wilt. Four effectors were found to trigger plant defenses in some of the lines. This is the first step in cloning the genes coding for the responsible resistance proteins. These genes may be used in the future for engineering tomato and potato for resistance to bacterial wilt.

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DEDICATION

I dedicate this thesis to my parents, Nick and Zinaida Naumenko, who have all the will and power to support at every step of my life me despite of thousands miles between us. Thank you, Mom and Dad.

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List of abbreviations

Avr protein	Avirulence protein
EFR	Ef-Tu receptor
Ef-Tu	Elongation factor Tu
ETI	Effector-triggered immunity
EtHAn	Effector-to-host analyzer
flg22	Flagellin22
FLS2	*FLAGELLIN SENSITIVE2*
HR	Hypersensitive response
LRRs	Leucine-rich repeats
MAMPs(PAMPs)	Microbial/pathogen associated molecular patterns
PRRs	Pattern-recognition receptors
PTI	PAMP-triggered immunity

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CHAPTER 1. INTRODUCTION

PLANT IMMUNITY

The most important ability of immune systems is to distinguish between self and non-self. In plants and animals, the mechanisms of immunity were evolutionarily selected through many different host-pathogen interactions. In general, these interactions are based on the recognition of specific molecular patterns of the pathogens by multiple host receptors located on the cell surface or in the intracellular space. Both, plants and animals, share the ability to rearrange receptors (Rodriques et al., 2012). This trait has evolved as an effective response to pathogen evolution since pathogens re-arrange or lose genes coding for molecular patterns to avoid recognition (Rodriques et al., 2012).

Different from animals, plants do not have a circulatory system and do not move. Plants have not evolved an adaptive immune response either. Nonetheless, plants are challenged by multiple pathogens and are resistant to most of them. The immune system of plants is complex but can be dissected into two main branches.

The first branch consists in natural barriers between plants and attacking microorganism. Unlike mammalian cells, plant cells have rigid and thick cell walls, leaf hairs, and a hydrophobic and thick layer of wax covering plant organs (Freeman and Beattie, 2008). Moreover, plant cells produce toxic secondary compounds – chemicals that are essential for plant defense. Secondary compounds, such as alkaloids and glycosides, create a protective chemical barrier (Freeman and Beattie, 2008). In most cases, natural barriers are sufficient to avoid invaders.

To pass the natural barrier, pathogens developed different strategies, such as avoidance/resistance to chemical attack, fast invasion through stomata or open wounds, and/or simply increasing the quantity of pathogen cells (Freeman, Beattie, 2008). If a pathogen is able to pass the physical barriers, the major system, called plant innate immunity, needs to be activated.

Plants can only rely on their innate immunity to fight the disease; therefore, a complexity of this “plant under attack” system is more than reasonable. Several lines of active defense response possessed by plants can be described.

MAMP-TRIGGERED IMMUNE RESPONSE

The first branch of the plant innate immune system consists of transmembrane pattern recognition receptors (PRRs) which are able to activate immune responses by recognition of specific molecules – PAMPs. PAMPs (or MAMPs) (pathogen/microbial-associated molecular patterns) are small extracellular molecules common to many classes of microbes (Ali and Reddy, 2008). The best-studied molecule activating plant defense is flg22, a short 22 amino acid long peptide derived from flagellin, the main building block of a bacterial flagellum (Bardoel et al., 2011). The direct interaction between flg22 and FLS2 (FLAGELLIN SENSITIVE2), a leucine-rich receptor has been shown to elicit manifold immune responses in *Arabidopsis* (Chinchilla et al., 2006).

Besides flagellin, other molecules can be recognized as PAMPs. Some examples include lipopolysaccharides, chitin, and bacterial elongation factor Tu (EF-Tu). Recognition of PAMPs leads to a MAP kinase cascade. Interestingly, different PAMPs can activate the same kinase pathway (for example, flg22 upon binding to FLS2 and Ef-Tu upon binding to EFR). This can be explained by the interaction of both FLS2 and EFR with the same co-receptor, BAK1, and therefore the network is shared between multiple receptors (Sun et al., 2011). The immune response triggered after PAMP recognition includes immediate responses and delayed responses. Among immediate responses, an oxidative burst (production of reactive oxygen species, ROS) can be named (Bailey-Serres and Mittler, 2006). Delayed responses include thickening of cell walls, callose deposition in the cell wall and altered accumulation of defensive proteins such as proteases and chitinases. These components of immune response affect the pathogen and prevent further development of the infection.

EFFECTOR-TRIGGERED PLANT IMMUNITY

Successful plant pathogens efficiently suppress PAMP-triggered immune responses by secreting effector proteins (pathogen-encoded secreted proteins). Effector proteins manipulate host gene expression, affect cell signaling, and thus induce what is referred to as effector-triggered susceptibility (Howden et al., 2012). Pathogens can secrete both extracellular effectors (which

accumulate in the apoplastic space) and intracellular effectors, which, upon secretion, travel to different cell compartments and target specific processes in plant cells (Jones, Dangl, 2006).

For gram-negative bacteria, the most important secretion system is the Type 3 secretion system (T3SS), which injects virulence factors into the host cell. The T3SS delivers effector proteins through the bacterial inner membrane, periplasm, outer membrane, and plant cell membrane into the host cell. This injectisome (Fig.1) consists of a hollow tube, approximately 25A in diameter and 60 nm in length (Cornelis, 2009) and is activated when it comes into direct contact with the host cell membrane. It is still unclear upon which signals bacteria start assembling the T3SS (Enninga et al., 2009).

The most important function of effector proteins in the host cell is their interaction with the immune system of the plant and alteration of proteins, which are capable of triggering immune responses and thereby suppressing plant immunity (Deslandes and Rives, 2012). However, effectors can also elicit plant immunity. The well-known example of an effector blocking the plant immune response is AvrPto (Angot et al., 2007). This protein binds to FLS2 and blocks early immune responses by interfering with flagellin recognition by this PAMP receptor. Effector proteins can also target proteasome degradation pathways in the host cell (as it will be discussed for some *Ralstonia* effectors later). For example, the HopM1 effector protein of *Pseudomonas syringae* targets the host protein AtMIN7, mediating its subsequent degradation (Angot et al., 2007). By changing expression level and targeting host proteins for degradation, bacteria sufficiently evade immune responses and are able to colonize the plant.

However, some of the effectors (avirulence factors) can be recognized. Plants evolved R proteins (resistance proteins), which interact with avirulence factors and are activated upon that interaction. Most R proteins contain a nucleotide-binding site (NBS), which together with leucine-rich repeats of these proteins (LRR) work as an active domain, which activates various protein kinase (mitogen-activated (MAP-kinase), calcium-dependent) cascades after the recognition of the effector (Zhang et al., 2012). Recent works indicated that programmed cell death can be also activated by metacaspases (Spoel et al., 2012). The gene-for-gene hypothesis is strongly supported by R gene –effector pairs, but the direct interaction between effector protein and R protein is rarely found (Bent and Mackey, 2007, Dangl and Jones, 2011).

The guard model explains how R proteins can “guard” host proteins to avoid effector impact by either direct binding of the R protein to the targeted host protein or binding upon

effector recognition (Dangl and Jones, 2001). In both cases, the activation of defense genes leads to a massive immune response.

Pathogens and plants both take parts in the so-called “arms race”, which describes the evolution of the plant immune system. In this race, pathogens evolve new effectors, change the structure of old ones or eliminate old ones in order to avoid recognition by plants; at the same time, plants evolve new R proteins or old R proteins become capable of recognition of more than one effector.

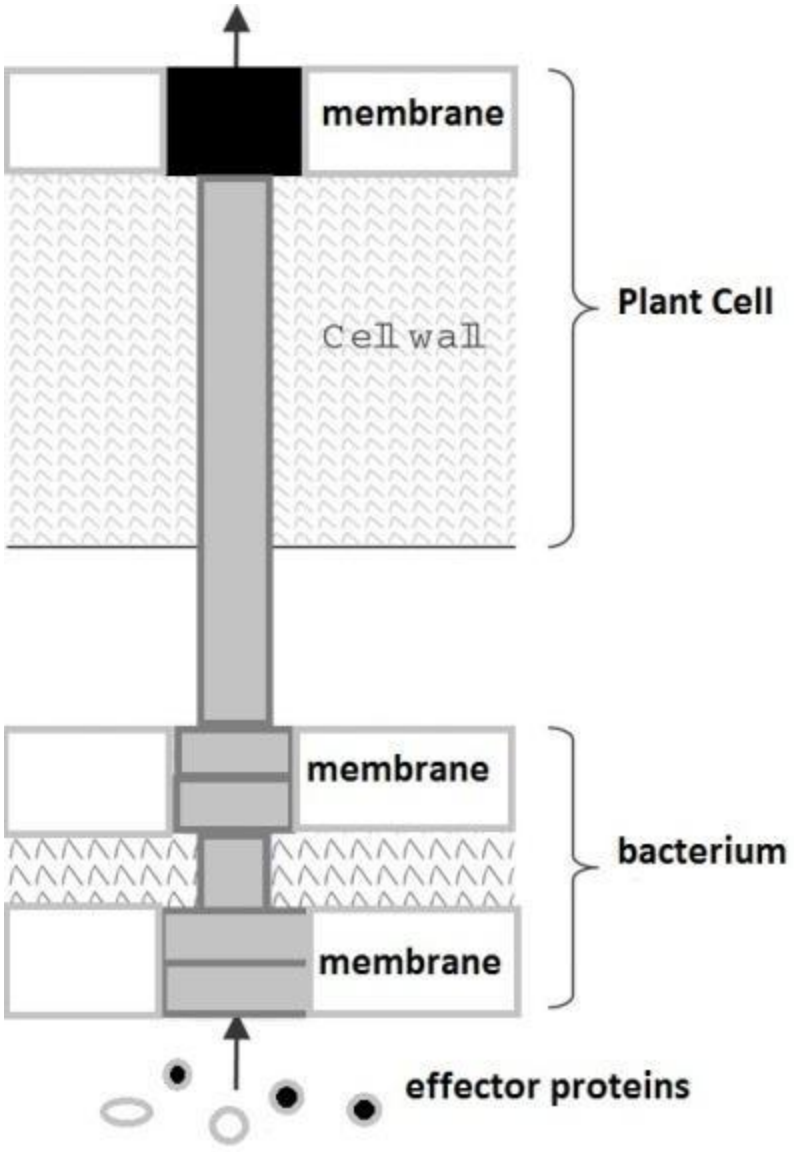


Fig.1. T3SS system of *Pseudomonas syringae*, schematic (adapted from Yang et al., 2010)

RALSTONIA SOLANACEARUM: A CASUAL AGENT OF PLANT BACTERIAL WILT

In 1995, the bacterium named *Ralstonia solanacearum* was described as a member of the family Ralstoniaceae included in the β-subdivision of the Proteobacteria (Yabuuchi et al., 1995).

Ralstonia solanacearum, previously known as *Pseudomonas solanacearum*, is a casual agent of bacterial wilt. This gram-negative, rod-shaped bacterium with polar flagella has a very high impact on economics worldwide, causing dramatic losses in yield. Affected crops range from tomato and potato to banana including more than 200 species in 53 different plant families (Alvarez et al., 2008). Broad host range, species composed of a large group of strains and fast development of disease symptoms probably make *Ralstonia* one of the most destructive plant pathogens worldwide (Mansfield et al., 2012). *R. solanacearum* is an endemic pathogen in tropical regions, where the range of disease and therefore economic losses are particularly dramatic. For quarantine areas, *Ralstonia* is also responsible for important restrictions on the production on contaminated land. It is difficult to estimate or quantify damages caused by *Ralstonia* because of its wide geographical distribution and multiple hosts but, for example, on potato only the estimated losses are over $1 billion per year worldwide (Gabriel et al., 2006).

Ralstonia solanacearum is a soil-born pathogen, which infects plants through roots, especially wounds and smaller cracks, and invades xylem. After infection, the pathogen rapidly colonizes the vascular system of the plant, invading the root xylem first and reaching stem and leaves through vessels then (Alvarez, 2008). There are several external and internal symptoms of the disease. External symptoms include wilting, stunting and yellowing of leaves and stems (Kelman, 1953). Frequently observed internal symptoms include tissue discoloration, xylem discoloration and degradation and cell death of infected areas. Biochemically, *Ralstonia* can block xylem vessels and alter water movement by producing extracellular polysaccharide (EPS1) (Genin et al., 2002). EPS1 might also contribute to *Ralstonia* virulence by minimizing contact of bacterial cells surface with the plant cell, therefore avoiding recognition (Schell, 2000).

Ralstonia has been extensively studied biochemically and genetically. The complete genomic sequence of one strain was published in 2002 (Salanoubat et al., 2002). The pathogen genome consists of a 3.7 Mb chromosome and a 2.1 Mb megaplasmid, with an average G+C content as high as 67% (Genin et al., 2002, Salanoubat et al., 2002). The chromosome carries genes necessary for the survival, and the megaplasmid contains genes required for virulence, including *hrp* (*harp*) genes, along with duplicates of metabolic genes. *Hrp* genes encode type III

secretion system pathways and are required in many phytopathogenic bacteria to elicit HR in plants (Zhu et al., 2000). The fitness of the bacterium and its ability to adjust to environmental changes are also determined by megaplasmid genes. A well-known phenomenon of Ralstonia is its genetic instability; rearrangements have been found in the GMI1000 genome (Genin and Boucher, 2002). These rearrangements have contributed to the evolution of *Ralstonia* strains.

The genes coding for the T3SS are called *hrp* (Hypersensitive response and pathogenicity) because mutations in the genes coding for T3SS lead to an inability to cause the hypersensitive response in non-host plants and reduce pathogenicity in host plants (Mukaihara et al., 2009). The T3SS injects effector proteins into the plants cell; more than 200 potential effector proteins were predicted in different *Ralstonia* strains based on the comparison to well-known ones (Mukaihara et al., 2009).

Ralstonia is now a model pathogen for the study of virulence determinants, particularly bacterial effector proteins. The pathogen delivers effectors into the plant cell via the T3SS, similarly to *Pseudomonas* and other Gram-negative plant pathogens (Mukaihara, 2010). *Ralstonia solanacearum* is defined as "species complex" and strains of *Ralstonia* belong, according to newest classification (Lebeau et al., 2011), to four different phylotypes based on accessible genome sequences. This phylogenetic diversity of *Ralstonia* strains provided an opportunity to evaluate the resistance of crops to different phylotypes of the pathogen and, therefore, find potential sources of resistance to use in future breeding or engineering of susceptible crops (Lebeau et al., 2011). In this recent work, a collection of breeding lines of tomato, eggplant, and pepper was challenged with *Ralstonia* strains belonging to different phylotypes. *Ralstonia* strains were chosen based on host specificity and geographical origin. Plants revealed different responses to *Ralstonia* infection. However, no tomato or pepper accession showed resistance to the most aggressive strains of the pathogen, while some resistance was found in eggplant accessions. In particular, strain GMI1000 was able to colonize both tomato and eggplant accessions, except for the T5, T6, T8 tomato breeding lines and the E1 and E2 eggplant breeding lines. Strain CFBP2957 was highly aggressive on tomato (except for line T4) but did not cause wilting or stem colonization in most eggplant accessions (E1-E5, E10). CMR15 infection of tomato caused wilting of all the lines tested, though some resistance was found in E1, E2 and E3 eggplant lines. Interestingly, this highly aggressive strain had almost no impact on pepper accessions; pepper breeding lines challenged by CMR15 showed resistance in

8 lines out of 10 and latent infection (high colonization but no wilting symptoms) in one accession out of the remaining two.

The well-studied effectors of *Ralstonia* include the GALA effector family of strain GMI1000 (phylotype I). The GALA effector family, which consistis of 7 proteins, was revealed based on its similarity with the F-box proteins (components of E3-ubiquitin ligase complexes) in plants. As bacteria do not have their own proteasome system, it has been predicted that GALA effector proteins manipulate the host-ubiquitin proteasome system, enabling interactions between the LRR (leucine-rich repeats) of the GALA effector and plant proteins targeted for ubiquitination (Remigi et al., 2011).

Another described effector, popP2, belongs to the YopJ-like family of cysteine proteases. Autoacetylation of the effector and subsequent interaction with the resistance protein RRS1-R in Arabidopsis prevents proteasomal degradation and triggers a defense response (Tasset et al., 2010).

The PopP1 effector shares amino acids characteristic of cysteine proteases (Orth et al., 2000) and is closely related to the avirulence proteins AvrRxv, AvrBsT, AvrXv4, and XopJ of *Xanthomonas* species, and to the AvrPpiG1 protein of *Pseudomonas syringae* pv. *pisi* (Corpet, 1988). PopP1 also belongs to the YopJ-like family of proteases (Lavie et al., 2002).

Interestingly, Hrp regulation of listed effectors may be conserved in all *Ralstonia* strains; also, most of predicted effector proteins were identified based on sequence comparison with known effector sequences. Overall, effectors share similarities between strains, and most of the known effectors require an Hrp-associated protein, HpaB, for their transfer into the plant cell (Mukaihara et al., 2009).

High genetic diversity within the *Ralstonia* species complex and the different ability of pathogens belonging to different phylotypes to cause disease in crops may be used as an efficient tool for screening crop breeding lines to reveal new genetic sources of resistance to this pathogen.

Agrobacterium-mediated transient assays are used to determine the role of effector proteins and find potential sources of resistance to a pathogen in different plant species as a good alternative to stable transformation and genetic complementation (Wroblewski et al., 2005). The method showed high efficiency and was reproducible in *Nicotiana benthamiana* (tobacco) and *Phaseolus vulgaris* (bean) (Vinatzer et al., 2006). Transient assays were later adapted for

various plant species (Bhaskar et al., 2009). In the assay, plants are challenged with the bacterial strain complemented with the effector under the control of the DEX promoter. This method allows identifying which effectors are recognized by the plant immune system based on the hypersensitive response caused by infiltration (Vinatzer et al., 2006).

However, *Agrobacterium* assays often need to be adapted to specific conditions and/or plants tested. To further investigate the function of virulence and avirulence proteins injected through T3SS systems, a new approach has been recently developed (Fabro et al., 2011). This approach is based on the natural way of effector delivery into cells through the T3SS system. In the system (EtHAn, Effector to Host Analyser), the complete *hrp/hrc* region of *P. syringae* was introduced into the soil bacterium *Pseudomonas fluorescens*; as a result, *P. fluorescens* can now inject individual effector proteins expressed in the same strain into plants to study them one at the time.

This project was mainly focused on (1). Identification and comparison of *FLS2* alleles from different tomato cultivars followed by subsequent transformation of tomato with different *FLS2* allele and (2). Determining an effector gene of *Ralstonia* which might be able to trigger immune response in pathogen-resistant eggplant breeding lines and thus identify the source of resistance to bacterial wilt.

CHAPTER 2. MATERIALS AND METHODS

Effector cloning

Effector sequences were used to design primers that amplify the entire open reading frames plus 15 bp upstream of the start codon and not including the STOP codon. Amplified sequences were then cloned into the Gateway™ (Life Technologies) entry clone pDONR221 (Fig.1) and from there into destination vectors.

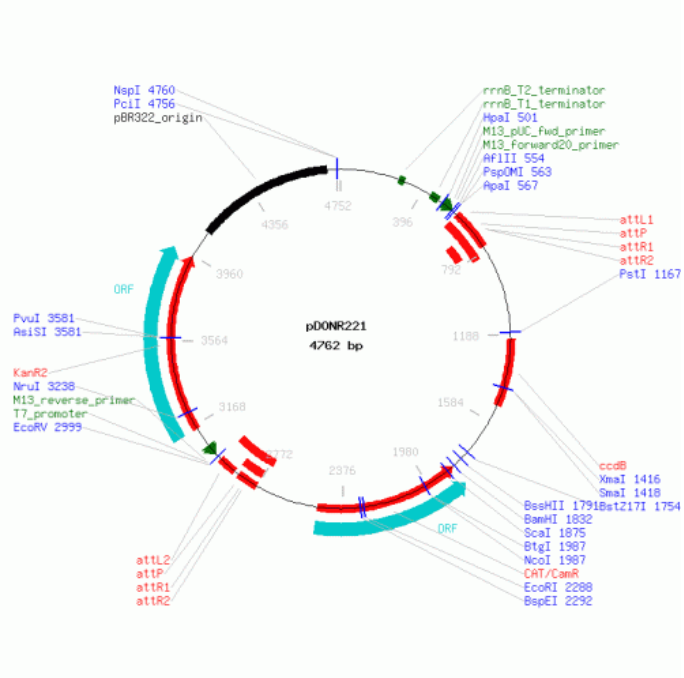


Fig.2. Circular map of pDONR221 entry vector used for Gateway cloning.

Due to the high GC content (up to 70%) and the limited choice of primer annealing sites, the following strategy was developed and successfully used to clone genes of interest.

Primers for effectors of the following four *Ralstonia* strains were designed: GMI1000, CMR15, MOLK2, CFBP. Primer sequences and are listed in Table1.

Table 1. Primer sequences designed for effector genes cloning.

DNA source	Effector name	Forward primer sequence	Reverse primer sequence
CMR15	GALA3-CMR15	AAAAAGCAGGCTACGCAGAGAGCGCAATGGGAAAC	AGAAAGCTGGGTAAATCCGCAGCGTCACGCCGAT
CMR15	popP2-CMR15	AAAAAGCAGGCTCGACCGTCGAGCGAATGC	AGAAAGCTGGGTAATCGCTATTCAATATGGAATTCT
GMI1000	Rsc0826 popP1	AAAGCAGGCTGGAATCTCGCAACGATGAAA	AGAAAGCTGGGTACGACTCCAGGGCATGTCGAA
GMI1000	Rsc0868 popP2	AAAAAGCAGGCTTCGAACGGATGGGTGTGGAT	AGAAAGCTGGGTAGTTGGTATCCAATAGGGAATCCT
GMI1000	Rsp0028 GALA3	AAAAAGCAGGCTAGCCACGGACGGAAATGGCTC	AGAAAGCTGGGTAAATCCGCAGCGTCACGCCGAT
GMI1000	Rsp0572	AAAAAGCAGGCTGCAACAACGACACGATGCT	AGAAAGCTGGGTATGCGTTGCGTGGCTTGTA
CMR15	Rsp1130 -CMR15	AAAAAGCAGGCTGGAACCCTCACGACATGG	AGAAAGCTGGGTAAGCCGCCTGCCGGATCG
CFBP	Rsp1130 -CFBP	AAAAAGCAGGCTAGCGCTCTCACGACATGG	AGAAAGCTGGGTAGGCTGCCAGCTCAGCGGCCTGCGT
GMI1000	Rsp1130	AAAAAGCAGGCTGGAACCCTCACGACATGGA	AGAAAGCTGGGTAAGCAGCCTGTCGGATCG
CFBP	Rsp1384 -CFBP	AAAAAGCAGGCTGGTCAATCCAGGCCATGAAA	AGAAAGCTGGGTAAGCGTGCCGGGCGCGGTAA
CMR15	Rsp1384 -CMR15	AAAAAGCAGGCTCCCCGCGTCCGGCGTTGGT	AGAAAGCTGGGTAAGTGTGCGGGCCGGGGCCGGGATACT
MOLK2	Rsp1384 MOLK2	AAAAAGCAGGCTGGTCAATCCAGGCCATGAAA	AGAAAGCTGGGTAAGCGTGCCGGCCGGCGTAACGGGCGCGCAGGG
GMI1000	Rsp1384	AAAAAGCAGGCTGGTCCATTCAGGCCATGAAAGTCAA	AGAAAGCTGGGTAAGCGTACGGGCCGGGGCCGGGAT

Polymerase chain reactions were first performed with IMMOMIX (Bioline) to determine whether the primers amplified sequences of the expected size. However, since the IMMOMIX enzyme does not have a proof reading function primers that gave a product of the expected size then needed to be amplified again with the iProof high fidelity polymerase (Bio-Rad) for cloning.

To improve PCR efficiency, 50% DMSO at a final concentration of 3.33% (1 µl per 15 µl reaction) was added to each PCR reaction. To avoid primer self-annealing and decrease the effect of diandry, three changes were made to the standard protocol of both IMMOMIX reaction and iProof mix: primer concentration was decreased 5 times (2 µl of 1mM stock), DNA concentration was increased 2-3 times, annealing temperature was increased to 59-60°C.

Adapter PCR for the Gateway™ BP reactions

Adapter PCR was performed in two separate steps with gel excision and purification after each step. For the first step, the following components were mixed in a standard PCR tube or in a 1.5ml tube to prepare a master mix:

iProof polymeraze 2x	10µl
Forward Primer	0.2µl of 10mM stock (final concentration 0.05mM)
Reverse Primer	0.2µl of 10mM stock (final concentration 0.05mM)
DMSO 50%	1µl (final concentration 3.33%)
DNA template	2-3µl
ddH2O	up to 20µl

PCR reaction steps were performed as listed in Table 2.

Table 2. PCR steps used for amplification with IMMOMIX and iProof polymerases.

IMMOMIX polymerase	iProof polymerase
1.Denaturation 95°C, 2min x 1	1.Denaturation 95°C, 2min x1
2. Denaturation 94°C, 15s x 35	2.Denaturation 94°C, 15s x 35
3. Annealing 59°C, 30s x 35	3. Annealing 59°C, 30s x 35
4. Elongation 72°C, 2min x 35	4. Elongation 68°C, 2min 30s x 35
5. Elongation 72°C, 10min x1	5. Elongation 68°C , 10 min x1
6. 4°C hold	4°C hold

Adding the final 10 minute long elongation step to the iProof PCR protocol significantly increased the amount of product. After this first step, the entire volume of PCR reaction was loaded onto a 1% agarose gel and run for 30 min along with DNA HyperLadder I (Bioline).

Bands were detected under fluorescent light and visually compared to the DNA ladder. Bands were then carefully excised without touching other bands (if present) to avoid contamination.

DNA was extracted from gel samples using the AccuPrep Gel Purification Kit (Bioneer) using the standard protocol described in the manual but using 25 µl (instead of the recommended 30-50µl) of buffer to elute the sample. 15µl of each sample was used in the next step using adapter primers (Vinatzer et al 2006), designed to anneal to the 5' end of the primers used in the first PCR step.

PCR mix included:

iProof mix	25µl
AttB forward10mM	2µl
AttBreverse10mM	2µl
DMSO 50%	2µl
Dd H_2O	4µl
Purified PCR product	15µl

Adapter PCR consisted of two separate steps listed in Tables 3,4.

Table 3. Conditions of adapter PCR, first step.

Reaction step	Temperature, °C	Step length	Number of cycles
Initial denaturation	95°C	2min	1
Denaturation	94°C	15s	5
Annealing	45°C	30s	5
Extension	68°C	2min30s	5

Table 4. Conditions of adapter PCR, second step.

Reaction step	Temperature, °C	Step length	Number of cycles
Denaturation	94°C	15s	30-35
Annealing	54-56°C	30s	30-35
Extension	68°C	2min30s	30-35
Hold	4°C	-	-

The entire PCR reaction volume was loaded on another agarose gel and cleaned again avoiding excision of any bands of unexpected size. The PCR product concentrations were measured and 7µl of the product (final concentrations 55-180ng/µl) was mixed in a 1.5 ml microcentrifuge tube

with 1 µl of the donor vector pDONR221(Invitrogen) (150ng/µl) and 2µl of BP Clonase Enzyme Mix according to the protocol supplied for the Gateway Reaction (Life Technologies). The reaction was mixed well by vortexing briefly twice, microcentrifuged briefly and incubated overnight at room temperature. The next day, 1µl of Proteinase K was added to each tube to terminate the reaction and samples were incubated at 37°C for 10 min. This step was followed by transformation of each reaction (1-2µl) into 50µl of *E.coli* DH5α entry clone: cells were incubated on ice for 30 minutes, heat-shocked by incubating at 42°C for 30s and shaken with 250µl of SOC (or LB) medium at 37°C for 1 hour. 250µl of cells were plated on selective plates for the vector containing the desired insert (LB supplemented with kanamycin at 100µg/mL) and incubated at 37°C overnight. 20-100 colonies per plate were usually obtained. 16-25 colonies were then re-streaked to LB plates with kanamycin and incubated at 37°C overnight. The next day, PCR on colonies was performed using IMMOMIX enzyme and the original DNA template as the positive control. Colonies giving bands of the expected size on the gel were put into culture in liquid LB medium containing the same concentration of selective antibiotic (kanamycin) and incubated at 37°C overnight with shaking. 2mL of the liquid *E.coli* culture was used to prepare glycerol stocks (stored at -80°C) and 1 mL was used for the plasmid extraction using a Plasmid Mini Extraction Kit (Bioneer). Plasmids were sequenced with M13 primers to confirm the presence of the insert. Sequences were analyzed and compared to the reference sequence using SeqMan (Lasergene DNAStar) software. iProof high fidelity mix demonstrated desirably low occurrence of mutations. Plasmids containing inserts lacking mutations were used to continue cloning into destination vectors by Gateway™ cloning.

Before proceeding to the Gateway™ LR cloning reaction to transfer inserts into the final plant expression vector, plasmids were digested. For the digestion, the following components were mixed in a PCR tube:

Plasmid	60ng/µl
NE Buffer3	1µl
BSA	0.2µl
*Eco*RV	1µl
ddH2O	up to 10µl

Samples were incubated 2-8h (most often 4) at 37°C. *Eco*RV was then heat-inactivated for 20 min at 80°C. 4µl of the reaction was loaded on a gel. For the LR reaction, the following components were added to a 1.5ml tube and mixed:

Entry clone after the digestion	1.5µl (90ng)
Destination vector *E.coli* (strain1284)	2.2µl (150ng)

2µl of LR clonase (Invitrogen) was added to each sample (using the standard Invitrogen protocol), mixed well by vortexing and incubated at room temperature for 1 hour. 1µl of Proteinase K was added to each sample to terminate the reaction. This step was followed by transformation of *E.coli* DH5α and selection on LB plates supplemented with kanamycin as described for the BP reaction. The success of the LR reactions was confirmed by IMMOMIX PCR on colonies after second day re-streaks.

Selected colonies were put into a liquid culture and used for a tri-mating into *Agrobacterium tumefaciens*. For tri-mating, *E.coli* strain RK600, *E.coli* with the gene of interest, and *A. tumefaciens* (BAV 1281) were plated together on a single LB plate and incubated for 2-3 days. Bacteria were then collected with a sterile loop from the bacterial loan grown on the LB plate and re-streaked onto LB plates supplemented with kanamycin and tetracycline to eliminate the *E. coli* strains. Ideally, *Agrobacterium* containing the new plasmid would form single isolated colonies. However, most of the time a second re-streak on LB plates supplemented with kanamycin and tetracycline was needed due to the high tri-mating efficiency. These colonies were re-streaked again and cultured in liquid LB media containing kanamycin and tetracycline overnight at 28°C. Plasmids were extracted the next day and sent for sequencing with primers specific to the expected insert effector to confirm the presence of the gene of interest. *Agrobacterium* strains containing effectors were stored at -80°C and further used for the transient assay below.

pENTR TOPO Cloning Strategy for cloning effectors into the pEDV6 vector.

To produce blunt-end PCR products, primers with a 3'-overhang CACC (corresponding to the GTGG overhang in the pENTR TOPO vector, Fig.2) were designed for two genes using Primer3

software. The forward primers were designed to anneal to the start codon and the reverse primer was designed to anneal to the 3' end of the gene ending immediately before the STOP codon (Table 5).

Table 5. Primer sequenced designed for cloning Rsc0868PopP2 into pENTR TOPO vector.

DNA source	Effector name	Forward primer sequence	Reverse primer sequence
GMI1000	Rsc0868popP2	CACCATGGGTGTGGATCATCCTTT	TCAGTTGGTATCCAATAGGGAAT
GMI1000	Rsp0028GALA3	CACCATGGCTCCGCCATCCAT	TCAAATCCGCAGCGTCAC

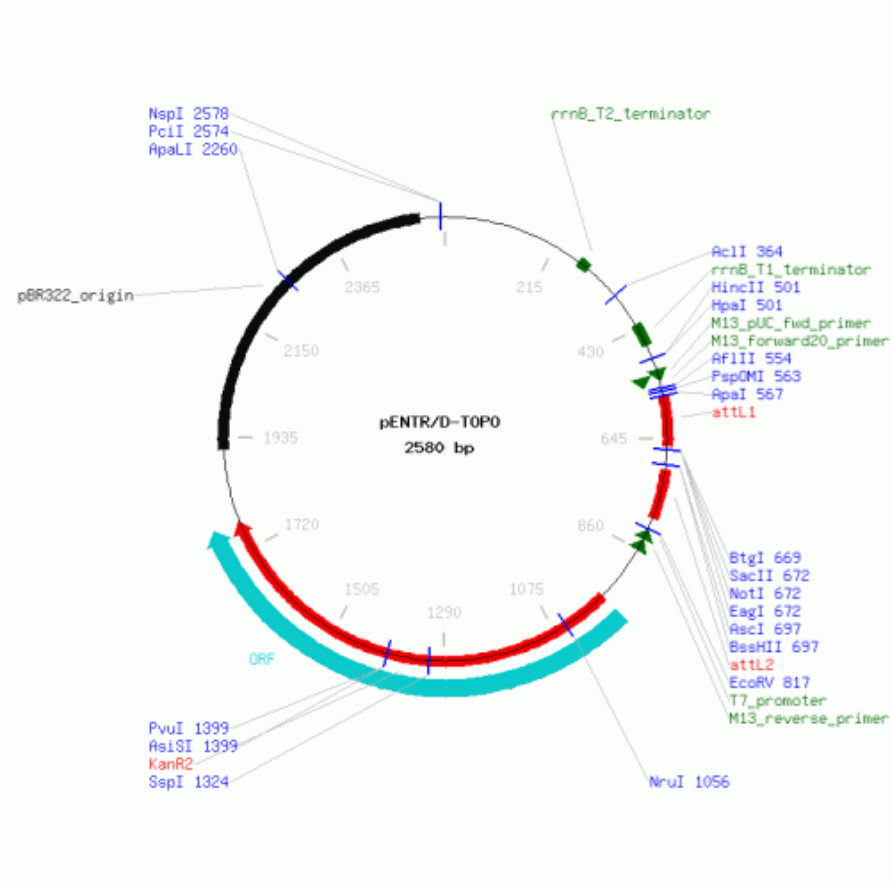


Fig.2. Circular map of pENTR/D TOPO entry vector.

Due to the complicated template (long and high GC content), the PCR protocol used for the directional TOPO cloning needed to modified as follows:

(1) The concentration of the enzyme (2x iProof High Fidelity Master Mix) was increased up to 16µl.

(2) The amount of the template (1:10 GMI1000 gDNA) was increased up to 2µl. This amount of template tended to give larger brighter bands on a gel compared to 1µl.

(3) The annealing temperature was lowered to 54°C according to PCR with complicated template instructions. PCR program used for directional TOPO cloning is described in Table 6.

(4) DMSO concentration (50%) in the PCR mix used was 3µl.

(5) Primer concentration (1µl) was not reduced. However, in the case of high diandry or self-annealing the concentration of primers could be reduced 5-10 times.

PCR Master Mix used:

2x iProof (Biorad)	16µl
forward primer	1µl
reverse primer	1µl
DMSO50%	3µl
DNA template (GMI1000)	2µl
ddH2O	2µl
total volume	25µl

Table 6. Amplification steps used for directional TOPO cloning.

Reaction step	Temperature, °C	Step length	Number of cycles
Initial denaturation	95°C	2min	1
Denaturation	94°C	15s	35
Annealing	54°C	30s	35
Extension	68°C	2min	35
Final extension	68°C	15min	1
Hold	4°C	-	-

3µl of PCR product of known concentration was added to 1µl of salt solution (Invitrogen pENTR TOPO Kit), 1µl of sterile water and 1µl of pENTR TOPO vector. The reaction was mixed gently and incubated for 5 minutes at 23°C. 2µl of cloning reaction was added to 50µl of DH5α chemically competent *E.coli* cells and stored on ice for 30 min. After this step, cells were heat-shocked at 42°C using a waterbath. 250µl of SOC medium was added to cells and incubated

at 37°C with shaking. 100µl and 200µl of the reaction was plated on LB supplemented with kanamycin. Colonies were re-streaked onto LB plates supplemented with kanamycin again and, after overnight incubation at 37°C, scanned for the insert with M13 forward primer (to confirm the correct orientation and the presence of the insert in the vector) and gene-specific reverse primer. To re-confirm the insert presence, the PCR was performed using forward gene-specific primer and M13 reverse primer. Corresponding DNA template (GMI1000) was used as the positive control with a gene-specific primer pair. PCR was performed using IMMOMIX enzyme with the following components:

IMMOMIX polymeraze 2X	9µl
M13 (forward OR reverse) primer	1µl
Gene-specific primer (reverse OR forward)	1µl
DMSO50%	1 µl
DNA template	1 µl
Sterile water	2 µl

Plasmids were extracted from LB-kanamycin overnight cultures and sequenced (Virginia Bioinformatic Institute Core Laboratory) with M13 forward and reverse primers.

Sequences were analyzed using MegAlign (DNAStar, Lasergene). After the absence of mutations was confirmed, bacterial cultures with the correct insert were further used for LR cloning into th epEDV6 destination vector.

Before LR reaction, plasmids were digested with *Not*I enzyme (cuts vector at position 652 but does not cut the insert). Digestion was performed using 600ng of extracted plasmid for a 10µl reaction. 1µl of NEBuffer3 was mixed with 0.2µl of BSA, 1µl of *Not*I enzyme, 3.5µl of plasmid and 4.3µl of sterile water. The reaction was placed at 37°C overnight. 4µl of the reaction was loaded on a gel to confirm the digestion and entry clone after digestion was used for an LR reaction with the destination vector at 1:1 ratio.

Gateway™ cloning into pEDV6 (Effector Detector Vector)

LR reactions included destination vector (pEDV6) at concentration of 30ng/µl, entry vector with the insert diluted to the same concentration, 2µl of LR clonase and TE buffer up to 10µl of total

volume. After 2h incubation at room temperature, 2µl of the reaction was used to transform 50µl of DH5α chemically competent cells as described above. After transformation, cells were plated on LB plates containing gentamycin and incubated at 37°C overnight. Colonies were re-streaked and scanned for the insert presence using pEDV6 vector-specific primer and gene-specific reverse primer using IMMOMIX (Bioline). Colonies of *E. coli* with the insert were then re-streaked and stored as glycerol stocks as for pENTR TOPO vector (Table 7).

Table 7. Glycerol stocks of *E.coli* containing Rsc0868popP2 construct.

Database ID	Host strain	Strain name	Resistance
2296	DH5α	pENTR TOPO+Rsc0868popP2	Kan
2297	DH5α	pENTR TOPO+Rsc0868popP2	Kan
2298	DH5α	pEDV6+Rsc0868popP2	Gent
2299	DH5α	pEDV6+Rsc0868popP2	Gent

Testing *Ralstonia solanacearum* pathogenicity on resistant and susceptible cultivars of tomatoes and eggplants

The ability of a Virginian strain of *Ralstonia solanacearum* (819) to cause disease on tomato was tested on 21-28 days old tomato lines WVA 700 and H7996 or 6 weeks old eggplants (accessions MM853 [E1] or MM738 [E8]). Plants were planted into approximately 100g of Metro Mix/Pro Mix(50/50) soil in 1h pots (4 plants per pot) and then grown in growth chamber/lab shelf at 18-22°C under long days (16h) . Bacteria from freshly streaked KB plates were grown overnight in liquid KB medium at 28°C with shaking. Liquid culture was centrifuged in 50 mL flasks for 15min at room temperature. The pellet has been re-suspended in 10mL of distilled water. Inoculum concentrations were determined spectrophotometrically. Fifty milliliters of bacterial suspension at an OD_{600} of 0.3 was poured over the dry (not watered 24h prior to infection) soil. For a control (8-16 plants per assay), 50 milliliters of distilled water was used instead of bacterial suspension. Pots with infected plants were bagged to prevent leakage but remained opened during the experiment. Plants were watered daily with 24h intervals. Pots were coded and plants

were inspected daily up to 9 days for wilting and were rated on a zero-to-four disease index scale as follows (after Tans-Kersten, J. et al.,1998):

0 no wilting
1 1 to 25% wilting
2 26 to 50% wilting
3 51 to 75% wilting
4 76 to 100% wilted or dead.

Each assay for tomato cultivars contained at least 13 plants per infection, and was repeated at least four times. At the end of each experiment, pictures were taken. Results were analyzed using JMP version 9.0 (SAS Institute Inc). The eggplant assay was repeated twice, with two plants in each assay. Eggplant stems were cut before the inoculation.

Agrobacterium Transient Assay, eggplants

5mL overnight cultures of kan-tet-resistant *Agrobacterium* containing the pDONR221+ effector construct (constructs are described in the table below) were pelleted in 15 mL Corning tubes at 2000rpm, 4°C for 15 min.

Table 8. Glycerol stocks of *A. tumefaciens* (database ID 1281) containing effector constructs.

Database ID	Strain name	Resistance
1972	Rsc0868popP2(5)	kan,tet
1973	Rsc0868popP2(5)	kan,tet
1974	Rsc0868popP2(6)	kan,tet
1975	Rsc0868popP2(6)	kan,tet
1976	Rsc0868popP2(16)	kan,tet
1977	Rsc0868popP2(16)	kan,tet
2269	Rsc0826popP1	kan,tet
2270	Rsp0028GALA3	kan,tet
2271	Rsp0028GALA3	kan,tet

2272	Rsp1130(GMI1000)	kan,tet
2273	Rsp1130(GMI1000)	kan, tet

Supernatant was discarded and the pellet re-suspended in 5 mL of MMA buffer (1.95g/L MES, 2.03g/L MgCl2, 200µm Acetosyringone). Tubes were incubated for 4 hours at room temperature and inoculum concentrations were determined spectrophotometrically using MMA buffer as blank. The ratio between tubes and MMA was calculated and bacteria were diluted in separate tubes so that tubes had 5mL of the culture at OD_{600} of 0.1 and 0.3. Six-week-old eggplant (*Solanum melongena*) breeding lines (E1, resistant to GMI1000, E2, resistant, E6, partially resistant, E8, susceptible, and E10, susceptible, see table below) were inoculated by leaf infiltration of the abaxial portion using a 2 mL disposable syringe. Plant leaves were coded and labeled with the tested effector, number and OD. Each leaf was also inoculated with an *Agrobacterium* strain containing *hopM1* (known avirulent effector) as positive control and *Agrobacterium* containing an empty vector as negative control. To activate the promoter, plants were sprayed with 30µm Dexamethason 48 hours after the infiltration (0.118g Dexamethason in 1ml of water supplemented with 0.1% Tween-20). Plants were inspected for symptoms 24h and 48 h after spraying with Dexamethason. Symptoms were rated on a zero-to-three cell death index scale (previously described by Hojo et al., 2008):

0 no symptoms;
1 discoloring at inoculated sites;
2 cell death at inoculated sites;
3 cell death at the periphery of the inoculated sites.

Within each trial, 3-6 leaves of each eggplant breeding line were treated. Results were statistically analyzed with JMP9 software. Five eggplant breeding lines (accessions MM853 [E1], MM643 [E2], MM960 [E6], MM738 [E8] and MM136 [E10]) were used. E1 and E2 lines demonstrated resistance to previously tested *Ralstonia* strains in wilting assays (Lebeau et al., 2011), E6 – partial resistance, E8 and E10 – susceptibility.

***FLS2* sequencing**

FLS2 (FLAGELLIN SENSITIVE 2) gene sequences from 5 different tomato cultivars (Chico III, 'Sunpride', 'Rio Grande', 'Roter Gnom', and 'M82') were obtained using a gene-specific primer set designed on the basis of known sequences of tomato cultivar 'Heinz' (Primer3 software). Sequences were analyzed for SNPs (single nucleotide polymorphisms) and translated into proteins using Lasergene software. Protein sequences were checked for amino acid changes using Lasergene (MegAlign) software. Leucine-rich domains of proteins were analyzed for conservative domains with RCM (conservational mapping) software (Bent et al., 2011).

Tomato Transformation Protocol

25 seeds of tomato cultivars 'ChicoIII', 'Rio Grande', 'Sunpride' and 'M82' were sterilized in 1mL of 50% (V:V) commercial bleach in distilled water for 20 min. Tubes were flicked every 5 minutes to mix the solution. Bleach was removed using a sterile pipet 1mL tip. 1mL of autoclaved distilled water (HyPure Molecular Biology Grade water can be used instead) was added to each tube to rinse the seeds. Tubes were mixed by flicking for one minute. The step was repeated to provide a second rinse. Sterilized and rinsed seeds were plated into Magenta boxes containing 40 mL of tomato basal media (see Protocol Supplies Tables below). Five seeds were placed into each box. Lids were wrapped with micropore tape.

Primary leaf tissue from seedlings was harvested by cutting off the base and the tip of leaves using a sterile scalpel on sterile blotting paper. Leaf pieces (5-25 per one plate) were placed upside down onto plates containing Pre-culture Media. Plates were labeled with the date and cultivar name.

Agrobacterium strain containing construct (*Le-FLS2:GFP*, 1) was streaked onto plates of LB medium containing selective antibiotics (kanamycin, rifampicin and gentamycin). Plates were incubated at 28°C until single colonies were visible. Three flasks containing 15mL of liquid LB with selective antibiotics were inoculated with three single isolated colonies from LB plates. When the *Agrobacterium* cultures reached an optical density of approximately 0.8 (20-30 hours), 1 mL of each culture was centrifuged at 3000rpm for 10 min. LB media was poured off and the pellet was re-suspended in 1mL of Dilution Media. The suspension was added to 20mL of Dilution Media in a 50mL flack. One 20mL dilution was used for one plate of explants. Explants were infected by placing them into tubes containing *Agrobacterium* suspension for 30

minutes. Tubes were gently shaken every few minutes to completely expose leaves to *Agrobacterium*. After 30 minutes, explants were removed from the dilution and placed onto sterile filter paper to dry. Explants were then placed upside down back on the plates of Pre-culture Media. Plates were wrapped in parafilm, several layers of aluminium foil to keep *Agrobacterium* in dark and incubated at 25°C for 48 hours in the growth room. After 48 hours, leaf explants were placed into 50mL flacks containing 20mL of Wash Off Media, capped and shaken gently for 1 minute to remove *Agrobacterium*. The liquid was discarded and 20mL of Wash Off Media was added to each tube again. Tubes were shaken for one minute. The liquid was discarded again and tubes were shaken in 30mL of Wash Off Media for 20 minutes, then in 10mL of Wash Off Media for one minute. Liquid was discarded and explants were blotted on sterile filter paper. Leaves were placed upside down onto Shoot Regeneration Media containing double amount of cefotaxime but no selective antibiotic (kanamycin). All plates were wrapped in micropore tape and incubated in a growth chamber for one week.

After one week, explants were transferred to fresh Shoot Regeneration Media with the selective antibiotic (kanamycin). We found that doubling the amount of Cefotaxime was not enough to completely remove Agrobacterium and at least four-times more Cefotaxime is needed (it does not seem to be phytotoxic). Leaf explants were divided by cutting them along the major leaf veins using a scalpel.

Explants and/or callus masses were transferred every two weeks until shoots appeared (normally 6 weeks are needed). Once shoots appeared, explants were transferred to Magenta boxes containing 40mL of Shoot Regeneration Media to allow room for growth. Shoots transferred to Magenta boxes were composed of fully differentiated tissue and were a least 4 cm in diameter. At this step, shoots need to be transferred to fresh Shoot Elongation Media every two weeks. Double Magenta boxes were used if plants outgrew a single box. When plants reached at least 5cm and had 5-6 leaves, stem cuttings were done from the top of the growing plant, leaving most of the leaves behind on the main stem along with the callus mass. The stem cuttings were transferred to Rooting Media. This allows generating many transgenic plants from a single transformed callus, increasing the number of potentially transformed plants. Roots should begin to form in 8-10 days. Once roots became long enough (3-4cm) plants were transferred to peat plugs hydrated with water and autoclaved. Plants forming roots extensively can be transferred

directly to soil (50%MetroMix, 50%ProMix in 1 or 2 gallon pots) along with the agar and covered with Magenta boxes for two days to let the plant acclimate to the dryer air. Otherwise, plants are transferred to soil when roots are visibly emerging from peat plugs.

The most critical point in tomato transformation is avoiding contamination and therefore losses of explants caused by *Agrobacterium*. Adding 4x concentration of Cefotaxime to the Shoot Elongation Media and Rooting Media solved this problem. However, losses caused by *Agrobacterium* were as high as up to 40% of explants. No other contaminating agents were observed due to sterile technique used at each transfer. Cultivars Chico III and 'Sunpride' demonstrated enhanced ability to resist *Agrobacterium* infection; 'Rio Grande' and 'M82' cultivars were affected the most. The ideal solution to recover the necessary amount of plants was to keep growing them in Shoot Regeneration Media with constant stem cuttings followed by a transfer into Rooting Media.

Plants were checked for GFP expression before transferring them to Shoot Regeneration Media. No GFP was observed under the fluorescence microscope, however, the only reliable method to determine the transformation efficiency in tomato is to perform PCR with primers for *GFP* present in the construct after tomatoes are transferred to soil.

Protocol Supplies - Media Recipes

Table 9. Media recipes for tomato transformation.

Tomato Basal Media		
Ingredient	Concentration	Unit
MS + Vitamins	4.43	g/L
Sucrose	30	g/L
Thiamine HCl	0.9	mg/L
Phytagel	4	g/L

Pre Culture Media		
Ingredient	Concentration	Unit
MS + Vitamins	4.43	g/L
Sucrose	30	g/L
Thiamine HCl	0.9	mg/L
Phytagel	4	g/L
BA	1	mg/L
NAA	1	mg/L

Wash Off Media		
Ingredient	Concentration	Unit
MS + Vitamins	4.43	g/L
Sucrose	30	g/L

Wash Off Media		
Ingredient	Concentration	Unit
MS + Vitamins	4.43	g/L
Cefotaxime	500	mg/L

Shoot Regeneration Media		
Ingredient	Concentration	Unit
MS + Vitamins	4.43	g/L
Sucrose	30	g/L
Thiamine HCl	0.9	mg/L
Phytagel	4	g/L
IAA	0.1	mg/L
Zeatin	2	mg/L
Kanamycin	100	mg/L
Cefotaxime	500	mg/L

Shoot Elongation Media		
Ingredient	Concentration	Unit
MS + Vitamins	4.43	g/L
Sucrose	30	g/L
Thiamine HCl	0.9	mg/L
Phytagel	4	g/L
IAA	0.1	mg/L
Zeatin	0.2	mg/L
Kanamycin	100	mg/L
Cefotaxime	500 (x4)	mg/L

Rooting Media		
Ingredient	Concentration	Unit
MS + Vitamins	4.43	g/L
Sucrose	30	g/L
Thiamine HCl	0.9	mg/L
Phytagel	4	g/L
IAA	2	mg/L
Cefotaxime	500(x4)	mg/L
Kanamycin	100	mg/L

LB Media		
Ingredient	Concentration	Unit
LB Broth	25	g/L
Bacto Agar	8	g/L
Rifampicin	20	mg/L
Kanamycin	100	mg/L

CHAPTER 3. RESULTS

MAMP-TRIGGERED IMMUNITY

FLS2 gene sequence: variability among 6 tomato cultivars.

Three primer pairs were designed to amplify the full-length *FLS2* sequence of five tomato cultivars ('ChicoIII', 'Sunpride', 'Rio Grande', 'M82' and 'Roter Gnom'). Additional primers were designed for sequencing from within PCR products. Sequences were then assembled and compared to the *FLS2* sequence of cultivar 'Heinz', for which a complete genome sequence is available (Sato et al., Tomato Genome Consortium, 2012, http://solgenomics.net/locus/5561/view).

Two of the cultivars sequenced ('ChicoIII' and 'Rio Grande') have 14 nucleotide transversions compared to the *FLS2* sequence of cultivars 'Sunpride', 'Roter Gnom' and 'Heinz' (Table10).

'M82' cultivar has 15 nucleotide transversions: 14 of them were identical to those in 'ChicoIII' and 'Rio Grande' cultivars and an additional transversion was found at position 4500 (G - A). An insertion of the codon gaa at positions 3008, 3009 and 3010 was found in this cultivar also. However, non of the mutations were in the extracellular LRR domain of the protein.

Table 10. Nucleotide transversions of *FLS2* sequence in tomato cultivars 'ChicoIII', 'Rio Grande' and 'M82' compared to cultivars 'Sunpride', 'Roter Gnom' and 'Heinz'.

#of transversion	Position	Mutation
1	1061	A-G
2	1126	T-C
3	1130	G-T
4	1178	G-A
5	1376	C-A
6	1377	C-T
7	1802	C-T
8	1900	A-G
9	1989	C-T

10	2035	C-T
11	2126	A-G
12	3381	G-T
13	3865	C-T
14	4433	T-C

FLS2 protein sequence. Description of LRRs.

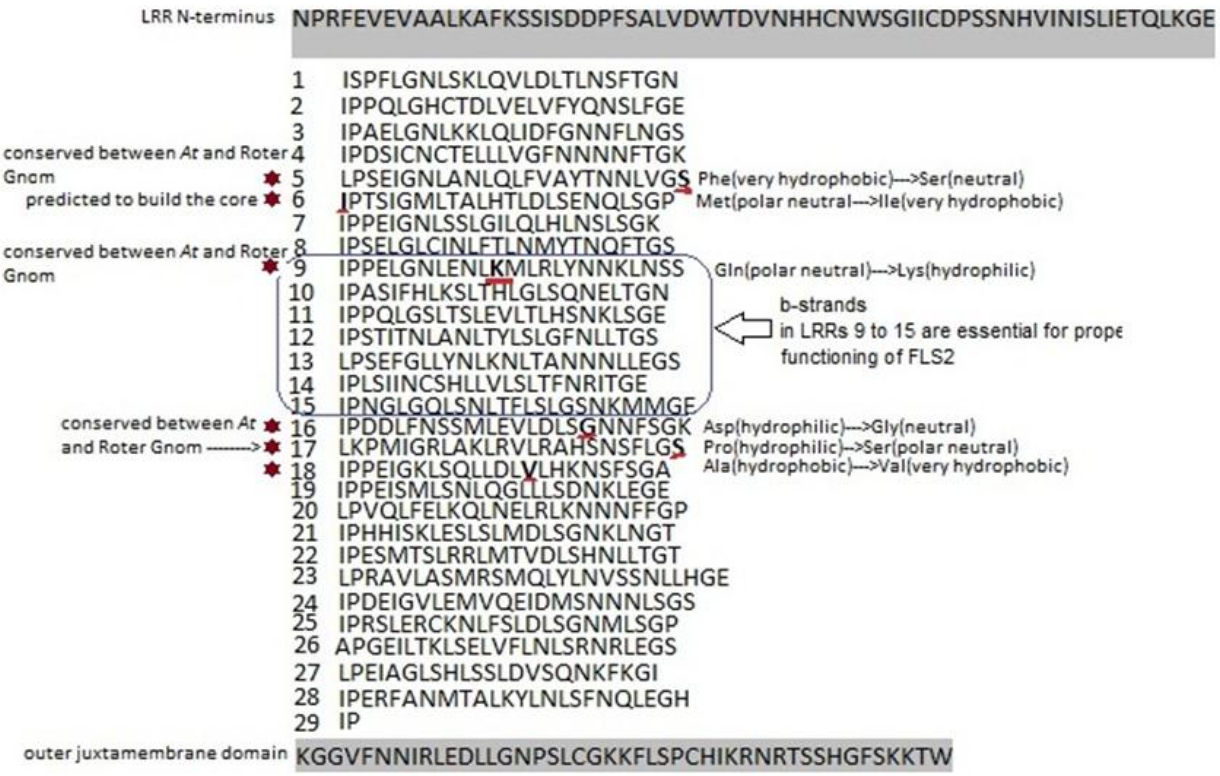
The *FLS2* sequences of the five tomato cultivars listed above were translated into protein sequences upon intron removal. The LRR (leucine-rich repeats) domain was identified within positions 62-738 of the translated protein sequences. After alignment, it was found that protein sequences of 'ChicoIII', 'Rio Grande' and 'M82' cultivars have 7 amino acid substitutions compared to 'Heinz', 'Sunpride' and 'Roter Gnom' cultivars. 6 of 7 substitutions were located within the LRR domain of the protein in positions listed in table 11.

Table 11. Amino acid substitutions in the FLS2 protein for different tomato cultivars.

Position	'Heinz', 'Sunpride', 'Roter Gnom'	'ChicoIII', 'Rio Grande', 'M82'
209	Phe	Ser
210	Met	Ile
293	Gln	Lys
467	Asp	Gly
497	Pro	Ser
512	Ala	Val

7 out of 14 transversions (50%) were non-synonymuos mutations and resulted in mutations in protein sequence (transversions at positions 1126, 1130, 1376, 1377, 1900, 1989 and 2035). These transversions correspond to the amino acid substitutions (Table 11) considering the intron removed. Other SNPs (single nucleotide polymorphisms) were synonymous and thus did not affect the protein sequence.

In cultivars 'ChicoIII', 'Rio Grande' and 'M82' phenylalanine (very hydrophobic amino acid) is substituted with serine (a neutral amino acid) at position 209. At position 210, methionine, a polar neutral amino acid is replaced by isoleucine (an amino acid with a very hydrophobic side chain). At position 293, glutamine (polar neutral amino acid) is substituted with lysine (a hydrophilic amino acid). At position 467, asparagine, hydrophilic amino acid, changes to glycine, which is neutral; at position 497, hydrophilic amino acid proline is replaced



by serine and finally, at position 512 hydrophobic alanine is substituted with very hydrophobic valine.

Fig.3. Predicted structure of the LRR domain of FLS2 protein. Mutations between 'Heinz'/'Sunpride'/'Roter Gnom' and 'ChicoIII'/'Rio Grande'/'M82' cultivars are highlighted with a red star.

Based on the comparison with known crystal structures and predicted structures of LRR-kinase receptors, the structure of the LRR domain of FLS2 is predicted as follows. The N-terminal part of FLS2 consists of 62 amino acids. The N-terminus is followed by a large LRR domain containing 676 amino acids. The LRR domain is followed by a short 45 amino acid outer juxtamembrane domain (Fig.1). In total, 28 LRRs were found within positions 63-738 of the protein sequence. The length of the LRRs varies from 23 to 26 amino acids, with an average length of 24 amino acids. The 23rd LRR contains 26 amino acids, the 26th LRR contains 25 amino acids and the 27th LRR contains 23 amino acids. The most common LRR motif found is IPXXLGXLXXLXXLXLXXXXLXGX, where X corresponds to variable amino acids.

Conserved domains. RCM mapping.

Conserved domain analysis is designed based on known structures of receptors with leucine-rich repeats (Helft et al., 2011). Known structures allow to predict amino acids potentially responsible for ligand binding (Chinchilla et al., 2006).

The RCM program used to predict biologically functional sites in a leucine-rich repeat (LRR) domain includes the identification of conserved surface regions on a model of the folded protein (Bent et al., 2011). As the input, orthologous sequences of the FLS2 proteins from the six tomato cultivars with similar number of LRRs were used. The program then rearranged protein sequences to roughly fit the folded example of known LRR protein structures. The program subdivided the LRR domains of the input sequences into clusters and predicted conserved amino acids based both on known sequences and FLS2 orthologues.

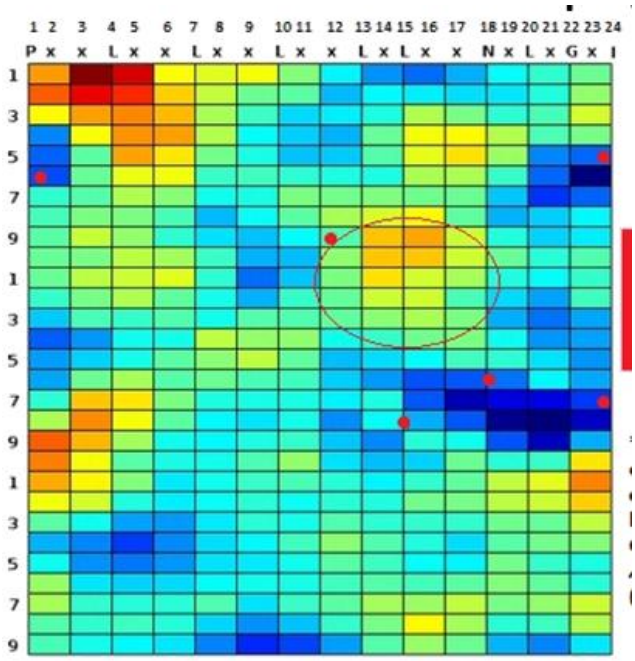


Fig.4. A color map that highlights predicted regions of evolutionary conservation or diversification, which frequently correspond to the key functional sites on the LRR.
Dark blue, blue and green color indicate the least conserved amino acids, yellow, orange, red and dark red color indicate the most conserved amino acids with highest conservation score for dark red color.

Conserved sites were predicted within LRRs 1-7 (amino acids 1-4 and 19-22 within LRRs 1-7, amino acids 12-17 within LRRs 9-13, 23rd amino acid within LRRs 20-22). When the sequence

of the FLS2 protein of *Arabidopsis thaliana* was included in the analysis (Figure not shown) conservative sites had the same pattern but were more extensive (because the FLS2 sequence of the *A.thaliana* orthologue has significantly more changes in amino acid positions compared to the FLS2 proteins of the tomato cultivars).

Transformation of Tomato with a *FLS2*:*GFP* construct.

Tomato cultivars 'Sunpride', Chico III, 'Rio Grande', 'M82' were grown following the tomato transformation protocol described in the Materials and Methods section. A total of 54 putative transformed plants were obtained from explants transformed with the *FLS2* gene of tomato cultivar ''Roter Gnom'' (Robatzek et al., 2007) fused to green fluorescent protein (GFP) and then tested for presence of the *FLS2* allele of ''Roter Gnom'' and for the kanamycin resistance gene. Survival rates during transformation were higher for Chico III and 'Sunpride' cultivars and extremely low for 'Rio Grande' and 'M82' cultivars (see Table 12 and 13). The majority of plants were lost during the 4-6 weeks after transformation because of *Agrobacterium* infection; in the case of ''Rio Grande'', two plants were lost because of drought, which might be explained by antibiotic selection against non-transformed cells. Plant cells, which are able to neutralize the toxic effect of antibiotic and therefore potentially have the construct, stay alive. However, a successful gene transfer does not guarantee construct expression. No FLS2 construct or kanamycin resistance construct were found in transformed plants. Low transformation efficiency could be explained by one of the following:

(1) Use of extensive amounts of cefotaxime which causes cell enlargement and additional water accumulation in cells. Water accumulation might cause low transformation rates.
(2) Use of a potato-specific transformation protocol instead of a protocol specific for tomato, for example, temperature and light conditions were optimized for potato and not for tomato.
(3) Use of cultivars which were not used for transformation of tomato before (the transformation rate depends on the cultivar used, according to many sources).

Based on the protocol used and a protocol obtained later from Katharine Genie (University of Tubingen), a new protocol for tomato transformation was devised but has not been used (Supplementary Material).

Table 12. Regeneration rates and plants obtained during transformation in various cultivars.

Cultivar	N of seeds planted	N of regenerated plants	N of survived plants	N of plants obtained and screened	Transformed
'ChicoIII'	25	20	10	22	0
RioGrande	25	20	1	1	0
'Sunpride'	25	20	16	30	0
'M82'	25	20	1	1	0

Table 13. Loss rates in transformed explants. Data shown in weeks post transformation.

Cultivar	N of regenerated plants	Survival rate, 2^{nd} week	Survival rate, 4^{th} week	Survival rate, 6^{th} week	Survival rate, 8^{th} week
'ChicoIII'	20	20	14	12	10
'Rio Grande'	20	18	16	5	1
'Sunpride'	20	20	18	18	16
'M82'	20	9	5	1	1

EFFECTOR-TRIGGERED IMMUNITY

Effector cloning

Four potential avirulence genes (effectors which may trigger immunity in eggplant breeding lines) were cloned and then transformed into *Agrobacterium* and further tested in eggplant

breeding lines, which were known to have differential resistance to the *R. solanacearum* strain GMI1000 (Lebeau et al., 2011).

Cloning efficiency varied depending on the effector: due to restrictions in primer design (the entire gene sequence including 15 bp of upstream sequence needed to be amplified), cloning required additional gel purification and a second PCR with the purified product and the same primer set to increase the amount of the product before cloning into *Escherichia coli* for every effector except for Rsc0868 (popP2). After initial PCR, additional bands of different sizes were often visible. Therefore, gel excision was necessary (Fig. 3, Fig. 4). Also, the amount of product was not sufficient for successful cloning into the entry vector. An increase in product yield was achieved by the additional PCR step. Finally, four effectors (all from strain GMI1000: Rsc0826 (*popP1*), Rsc0868 (*popP2*), Rsp0028 (*GALA3*) and Rsp1130) were cloned into *E.coli* DH5a competent cells and then further transferred into *Agrobacterium tumefaciens*.

Fig.5. Effectors Rsc0868(*popP2*), Rsp0028(*GALA3*) and Rsp1130 (from right to left) after the first step of adapter PCR for Gateway™ cloning

All cloned effector genes were checked for mutations due to PCR errors by Sanger sequencing. The enzyme used for PCR (High Fidelity iProof DNA Polymerase) provided amplification with almost no mutations. Plasmids with no mutations were then chosen for Gateway cloning into the plant expression vector (Gateway™ LR reaction).

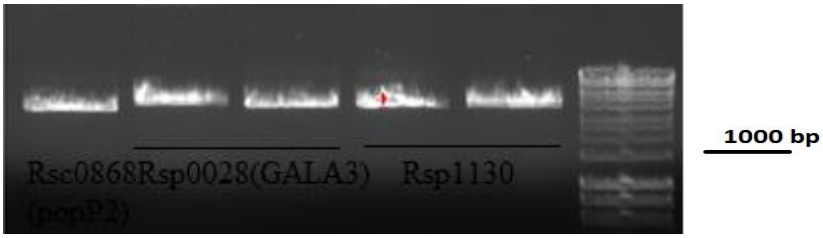


Fig. 6. Preparation of cloned effectors (Fig.3) for the LR reaction. Plasmids shown after digestion.

Transient *Agrobacterium*-based assays

Our hypothesis was that inoculation of resistant eggplant lines with the *Agrobacterium* strains containing constructs with potential avirulence factors that are recognized by eggplant resistance genes would lead to an immune response visible as leaf collapse due to cell death, called a hypersensitive response (HR). On the contrary, if the effector is not recognized by a resistance gene in an eggplant line, no signs of immune response to the particular *Ralstonia* effector would be observed.

Table 14. Cell death index scale used in *Agrobacterium* transient assays (adapted from Hojo et al., 2008).

Cell death index	Symptoms observed
0	No symptoms
1	Discoloring at the inoculated site
2	Cell death at the inoculated site
3	Cell death at the periphery of inoculated site

Each effector was tested in five eggplant breeding lines with known differential resistance to *R. solanacearum* in four independent experiments. HR caused by cloned effectors varied among eggplant breeding lines.

Transient assays with *Agrobacterium* containing effector constructs were performed at different inoculum concentrations (OD_{600} of 0.1 or 0.3) to find the optimal conditions for the experiment. The results obtained at the two different concentrations were not statistically different, though inoculation at an OD_{600} of 0.3 gave more consistent results. Leaves were scored for the presence of an HR 24 and 48 h after spraying with Dexamethasone (Dex), which induces

the promoter in the constructs used for effector expression (Vinatzer et al., 2006). In most replicas, the observations were continued for additional days due to delayed response. In some cases, symptoms of cell death developed only after 48 h, but never later than 72 h.

Five eggplant breeding lines (accessions MM853 [E1], MM643 [E2], MM960 [E6], MM738 [E8] and MM136 [E10]) were used. E1 and E2 lines showed resistance to previously tested *Ralstonia* strains in wilting assays, E6 had shown partial resistance, while E8 and E10 had shown susceptibility (Lebeau et al., 2011).

Preliminary results showed variability in immune responses to different effectors and different ability of the same effector to cause cell death depending on the eggplant line.

Ability of Rsc0868 (*popP2*) to cause an HR response in eggplants

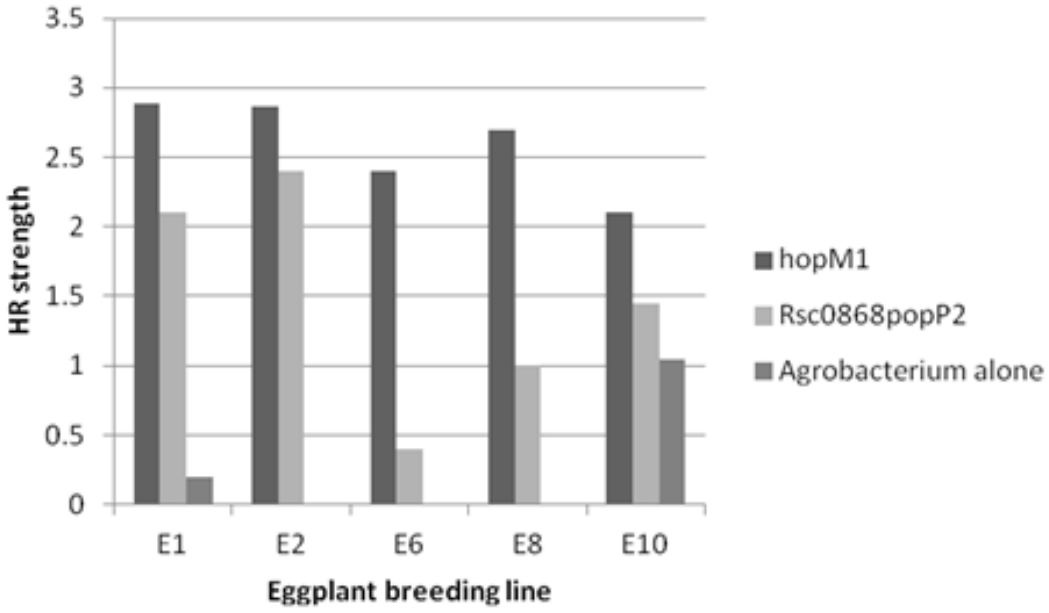


Fig. 7. Strength of the HR (on a scale from 0 to 3 based on Hojo et al., 2008) caused by the PopP2 construct in *Agrobacterium*. Data shown were obtained at 48 h after spraying with Dex after combining all replicas. The dark grey bar shows the strength of the HR caused by HopM1, which was used a positive control since it was found to cause a strong HR in eggplant previously (Clarke et al, in preparation). The very light grey bar shows the average strength of the HR caused by PopP2, the medium grey bar shows the strength of the HR induced by *Agrobacterium* not containing any construct.

Preliminary results showed that the PopP2 effector caused an HR in E1 and E2 eggplant breeding lines. In all replicas, line E10 showed the most inconsistent response to inoculum infiltration. In particular, leaf size and age seemed to influence the strength of the HR. The leaf response in line E10 caused by *Agrobacterium* not containing any effector construct is shown as negative control (Fig.7).

Line E1 showed a consistently strong HR for the *hopM1* and *popP2* constructs at both inoculum concentrations (OD 0.1 and 0.3, OD 0.1 not shown), but line E2 showed stronger HR at OD 0.3 only. However, combined data shown higher (but not significantly higher) HR in line E2.

Rsc0826 (*popP1*) ability to cause hypersensitive response in eggplants.

Other effectors tested demonstrated various responses in eggplant breeding lines, with some hypersensitive response in both resistant and susceptible eggplant breeding lines (Fig. 5, Fig. 6). Interestingly, the response varied dependent on the leaf size, leaf morphology and leaf age. When testing effectors Rsc0826 (*popP1*), Rsp0028 (*GALA3*) and Rsp1130, HR varied dependent on the replica.

The *popP1* construct showed various responses in eggplant lines, giving a stronger HR in lines E1 and E2 (Fig.8). However, the HR varied with each replica; e.g., the construct did not cause significant HR in the E1 line in replica 3. However, at least some necrotic areas could be observed in every replica.

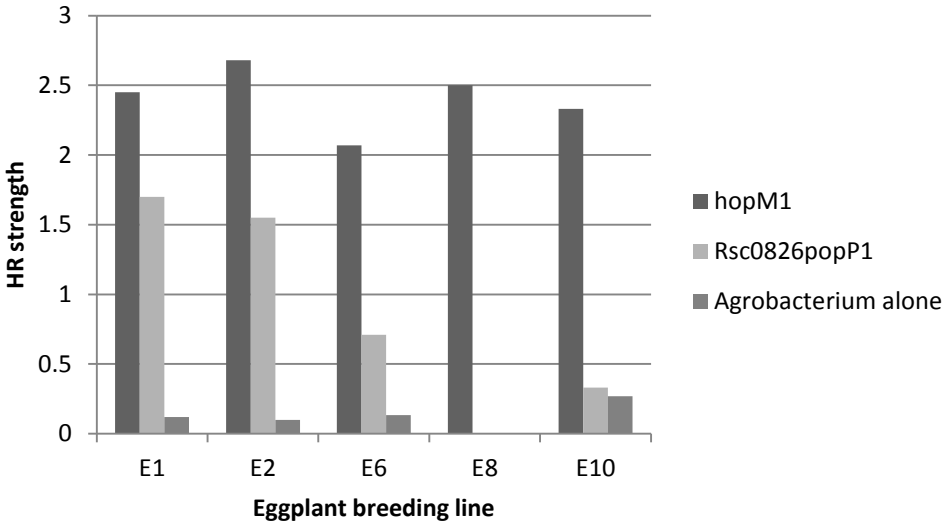


Fig. 8. Strength of the HR (on a scale from 0 to 3 based on Hojo et al., 2008) caused by the PopP1 construct in *Agrobacterium*. Data shown were obtained at 48 h after spraying with Dex after combining all replicas. The dark grey bar shows the strength of the HR caused by HopM1, which was used a positive control since it was found to cause a strong HR in eggplant previously (Clarke et al, in preparation). The very light grey bar shows the average strength of the HR caused by PopP1, the medium grey bar shows the strength of the HR induced by *Agrobacterium* not containing any construct.

Rsc0826 (*popP1*) ability to cause hypersensitive response in eggplants

Rsp0028 (*GALA3*) construct caused relatively low cell death in resistant lines E1 and E2 (Fig.9), but high (through inconsistent) level of cell death observed in two replicas for E6 (partially resistant) cultivar.

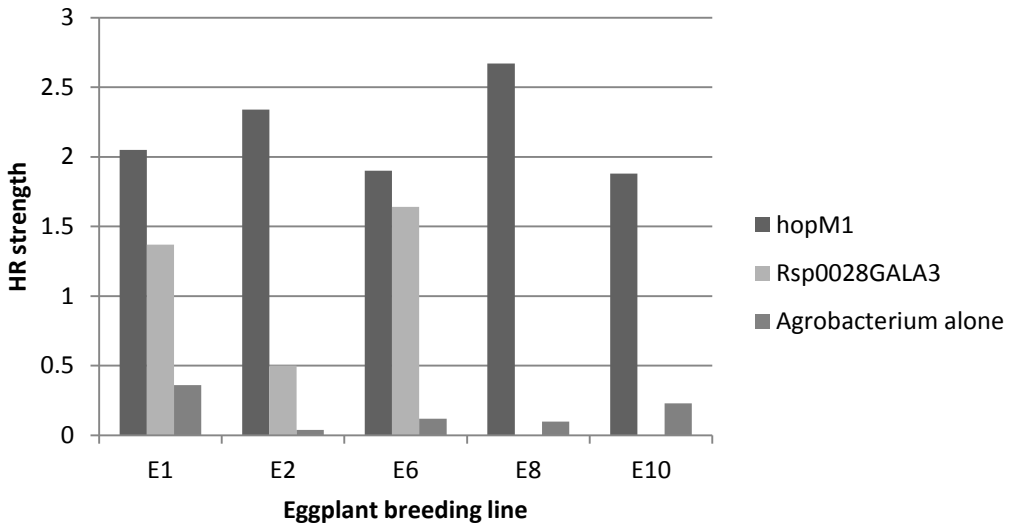


Fig.9. Strength of the HR (on a scale from 0 to 3 based on Hojo et al., 2008) caused by the GALA3 construct in *Agrobacterium*. Data shown were obtained at 48 h after spraying with Dex after combining all replicas. The dark grey bar shows the strength of the HR caused by HopM1, which was used a positive control since it was found to cause a strong HR in eggplant previously (Clarke et al, in preparation). The very light grey bar shows the average strength of the HR caused by GALA3, the medium grey bar shows the strength of the HR induced by *Agrobacterium* not containing any construct.

Rsp1130 ability to cause an HR in eggplants

Rsp1130 did not trigger a strong HR in any replica, neither in resistant nor susceptible cultivars. However, discoloration and sometimes small necrotic spots were observed at inoculation sites, especially for lines E1, E2 and E10 (Fig.8).

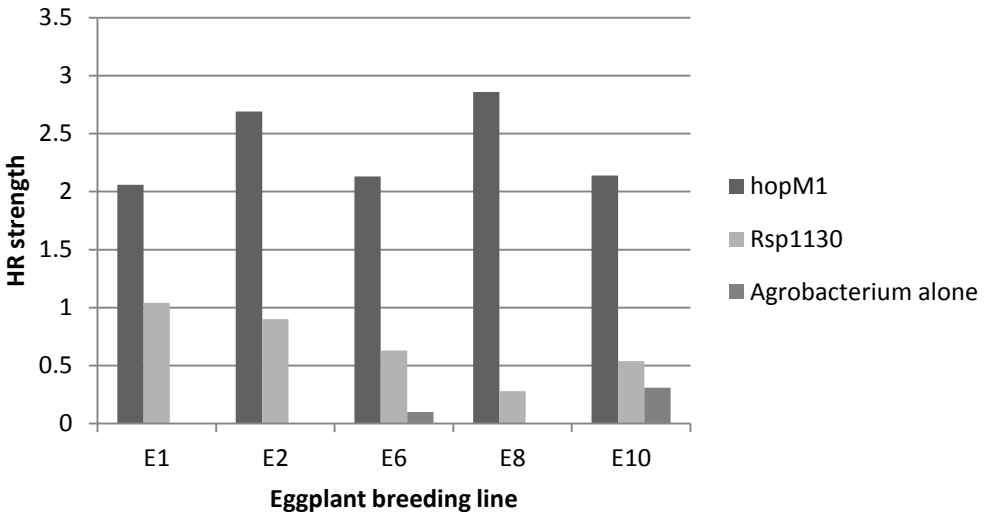


Fig.10. Strength of the HR (on a scale from 0 to 3 based on Hojo et al., 2008) caused by the Rsp1130 construct in *Agrobacterium*. Data shown were obtained at 48 h after spraying with Dex after combining all replicas. The dark grey bar shows the strength of the HR caused by HopM1, which was used a positive control since it was found to cause a strong HR in eggplant previously (Clarke et al, in preparation). The very light grey bar shows the average strength of the HR caused by Rsp1130, the medium grey bar shows the strength of the HR induced by *Agrobacterium* not containing any construct.

In general, replicas performed at OD_{600} of 0.3 showed approximately the same level of hypersensitive response in lines E1 and E2. However, some leaves of E1 and E2 did not develop any HR in a response to infiltration with *Agrobacterium* constructs although the HR in response to hopM1 was observed in all leaves tested (not less than 2 using cell death scale index). No more critical differences between data at two different concentrations were observed, except for line E10 (Rsc0868 popP2 effector), where the strength of the HR varied widely from 2 to 3 for hopM1, 0 to 3 for the effector, and 0 to 2 for the empty vector at OD_{600} 0.3. Hypersensitive response to an empty vector in all replicas was close to 0 except for a few leaves where some necrotic cells at infiltrated areas could be observed.

The HR for all effectors varied depending on leaf morphology in the following way: leaves older than 6 weeks (or darker thick leaves, especially hairy and/or with spines) had an inconsistent response to the tested effectors as well as to the positive control *hopM1*. Leaves with

the type of morphology described above taken from the same plant could develop strong HR or, on the contrary, not give an HR in a response to HopM1 and/or effector inoculation.

Interestingly, smaller leaves (less than 2 x 2 cm) for all the tested lines shared the tendency to develop senescence of the whole leaf instead of the cell death at inoculated sites. This might be a possible variation of cell death as a massive immune response to expression of HopM1.

Wilting assay: testing a *Ralstonia solanacearum* strain isolated in Virginia for virulence

Two tomato lines (WVA700 and H7996) were tested using a soil soaking assay (Tans-Kersten et al., 1998) to evaluate the aggressiveness of a Virginian strain of *Ralstonia* (strain 819). In six replicas that were performed, the H7996 line demonstrated strong resistance to *Ralstonia* infection. Between 0 and 1 plants wilted or developed latent infection in each assay. Latent infection was characterized by a delayed growth and affected plant morphology (dwarfism) but less than in other tomato cultivar tested. For WVA 700, the assay showed the highest percentage of wilting (only 0 to 1 plants out of the tested plants survived in the combined assays, Fig. 11).

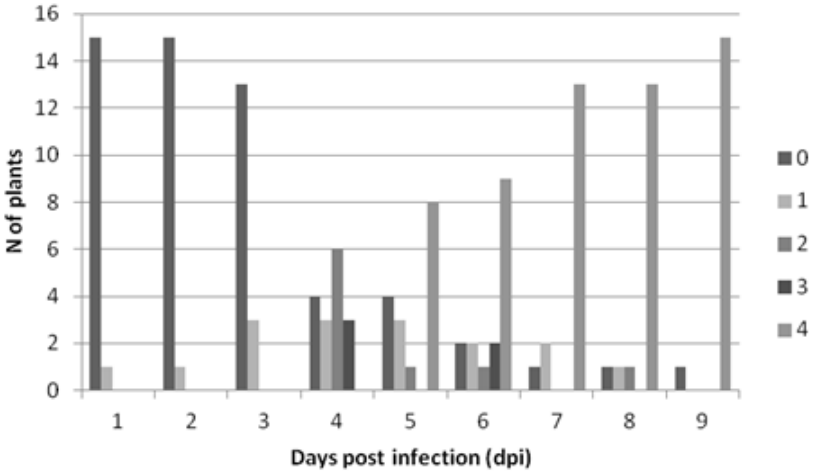


Fig. 11. Wilting assay, WVA700 cultivar. Data represents N of wilted plants at 1 to 9 days (X axis) post inocuation using 0 to 4 wilting index scale (Tans-Kersten et al., 1998), where 0 corresponds to no wilting symptoms and 4 to more than 75% of plant wilted.

For the WVA 700 cultivar, plants exhibited wilting symptoms the first day after soaking with *Ralstonia*, but the majority of plants started wilting at the 4th and 5th day post inoculation. On

the 7th day post inoculation, most infected plants were completely wilted. Once the plant started wilting, it could not overcome the infection. Plants that showed no wilting up to the end of the experiment exhibited changed morphology: dwarfism (approximately ½ of control plants size) and an enormously enlarged root system (from 4 to 6 times compared to the roots of uninfected plants). Some roots reached 31 cm in length (compared to 3-6 cm in the control group of the same cultivar).

H7996 plants affected by *Ralstonia* exhibited wilting symptoms at 4-5 days post infection, with complete wilting at day 6 to 7. However, most of the H7996 plants tested demonstrated high resistance to infection, without signs of latent infection or changes in plant size.

PopP2 delivery to plants through the *P. syringae* type III secretion of *P. syringae* and *P. fluorescens*

Results obtained by *Agrobacterium* transient assays had shown that the effector PopP2 triggered an HR in resistant breeding lines. This effector was thus cloned into the pEDV6 vector in which it is expressed as a C-terminal fusion to a *P. syringae* effector so that it can be delivered into plant cells through a *P. syringae* type III secretion system (T3SS) from either *P. syringae* or *P.fluorescence* EtHaN (Effector-to-Host Analyzer). Unfortunately, transfer of the pEDV6 popP2 construct into EtHaN was unsuccessful. However, the construct was transferred to *P. syringae* strain DC3000 and tested in five eggplant lines used before.

Breeding lines infiltrated with *P. syringae* strain DC3000 either expressing or not expressing *popP2* showed various responses. E1, E2, E6, E8 and E10 lines showed the strongest HR (corresponding to 3 on the cell death, Table 3) at the sites of infiltration with the *P. syringae* DC3000 strain with and without the *popP2* construct in all five replicas.

CHAPTER 4. DISCUSSION

MAMP-TRIGGERED PLANT IMMUNITY

The ability of plant cells to recognize MAMPs (Microbial-associated Molecular Patterns) is the most important step in developing immune response and overcoming pathogen attack. The "address-message" concept, introduced originally as a way of activation of receptors for neuropeptides in animals (Schwyzer et al., 1980) was proposed as the actual way of activation of FLS2 by flagellin (Chinchilla et al., 2006). According to the concept, the ligand (flg22) first binds to the N-terminal part of receptor (address) and further activates the C-terminal part (message) (Meindl et al., 2000). Conserved domains of receptors are most likely to be sites for ligand binding. Covalent high-affinity binding of flg22 to the N-terminus of FLS2 was shown to be the first step of the flg22-FLS2 interaction (Meindl et al., 2000). AtFLS2 and SlFLS2 (FLS2 of *Arabidopsis thaliana, Solanum lycopersicum*, respectively) were hypothesized to function according to the address-message concept (Chinchilla et al., 2006).

Furthermore, direct binding of flg22 to FLS2 has been demonstrated (Chinchilla et al., 2007). Detailed analysis of FLS2 protein function using site-directed mutagenesis showed that the conserved part of the protein across the β-strand/b-turn region of repeats 9 to 14 of the FLS2 LRR is most likely to be the binding region for the flg22 peptide (Dunning et al., 2007).

According to the data presented here that were obtained from RCM mapping, the region consisting of approximately 11 amino acid residues on the protein surface (amino acids 12-17 within LRRs 9-13) was predicted to be conserved. This result is approximately consistent with previous findings in regard to FLS2 function, whereby the LRRs 9-14 were shown to contribute to flagellin binding. However, more conservative regions (potentially responsible for ligand binding) have been identified in the N-terminal LRRs of FLS2. We lack the data on other ligands potentially binding to the FLS2 receptor.

Interestingly, the FLS2 receptors in Arabidopsis and tomato are conserved at least in correspondence to the β-sheets. However, β-sheet- β -turn residues (which are often solvent-exposed and therefore can carry the function of ligand binding) are most likely to be under positive evolutionary selection (Dunning et al., 2007). The recognition of flg22 in tomato

cultivars and *Arabidopsis* varies: ROS (reactive oxygen species) production depends on the plant species and varies even among tomato cultivars (Clarke et al., unpublished).

Based on bioassays with truncated flagellin, Mueller et al. (2012) showed the importance of the initial 10 LRRs of SlFLS2 between the amino acids 32 and 337 and, especially, the importance of the region 236-337. Interestingly, LRRs 19 to 24 were shown to recognize the C-terminus of the flagellin derivatives (Mueller et al., 2012).

Three of six mutations found in the LRR domain of different tomato cultivars were located at positions 209, 210 and 293. Other mutations were located in LRRs 16, 17 and 18. RCM mapping using *SlFLS2* alleles identified several conserved domains (not one), which might correspond to several ligand-binding sites and therefore suggest that FLS2 binds more than one ligand. flg22 is the only confirmed MAMP binding to FLS (Chinchilla et al., 2006). Recent work suggested binding of AtFLS2 to Ax21, a short functional MAMP of *Xanthomonas oryzae pv. oryzae* (Danna et al., 2011).Furthermore, the competitive assay indicated that probably the same site of AtFLS2 is responsible for Ax21 binding (Danna et al., 2011). CLV3p was also found to interact with FLS2 (Lee et al., 2011). However, results from both reports were questioned by others (Segonzac et al., 2012, Mueller et al., 2012). These authors, on the contrary, report Arabidopsis FLS2 blindness to CLV3 and axY(s)22 peptides.

The only crystal structure identified for a plant LRR protein involved in the immune response is that of polygalacturonase inhibiting protein 2 (PGIP2) from *Phaseolus vulgaris* (Di Matteo et al., 2003). The crystal structure revealed a solenoid form, typical for LRR proteins, with two β-sheets formed by β-strands. A second β-sheet is unique for plant LRRs and is formed by a LT/sGxIP motif. Hydrophobic leucine residues face the core of the solenoid, and solvent amino acid residues are exposed to the surface. In many cases, solvent-exposed residues have been predicted to interact with ligands (Bent et al., 2011, Mueller et al., 2012). Consequently, when the amino acid residue is replaced by a residue with different polarity, the structure of the β-strand can be affected in a way so that the hydrophobic residue goes to the concave side of the solenoid structure and, on the contrary, hydrophilic residue becomes solvent-exposed. There is evidence that even single mutations can dramatically change the binding capacity of FLS2. For example, a single mutation in AtFLS2 (LRR10, position 318, G → R) has been shown to disrupt the binding of flg22 (Dunna et al., 2011).

The character of amino acid changes observed for tomato cultivars used in this project was, in most cases, linked to changes in polarity. Three mutations within positions 209-293 were: very hydrophobic Phe to neutral Ser (209), polar neutral Met to very hydrophobic Ile (210) and polar neutral Gln to hydrophilic Lys (293). Interestingly, mutations in positions 209 and 210 were located in the secondary β-sheet, which is the loop-turn region connecting the front and the back of the structure. Any changes in the polarity of residues in this region could affect the structure of the solenoid; however, these two mutations could also potentially balance each other, reorganizing the structure but restoring the solenoid by acquiring hydrophobicity through the Ile residue that is replacing the Phe residue (forms concave side) and thus "flipping" the structure.

Theoretically, any of the mutations located within the functional domain of the protein could affect its ability to bind ligands by simply changing the 3D structure and exposing different residues to a ligand. We propose that *SlFLS2* might undergo high selective pressure to adapt to mutated ligands (because of the high ratio of non-synonymous to synonymous mutations that might change the protein structure). The concentration of mutations in the functional domain of the protein might indicate positive selection for sequence diversification to recognize new flagellin alleles evolving in pathogen populations and be an example of the plant-pathogen arms race. This selection leads to changes in protein structure and to the exposure of different amino acid residues to the ligand. Depending on the receptor structure, the ability of FLS2 to recognize flagellin and/or other ligands might be affected.

Many more experiments are needed to confirm this possibility. First of all, transformation of tomato cultivars with different alleles of *SlFLS2* might affect the ability to recognize flagellin. If stable transformants show different levels of flagellin perception compared to wild-type, the approach could be used further for obtaining transgenic lines with increased ability for PAMP recognition. On the other hand, the transformation approach might have potential pitfalls in regard to different levels of expression of native and introduced alleles of *FLS2* and possibly disrupt interaction between receptors. Secondly, the FLS2 protein structure needs to be further investigated as various LRRs have been proposed to be involved in binding flg22 (Mueller et al., 2012, Mueller et al., 2011).

EFFECTOR-TRIGGERED PLANT IMMUNUTY

A Virginian strain of *Ralstonia solanacearum* has been shown to be highly aggressive on tomato cultivar WVA700 but not on H7996. The development of the disease was similar in all replicas and included two stages. During the first stage, the plants started to develop symptoms of wilting on leaf edges, during the second stage (2^{nd}-4^{th} days from appearance of the first symptoms) the plant completely wilted. Interestingly, the root system of the WVA 700 plants that survived and had latent infection was larger than in control plants. This can be explained by a plant strategy to overcome the disease not by using genetic resistance but by developing a larger root system to compensate for the reduced flow through infected xylem vessels.

The cell death observed in transient assays with *Agrobacterium* was a useful tool to determine whether an effector has potential avirulence activity. The highest rate of cell death in all replicas was observed for *Ralstonia* effector Rsc0868popP2. The family of popP2 effectors was previously shown to trigger HR in *Arabidopsis* (Tasset et al., 2010). In our experiments, the PopP1 effector was a second potential candidate to be an avirulence factor as it elicited a somewhat stronger HR in two resistant eggplant lines (E1 and E2) and a partially resistant line (E6) compared to the susceptible lines E8 and E10. Though other effectors tested (GALA3 and Rsp1130) elicited some HR, the HR was inconsistent in all eggplant lines except for E8 (where it did not cause any HR).

Eggplant breeding lines challenged with the four cloned effectors demonstrated variability in their response when challenged with the same effector. Though the tested resistant breeding lines (E1 and E2) shared the tendency to have a higher cell death rate in almost all the experiments, other lines (except for E8 which showed the lowest level of cell death in all the experiments) had different levels of cell death even in the same experiment. This variability could be due to different factors. First of all, the conditions of the experiment (temperature, light exposure, time of day of infiltration) can potentially affect the level of HR. Secondly, it has been found that leaf morphology has certain effects on the assay results. In bigger upper leaves (6-8 weeks old) the HR was variable even in the case of the *hopM1* effector (which was used as a positive control). Ideal leaves to use in the assay were 4-6 weeks old. Younger leaves (not larger than 2X4 cm) shared the tendency to develop leaf senescence in 2-3 days, even if larger leaves were not showing cell death at the infiltration sites, probably due to the mechanical response to

the infiltration (or the massive cell death as a response to effector recognition). The senescence started at all inoculated sites (except for the negative control in most cases, but not always) and spread over the leaf shortly. Interestingly, a weaker response was observed if the infiltration site included the large vein, probably because of the higher water concentration and movement of the effector from the area. Line E10 (previously reported to be susceptible to *R. solanacearum* strain GMI1000) showed the most variable response depending on the leaf and sometimes depending on the location of the infiltration site. The HR caused by the same effector varied from 0 to 2 which was observed for PopP2 and PopP1 effectors. For GALA3 and Rsp1130 effectors the HR was constitutively low in leaves tested.

HR variability can therefore be explained by different reasons. First, eggplant lines showing HR might have different sources of genetic resistance to the pathogen; however, this resistance can depend on the environment, such as temperature, light exposure and humidity. Secondly, resistance can be partial and thus the effector can cause HR only under certain conditions. Thirdly, the expression of R genes could be environment-dependent and thus alter the strength of the HR.

Previous results for *Ralstonia* strain GMI1000 demonstrated that the resistance to this strain varies widely between species and even within species. Two effector systems, AvrA and popP1, were limiting the host range of GMI1000 in tobacco, with AvrA eliciting HR in *N. benthamiana* and *N. tabacum* and popP1 eliciting HR in *N. glutinosa*. Therefore, differences in the ability of different effectors to trigger HR may correspond to presence/absence of R genes in various eggplant cultivars.

Undoubtedly, the role of *Ralstonia* effectors (especially those belonging to popP2 and popP1 families) need to be further investigated for their ability to trigger HR and their role in genetic resistance of eggplant breeding lines tolerant to a bacterial wilt. Knowledge of resistance genes (such as *RRS1-R* gene of Arabidopsis, Tasset et al., 2010) contributes to further similarity-based identification of resistance genes in different plant species. The system of double-checking with consequent Agrobacterium transient assay followed by *P. fluorescence* effector delivery through T3SS system is an example of a useful tool for the detection of breeding lines containing R genes to a specific effector. PopP2 effector introduced into DC3000 *P.syringae* strain caused high HR in 12 h in all the cultivars; however, the response might be explained by

an HR caused by DC3000 itself. An useful assay would be to deliver the construct through EtHaN strain which does not cause HR if no construct is introduced. In my experiments eggplants inoculated with EtHaN strain shown delayed HR (or developed disease symptoms) in 48h. However, this system needs to be further improved to avoid artifacts and adjusted to lab conditions to reduce the possibility of false negative/false positive results.

Some breeding lines (E2 in my experiment) may carry the allele of resistance to *Ralstonia* effector (Rsc0868popP2) and this allele might contribute to the overall resistance of this breeding line to GMI1000 strain. Other resistant eggplant lines, like E1, might have an allele responsible for recognition of another effector, as well as partially resistant eggplant line (E6). Interestingly, E10 line (previously reported to be susceptible) developed HR in some leaves in a response to all four effectors which might be explained by partial resistance.

In broad terms, resistances of eggplant lines tolerant to bacterial wilt can be due to the recognition of multiple effectors. In this case, different effectors can trigger different levels of HR depending on the contribution of the particular effector to immunity. When exposed to a real pathogen, plant lines/species recognizing more than one effector have advantages compared to others. By introducing R genes known for their ability to interact directly or indirectly with different bacterial effectors into new breeding lines may be a promising avenue to obtain disease resistant high yielding cultivars. Multiple resistance genes introduced into plant breeding lines confer more durable resistance to the specific pathogen because the pathogen would have to evolve ways to avoid recognition by multiple resistance genes (instead of few), which would necessitate multiple evolutionary events to occur.

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SUPPLEMENTARY MATERIAL

Tomato transformation protocol (based on Branchato protocol, ZMPB Tubingen, modified)

1. Seed sterilization – 2-3 weeks prior to transformation. All solutions are sterile:
   70% ethanol for 3 min (shaking)
   1.5% hypochlorite (bleach) with few drops of 0.001% Triton W-100 (for better absorbance) for 10 min, shaking
   3 x200mL sterile water (in 2 mL tubes)
   Keep seeds for two days at 4°C in the dark.
2. 14 days before the transformation: plant seeds on Germination medium, 25 seeds of cultivars Money-Maker, Micro Tom, 'ChicoIII', 'Rio Grande', 'Sunpride', 'Roter Gnom'. Keep in the dark (foil wrapped) at 22 °C.
3. Use only primary leaves that are not rolled. Lay them adaxial side down on plates containing Conditioning Medium. 20 leaves per Petri dish, incubate for 2 days in darkness at 22°C.
4. Agrobacterium: 4 days before the transformation, the recombinant Agrobacterium strain (Le-FLS2+ maybe 2 constructs Nan Lu has) should be streaked out on plates with LB-media and selective antibiotics (Rif, Kan, Gent). 2 days before the transformation, inoculate 3mL low-salt LB medium with one single colony of Agro. 1 day before the transformation, 100mL culture with 1mL subculture should be started in a Bacteria-Growth medium. Shake overnight at 28 °C. Before using culture, bring OD to 1.0 with 10mM MgSO4.
5. Drop the Agrobacterium suspension on leaves (about 2 drops so that the leaf is fully covered). Incubate for 2 days in the dark at 22 °C.
6. 2 days after the transformation, leaves can be put on plates with Selection Medium (35 mg/L Kan) with antibiotics against Agrobacterium (tricarcillin=amoxycilline, 250mg/L), leaf surface up, in the light (14h light and 10h dark at 23 °C, 50%humidity), leave them for 3 days on it.
7. Transfer leaves every week. 2x7 days – 50mg/L Kan, from the 4th week 100mg/L Kan

8. After 14 days, the first shoots are forming. After approximately 2 months, the shoots with apical meristems can be cut and transferred to Rooting Medium. If problems with rooting, remove the Kan. The callus should be kept.
9. Medium Recipes:

LB-Medium:

10 g/l Bacto-Tryptone

5 g/l Bacto-Yeast Extract

10 g/l NaCl (5 g/l for low salts medium)

For plates add 15 g/l Bacto Agar Difco direct into the bottle

Autoclave

Bacteria-growth medium (BGM)

10 g/l Yeast-Extract

10 g/l Bacto-Peptone

5 g/l NaCl

Autoclave

0,2 mM Acetosyringone (from 400 mM stock-solution in DMSO)

Add antibiotics to 60°C warm medium, stir well and put immediately in plates.

If plates contain antibiotics, they should not be kept longer than a month.

- ampicillin 100 mg/l (stock 100 mg ddH_2O)
- rifamycin 100 mg/l (stock 50 mg DMSO)
- kanamycin 25 mg/l (Stock 50mg/ml ddH_2O)
- gentamycin 40 mg/l (stock 10 mg/ml ddH_2O)

Medium for plants

Germination Medium (GM - 2 Liters for about 30 0,5 liter-glasses)

dissolve in 900 ml ddH_2O:

4,4 g/l Murashige & Skoog + minimal organics (MSMO Sigma, M 6899, store at 4°C, 10 liter = € 54,00)

30 g/l sucrose

adjust pH to 5.8, with about 8-10 droplets of a KOH stock1M and fill up to 1 l

Phytagel 4g/L

Autoclave, stir well and pour immediately in glassware (0,5 l).

Liquid Germination Medium (LGM – 1 liter in two bottles)

dissolve in 450 ml ddH_2O:

2,2 g/l Murashige & Skoog + minimal organics

15 g/l sucrose

adjust pH to 5.8, with about 5 droplets of a KOH stock1M and fill up to 0,5 l

autoclave

Conditioning medium (CM – 1 liter for about 30 Petri-dishes)

dissolve in 900 ml ddH_2O:

4,4 g/l Murashige & Skoog + minimal organics

30 g/l sucrose

adjust pH to 5.8, with about 8-10 droplets of a KOH stock1M and fill up to 1 l

Phytagel 4g/L

Autoclave, cool to 60°C (hand warm), add hormones: 0,1 mg/l BAP, 1 mg/l NAA,

stir well and pour immediately in Petri dishes (9 cm ∅)

Selection medium (about 10 liters)

dissolve in 900 ml ddH_2O:

4,4 g/l Murashige & Skoog + minimal organics

1 ml/l NPT Vitamins stock-solution (= 10,0 mg/l Thiamine + 1,0 mg/l Nicotinic acid + 1,0 mg/l Pyridoxinic acid)

30 g/l sucrose

adjust pH to 5.8, with about 8-10 droplets of a KOH stock1M and fill up to 1 l

Phytagel 4g/L

Autoclave, cool to 60°C (hand warm), add hormones: 1 mg/l trans-Zeatin (Sigma, Z0876, -20°C), add antibiotics against Agrobacterium: 250 mg/l Ticarcillin-clavulanate (Duchefa, T0190, 4°C);

add antibiotics to select:Kanamycin: 35, 50 or 100 mg/l Kanamycin. Stir well and pour immediately in 0,25 l glassware.

Rooting medium (about 5 liters)

4,4 g/l Murashige & Skoog + minimal organics

1 ml/l NPT Vitamins stock-solution (= 10,0 mg/l Thiamine + 1,0 mg/l Nicotinic acid + 1,0 mg/l Pyridoxinic acid)

30 g/l sucrose

adjust pH to 5.8, with about 8-10 droplets of a KOH stock1M and fill up to 1 l

Phytagel 4g/L

Autoclave, cool to 60°C (hand warm), add hormones: 0,1 mg/l IAA, add antibiotics:

20 mg/l Kanamycin, 500 mg/l Vancomycin (Douchefa V0155)