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Investigating the expression of two protein kinase genes involved in the tomato immune response

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Introduction

As sessile organisms, plants are literally "rooted" in place; this means they cannot avoid stresses or dangers by simply evading them. Instead, they have evolved intricate signaling pathways and strategies to deal with the many threats they face, including pathogens. Regarding defense against pathogens, plants also lack circulating cell populations and consequently do not have a specialized mobile cellular defense as many animals have. They lack for example T and B cells to protect them from intruding microorganisms.

Instead, plants evolved several unique lines of defense to prevent colonization by pathogens. The first of these is formed by components of their normal morphology, even though they can be modified / strengthened in response to an infection (see below). This can be referred to as “integral” defense. It consists mainly of physical barriers that minimize direct exposure of susceptible plant cells to the environment to limit potential points of pathogen attack. Examples are the cell wall, waxy epidermal cuticles and bark.

In contrast to this integral defense, which is to a certain extent always present as part of the normal plant body plan, the other two defense lines are activated in response to certain stimuli. They can consequently be referred to as “inducible defenses” and are parted into two types referred to as PAMP-triggered immunity (PTI) or effector-triggered immunity (ETI) [1], [2].

PTI is triggered in response to so-called pathogen associated molecular patterns (PAMPs; sometimes also referred to as microbe associated molecular patterns or MAMPs); which consist of integral parts of the morphology of potential pathogens that are under a high selective pressure and therefore highly conserved. Examples are bacterial flagellin [3] and elongation factor Tu (EF-Tu) [4], or chitin [5] as an integral part of fungal cell walls. As these molecules are tremendously important for the viability of their organisms, they can not be easily shed and serve as telltale signs that, when sensed by the plant through specialized receptor molecules termed PAMP

recognition receptors (PRRs) [6], are taken as indication that a potential pathogen is in close vicinity. As these PAMPs are conserved across the respective kingdoms, their presence does not necessarily indicate a pathogen; they are also associated with non-pathogenic microbes that pose no direct danger to the plant. Consequently, the defensive actions initiated by the plant cell are relatively mild; they consist of, amongst others, cell wall fortification at the site of PAMP detection and release of reactive oxygen species (ROS) to the outside of the cell. By these measures, the plant inhibits microbial growth and protect cellular integrity.

The last line of defense is now known as effector-triggered immunity or ETI. Effectors are proteins that pathogens deliver into cells of host organisms to manipulate host cellular pathways or metabolism to their own advantage. Different models exist as to how plants' cells are able to detect the presence of these proteins inside of their cells (see below). In any case, the detection of an effector is a highly alarming signal for the host, as its presence is indicative of an attack by a pathogen with an arsenal of effector proteins and the means to deliver those. The strong response often involves programmed cell death (PCD) as part of a process called hypersensitive response (HR). Basically, plant cells that detect the presence of effectors commit suicide to inhibit or halt microbial proliferation. This strategy is obviously only effective against obligate biotrophic or semi-biotrophic pathogens [7]. Both PTI and ETI will be discussed in more detail below.

During evolution, plants have been exposed to a multitude of pathogens like viruses, bacteria and fungi. While they developed defenses to keep infectious agents at bay and protect themselves, the pathogens in turn evolved their own repertoires of tools in order to circumvent these defensive mechanisms and allow infection, particularly the aforementioned effectors. As a consequence of this constant back and forth in between plants and their respective pathogens, the two share a long history of co-evolution. This – still ongoing – evolutionary arms' race in between host and (potential) pathogen can be represented in a zigzag model [2], which is shown in Figure 1.

Initially, plants developed PRRs to detect the presence of potential pathogens

by means of conserved PAMPs/MAMPs; having the cognate PRR to a given PAMP allows the host to detect invading microbes at very low concentrations and fortify their defenses as a response to attack. Using molecules that are essential to a very large number of pathogens enables plants to efficiently detect many different microorganisms with a limited number of receptors, while limiting the possibilities of the pathogens to evade detection by changing or shedding the detected structures.

To circumvent these defenses, would-be pathogens had to develop tools that would allow them to undermine this surveillance system [8]. They achieved this by evolving effectors, proteins that are injected into the cells to inhibit PAMP-induced signaling and promotes disease; this allowed them to once again become pathogenic on plants with PTI, a state that has been termed “effector triggered sensitivity” or ETS.

The next “zig” in the model represents the development of dedicated detector molecules by plants encoded by resistance genes (R-genes). The products of these genes are proteins that can detect the presence of microbial effectors and again mount a defense response resulting in resistance to the pathogen. This response is a much stronger and more rapid form of defense, often resulting in induced cell death at the site of infection to halt microbial invasion. The reconstituted resistance is called ETI. As often one detector gene on the plant side corresponds to detection of one effector from the pathogen side, this interplay is also known as gene for gene interaction [9].

As a consequence, the pathogens again had to devise means to disarm this new defensive mechanism. One way they achieved this was the evolution of novel sets of effectors that can interfere with and prevent ETI signaling, reestablishing a state of ETS.

The continued rounds of co-evolution of plants and their pathogens lead to sophisticated arsenals of detectors and effectors in plants resp. pathogens. This can in part explain why successful infection of a host is rare: the pathogen needs just the right effectors to suppress defense response, but must not have any that are recognized and trigger ETI. In an ongoing

competition, these processes are still permanently unfolding, driving the continued evolution of novel effectors and resistance mechanisms.

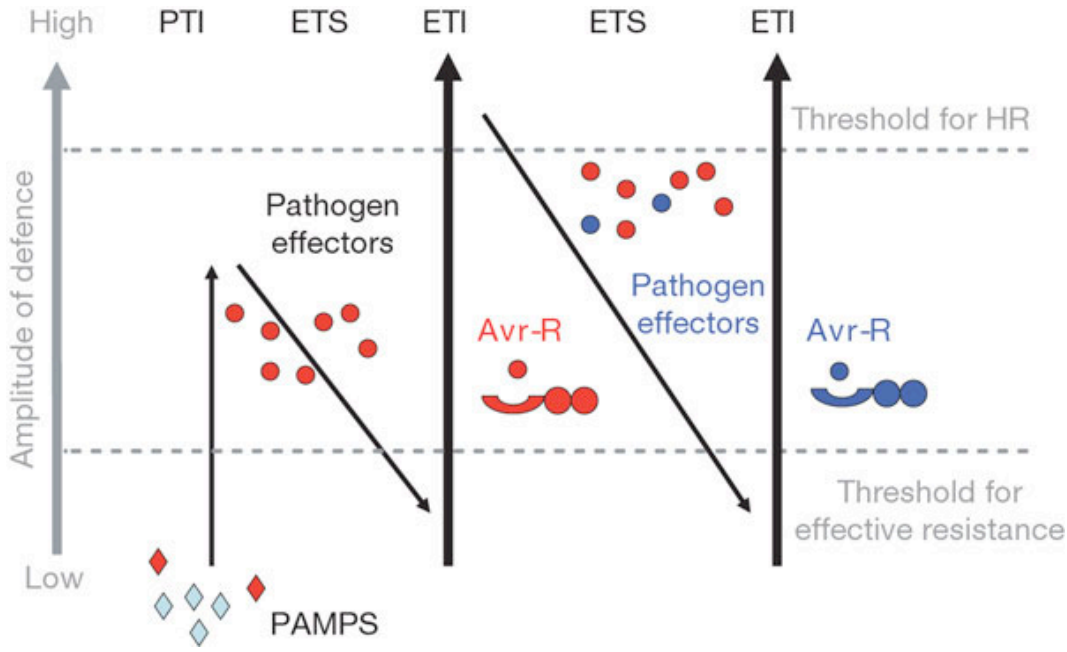

Figure 1: Zig-Zag model proposed by Dangl and Jones for ETI and PTI (Jones 2006)

PAMP triggered immunity

PTI constitutes the basal defense mechanism in plants and is activated by the recognition of standard molecular patterns, called PAMPs, through pattern recognition receptors, PRRs [1]. As mentioned above, PAMPs are conserved molecules associated with a wide spectrum of pathogenic as well as non-pathogenic microbes [10].

A well studied PTI elicitor is flagellin; this protein represents a main component of the bacterial flagellum and is recognized by the pattern recognition receptor FLAGELLIN-SENSING 2 (FLS2) in many higher plant species [11]. Prokaryotic flagella are structures on the outside of bacterial

cells that, by utilizing a proton gradient to generate a rotational force, allow the organisms to propel themselves forward [12]; [13]. Flagellin proteins from different bacterial strains have highly conserved amino- and carboxyl–termini, but variable central regions [14]. Motility is of central importance for the viability of microorganisms, as it allows them to seek favorable and avoid unfavorable environments; in the case of pathogens, it is in many cases indispensable for pathogenicity. Consequently, flagellin appears to be an ideal PAMP for any organism that wants to be alerted of the presence of motile prokaryotes. This is illustrated by the fact that flagellin serves as a PAMP not only in the *planta* kingdom, but is also a recognized in animal cells by the immune receptor TLR5, triggering a defense response. While there are functional and structural parallels between FLS2 and TLR5, the two receptors perceive different parts of the flagellin protein [15]. A synthetic 22 amino acid peptide (flg22) that originates from the most conserved N-terminal part of bacterial flagellin is sufficient to induce PTI [3]. Recognition of this highly preserved part of the protein allows plants to recognize flagella from many different bacterial species with the same receptor, while giving the microbe the lowest possibility of evading detection through mutation of the PAMP. However, it has been observed that responses to flagellin from different bacterial species can vary in strength [3]. Arabidopsis mutants lacking the FLS2 receptor exhibit higher sensitivity against *Pseudomonas syringae pv. tomato* DC3000 in experiments in which the bacteria are applied by spraying, but not when the pathogen is syringe infiltrated into the leaf apoplast [16] [17]. This suggests that FLS2-mediated recognition of flagellin and the ensuing defense response might play a more important role in the early stages of pathogen invasion and are less effective once the bacteria have reached the apoplast.

Another known PAMP that triggers a defense response similar to flg22 is the bacterial Elongation Factor Thermo Unstable, EF-Tu [4]. The prokaryotic EF-Tu protein mediates the entry of a charged t-RNA into the ribosome. EF-Tu binds to the tRNAs in the cytoplasm and the complex enters the free A-site in the ribosome. If the pairing between codon and anticodon is correct, EF-Tu hydrolyzes GTP, which induces a conformational change and its dissociation

from the tRNA; only then the tRNA can progress to the next (P-) site in the ribosome. In case the codon-anticodon pairing is not perfect, hydrolyzation of GTP by EF-Tu is delayed, which makes it likely that the “wrong” tRNA dissociates from the ribosome before the initiation of the next step of protein chain elongation. Hence, EF-Tu is a central component in translational quality control in prokaryotes [18]. Like flagellin, the function of EF-Tu is paramount to the viability of the cell, once again demonstrating that proteins of crucial importance are utilized as PAMPs to give the microbes no opportunity to evade recognition through mutations. EF-Tu is recognized by an Arabidopsis LRR-RLK (Leucine-rich repeat receptor-like kinase) called EFR [19]. That a cytoplasmic bacterial protein is utilized as a PAMP is somewhat surprising, as it is not usually presented on the outside of the cell or secreted; it is typically assumed that a certain number of bacterial cells always lyse in the course of an infection and that the EF-Tu that is released into the apoplast from these is detected by EFR.

A large number of molecular, morphological and physiological changes are triggered by the perception of PAMPs [20]. Ion flux across the membrane, oxidative burst, mitogen activated protein (MAP) kinase activation and protein phosphorylation [21] are all events that happen few minutes after PTI has been induced by pathogen recognition. Finally, PTI leads to major transcriptional reprogramming of the cell, as for example in Arabidopsis, where it has been shown that up to 3% of total transcripts can change. Later adjustments help the plant to prevent the possible continued entrance of pathogens by closing of the stomata [22] and enhancing the amount of callose in the cell walls, thereby building a stronger physical barrier.

The evolution of PTI appears to have occurred early, since there has been found through genome sequencing a homologue of FLS2 in all higher plants [10]. Also, functional conservation has been shown between FLS2 homologs from different plants. fls2 mutants are not longer able to detect flg22, and therefore no PTI is induced. It was shown that by complementing fls2 mutant *A. thaliana* with the rice FLS2, OsFLS2, the mutants were again able to detected flg22 and trigger defense [23], demonstrating that not only the

receptor, but also the complete signaling pathway is conserved. However, not all PAMPs are recognized equally strong or by all plant species. For example, flagellin from *Agrobacterium tumefaciens* or *Sinorhizobium meliloti* is less active than that from *P. syringae* [3]. In fact, individual plant species recognize only a subset of potential PAMPS. EF-Tu for example has to date only been shown to trigger defense in *Brassicacea* [4]. Nevertheless, transfer of the receptor that recognizes EF-Tu from *Arabidopsis thaliana* into tobacco, a plant species from the *Solanaceae* family that normally lacks a response to EF-Tu, resulted in activation of PTI [19]. This shows that the PTI signaling pathway is conserved downstream of the PRR. In fact, in Arabidopsis treatment with a conserved EF-Tu peptide induces activation of a gene set almost identical to the one induced by flg22, including upregulation of FLS2 [19]. *Vice versa*, elicitation by flg22 also leads to an upregulation of EFR. Therefore, it seems likely that different, but not all, PAMPs like flg22 and EF-Tu converge on a limited number of downstream signaling pathway components involving a MAP kinase cascade and the leucine-rich repeat receptor-like kinase BAK1 (BRI1-associated receptor kinase 1) [24], [25] [19]. On the other hand, chitin, a fungal PAMP, appears to use at least some different downstream components, since it is BAK1 independent, but it still activates the MAP kinase cascade [26]

Chitin

Chitin is a polymer of β-1,4-linked N-acetyl-glucosamine (GlcNAc) that is a major component of fungal cell walls and has long been known as a PAMP able to trigger various defense responses in plants [5]. In response to fungal attack, plant cells express chitinases and release these enzymes to the site of infection [27]. Not only is the infection inhibited by the resulting degradation of the fungal cell wall, but the released chitin and chito-oligosaccharides can also be recognized by PRRs and elicit PAMP triggered immunity in plants [5] [28] [29].

The first receptor that was shown to bind chitin was CEBiP (chitin elicitor binding protein). CEBiP was found as a plasma membrane localized protein in rice cells. It has an extracellular domain containing two Lysin Motifs involved in chitin perception and a transmembrane domain, but it lacks an intracellular kinase domain that would be required for signal transduction. It was possible to show that this receptor specifically binds chitin oligosaccharides and that knock down of the CEBiP gene using RNAi results in the suppression of defense responses activated by chitin [30]. However, the finding that the receptor does not have a kinase domain suggests the need for at least one extra protein to allow signal transmission across the plasma membrane into the cytoplasm. Recent studies have confirmed that at least one other protein, OsCERK1, is required for chitin perception. OsCERK1 is a plasma membrane protein containing one LysM motif in the extracellular domain and an intracellular Ser / Thr kinase domain. OsCERK1 knockdown cell lines are compromised in their defense response to chitin. However, a minor response remains, which could be explained either as a result of incomplete suppression of OsCERK1 by RNAi or by the presence of other OsLysM-RLKs playing partly redundant roles in chitin signaling [31]. This has still to be clarified. Also, it was not possible to detect direct binding of OsCERK1 to chitin, leaving the possibility that CEBiP plays an important role in the binding of chitin and that OsCERK1 functions as a signal transducer through its Ser / Thr kinase activity in rice. Using yeast two hybrid assay, it was demonstrated that the two proteins have the potential to form homo- and heterodimers through interactions between their extracellular domains [31].

Recently, two independent groups identified a chitin recognition receptor in Arabidopsis (Figure 2). A collection of Arabidopsis insertion lines carrying insertions in genes encoding LysM containing proteins was tested for their ability to react to chitin. This screen allowed the identification of two independent mutants, a Ds-transposon and a T-DNA insertional mutant, that had completely lost their ability to respond to GlcNAc, but not to other PAMPs. Both of these mutants harbored insertions in the same gene, a lysine motif receptor like kinase containing three extracellular LysM motifs, a transmembrane domain, a juxtamembrane domain and an intracellular Ser /

Thr kinase domain. The protein was named CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1) by one group and LysM RLK1 by the other [32] [33]. Using complementation approaches, both groups were able to demonstrate that the disruption of the gene was the reason that all elicitor-induced responses were completely impaired [32] [33]. The complete loss of reaction indicated that CERK1 might be the only or at least the most important receptor in the chitin signaling cascade and, as there is still response to other PAMPs, it also indicates that CERK1 is specific for chitin recognition. Neither group was able to directly show the actual binding of chitin to the receptor, leaving open to discussion the presence of another protein that binds chitin. Recent studies with CERK1 protein fused to yEGFP expressed in yeast cells gave the first evidence of direct binding between receptor and elicitor. It was shown in a ligand specificity assay that CERK1-yEGFP was largely specific for chitin; the interaction was visualized using fluorescence microscopy [34]. Last year, a group using an affinity purification approach was able to demonstrate that CERK1 can bind chitin directly and without the need for interaction with other proteins *in vitro*. Their results also suggest that CERK1 binds stronger to polymeric chitin than to oligomers. It was also shown that CERK1 needs all three LysM domains for the binding of the elicitor [35].

There are still many open questions about this pathway, especially regarding specificity, i.e. whether only chitin or also chitosan, a deacetylated version of the GlcNAc, can be recognized by CERK1/ CEBiP. Studies *in vitro* only detected strong binding of CERK1-yEGFP to chitin, but not to chitosan [34]. On the other hand, Petutschnig et al. were able to show *in vitro* that CERK1 can also bind chitosan polymers, but not oligomers. One possible reason for the recognition of chitosan polymers, but not to oligomers, is that polymeric chitosan contains a significant amount of acetylated glucosamine residues, whereas the chitosan oligomers used were completely deacetylated. These results might indicate that the acetyl group in chitin plays an important role in its recognition by CERK1 [35].

It is interesting to note the differences in chitin / chitosan recognition between rice and Arabidopsis. In rice, CEBiP plays a central role in binding the elicitor,

but depends on OsCERK1 for signaling, as it lacks an intracellular kinase domain. For Arabidopsis, CERK1 has been shown to be able to directly bind chitin / chitosan in yeast and *in vitro*. It brings its own functional kinase domain; mutations rendering this kinase domain inactive abolish CERK1-mediated PTI signaling *in vivo* [35]. This opens the possibility that AtCERK1 might not be dependent on an accessory CEBiP like protein for PTI signaling. However, addition of elicitor to isolated AtCERK1 protein *in vitro* does not induce a change in phosphorylation status [35], which might indicate that additional factors are necessary also for CERK1 function in Arabidopsis. Consequently, there might be distinct differences in chitin signaling between rice and Arabidopsis. Alternatively, Arabidopsis could express a so far unknown CEBiP homolog that cooperates with AtCERK1 to initiate signaling.

Recently, it has been reported that chitosan might be perceived through a CERK1-independent pathway that potentially partially converges on the CERK1 signaling cascade downstream of CERK1. Treatment of Arabidopsis seedlings with chitosan followed by transcript profiling using DNA microarrays showed only limited overlap compared to seedlings treated with chitin. However, the chitin treatment experiment had not been conducted in parallel but instead stems from an earlier publication by another group, leaving the possibility that some of the effects result from uncontrolled differences in other factors. Interestingly, cerk1 plants still respond to chitosan treatment [36], indicating that there might indeed be chitosan perception independent of CERK1. Another surprising observation is that not only CERK1 is targeted by the bacterial effector AvrPtoB [37], but also contributes to Arabidopsis resistance against Pst [37]. As bacteria do not produce chitin, this might indicate that AtCERK1 is involved in the perception of PAMPs other than chitin.

Figure 2: Predicted structure of CERK1 protein (Miya 2007)

Effector Triggered Immunity

To infect a plant, pathogens must be able to evade or overcome the first level of plant defense formed by PTI. Successful pathogens secrete effectors to suppress PTI, resulting in effector-triggered susceptibility (ETS). Gram negative plant pathogenic bacteria, for example, deliver many such effectors into the host via a type III secretion system (T3SS) [38] [39]. It has been demonstrated that *Pseudomonas syringae* strains that have lost the ability to deliver effectors by means of the type III secretion system are recognized faster and stronger by the plant defense system [40]. Hence, the delivered effectors must collectively dampen the basal defense to an extent that allows colonization. For example, AvrPto and AvrPtoB, two unrelated proteins that are delivered by the T3SS, contribute to virulence by suppression of early steps in PTI, preventing the phosphorylation of MAPKKK and consequently signaling [41] [25] [42]. Furthermore, it has been shown that AvrPtoB, like other type III effectors, is a modular protein. Its amino-terminus can bind and inhibit the kinase domains of many proteins involved in PTI signaling, for example FLS2, BAK1 and CERK1 [43] [26]. By blocking these kinase activities, AvrPtoB can efficiently suppress PTI. Its carboxy-terminus encodes a domain that has been shown to form an active E3-ligase domain. By adding ubiquitin to host proteins, AvrPtoB can mark these for degradation by the proteasome, effectively “hijacking” the cellular protein degradation machinery and using it to the advantage of the pathogen [44] [8] [26]). It has been demonstrated that this function of the effector suppresses ETI by degrading the tomato R gene product Fen [45], which would otherwise detect the presence of AvrPtoB and elicit HR, but the question whether this mechanism also plays a role in PTI suppression by marking PRRs for degradation is controversially discussed. While in virulence assays a C-terminally truncated version of AvrPtoB lacking the E3-domain is sufficient to confer enhanced virulence, it has been reported that the E3-activity is involved in the degradation of PRRs in plant cells [46] [26].

Effectors from eukaryotic plant pathogens, like fungi, are poorly understood. It is known that Avr4 (Avirulence Gene 4), an effector from the fungal tomato pathogen *Cladosporium fuluvum*, helps the pathogen to evade being recognized by shielding the fungal cell wall from plant chitinases [47]. Recently, another effector from *Cladosporium fulvum* has been found that is very effective in inhibiting PTI. This effector is Ecp6 (Extracellular Protein 6), a protein that, like the plant chitin receptors, also contains LysM domains [48]. It was found to compete with the plant receptor CEBiP for chitin fragments, sequestering them away from the PRR to suppress chitin triggered immune responses in the plant [48]. In the case of fungal effectors that act inside the host cytoplasm it is still unclear how fungal effectors are delivered into the cells.

Even though with effectors pathogens seem to have evolved a strong "weapons arsenal" to attack the plant immune system, infection is still a rare occurrence This is in part because effectors that enable the plant pathogen to overcome PTI can themselves be recognized by specific disease resistance (R) genes [2]. This second part of the immune system has consequently been termed Effector-Triggered Immunity (ETI). Many R proteins that confer resistance against viral, bacterial and fungal pathogens and even to nematodes and insect pests have been identified in plants. Most known R proteins belong to the nucleotide binding site - leucine rich repeat (NB-LRR) group of proteins or, less common, the leucine rich repeat receptor like kinases (LRR-RLKs). Both groups share the leucine rich repeat domain; NB-LRRs encode an additional nucleotide binding domain N-terminal to the LRR domain, whereas LRR-RLKs have a kinase domain C-terminal of the LRR. NB-LRRs can be further assigned to subgroups according to the domains they encompass N-terminal of the NB-domain. These can consist of sequences carrying a homology to the Toll and Interleukin 1 receptor (TIR-NB-LRRs), a leucine zipper (LZ-NB-LRR) or a coiled coil motif (CC-NB-LRR) [49]; [50]. However, R genes that do not encode for a LRR domain have also been described. For example, the tomato R genes Pto and Fen encode kinases that can confer resistance to certain *Pseudomonas syringae* strains.

ETI has been described as a faster and stronger version of PTI [51] [52] [53]. It often results in a form of host cell death called the hypersensitive response (HR) [54]. As it is often possible to determine pairs of effector and responsive host genes, this plant pathogen interaction is also known as the “gene-for-gene” interaction [9].

R proteins can detect effectors either directly or indirectly [2]. In the case of direct recognition the R gene product simply acts as a protein / protein interaction partner for the effector. Basically, the R protein is the receptor and the effector constitutes the ligand. One of the best-known examples for a direct interaction is between flax (*Linum usitatissimum*) and flax rust (*Melampsora lini*). Yeast two hybrid studies have shown that the flax L locus encodes NB-LRR proteins that can interact with the reciprocal AvrL proteins [55]. Another example for direct interaction between R proteins and cognate effector are tomato Pto and Fen and their corresponding effector(s) AvrPto and AvrPtoB [56]. It has been proposed that the directly interacting plant R genes evolved to mimic the real targets of the effectors to lure their cognate effectors into interacting with them; this has been formalized as the “decoy model” [57].

Even though there are well-documented cases of direct interactions between R gene products and effectors, this is not the only mechanism by which plants can detect bacterial proteins. In many cases R proteins detect the presence of effectors without physically interacting with them. This led to the formulation of the “guard hypothesis” [2] [1], which states that these R proteins monitor the status of key cellular targets of effectors. If effectors temper with the monitored proteins, the R proteins can sense this so called “modified self” state and initiate a response. One example for indirect recognition of effectors is the interaction between *Arabidopsis thaliana* and *P. syringae*. RIN4, a plasma membrane associated protein from Arabidopsis is a target of the *Pseudomonas* effectors AvrRpt2, AvrB and AvrRpm1 [2]. However, modification of RIN4 is sensed by and activates RPT2 and RPS2, two different R genes in *A. thaliana*, which trigger a response resulting in plant resistance to the pathogen [58]. Assuming that many different effectors act on

only a limited number of host proteins (mostly those involved in immunity), the advantage of this indirect mechanism lies in the fact that plants only had to evolve comparatively few R genes monitoring the key host targets that many diverse effectors act upon.

Pto

The tomato gene Pto (resistance to *Pseudomonas syringae* pathovar (pv.) tomato, *Pst*) represents one of the best-described examples of R-gene mediated ETI. The Pto gene encodes a cytoplasmic Ser / Thr kinase that confers resistance against strains of *Pst* that express the effector genes AvrPto and / or AvrPtoB. The Pto gene was discovered originally in *Lycopersicon pimpinellifolium*, a wild tomato species, and isolated by map-based cloning [59]. Pto interacts with Prf, a nucleotide binding and leucine rich repeat protein. Upon interaction with either AvrPto or AvrPtoB, Pto triggers ETI and a resultant HR, which can be observed as rapid tissue collapse at the site of infection [60] [56]. This response is dependent on the ability of Pto to auto-phosphorylate and also on functional Prf. Mutations in the auto-phosphorylation sites Thr38 and Ser198 of Pto or nonfunctional Prf abolish Pto mediated HR. Thr38 is located outside of the kinase domain and involved in the direct physical interaction of Pto with AvrPto, whereas Ser198 is inside the kinase activation domain. While it seems to be dispensable for the interaction of Pto with its cognate effector or kinase activity, it appears to be involved in its interaction with downstream signaling partners, particularly Pti3 and Pti10 [61]. Prf constitutes a crucial signaling component that directly interacts with Pto *in vivo* [62]; mutations in Prf are epistatic to Pto, completely suppressing Pto mediated ETI [63]. Interestingly, Pto-related genes have been found in many diverse plant species, from dicotyledons and monocotyledons, raising the possibility that Pto-like signaling pathways might be a widely conserved mechanism within the plant kingdom [59].

Pseudomonas syringae / tomato interaction as model system

Pseudomonas syringae is a rod-shaped, gram-negative bacterium that is known as a common plant pathogen. It is able to infect a wide range of different plant species, and more than 50 pathovars have been described. To successfully infect its hosts, it depends on a diverse array of effectors, which are delivered by means of a T3SS; only a handful of those have been characterized to date; some of the better-described act by mimicking or inhibiting the function of eukaryotic proteins to promote bacterial proliferation inside of plant tissues [64] [65].

The interaction between *Pseudomonas syringae pv. tomato* and tomato plants has long been an active area of research. The presence of virulent and avirulent bacterial strains on one side and resistant and susceptible tomato species on the other side made it a perfect model for the investigation of plant pathogen interactions and the basis of gene-for-gene resistance. The role of Pst strain DC3000 as the prevalent model for bacterial plant pathogens was further enhanced when it was discovered to also be able to infect the model plant *Arabidopsis thaliana*, allowing the use of the extensive array of genomic and technical resources that had been established for this plant. The complete genome sequence of Pst DC3000 was published in 2003 [66]; it contains a set of approximately 40 effectors that play a role in pathogenicity [67]. As a natural non-host, *Arabidopsis thaliana* accession Col-0 recognizes none of these effectors, making it a perfect model to investigate their effect on conserved constituents of PTI [68]. Tomato, on the other hand, has been co-evolving with the pathogen for a long time, producing resistant tomato species that allow the investigation of ETI and provide insights into the evolutionary arms' race that unfolded in between the pathogen and its host [45]. In summary, Pst DC3000 in combination with the host tomato and the non-host Arabidopsis provide us with a powerful model system that is the basis of many important discoveries in the field of plant immunity.

Material and Methods

Microbial techniques

Bacterial strains and vectors used for cloning

The bacterial strains of *E. coli* and *A. tumefaciens*, and the cloning vectors used in this work are listed in the following tables (**Table 1** and Table 2)

Strain	Genotype
E. coli	
DH5α	F^{-}, ø80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rK^{-}, mK^{+}), phoA, supE44, λ–, thi-1, gyrA96, relA1
One Shot® Mach1™ T1 Phage-Resistant Chemically Competent E. coli	DrecA1398 endA1 tonA P80DlacM15DlacX74 hsdR(rK-mK+) from Invitrogen
DB3.1	F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1
One Shot® TOP10 Chemically Competent E. coli	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 $λ^-$) from Invitrogen
A. tumefaciens	
ASE	

Table 1: Bacterial strains used

Plasmid	Resistance
pGEM-Teasy	Ampicillin
PCR2.1	Ampicillin & Kanamycin
pFK209	Chloramphenicol & Spectinomycin
pJLSmart	Kanamycin

Table 2: Cloning vectors used

Culture Media

Culture media and their composition are listed in Table 3

Bacteria	Growth Media	Composition
E. coli and *A. tumefaciens*		
Routine culture	Luria Bertani (LB)	Tryptone 10g; yeast extract 5 g; NaCl 5 g; water 1L
Liquid culture after transformation	SOC (Super Optimal broth with Catabolite repression)	2% tryptone; 0.5% yeast extract; 10mM NaCl; 2.5 mM KCl; 10mM $MgCl_2$; 10mM $MgSO_4$; 20mM glucose

Table 3: Culture Media used in the lab

All media were prepared by the Boyce Thompson central services facility.

Antibiotics

Antibiotics were added to culture media from concentrated stock solutions prior to inoculation. Stock solutions were diluted in distilled water / methanol / ethanol and sterilized through pore filters. Final concentrations of the different antibiotics are indicated in the Table 4.

Antibiotics	***E. coli***	***A. tumefaciens***
Concentration (µg/ml)		
Ampicilin (Amp)	100	-
Chloramphenicol * (Cm)	25	-
Gentamycin (Gen)	25	25
Kanamycin (Kan)	50	50
Rifampicin * (Rif)	-	100
Spectinomycin (Spec)	100	100
Tetracyclin (Tet)	10	10

Table 4: Antibiotics table with the different concentrations for *E. coli* and *A. tumefaciens*

*The concentrated stock solution of chloramphenicol was prepared in ethanol and the rifampicin stock in Methanol.

Enzymes

Enzyme	**Provider**
AatII	New England Biolabs (NEB)
BsrGI	NEB
DpnI	NEB
EcoRI	NEB
LR Clonase II	Invitrogen
NdeI	NEB
Phusion Polymerase	Finnzymes
RNase H	Invitrogen
RNaseOUT	Invitrogen
RQ1 DNase	Invitrogen
SalI	NEB
SuperScript III RT	Invitrogen
SYBR Green	Applied Biosciences
T4 DNA Ligase	NEB
Taq Polymerase	NEB
XbaI	NEB
XhoI	NEB

Table 5: Enzyme list

Long term storage of bacterial strains

Bacterial strains were stored in tubes containing 1.5 ml bacterial culture with 20% final concentration of glycerol and kept at -80°C.

Growth conditions

Liquid and solid aerobic cultures of *E. coli* and *A. tumefaciens* were grown at 37°C and 30°C, respectively.

Molecular Biology Methods

DNA Purification from bacteria

DNA war purified using Wizard Plus DNA Midiprep DNA Purification System or Wizard Plus SV Miniprep DNA Purification System according to manufacturer's guidelines. The DNA concentration was determined using the NanoDrop photometer according to the manufacturer's manual.

Bacterial transformation

Transformation of Agrobacterium by electroporation

Electro-competent cells were thawed on ice. Cells were transferred to a 2 mm electroporation cuvette and electroporated using the pre-set Agrobacterium program and the BioRAD electroporation apparatus. 1 ml SOC medium was

added and the cells incubated shaking at 30°C for 1-2 hrs for regeneration. Cells were then plated on LB plate with antibiotics corresponding to plasmid selection markers and grown for 2-3 days at 30°C.

Transformation of chemically competent *E. coli*

Chemical competent cells were thawed on ice. 5 µl plasmid DNA were added, mixed gently and incubated on ice for 30 min. Cells were heat shocked at 42°C for 1.5 min and cooled down for 2 min on ice. 1 ml SOC medium was added and cells were incubated shaking at 37°C for 1 hr. Cells were then pelleted by centrifugation for 1 min at 14000 rpm at RT. Supernatant was decanted and cells were resuspended in the remaining supernatant. Cells were plated on LB plates with antibiotics corresponding to plasmid selection markers.

Restriction enzyme digests

Digestion of DNA with restriction enzymes was set up at the optimal conditions for each enzyme regarding temperature and buffer, as indicated by the suppliers.

Ligation

Inserts digested with restriction enzymes were integrated into the appropriate vectors. The reaction resulted in a closed circular plasmid with the respective insert and was catalyzed by the T4 DNA ligase. The ligations were incubated over night at 16°C or 1 hr at RT.

Reaction setup according to manufacturer's guidelines:

1 µl 10x T4 Ligase Buffer

x µl Vector (150 ng / µl)

x µl Insert (50 - 100 ng / µl)

1 µl T4 Ligase

10µl

A-tailing

For adding a 3' terminal A overhang onto blunt PCR products. The ATP overhang was used for PCR based cloning into pGEM-Teasy vector. PCR product was resolved on an agarose gel to extract the right band and remove the proofreading enzyme from the reaction. After DNA purification from the gel, a PCR reaction using non proofreading GoTaq was performed.

Reaction setup according to manufacturer's guidelines:

7 µl DNA

1 µl dATPs (2 mM)

1 µl Buffer (no Green)

1 µl GoTaq (non proofreading)

30 min at 72°C (PCR)

Site directed mutagenesis

Template DNA was purified from *E. coli*, to ensure that all GATC sites are methylated. This is important for later digestion with DpnI. Non-phosphorylated forward and reverse primers containing the wanted modification were designed and used in a PCR reaction with Phusion® High-Fidelity DNA Polymerase. Following the PCR, 1 µl DpnI was added and incubated at 37°C for 1 hr. DNA was then transformed into competent DH5α cells. Selected clones were verified by control digestion and sequencing.

Reaction setup

10 µl Phusion HF buffer (5x)

5 µl dNTPs (2 mM)

1 µl P_{for} (10 µM)

1 µl P_{rev} (10 µM)

1 µl Plasmid DNA (50 ng)

0.5 µl Phusion

31.5 µl H_2O

50 µl

95°C	2 min	1x
95°C	0.30 s	16x
55°C	1 min	16x
72°C	15 s/kb	16x
72°C	10 min	1x
10°C	Forever	

Polymerase chain reaction (PCR)

PCR was performed to amplify DNA and to check clones. Annealing temperatures and cycle duration were modified according to the primers and Polymerase that was used in the reaction.

Reaction setup according to manufacturer's guidelines:

Phusion PCR

10 µl Phusion HF buffer (5x)

5 µl dNTPs (2 mM)

1 µl P_{for} (10 µM)

1 µl P_{rev} (10 µM)

50 ng Plasmid DNA

0.5 µl Phusion

xx µl H_2O

50 µl

98°C	30 s	1x
98°C	10 s	35x
55°C	30 s	35x
72°C	15 s/kb	35x
72°C	5 min	1x
10°C	Forever	

GoTaq PCR

4 µl Green GoTaq buffer (5x)

2 µl dNTPs (2 mM)

1 µl P_{for} (10 µM)

1 µl P_{rev} (10 µM)

0.2 µl GoTaq

2 µg Plasmid DNA

xx µl H_2O

20 µl

95°C	5 min	1x
95°C	30 s	35x
55°C	30 s	35x
72°C	1 min/kb	35x
72°C	5 min	1x
10°C	Forever	

Colony PCR

Colony PCR was used after a transformation to screen for positive colonies (from a plate or a liquid cultures). Primers were used to generate a PCR product of known size, confirming the presence of the desired colonies. A sterile tip was used to pick single colony from a plate, partly streaked on a fresh plate and the remaining bacteria resuspended in the PCR tube containing the master mix by pipetting up and down with a micropipetter to ensure that cells were transferred well.

When using a liquid colony, 100 µl overnight culture were boiled for 5 min and debris pelleted by centrifugation for 1 min at 15.000 g. 1-2 µl of the supernatant was used for the reaction

4 µl Green GoTaq buffer (5x)

2 µl dNTPs (2 mM)

0.5 µl P_{for} (10 µM)

0.5 µl P_{rev} (10 µM)

0.2 µl GoTaq

12.8 µl H_2O

20 µl

95°C	5 min	1x
95°C	30 s	35x
55°C	30 s	35x
72°C	1 min/kb	35x
72°C	5 min	1x
10°C	Forever	

LR-Reaction for Gateway cloning

LR-reaction was performed following the Invitrogen protocol. 1 µl 5x LR Clonase II enzyme was added to 100-200 ng Entry clone (containing the fragment that is going to be introduced into the vector) and 100-200 ng of Destination vector. An amount of water was added to reach a final volume of 4 µl. The tube was then incubated for one to two hours at 25°C. Finally, 1 µl of

Proteinase K was added and incubated for 10 min at 37°C before transformation into *E. coli* cells.

Electrophoresis of DNA

All DNA samples were analyzed on 1% to 2% agarose gels. Ethidium bromide was added for visualization of the band under the UV light. The samples were loaded into the gel after addition of 1/5 volume of loading buffer. Electrophoresis was performed in 1% TBE buffer at 90-120 V.

10x TBE-Buffer	108 g Tris base [tris(hydroxymethyl)aminomethane]; 55 g Boric acid; 7.5 g EDTA; 1 L distillated water
Loading Buffer	50% (v/v) glycerol, 50% (v/v) water, 0.00025% (w/v) each Bromphenolblue and Xylenxyanol

Table 6: Buffers used for DNA electrophoresis

Purification of DNA fragments from agarose gel

DNA fragments of interest were excised and DNA was extracted using spin columns from Promega. The gel slice was dissolved in 1 µl DNA extraction buffer per mg gel for 10 min at 55°C. After dissolution, the solution was applied to a spin column and spun down for 90 seconds full speed. After two washes with washing buffer followed by 90 seconds spin down, the DNA was eluted in 25 µl of water into a fresh 1.5 ml Eppendorf tube.

Sequencing

The Sequencing Reaction was set up according DNA Sequencing Facility at Cornell University:

1 µg plasmid DNA

8 pmol Sequencing Primer

xx µl ddH2O

18 µl

For PCR products, the following formula was used to determine the correct amount of DNA:

#base pairs / 5.0 = amount of PCR product in ng needed.

The sequences were analyzed using the Sequencher Software version 4.10.1

Genomic DNA isolation from plant tissue

1-2 young leaves were collected in a 2.2 round bottom tube containing exactly 2 copper beads and placed in liquid nitrogen. Then they were ground to a fine powder using a TissueLyser II from Quiagen. The right amount of pre-warmed 3x CTAB buffer was added (220 µl for *A. thaliana* and 550 µl for tomato) and incubated at 65°C for 1 hr. After addition of an equal amount of Chloroform-isoamylalcohol in a ratio 24:1, the tubes were vortex for 15 s and centrifuged at full speed for 10 min to separate the suspension into to phases. The upper aqueous phase containing the DNA was transferred to a fresh tube, mixed with an equal amount of isopropanol and precipitated for 1-2 hrs at 4°C. DNA was pelleted by centrifugation at full speed for 15 min and the pellet washed with 1ml 70% ethanol followed by centrifugation for 1 min. After air-drying, the

pellet was resuspended in 25 -100 µl TE, depending on the pellet size. The tubes were kept over night at 4°C for a complete dissolution of the DNA. The next day, 1 µl of 10 mg / ml RNaseA (Sigma) was added and incubated at 37°C for 1 hr.

3X CTAB	3% CTAB (Cetyltrimethylammoniumbromid), 100 mM Tris pH 8.0, 20 mM EDTA, 1.4 M NaCl, in water. Immediately before use, 3.89 g/l Sodium Bisulfite, 1% (w/v) Polyvinylpyrolidon and 1% (v/v) β-Mercaptoethanol were added.

Table 7: CTAB medium for DNA extraction

DNA precipitation

0.1 volume of 3 M Sodium acetate pH6 and 0.5 - 1 volume isopropanol were added to the DNA solution and incubated for 2 hrs at room temperature or longer at 4°C. Next, the tubes were centrifuged at full speed for 20 min followed by two washes with 70% ethanol, intercalated by a 1 min centrifugation full speed. After air-drying, the pellet was resuspended in TE. The tubes were left over night at 4°C for a complete dissolution of the DNA. DNA amount was measured with NanoDrop and then stored at -20°C

RNA isolation

Approx. 1 g of young leaves was collected in a 2.2 round bottom tube containing exactly 2 copper beads and placed in liquid nitrogen. Then they were ground to a fine powder using a TissueLyser II from Quiagen. 0.5 ml cold Plant RNA Reagent from (Invitrogen) was added mixed by vortexing until the sample was thoroughly resuspended, and incubated for 5 min at room temperature by keeping the tubes horizontally. After centrifugation for 2.5 min

at full speed, the supernatant was transferred to new RNase free tubes. 100 µl of 5 M NaCl and 300 µl Chloroform-isoamylacohol in a ratio 24:1 were added and mixed well after by vortexing. Phases were separated by subjecting tubes to centrifugation at full speed for 10 min at 4°C. The upper aqueous phase was transferred into a new RNase free tube and mixed with an equal volume of isopropanol. Tubes were incubated at room temperature for 10 min. RNA was precipitated by centrifugation at full speed at 4°C for 10 min. The supernatant was discarded and the pellet was washed with 1 ml 70% ethanol and centrifuged at full speed for 1 min. After air-drying, the pellet was resuspended in 30 µl DEPC treated water. RNA concentration was measured with NanoDrop.

The RNA was treated with RQ1 DNase from Promega for 1 hr at 37°C followed by a measurement with NanoDrop.

Reaction setup according to manufacturer's guidelines:

15 µl	RNA
10 µl	10x DNase buffer
10 µl	DNase (Promega; 1u/ mg RNA. Each unit digests 1 µg DNA in 1 hr)
65 µl	DEPC-treated H_20
100 µl	

This was followed by RNA clean up using the RNeasy Mini Kit from Qiagen and by a measurement with NanoDrop.

cDNA synthesis

2 µg RNA were used to prepare cDNA using Superscript III and oligo dT primer from Invitrogen. Reactions were prepared according to the manufacturer's manual. cDNA was then stored at -80°C.

Quantitative real time PCR

qRT-PCR was performed using 150 nM sequence specific primers and SYBR Green from Applied Biosystems. The PCR was run on CFX Manager (BioRAD). Primers were designed in such a way that each amplicon spans an intron in the genomic sequence, allowing the detection of contamination with genomic DNA by the presence of a larger band. Each reaction was performed in triplicates. Dissociation curve analysis was performed at the end of each run to ensure that unique products were amplified. Data was normalized to the EF1α (Elongation Factor 1 alpha) control gene. The efficiency of all primer pairs used was calculated and taken into consideration.

Quantitative Real Time PCR reaction was set up on ice:

10 µl 2x SYBR Green

0.5 µl Primer I

0.5 µl Primer II

2 µl DEPC water

7 µl cDNA

Standard program setup

10 min	95°C	1x
30 s	95°C	39x
30 s	62°C	39 x
40 s	72°C	39x
7 min	72°C	1x

SDS PAGE and Western Blot

SDS Polyacrylamide Gel Electrophoresis (PAGE)

Stacking Gel for 1 gel	
Water	1.2 ml
0.5 M Tris pH 6.8	0.5 ml
Acrylamide 30%	250 µl
SDS 10%	20 µl
APS 10%	20 µl
TEMED	2 µl
Resolution Gel for 1 10% gel	
Water	2.1 ml
1.5 M Tris pH 8.8	1.25 ml
Acrylamide 30%	1.6 ml
SDS 10%	49.2 µl
APS 10%	24.6 µl
TEMED	2.46 µl

Table 8: SDS Gels consist of two parts: Stacking and Resolution gels. After resolution by PAGE, proteins were transferred to a membrane by Western Blotting for further analysis.

All gels were run in a BioRad SDS-PAGE / W-blotting setup.

The transfer from the gel to the PVDF-membrane was performed at 100 V for 1 h with Tris /Glycine buffer containing 20% methanol. After the transfer, membranes were blocked in blocking buffer for 60 min at RT. Thereafter, the membrane was incubated with primary antibodies over night at 4°C or for 1 h at RT. The membranes were washed 3x 15 min with 1x PBS 0.05% Tween20

and incubated with a secondary antibody coupled with HRP. Finally, membranes were washed with 1x PBS 0.05% Tween20 for 3x 15 min, rinsed with 1x PBS and ECL solution (GE healthcare) applied according to the manufacturers recommendations. The protein bands were visualized on films (GE healthcare).

SDS-PAGE Running buffer (1L)	250 mM Tris base pH 8.8; 1.9 M Glycine; 1% SDS
Western Blot Transfer buffer	1x Running buffer; 20% MeOH
1x TAE DNA Running buffer	40 mM Tris-Acetate; 1 mM EDTA pH 8.5
5x Protein Sample buffer	125 mM Tris HCl pH 6.8; 900 mM Glycerol ; 1 mM EDTA; 6% SDS; 6 M Urea; 10% β-Mercaptoethanol; 0.01% Bromphenol Blue
Blocking buffer	5% milk powder in 1x PBS

Table 9: Buffers used for Western Blotting

Reactive oxygen Species (ROS) assay

Leaf discs were punched out of fully extended Arabidopsis leaves from 3 week old LD grown plants and incubated in a 96 well plate in 100 µl sterile deionized H_2O for 16 hrs at room temperature to allow the wounding response to subside. The water was discarded and replaced by 100 µl fresh water containing 34 mg/l Luminol, 10 mg/l horseradish peroxidase and the PAMP (flg22: 100 nM, chitin: 100 µg/ml). Immediately after the addition of the elicitor mix, the 96 well plate was inserted into the Synergy2 plate reader (BioTek) to monitor the ROS induced luminescence. The generated data was analyzed using the BioTek Gen5 analysis Software.

Plant related Methods

Ethanol seed sterilization

Seeds were placed in Eppendorf tubes and immersed in 75% ethanol containing 0.5% Tween20 for 2 min at room temperature under constant movement on a tilting shaker. After allowing the seeds to sink to the bottom of the tube, the ethanol was removed with a pipette, followed by two washing steps with 100% ethanol to remove residual Tween20. Seeds were then pipetted on sterile filter papers and air dried before being spread on MS plates. The plates were sealed with Micropore surgical tape (3M), covered with tin foil and stored at 4°C for 3-5 days for vernalization and synchronization of germination. After this period, the foil wrap was removed and the plates were transferred to a growth cabinet with the relevant growth conditions.

Chlorine gas seed sterilization

Seeds in open Eppendorf tubes were placed into a desiccator in a fume hood. A beaker containing 100 ml of chlorine bleach was placed in the desiccator next to the seeds. Before quickly sealing the desiccator, 3 ml of concentrated HCl were added to the bleach to create chlorine gas. The desiccator was maintained closed for at least 12 hrs. Following release of the gas, the tubes were closed and moved into a laminar flow hood where the seeds were transferred to MS plates. The plates were then sealed with Micropore surgical tape (3M), covered with tin foil and stored at 4°C for 3-5 days to imbibe seeds. After this period the foil wrap was removed and the plates were transferred to a growth cabinet with the relevant growth conditions.

MS plates	1 g / l Murashige and Skoog salts, 10 g / l agarose or agar. Sterilize by autoclavation.

Table 10: Media used for plant propagation.

Arabidopsis transformation with *Agrobacterium tumefaciens*

A single colony was inoculated into 5 ml of LB $_{Kan,\ Chlor,\ Tet,\ Spec}$ and grown for approx. 2 days at 30°C shaking. 1 ml of the starter culture was used for the inoculation of 200 ml LB $_{Kan,\ Chlor,\ Tet,\ Spec}$ and cells were incubated shaking at 30°C for 24 hrs. The following day, the cultures were centrifuged for 15 min at 4000 rcf and the pellet was resuspended in Infiltration medium. Before dipping Arabidopsis into the bacterial suspension, 200 µl Silwet were added to the solution. The plants were dipped for at lest 1 min and then kept on the side on a tray and covered up over night outside the growth chambers over night. The next day, the trays were shifted into a growth chamber with the proper growing conditions.

Infiltration medium	1g MS Salts, 50 g sucrose, add water to 1 l

Table 11: Infiltration medium used for plant transformation

Transient protein expression in *Nicotiana benthamiana* using *A. tumefaciens*

For a more detailed description of the infiltration procedure, see [69]. Briefly, a single colony was inoculated in 5 ml of LB $_{Kan,\ Chlor,\ Tet,\ Spec}$ and grown for approx. 2 days at 30°C. Then, the cells were harvested by centrifugation at full speed for 7 min and the pellet was resuspended in the same volume of liquid Induction Medium as the original culture. Centrifugation and resuspension steps were repeated two more times. A 1:25 dilution was added

to 4 ml Induction Medium containing antibiotics and 200 µl acetosyringone and incubated shaking at 30°C. The next day, the cells were centrifuged at full speed for 7 min and the pellet resuspended in Infiltration buffer. Centrifugation and resuspension steps were repeated two more times. OD_{600} was determined using a 1/10 dilution. An appropriate amount was aliquoted to generate 10 ml of OD_{600} = 0.3, spun down at 3000 x g for 5 min and resuspended in Infiltration buffer containing acetosyringone at a final concentration of 200 µM. Finally, the abaxial side of the leaf was infiltrated with a blunt 1 ml syringe and the plants were put in a grow chamber for 1-2 days before harvesting the tissue.

Histochemical Staining with X-Gluc

Plant leaves were sampled and immediately placed in a 15 ml Falcon tube containing the GUS staining solution (5 ml solution). Thereafter, the tubes containing the samples were vacuum infiltrated for 15 min and incubated for 24 hrs at 37°C. Finally, the staining solution was replaced by a destain solution. The tissues were left in the destain solution for at least 24 hrs or until the tissues had cleared.

Staining solution (was made fresh prior to use)	
X-Gluc	1 mM
Sodium Phosphate Buffer pH 7	100 mM
Triton X-100	0.1 % (v/v)
EDTA	1 mM
Water	
Destain solution	
Ethanol: Acetic acid	3:1

Table 12: Solution used for Histochemical staining of GUS.

Results

Bti9, the closest known tomato homologue to the gene CERK1

CERK1 was discovered to be the chitin recognition receptor in *Arabidopsis thaliana*. CERK1 is a lysin motif (LysM) receptor like kinase containing three extracellular LysM motifs, a transmembrane domain, a juxtamembrane domain and an intracellular serine/ threonine kinase domain. Recent studies have shown that CERK1 is able to bind chitin directly and specifically *in vitro*.

The closest known homologue to CERK1 in tomato is Bti9. It was identified in a yeast two hybrid screen using the type III effector protein AvrPtoB from *Pseudomonas syringae pv. tomato* as a bait. Like CERK1, the gene encodes a protein with an extracellular region consisting of three LysMs domains, a putative transmembrane domain and an intracellular serine / threonine protein kinase domain (L. Zeng, unpublished). Interestingly, CERK1 has also has been reported to interact with AvrPtoB [37].

In contrast to CERK1, two different splice forms, which differ in their first exon, are generated from the Bti9 locus. The one initially described resides directly upstream of the invariable second exon and will be referred to as exon 1b; in contrast, the alternative first exon is approx. 2.5 kb upstream of the described transcriptional initiation site; we will refer to this exon and the resulting splice form as exon 1a (A. Velasquez, unpublished). The nucleotide sequences of the two exons differ considerably (“no significant similarity” according to a standard BLAST2SEQ alignment – this is surprising given the amino acid similarity is pretty high), while amino acid sequences are fairly conserved (68% identities, 81% similarities). Neither one of the splice forms is more closely related to AtCERK1 than the other, but Bti9 exon 1a contains approximately 20 amino acids at its C-terminus that are conserved in

AtCERK1, but absent from splice form 1b. ESTs have been found for each splice form in public databases, indicating that both are expressed.

CERK1 and both splice forms of Bti9 encode ORFs of about 1.8 kb in length, and all variants found so far consist of 12 exons. Based on the sequence similarity between CERK1 and Bti9, we hypothesized that Bti9 might function as a pattern recognition receptor (PRR) in tomato. This would be consistent with an earlier finding of L. Zeng that a Bti9-GFP fusion protein localizes to the plasma membrane when expressed in protoplasts (L. Zeng, unpublished). Given the high similarity to CERK1, it is possible that Bti9 might recognize chitin from fungi or related molecules from microbes, possibly peptidoglycans, as its corresponding PAMP. In this context, it is interesting to note that the first exon of CERK1, as well as the two alternative exons of Bti9, encode the LysM domains, which in the case of CERK1 have been demonstrated to be involved and necessary for chitin binding [35]. This might result in different specificities for or affinities to different PAMPs between the two splice forms.

As we were able to retrieve ESTs (expressed sequence tags) for both splice forms of Bti9 from databases, it is reasonable to assume that both are expressed at least to a certain degree. However, nothing more was known about expression levels or pattern of either splice form. To determine expression and behavior of the different transcripts *in planta*, quantitative Real-time polymerase chain reaction (qRT-PCR) was performed to quantify the transcript abundance of the two forms across developmental stages, tissue types or in response to PAMPs or bacteria. While qRT-PCR will provide quantitative data regarding transcriptional levels and changes in those in response to stimuli or across developmental stages, its resolution is limited to tissue level.

Consequently, this approach was complemented by the generation of transgenic tomato lines containing Bti9 promoter-GUS constructs that will enable us to visualize expression and localization of both forms *in vivo*. Visualization of the expression pattern *in vivo* with reporter GUS lines allows us to determine expression at single-cell resolution.

Expression profiling of the two splice forms

While a standard BLAST does not find a significant similarity across the full length two spliceforms, a pairwise alignment shows that there are sequence-identical stretches of more than 20 consecutive nucleotides distributed all along the two first exons (see Figure 3B). To ensure that a qPCR product only originates from one specific spliceform, but not from the other, different primers had to be tested thoroughly for specificity and efficiency. The alternative first exon sequences were compared by BLAST, and the regions with the greatest differences between them where chosen for oligo design. We aimed to engineer the primer pairs in such a way that, besides maximal divergence in the chosen sequence between exon 1a and exon 1b, the 3' end of each primer would not be present in the divergent first exon. Also, each corresponding primer pair was designed spanning at least one intron. Because of the resulting size difference between cDNA and genomic DNA, any genomic contamination can be detected by the presence of two differently sized amplicons. As this required the reverse oligos to be located in exons that are not divergent in between the different splice forms, the specificity of the resulting oligo combinations has to stem from the forward primer only. The size of the generated amplicons was designed to be approximately 400 bp, to be comparable to the size of an EF1a amplicon using a primer pair tested and used in lab (J. Munkvold, personal communication)

Figure 3: Primer design for the two Bti9 splice forms A. Overview of the position of specific forward and the general reverse oligos in Bti9. Each corresponding oligo pair was designed spanning at least one intron. White: introns; Blue: exons. **B.** BLAST sequence alignment of Bti9 exon 1a against Bti9 exon 1b. Specific oligos are indicated by different colors: oTK 82; oTK 81; oTK 97.

To conserve resources, initially the specificity of the designed oligo combinations was verified by standard PCR using plasmids pTK20 and pTK30 as template. 11 pairs for Bti9 exon 1a and 13 pairs for exon 1b were tested. Plasmid pTK20 contains the Bti9 splice form with exon 1b and pTK30 contains Bti9 with splice form exon 1a.

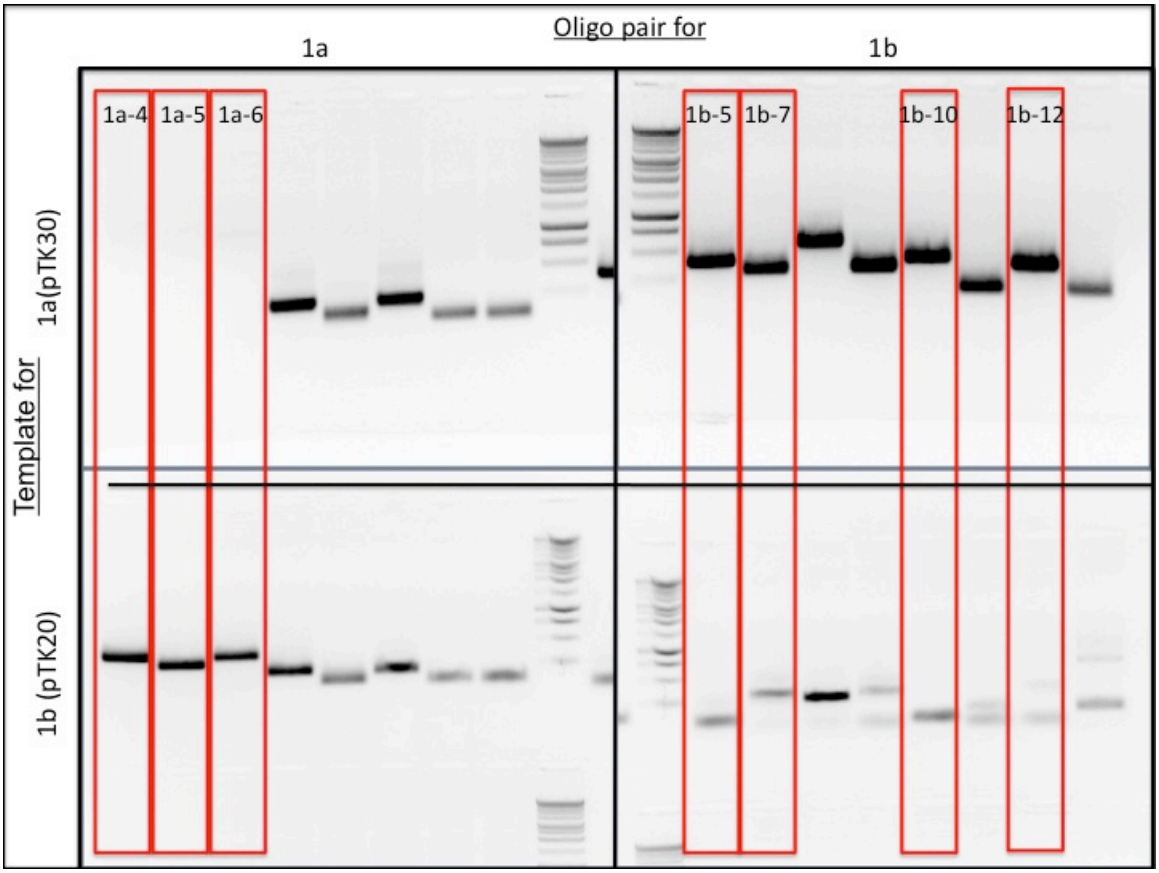

Figure 4: Primer pair specificity. Agarose gel electrophoresis of PCR products for oligo pairs for Bti9 exon 1a and Bti9 exon 1b. Splice form specific combinations are marked by red boxes.

1a specific oligo	Forward	Reverse	Size in bp	Specificity
1a-1	oTK 54	oTK 71	391	Specific
1a-2	oTK 53	oTK 71	492	Unspecific
1a-3	oTK 78	oTK 71	610	Specific
1a-4	oTK 82	oTK 71	400	Specific
1a-5	oTK 82	oTK 96	320	Specific
1a-6	oTK 82	oTK 98	392	Specific
1a-7	oTK 99	oTK 71	238	Unspecific
1a-8	oTK 99	oTK 96	158	Unspecific
1a-9	oTK 99	oTK 98	230	Unspecific
1a-10	oTK 100	oTK 71	147	Unspecific
1a-11	oTK 100	oTK 98	139	Unspecific

Table 13: Specific oligos for Bti9 exon 1a and corresponding general reverse oligos. Also size and specificity are given.

1b specific oligo	Forward	Reverse	Size in bp	Specificity
1b-1	oTK 51	oTK 71	411	Unspecific
1b-2	oTK 77	oTK 71	446	Unspecific
1b-3	oTK 79	oTK 71	604	Unspecific
1b-4	oTK 80	oTK 71	388	Unspecific
1b-5	oTK 81	oTK 71	400	Specific
1b-6	oTK 83	oTK 71	400	Unspecific
1b-7	oTK 81	oTK 93	316	Specific
1b-8	oTK 81	oTK 95	582	Unspecific
1b-9	oTK 81	oTK 96	320	Specific
1b-10	oTK 81	oTK 98	392	Specific
1b-11	oTK 97	oTK 71	162	Specific
1b-12	oTK 97	oTK 95	344	Specific
1b-13	oTK 97	oTK 98	154	Specific

Table 14: Specific oligos for Bti9 exon 1b and their respective general reverse oligos. Also size and specificity are given.

Of the tested oligos, pairs 1a-4, 1a-5 and 1a-6 were specific for exon 1a (i.e. no amplification using a exon1b-cDNA as template) and pairs 1b-7, 1b-10 and 1b-12 were specific for exon 1b (no PCR product when using exon1-cDNA).

Consequently, the amplification efficiency of these oligo combinations was tested under qRT-PCR conditions. Initially, cDNA generated from Rio Grande tomato RNA was used as template. To control for template quality, we also included an alternative oligo pair for EF1a, which had been determined to have an amplification efficiency of 100% (A. Velasquez, personal communication). As the very short amplicon resulting from these primers does not spam an intron, this combination cannot be used to perform the actual qRT-PCRs later on, as contamination with genomic DNA would be indistinguishable from cDNA.

Quantitative real-time PCR was performed in triplicates on a cDNA dilution series from 10^0 to 10^{-4}; template concentration was determined using the Qubit fluorometer (Invitrogen). Measurements, quantification and efficiency computation were performed using the CFX Manager software (BioRAD). While this worked reliably for the relatively high expressed Bti9-exon1b splice form, expression of the splice form incorporating exon 1a in this tissue was

too low to determine amplification efficiency. Therefore, we decided to use purified plasmid DNA from pTK30 as template. We also included the exon1b specific oligo pair 1b-12 and pTK20 in these experiments to confirm that the determined amplification efficiency would not change using plasmid DNA compared to using cDNA. As expected, the determined efficiencies for exon 1b were comparable between cDNA (69.4%) and plasmid (67.2%) templates. The determined efficiencies are represented in Table 15 and Table 16. All experiments have been repeated at least 3 times, and the given numbers represent the average of all determined efficiencies across all experiments using the respective primer combination. All qPCR products were resolved on agarose gels to confirm correct amplicon size and the absence of a higher species resulting from genomic contamination (even though this should also have been apparent in the melting curves). Additionally, the qRT-PCR products of the primer pairs used in the remainder of this work were isolated following gel electrophoresis and sent for sequencing once to confirm amplification of the correct sequence.

1a specific oligo	Forward	Reverse	Size in bp	Specificity	Efficiency
1a-4	oTK 82	oTK 71	400	Specific	43.9
1a-5	oTK 82	oTK 96	320	Specific	46.2
1a-6	oTK 82	oTK 98	392	Specific	33.8

Table 15: Specific pairs of primers for Bti9 exon 1a that were tested for amplification efficiency. The stated efficiency is the average of all determined efficiencies across all experiments using the respective primer combination.

1b specific oligo	Forward	Reverse	Size in bp	Specificity	Efficiency
1b-5	oTK 81	oTK 71	400	Specific	59.5
1b-7	oTK 81	oTK 93	316	Specific	69.5
1b-10	oTK 81	oTK 98	392	Specific	63.5
1b-12	oTK 97	oTK 95	344	Specific	65.0

Table 16: Specific pairs of primers for Bti9 exon 1b that were tested for their amplification efficiency. The stated efficiency is the average between all the experiment using cDNA and the ones using plasmid DNA as a template.

For the determination of exon 1a expression levels, we decided to use primer pair 1a-5, as it has the highest amplification efficiency of the three specific combinations. For exon 1b, pair 1b-12 was chosen over 1b-7 even though it has slightly lower amplification efficiency. The reason for this is that the melting curves performed on the qRT-PCR products showed a tendency of oTK81 to form dimers, which was not the case for oTK97.

Unfortunately, the efficiency of these oligonucleotides is far from perfect (100%), but as the efficiencies for both are similar, we should still be able to get comparable results. Also, the formula used for the determination of product levels takes the individual amplification efficiencies into account and compensates for them [70].

A. Plant age

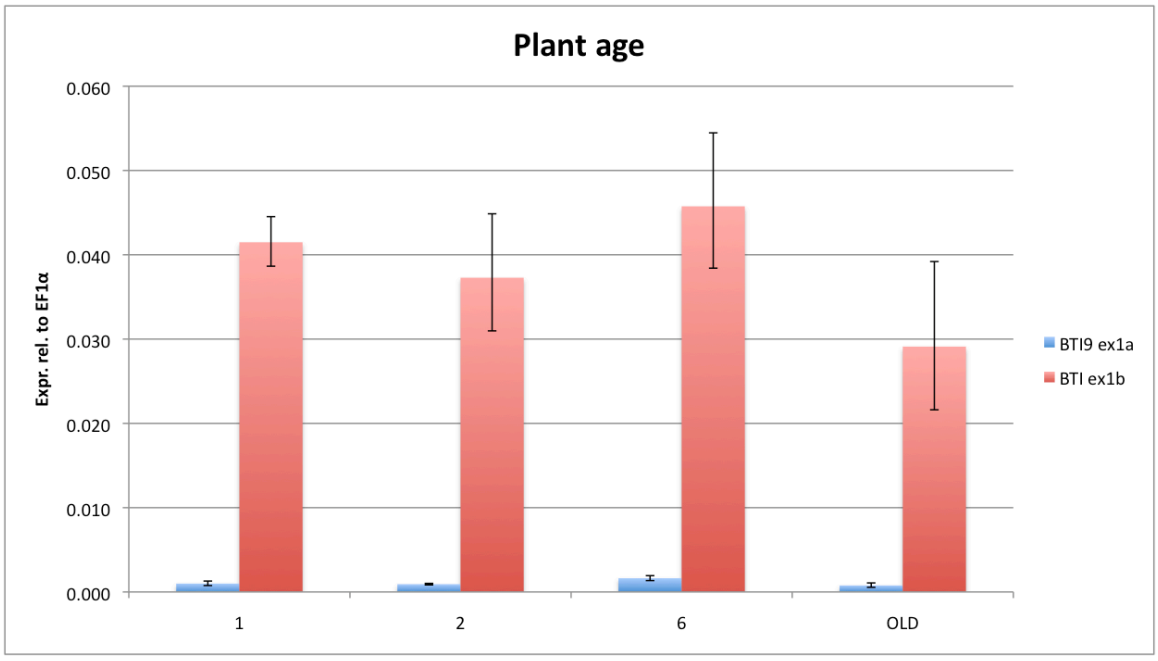

Figure 5: Bti9 exon 1a and 1b abundance between plants of different ages. Splice form 1a is shown in blue and 1b in red

To investigate whether there are differences in transcription abundance between plants of different ages, RNA was isolated from 1, 2, 6 week old and

from fully developed RG prf3 tomato plants. To avoid effects resulting from differential expression across different tissue types, samples were always taken from the second leaf from above. Bti9 splice form 1b is robustly expressed in leaf tissue across all plant ages; expression levels are not significantly changing while the plants become older. In contrast, while transcripts of splice form 1a are detectable, expression levels are marginal. Like in the case of splice form 1b, we were unable to detect any significant change in Bti9 exon1a expression levels in plants of different ages.

B. Tissue specificity

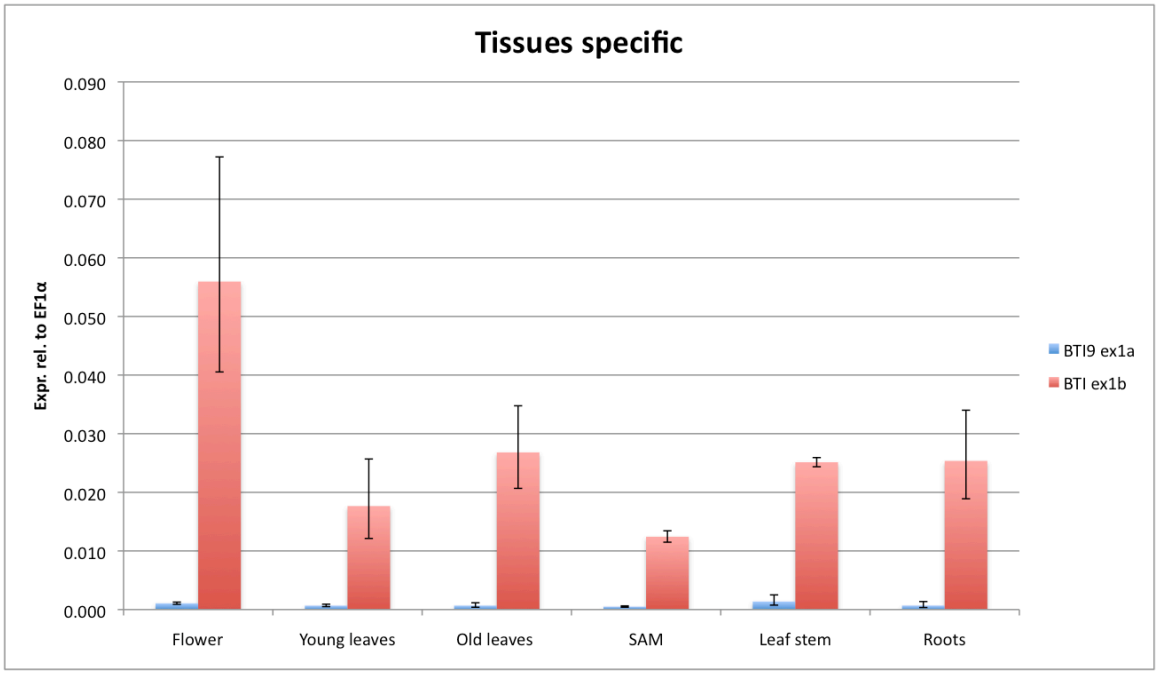

Figure 6: mRNA levels of the two different Bti9 splice forms across different tissue types. Splice form 1a levels are shown in blue and 1b in red

Next, we determined expression levels of the two splice forms in different tissue types. As the individual organs of a plant are exposed to different environments (e.g. roots versus leaves), they also face different biotic and abiotic stresses; consequently, a functional divergence between the two splice

forms might lead to distinct patterns of expression. Samples were collected from whole flowers, young leaves, fully extended old leaves, shoot apical meristems (SAMs), leaf stems and roots. For all samples, tissues were collected from 3 different fully grown Rio Grande prf3 tomato plants (approximately 8 weeks old).

As in leaves across plants of different ages, Bti9 splice form 1b is strongly expressed across all tissue types tested, while expression of splice form 1a is minimal. Expression of 1b is highest in flowers and lowest in the SAM, with the expression being approximately 5x higher in flowers than at the meristem. Leaves, stems and roots fall in between with comparable expression levels. The differences in the expression of 1a are not as pronounced as those of 1b; the highest expression is approximately 2.5x higher than the lowest one. As for splice form 1b, expression of 1a is lowest at the SAM. In contrast to splice form 1b, the highest expression is detectable in leaf stems. However, the very low expression levels of 1a and the resultant relatively high standard deviation make a reliable interpretation of the data for splice form 1a difficult. Also, this experiment has been performed only once and should be repeated to confirm the results.

C. Response after induction with PAMPs

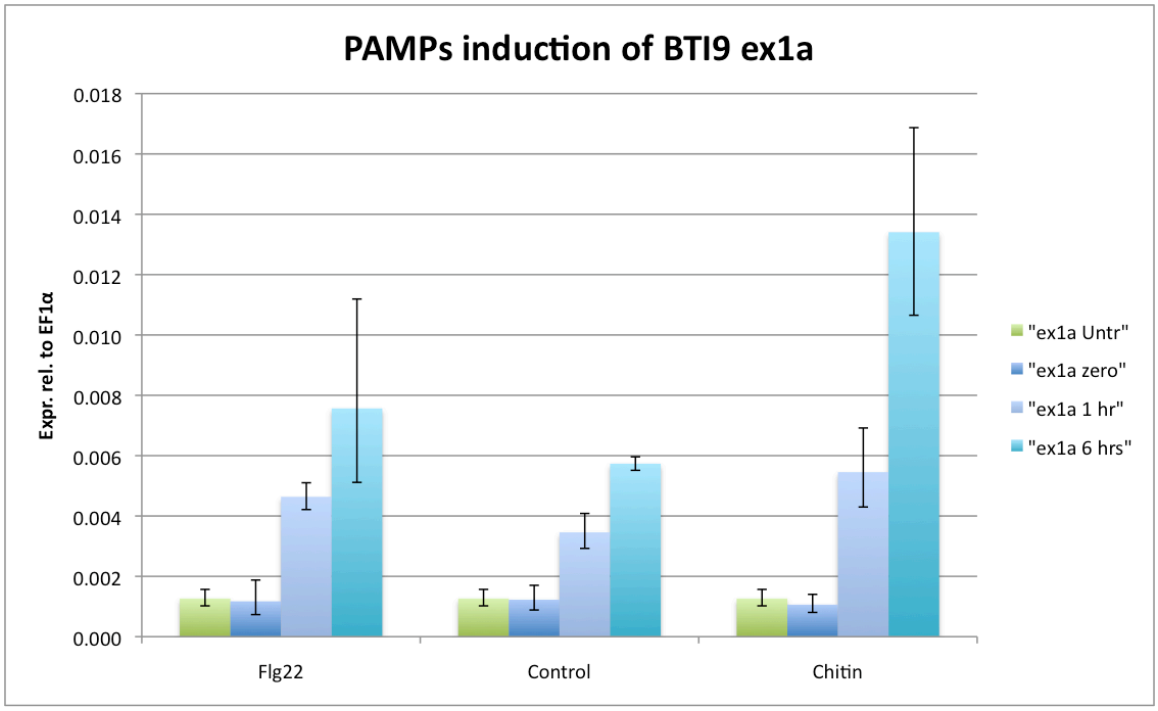

Figure 7: Bti9 exon 1a expression in response to PAMP treatment. Different time points are indicated by the differently colored bars.

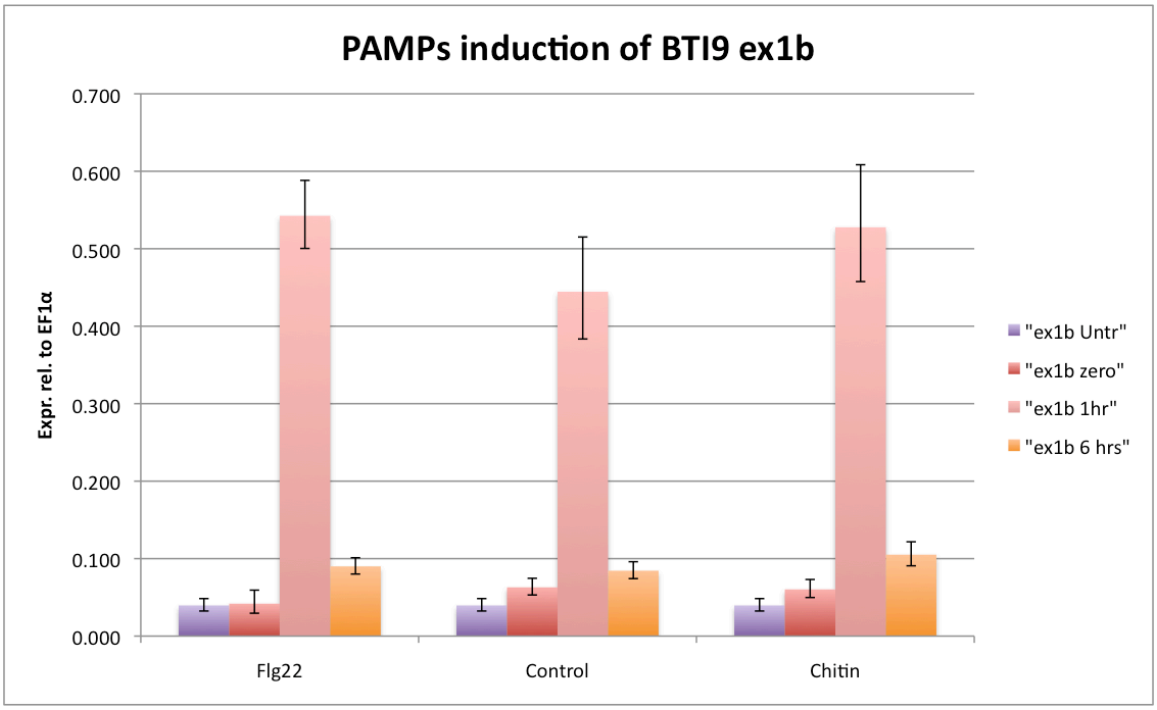

Figure 8: Bti9 exon 1b levels in response to PAMP treatment. The differently colored bars indicate different time points.

It has previously been demonstrated that, as part of a PTI response, the known PRRs are upregulated. For example, FLS2 transcription levels increase in response to stimulation with elf18, the cognate peptide ligand for EFR. As the closest homolog to CERK1, Bti9 might function as a PRR, possibly for chitin, and consequently might also be upregulated in response to other PAMPs. To test this, we challenged leaves of 4 week old Rio Grande prf3 tomato plants by syringe infiltration with 1 µM flg22, 100 µM chitin or water and collected leaf tissue one and 6 hours post infiltration to test for changes in Bti9 expression levels in response to PAMPs. The infiltrations were performed 6 hours resp. one hour before collection, to allow simultaneous harvesting of all samples (including the “untreated” control) at the same time and avoid differences that might caused by circadian regulation.

Both splice forms are strongly induced in response to the treatment, up to 10 fold. Interestingly, Bti9 splice form 1a is most highly induced in response to chitin, even though the difference to the induction by flg22 is not significant. Bti9 exon1b is equally induced in response to both flg22 and chitin. However,

both splice forms are also strongly induced in response to treatment with water; this indicates that the transcriptional activation is more in response to the wounding that is associated with the infiltration of the PAMPs than to the PAMP itself. It is interesting to note that the induction profiles of the two splice forms differ substantially; while splice form 1b is highly induced after one hour and drops back almost to the basal levels of untreated plants 6 hours after infiltration, expression levels of splice form 1a are still increasing from one to 6 hours after infiltration. As 6 hours represents the last time point sampled, it is possible that exon1a expression continues to rise at even later time points.

D. Response after induction with bacteria

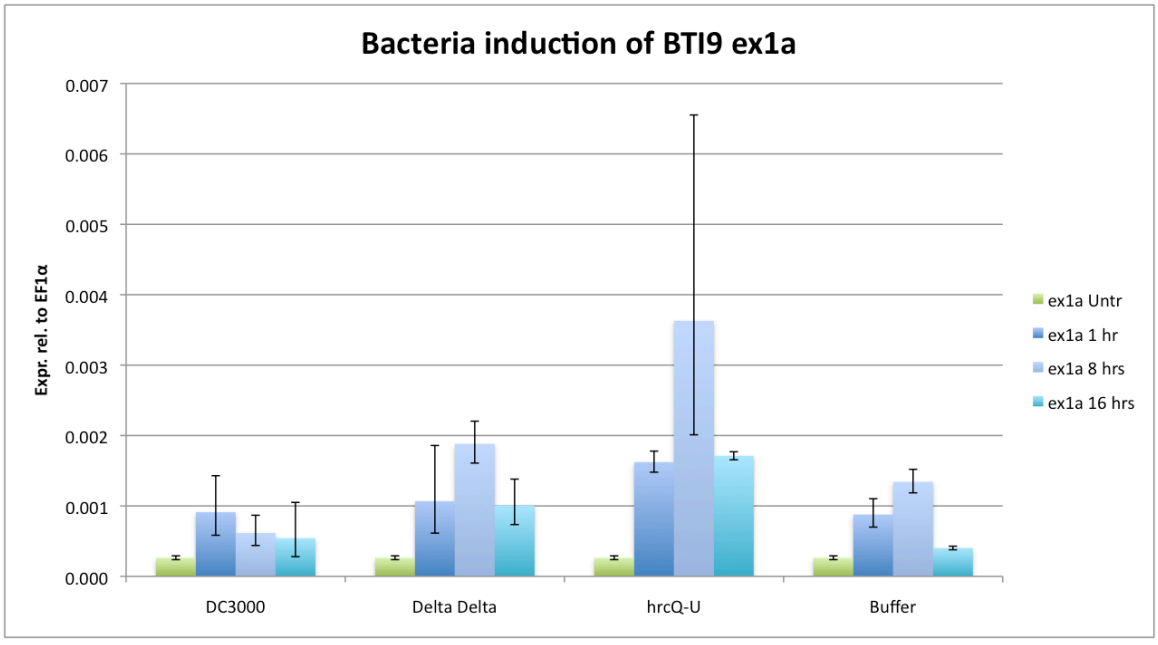

Figure 9: qRT-PCR results for Bti9 splice form 1a after bacterial induction. The differently colored bars indicate different time points.

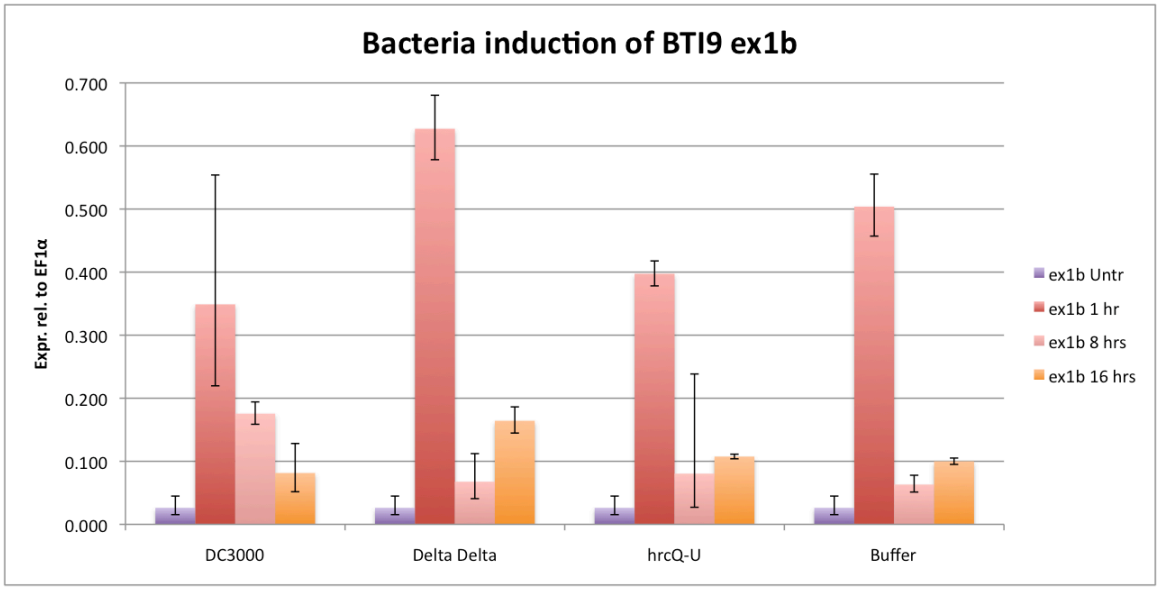

Figure 10: qRT-PCR results for Bti9 splice form 1b after bacterial induction. The differently colored bars indicate different time points.

To extend the range of PAMPs beyond the few ones with known PRRs, we decided to also test the response of Bti9 expression levels to infiltration with live bacteria. Again, 4-week-old Rio Grande prf3 plants were used for syringe infiltration. The *Pseudomonas syringae DC3000* strains were used at $1x10^7$ CFUs / ml and samples taken 1, 8 and 16 hours post inoculation. As before, infiltrations were done consecutively so that all samples including the negative controls could be taken at the same time. Besides wild type (wt) Pst, dAvrPto / dAvrPtoB double knockout mutants and hrcQ-U mutant strains were included. The double knockout strain is deficient in two effectors that have been described to have a very strong effect in suppressing PTI (i.e. the double knockout has a strongly reduced virulence). Their absence might make the potential PTI-mediated induction more easily visible. This hold true even more for the hrcQ-U strain, which is generally impaired in effector delivery and should allow for the full PTI response without the attenuating interference of bacterial effectors.

The observed response is largely similar to what we observed using isolated PAMPs. Both splice forms are clearly induced in response to injection, and again the timing of the expression peaks differs between splice form 1a and splice form 1b, in that 1b peaks after 1 hour and subsequently returns to basal

expression levels, whereas the main peak of splice form 1a is at 8 hours post infiltration. In this experiment we had included a 16 hrs time point to test whether Bti9 splice form 1a would continue to accumulate; this is not the case, as there is a clear decrease in transcript abundance from 8 to 16 hrs. As in the isolated PAMP treatments, the observed upregulation does not seem to be a specific response to a molecule derived form bacteria, but more likely a wounding response, as infiltration with buffer elicits a similar response as infiltration with bacteria.

Generation of the Bti9-GUS constructs

In order to visualize expression and localization of the two Bti9 splice forms *in vivo*, transgenic tomato lines carrying Bti9-GUS constructs will be produced. In contrast to qRT-PCR, visualization of the expression pattern *in vivo* with reporter GUS lines allows determination of expression patterns down to single-cell resolution.

The genomic region comprising approx. 1.9 kb upstream of exon 1a and the coding region down to exon 7 were subcloned, amounting to a total fragment size of more than 9 kb. The genomic fragment was designed as large as possible to include not only a substantial portion of the upstream region, but also the first introns, as they might contain additional regulatory elements. After subcloning, the two alternative first exons were to be individually replaced by GUS (beta-glucoronidase). As no information regarding potential regulatory elements was available, it was not possible to integrate new restriction sites into the sequence flanking exons 1a respectively 1b, as these changes might interfere with normal regulation. Instead, unique restriction sites already present in the natural sequence had to be identified and used to exchange the exons. For exon 1a, SalI and NdeI are present in the sequence, and exon 1b is flanked by BsrGI and AatII. This allowed subcloning of shorter fragments flanked by those restriction sites, replacement of the individual exon with GUS and reinsertion of the novel fragment now containing GUS into the larger genomic construct. After completion, the finished constructs were to be transformed into tomato plants by the core facility at BTI.

Initially, the constructs bordered by SalI / NdeI (exon1a) resp. BsrGI / AatII (exon1b) containing GUS replacing the first exons of Bti9 were generated by fusion PCR. Outer oligos were designed in such a way that they would generate fragments that contain the respective restriction sites in the 5’ or 3’ ends of the amplicon. 4 inner oligos were engineered: One pair amplifies the GUS ORF with added overhangs 5’ and 3’ encoded in the oligonucleotides that match to the genomic tomato sequence flanking the exon to be replaced.

The other two oligos amplify the genomic sequence fragments upstream and downstream of the first exon together with the outer oligos flanking the unique restriction sites. These oligos also add overhangs that match the GUS ORF. Consequently, a total of 3 separate PCR reactions were performed: one for GUS and one each for the sequence 5'- and 3' of the target gene. Each of the generated PCR fragments contains overhangs added by the oligonucleotides that are complementary to the neighboring piece: GUS on both the 5' and 3' ends, the 5' genomic fragment on its 3' end complementary to the GUS 5', and the 3' genomic fragment on its 5' end complimentary to the 3' of GUS.

PCR #	Product	Primer fr.	Primer rev.	Size
1	Exon 1a 5' with GUS overhang	oTK 1	oTK 6	473
2	GUS with 5' and 3' overhang for exon 1a	oTK 5	oTK 8	1851
3	Exon 1a 3' with GUS overhang	oTK 7	oTK 2	1525
4	Exon 1ab5' with GUS overhang	oTK 3	oTK 10	667
5	GUS with 5' and 3' overhang for exon 1b	oTK 9	oTK 12	1848
6	Exon 1b 3' with GUS overhang	oTK 11	oTK 4	177

Table 17: Fusion PCR fragments for GUS replacement of exon 1a resp. 1b. Fragments 1, 2 and 3 were used as templates for the exchange of exon 1a and fragments 3, 4 and 5 for exon 1b.

The different products were resolved by agarose gel electrophoresis; the observed fragment sizes matched the predicted sizes given in the Table 17. The fragments were purified from the gel and used as templates for fusion PCRs. Here, the overlaps in between the different fragments that had been introduced by the first PCRs would allow a "sewing together" of the three individual pieces (PCRs # 1, 2, 3 for exon 1a and PCRs # 4, 5, 6 for exon 1b) in a PCR with the three products as template and the two outermost oligos (oTK1 and oTK2 for exon 1a and oTK03 and oTK04 for exon 1b) as primers. These PCRs produce fragments which have 5' and 3' regions that are identical to the genomic regions, but with GUS in place of the first exon. PCR products of the expected size were identified by electrophoresis and purified from the gel, followed by "A-tailing" and ligation into the commercially

available pGEM-Teasy vector (Promega). The vector was then transformed into DH5α cells and plated on ampicillin plates. Positive colonies were miniprepped, verified by control digestion and sent for sequencing. Because there are two BsrGI restriction sites in the GUS sequence, we had to implement site directed mutagenesis PCR to disrupt these, as BsrGI had to be used to reintroduce the exon 1b – GUS fragment. We eliminated the unwanted sites by introducing “silent” mutations, which means that although we changed one base to abolish the restriction site the amino acid sequence of the protein still remained the same. This approach is based on the redundancy of the gentic code, where most amino acids are encoded by several different codons. The first BsrGI restriction site was abolished by changing the codon for leucine from CTT to CTA, and the second by mutating a tyrosine codon from TAC to TAT.

Figure 11: Schematic illustration of the two alternative first exons replaced by GUS cassettes as marker, with the localization of the unique restriction sites used for swapping annotated.

Next, the complete genomic region encompassing 1.9 kb upstream and the coding region down to exon 7 had to be subcloned from genomic DNA of PtoR tomatoes into pGEM-Teasy to subsequently shuttle the fragments generated above inside to produce the final constructs. Since this fragment is

more than 9 kb long it was cloned in two fragments; the 5' fragment is 4.6 kb and the 3' fragment is 5.2 kb long. The two fragments share an overlapping sequence fragment containing a unique restriction site, allowing them to be subsequently combined by ligation in pGEM-Teasy. The used oligonucleotides are summarized in Table 18. The sequence of the two vectors containing the fragments was confirmed by sequencing. Both fragment were digested with BsrGI (internal) and SphI (vector) and resolved by agarose gel electrophoresis. Bands of the right size were then purified and ligated. As the size of the vector combining the two fragments was exceeding 10 kb, commercial available Top10 cells were used for transformation instead of the lab-made DH5α to take advantage of their higher transformation efficiency. Colony PCR was performed as a screening method to identify positive clones amongst the resulting colonies, and the isolated vector sent for sequencing to confirm the correct sequence.

PCR #	Product	Primer fr.	Primer rev.	Size
1	Fragment 1	oTK 73	oTK 56	4605
2	Fragment 2	oTK 23	oTK 70	5279

Table 18: Primers used to clone the two fragments comprising the 1.9 kb upstream and the coding region down to exon 7 of Bti9

After confirmation of the sequence, the genomic fragment was excised and pasted into pFK209 [71], a modified pGreenII vector with an extra gateway cassette. To generate the backbone of the binary vector, the originally present gateway cassette and preceding 35S promoter were removed by digestion with SalI and SacI. After digestion, the linearized vector was treated with Mungbean nuclease for blunting (this also removed the remaining Sal restriction site). The use of a binary vector allows transformation into *E. coli* for cloning and amplification and subsequent transfer into *A. tumefaciens* for transient and stable transformation of plants.

Unique restriction enzyme sites (shown in Figure 11) present in genomic sequence were used to excise the exons and exchange them individually for

the fragments containing GUS in place of the first exons described above. For exon 1a, SalI and NdeI were used and for exon 1b BsrGI and AatII. As before, colony PCRs was used to identify positive colonies after transformations. The generated constructs were confirmed by control digests, followed by sequencing. The completed reporter GUS constructs were then transferred into *Agrobacterium tumefaciens* to allow their transfer into plant cells.

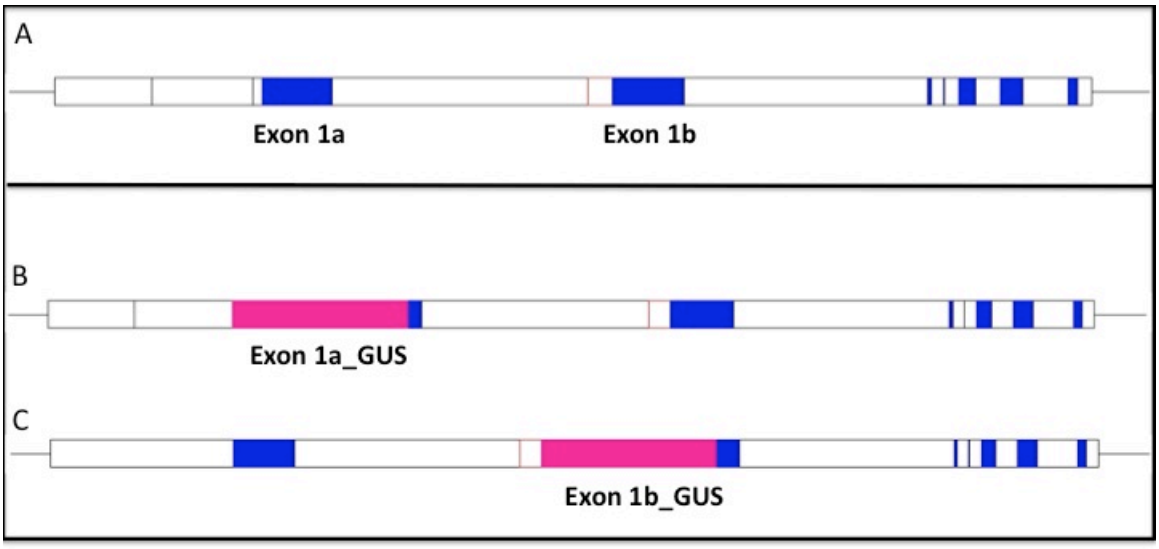

Figure 12: Schematic illustration of the cloned Bti9 fragment before and after GUS exchange Exons are annotated in blue, introns and promoter regions are shown in white **A**. Cloned Bti9 fragment in modified pFK209 vector after Mungbean nuclease treatment, **B:** Exchange of exon 1a with GUS, **C**: Exchange of exon 1b with GUS.

The vectors were then tested transiently in the model plant *Nicotiana benthamiana.* Transformed *A. tumefaciens* were used at an OD_{600} of 0.3 for syringe infiltration on the abaxial side of the leaves. Samples were taken after 2 days of induction. A GUS gene under an estradiol inducible promoter was used as a positive and buffer as negative control. Leaves were induced one day after infiltration and all samples were collected at the same time. The GUS gene under the inducible promoter contains an intron that has to be spliced in order for the GUS protein to be synthesized. Since bacteria lack splicing machinery, this process can only be performed in plant, but not by

agrobacterial cells. The presence of an intron in the DNA is an assurance that the transient transformation has worked and that the GUS protein is not being produced by the bacteria. Two days after the infiltration the samples were collected for histochemical staining with X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid). For that the samples were submerged in a falcon tube containing the staining solution, vacuum infiltrated for 15 min and incubated for 24 hrs at 37°C. The following day, the staining solution was removed and destain solution was added for at least 24 hrs or until the tissues had cleared.

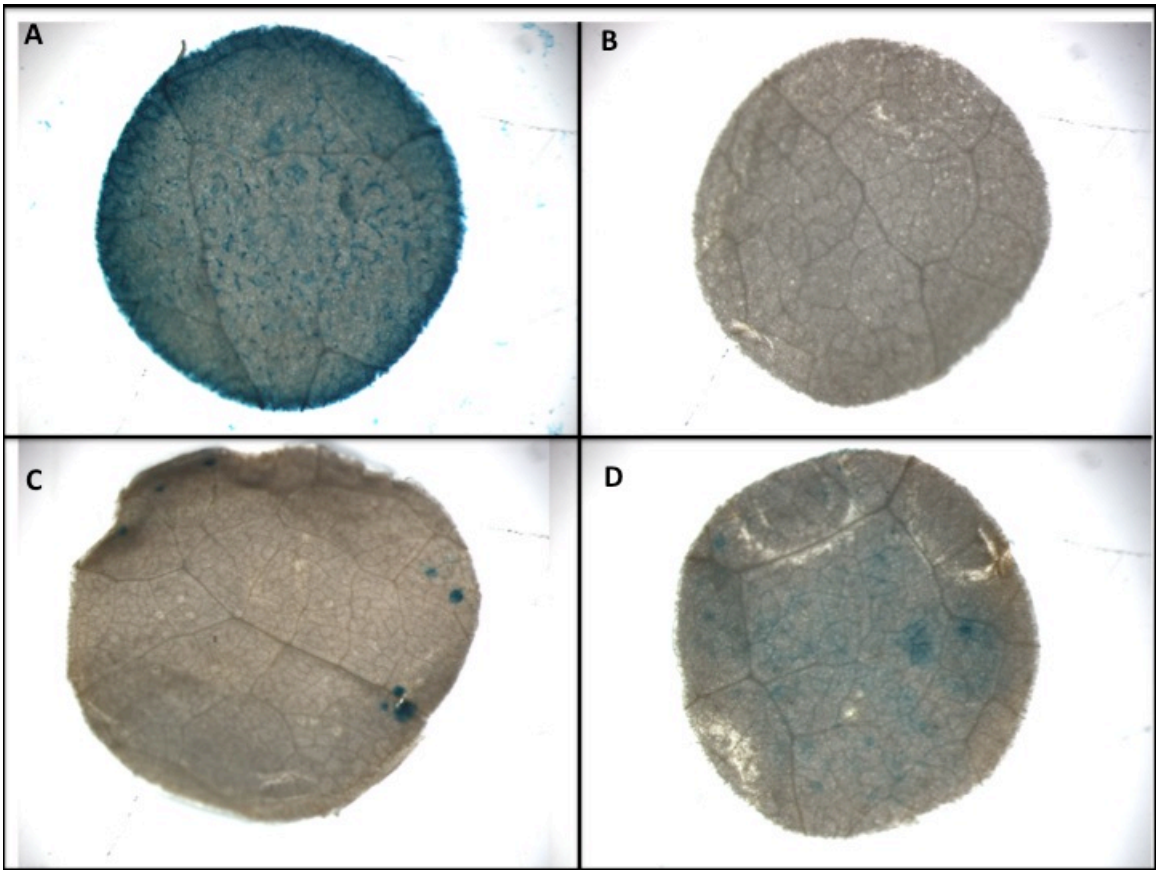

Figure 13: GUS-staining in *N. benthamiana* leaves. A GUS gene under control of an estradiol inducible promoter as a positive control; **B** Negative control. Leaves infiltrated with buffer; **C** pTK49 = exon1a-GUS; **D** pTK51 = exon1b-GUS

The positive control shows solid staining after induction, and the negative control is completely white, confirming that both transient transformation of the

N. benthamiana cells and the staining have worked. While pTK49 does not produce any significant staining in plant cells (the dots on the surface most likely result from a microbial contamination), pTK51 demonstrates clear staining inside of the leaf tissue. While it was not possible to take higher-resolution pictures of sufficient quality with the used setup, a closer examination by eye seemed to indicate that the coloration mostly originates from leaf vasculature cells.

Complementation of *A. thaliana cerk1-2* with Bti9 splice forms

The finding that Bti9 from tomato is the closest homologue of the chitin receptor CERK1 from Arabidopsis might indicate that both have the same function in PAMP recognition. *A. thaliana* knock out mutants for *CERK1* are no longer able to respond to chitin as PTI elicitor. The complete pathway is compromised, including MAPK activation, reactive oxygen species (ROS) generation and downstream gene regulation. We aimed to use complementation of Arabidopsis *cerk1-2* by Bti9 to test whether one or both splice forms are able to restore response to chitin, which would demonstrate that the respective splice form could function as a PRR for chitin. It has also been shown that AtCERK1 is involved in defense against bacterial infection, even though the sensed PAMP in this case is unknown. In a broader approach, the complemented plant lines will also allow us to test whether Bti9 can function to restore this CERK1 dependent defense against bacteria by assessing pathogen growth on complemented versus CERK1 knockout lines.

Generation of P_CERK1::Bti9 construct

The two splice forms from Bti9 were first cloned into the pJLSmart Gateway entry vector. cDNA generated from RNA that had been isolated from Rio Grande tomatoes was used as template for generating the two Bti9

constructs. Two different entry clones were generated for each, one containing a STOP codon to allow the expression of an untagged protein and one without STOP to allow the generation of C-terminally tagged versions by Gateway recombination. The two versions of the entry vector for Bti9 exon 1b, with and without stop codon, were already available in the lab (J. Mathieu, unpublished). Cloning both forms for each cDNA was necessary because it was not clear whether a C-terminal tag would interfere with protein function; preliminary data indicated that this might be the case at least for C-terminal GFP fusions (L. Zeng, personal communication). N-terminal protein fusions were not possible, as the membrane targeting signal forms the N-terminus of the protein.

Vector	Product	Stop codon
pTK18	cDNA Bti9 ex1a	No
pTK26	cDNA Bti9 ex1a	Yes
pJM56	cDNA Bti9 ex1b	No
pJM55	cDNA Bti9 ex1b	Yes

Table 19: Vectors containing the two Bti9 splice forms using Rio Grande cDNA as template. Two different versions of the cDNAs were cloned with and without stop codon for further use in a modified pFK209 vector containing AtCERK1 promoters

As Gateway destination vectors we generated modified versions of pFK209 with and without N-terminal protein fusion tags. pFK209 version without tags or with either Flag, His or AcV5 tags were already available in lab. In each of these vectors, the 35S promoter was replaced by 2 different versions of the AtCERK1 promoter: a longer version (approx. 1.1 kb) which is identical to a previously published construct that rescued the cerk1 knockout phenotype [32] and a shorter version (approx. 300 bp upstream of CERK1) that encompasses the complete upstream sequence to the end of the 3' UTR of the preceding gene (the Miya construct contains the 3' UTR as well as several introns and exons from the preceding gene). Both promoter versions were amplified from genomic Arabidopsis Col-0 DNA using oligonucleotides that added a 5' EcoRI and a 3' XbaI restriction site. The resulting PCR products

were purified, ligated into pGEM-Teasy, transformed into DH5α cells and plated on medium containing ampicillin. From growing colonies, DNA was purified by mini-preps, verified by control digestion and sent for sequencing. From positive clones, the two promoters were excised using the two restriction enzymes and ligated into the different tagged and untagged versions of pFK209, from which the 35S promoter had been removed EcoRI / XbaI.

PCR #	Product	Primer fr.	Primer rev.	Size in bp
1	CERK1 promoter long	oTK40	oTK41	1100
2	CERK1 promoter short	oTK44	oJM43	300

Table 20: PCRs to clone the two versions of the CERK1 promoter. Also included are the used primers and the expected product sizes.

Bands corresponding to the CERK1 short and long promoters and the pFK209 tagged and untagged backbones were then purified from the gel and ligated using T4 DNA ligase. After transformation into DB3.1 cells the ligations were plated onto chloramphenicol and spectinomycin plates. DNA from colonies carrying the vector was purified by mini-preps, verified by control digestion and sent for sequencing. The resultant Gateway destination binary vectors containing the AtCERK1 promoters are listed in Table 21.

pTK #	Product	Tag
11	CERK1 promoter short	No tag
12	CERK1 promoter long	Flag
13	CERK1 promoter long	Myc
14	CERK1 promoter long	AcV5
15	CERK1 promoter short	Myc
16	CERK1 promoter short	Flag
17	CERK1 promoter short	AcV5
36	CERK1 promoter long	No tag

Table 21: Generated Gateway vectors containing the two AtCERK1 promoters

Subsequently, LR-recombination reactions were performed between the entry vectors containing the different splice forms with and without STOP codons and the P_CERK1-promoter destination vectors. As before, the vectors were transformed into DH5α cells and plated on spectinomycin plates. Positive colonies were miniprepped, verified by control digestion and sent for sequencing. The sequence-verified constructs, containing the P_CERK::Bti9 constructs, were then transformed into *Agrobacterium tumefaciens* to allow their transfer into plant cells.

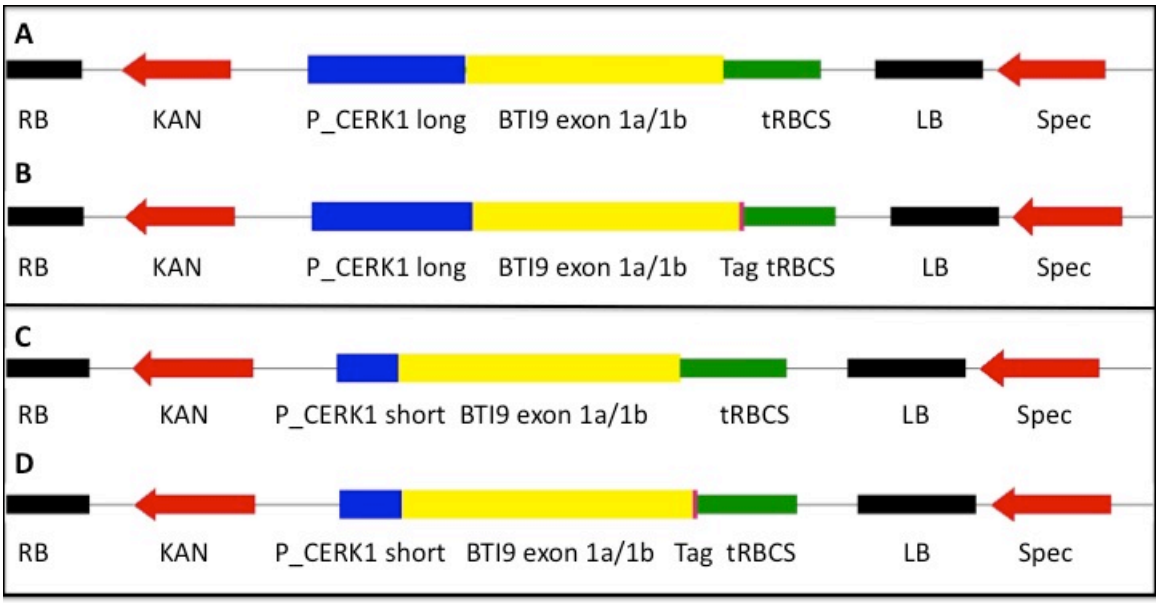

Figure 14: Schematic illustration of the P_CERK1::Bti9 constructs. A: CERK1 promoter long followed by the splice forms of Bti9; **B.** CERK1 promoter long followed by one of the splice forms of Bti9 without stop and C-terminal tags; **C**: CERK1 promoter short followed by the splice forms of Bti9; **D.** CERK1 promoter short followed by the splice forms of Bti9 without stop and C-terminal tags.

To test if expression of the Bti9 splice forms is possible in Arabidopsis cells, protoplasts were transiently transformed with Haemagglutinin (HA) tagged vectors containing either Bti9 exon 1a or 1b cDNA under the control of a cauliflower mosaic virus 35S constitutively active promoter and the protein visualized by Western Blot.

A LR recombination was performed between the entry vectors pTK18 (containing Bti9 exon 1a cDNA without STOP) or pJM56 (containing Bti9 exon 1b cDNA without STOP) and the C terminally tagged pHBT95:3xHA (J. Munkvold, personal communication) destination vector. This vector contains a 35S promoter and was used to test whether it is in principle possible to express Bti9 in Arabidopsis. The constructs were then transformed into DH5α and plated on ampicillin plates. Colonies were tested by control digestion and sequencing. The used plasmid DNA was purified using the Midiprep kit from Qiagen and concentrated by isopropanol precipitation. HBT95::MPK6:3xHA was used as a positive control for the transformation, as it expresses to very high levels in protoplasts (K. Munkvold, personal communication). Protoplasts were transformed with the maximal amount of plasmid DNA possible based on the concentration of the DNA preps, harvested 6 hours post-transformation, resuspended in 20µl 1x Laemmli buffer and boiled for 5 min.

pTK #	Expressing	DNA amount used
Lab	P_35S::MPK6:3xHA	20 µg
40	P_35S::Bti9[1b]:3xHA	19.8 µg
39	P_35S::Bti9[1a]:3xHA	39.9 µg
21	P_CERK(long)::BTI[1a]:FLAG	40 µg
35	P_CERK(long)::BTI[1b]:FLAG	39.6 µg
24	P_CERK(short)::BTI[1a]:FLAG	30 µg
	H2O	

Table 22: Constructs used for the transient transformation of *Arabidopsis thaliana* protoplasts.

Western Blot was performed using commercial antibodies against HA (monoclonal 3F10, Roche) or FLAG (monoclonal M2, Sigma) to verify protein expression in the protoplasts. The signal was visualized using HRP-coupled secondary antibodies from Santa Cruz (goat anti mouse / anti-rat).

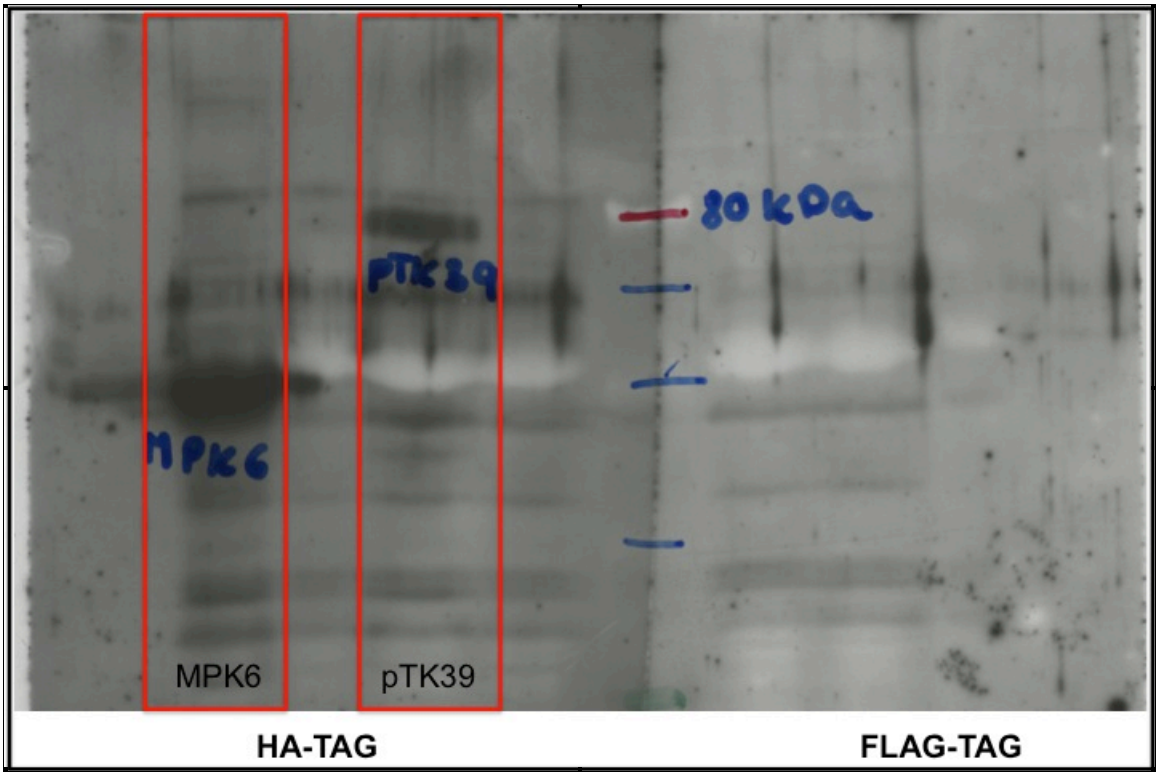

Figure 15: Western Blot analysis of the expression of MPK6 and the two Bti9 splice forms. On the left side, antibodies against the HA-tag were used and on the right site anti-FLAG antibodies.

As shown in Fig. 13, both the MPK6 positive control and Bti9 exon1a HA fusions are clearly expressed in Arabidopsis, whereas Bti9 exon 1b appears to be negative. However, the quality of the vector preparation for exon 1b used in this experiment was much worse than that of exon 1a (compare total DNA amounts in Table 22). Hence, the lack of expression for splice form 1b might result either from an unsuccessful transformation or from it not being expressed in Arabidopsis cells under these conditions. None of the Flag-tagged versions under control of the different CERK1-promoters show visible expression in this experiment. However, as no positive control was included, it remains unclear whether this is due to the proteins not being expressed at all, not being expressed high enough to be detectable or problems with the detection itself. While this experiment would need to be repeated to produce better results, the fact that at least Bti9 exon1a expresses nicely was taken as indication that the complementation approach in Arabidopsis might work.

Transformation of *Arabidopsis thaliana* cerk1-2 for complementation

To generate transgenic complementation lines, approx. 5 old weeks *Arabidopsis thaliana* cerk1-2 plants were transformed using the floral dip method with all constructs listed in Table 23.

pTK	Promoter	cDNA	Tag
20	Short	Bti9 exon 1b	-
21	Long	Bti9 exon 1a no stop	Flag
22	Long	Bti9 exon 1a no stop	AcV5
23	Short	Bti9 exon 1a no stop	Myc
24	Short	Bti9 exon 1a no stop	Flag
25	Short	Bti9 exon 1a no stop	AcV5
30	Short	Bti9 exon 1a	-
31	Long	Bti9 exon 1b no stop	Myc
32	Long	Bti9 exon 1b no stop	AcV5
33	Short	Bti9 exon 1b no stop	Myc
34	Short	Bti9 exon 1b no stop	Flag
35	Short	Bti9 exon 1b no stop	AcV5
37	Long	Bti9 exon 1a	-
38	Long	Bti9 exon 1b	-

Table 23: List of constructs used for Arabidopsis transformation. The different tags are also shown.

Plant were then grown in a long day chamber at 23°C for several weeks until the siliques with the seeds were completely dried. The dry seeds, which represent the F1 generation, were then collected. After approx. 1 week, the seeds were sterilized with ethanol and spread on MS-plates containing 25 mg / ml kanamycin for selection. Transformed seedlings contain a kanamycin resistance gene that allows them to grow on these plates beyond the cotyledon stage and producing real leaves. (Non-transformed seedlings die before producing real leaves). The plates were incubated in a light shelf for approximately two weeks (long enough to see a different between transgenic and non transgenic seedlings) followed by careful transplantation onto soil. Initially, only very few seedlings survived the selection on plates and most of

these died briefly after transplantation on soil. The reason for this was that the temperature on the lab light shelf was too high (the room containing the shelf was not climatized, causing temperatures to peak to over 30°C); the resultant heat stress combined with the selection obviously cause lethality even in resistant transgenic T1 plants. After moving to a light shelf in the transformation core facility at the Boyce Thompson Institute for Plant Research, transgenic plants survived in more typical numbers (>5/500 µl T1 seeds). Seeds from individual F1 plants were collected; each of these seed stocks represents an independent, segregating F2 generation for the respective transgene. After 1 week to allow the collected seeds to dry completely, some of the T2 lines were sterilized by chlorgas sterilization and sowed on soil. At the end of this thesis project, these F2 plants were growing; by now, seeds for many of the individual plants have been collected. Later, aliquots of the F2 seed batches will be plated on selective plates to determine their insertion numbers (single insertion lines will segregate in a simple Mendelian fashion with 25% lethality). Subsequently, aliquots of the F3 line seed batches originating from F2 lines determined to carry a single insertion can also be sowed on selective plates to identify homozygous single insertion lines.

Latest when we established defined homozygous single insertion lines, the expression of the transgene will be tested either by Western Blotting (constructs with protein fusion tags) or RT-PCR (untagged versions). The verified lines can then subsequently be used to test complementation of the cerk1-2 knockout phenotype by the Bti9 splice forms. Complementation of either one or both known AtCERK1 defense responses, ROS or suppression of bacterial growth, would strongly indicate that the complementing splice form(s) are performing similar functions in tomatoes, demonstrating that Bti9 is indeed a functional homolog of CERK1 in tomato.

Chitin-induced ROS response in Arabidopsis thaliana

As we plan to use the response to chitin in Arabidopsis to test the complementation of cerk1-2 by our Bti9 constructs, the chitin ROS assay had to be established in lab. While there are some contradicting reports in the literature, some groups reported chitin from crab shells (Sigma-Aldrich) was successfully used as elicitor to induce the production of reactive oxygen species in Arabidopsis. According to this published data, ROS production should peak approximately 30 min after treatment with ground chitin. We initially tried to reproduce these results by grinding chitin in a mortar and infiltrating at concentrations of 100 µg / ml. One problem with this approach is that at these concentrations, chitin does not go into solution, but forms a suspension in aqueous solutions at neutral pH. We tried to grind chitin even finer by adding liquid Nitrogen, but this did not enhance solubility. Chitin does go into solution at low pH, and we managed to achieve complete dissolution resulting in a viscous solution by titration with HCl; unfortunately, the low pH also inhibited the ROS assay. We also tested sonication of the chitin, hoping to break the crystals down to finer particles that would stay in solution better. In all infiltrations, we tried to keep the chitin particles in suspension as good as possible by rigorous vortexing before adding the elicitor to the leaf discs. None of the preparations mentioned above resulted in any measurable ROS response in our assays, while the included flg22 positive controls worked as expected. We also attempted to enhance penetration of the leaf discs by either adding Silwet-L77 as a detergent or using vacuum to better infiltrate the tissue; however, also in these cases we were never able to detect any ROS response.

Chitosan is a commercially available derivate of chitin, in which the acetyl groups are removed by treatment with concentrated acids; in contrast to chitin, it is soluble in water. It has been reported that in CERK1 can bind to chitosan polymers, but not oligomers, *in vitro* [35]. Consequently, we also tested chitosan for its potential to elicit a ROS response in Arabidopsis by infiltrating leaf discs with chitosan polymers (Sigma) at concentrations of 100

µg/ml, but as in the case of chitin were not able to detect ROS production. One possibility why some groups report successful induction of ROS production with the Sigma chitin, while others, including us, are not able to reproduce these results, might be differences between the chitin preparations obtained.

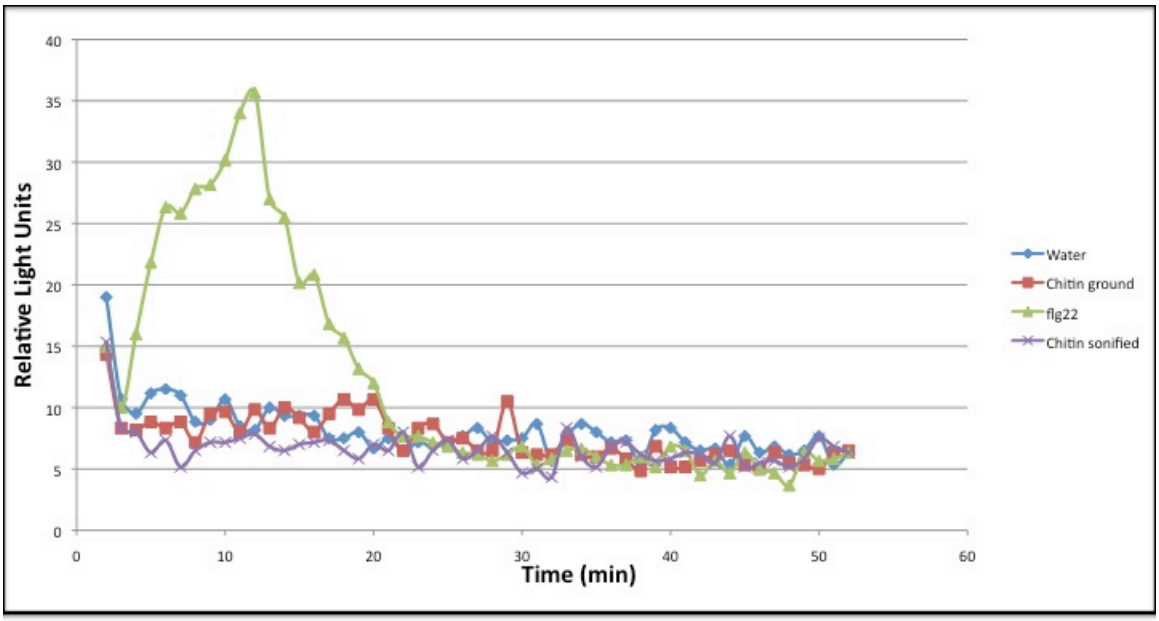

Figure 16: ROS assay using chitin or flg22

As the used chitin is produced from a biological source, (crab shells), variations in the quality or contaminations with other compounds cannot be ruled out. To evaluate whether our failure to produce detectable ROS responses with chitin lies in the elicitor we used or in our performing of the assay, we aimed to get a more uniform elicitor preparation to verify that the assay is in principle working in our hands with chitin (flg22 mediated elicitation of ROS was always successful across our experiments). N. Shibuya generously provided us with small aliquots of fully acetylated and purified chito-hexaose and chito-octaose. As shown in Figure 17, using these elicitors we were able to get a robust ROS burst in response to chito-octaose, but not chito-hexaose. Quantitatively, the response is comparable to flg22, but longer lasting. These results demonstrate that our chitin response ROS assay is in

principle working; obviously, the problem lies in the nature of the used elicitor and not in the assay itself. Unfortunately, we were only able to obtain very minimal amounts of chito-octaose at this time. To confirm the results and routinely use ROS production in response to chitin as a PTI assay in lab we will have to find a way to obtain larger amounts of well defined elicitor in the future.

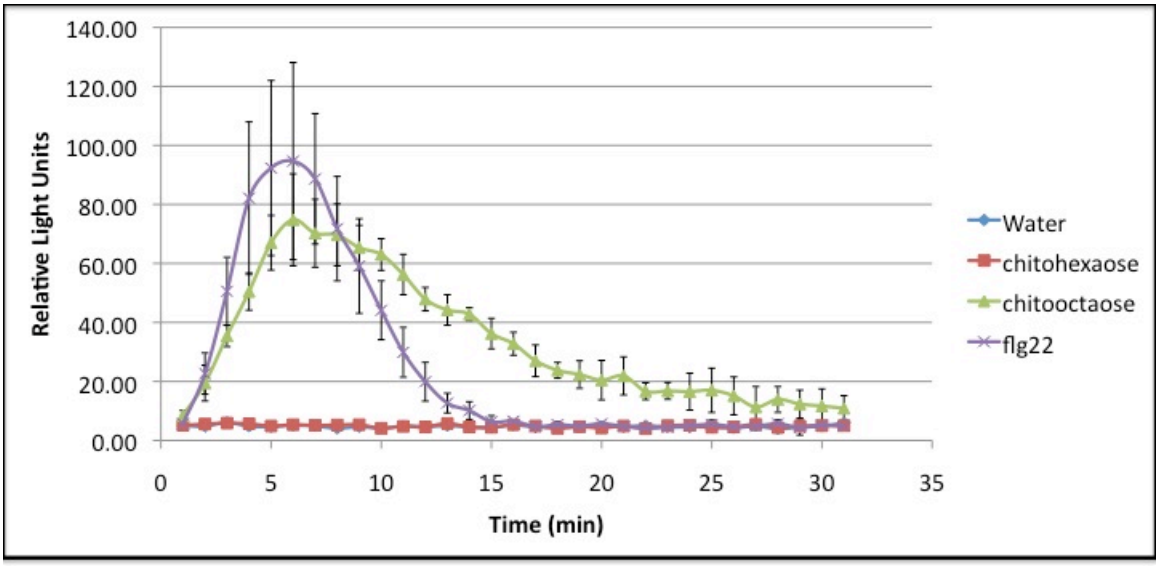

Figure 17: ROS assay using chito-oligomers

Development and characterization of transgenic tomatoes containing Pto promoter-GUS fusions

Pto (resistance to *Pseudomonas syringae* pathovar (pv.) tomato, *Pst)* was the first disease resistance gene cloned from a plant and represents one of the best-described elicitors of R-gene mediated ETI. The *Pto* gene encodes a cytoplasmic Ser/Thr kinase that confers resistance against strains of *Pseudomonas syringae pv tomato* that express the effector genes AvrPto and / or AvrPtoB. *Solanum pimpinellifolium*, a wild tomato species, is the plant where *Pto* was first discovered and isolated by map-based cloning [72]. The Pto-like pathway might be a highly conserved mechanism in the plant immune system, since Pto-related genes have been found in diverse plant species [72].

Despite Pto being such an important example for a protein involved in the plant immune system, not much is known regarding its expression, especially on a single cell resolution level *in vivo*. For that, transgenic tomato lines carrying the Pto promoter::GUS (P_Pto::GUS) construct were produced. This will allow us to visualize Pto expression patterns down to single-cell resolution.

Generation of the Bti9-GUS constructs

The genomic region comprising approx. 2.2 kb sequence upstream of the Pto gene had previously been cloned into pGEM-Teasy and verified by sequencing by Zhilong Bao during his rotation in the Martin lab. As Pto is a single exon gene, it is reasonable to assume that most or all relevant regulatory elements should be contained in this region. It was now to be recloned into pFK209. After recloning, a GUS cassette was recombined in behind the promoter by LR-recombination.

Initially, the 35S promoter contained in the pFK209 binary vector was replaced by the Pto promoter. XbaI and BamHI were used for removal of the promoter and its exchange with the fragment containing the 2.2 kb Pto promoter fragment (P_PTO). Both pGEM-Teasy-P_PTO and pFK209 were digested with BamHI and XbaI and resolved by agarose gel electrophoresis. Bands corresponding to the vector backbone (pFK209) resp. the promoter element (pGEM-Teasy-P_PTO) were then purified from the gel and ligated using T4 DNA ligase. The ligation was then transformed into *E. coli* DB3.1 cells and plated on medium containing chloramphenicol and spectinomycin. From growing colonies, DNA was purified by mini-preps, verified by control digestion and sent for sequencing. The resultant Gateway destination binary vector containing the Pto-promoter was termed pTK03. Subsequently, a LR-recombination reaction was performed between pENTR-GUS (Invitrogen), a Gateway entry clone containing the GUS ORF inside the Gateway cassette, and pTK03 as destination vector. This time, the vector (pTK05) was transformed into DH5α cells and plated on spectinomycin plates. Positive colonies were miniprepped, verified by control digestion and sent for sequencing. The sequence-verified pTK05, containing the reporter GUS construct, was then transferred into *Agrobacterium tumefaciens* to allow its transfer into plant cells.

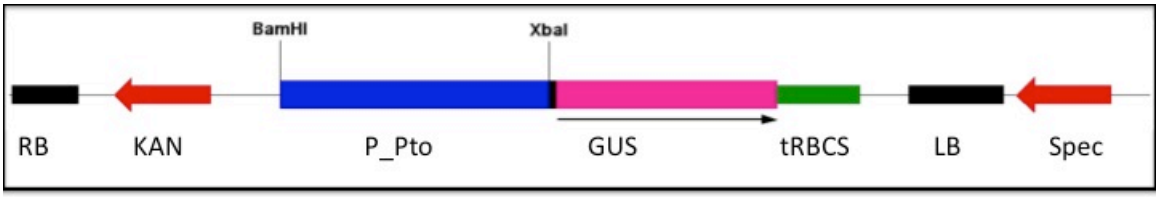

Figure 18: Schematic illustration of pTK05 a Gateway vector containing the Pto promoter followed by a GUS cassette.

The vectors were then tested transiently in *Nicotiana benthamiana,* the same way as described above for Bti9_GUS constructs.

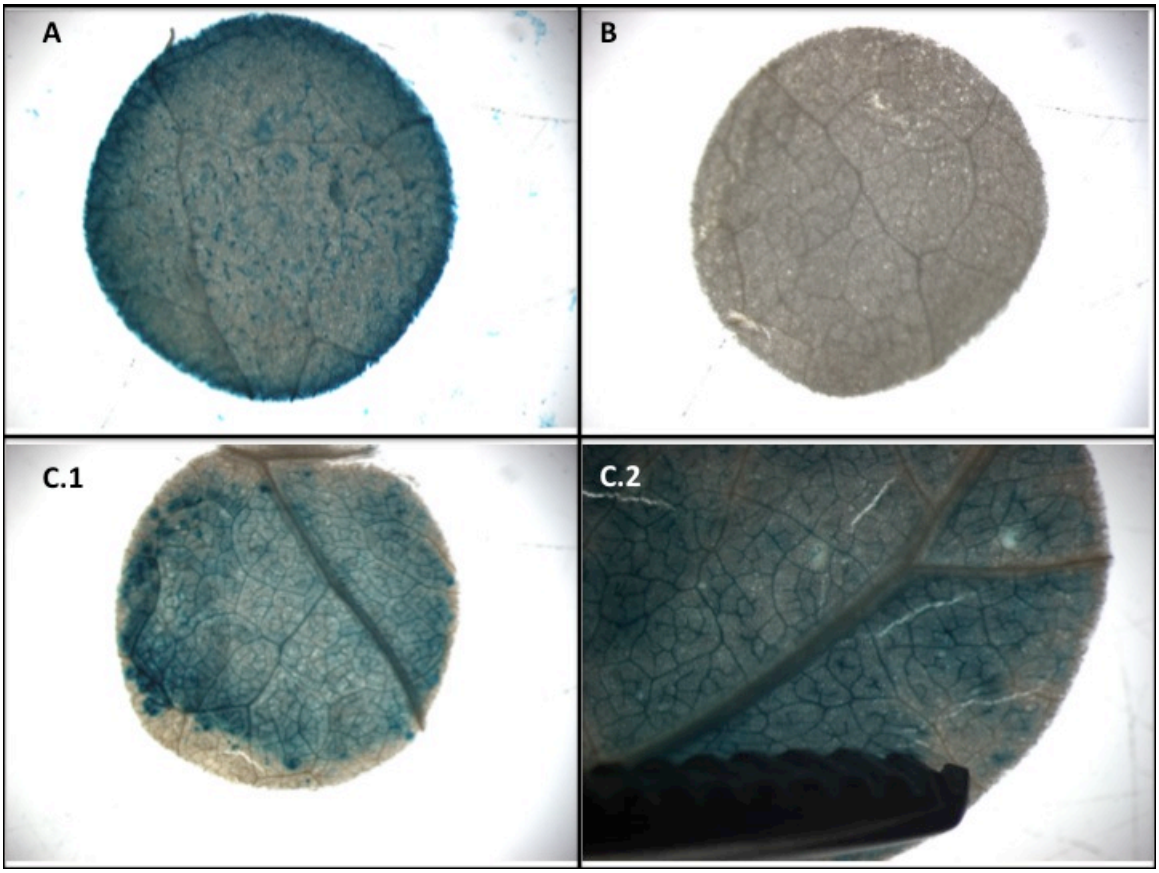

Figure 19: GUS-staining in *N. benthamiana* leaves. A GUS gene under control of an estradiol inducible promoter as a positive control; **B** Negative control. Leaves infiltrated with buffer; **C.1** pTK05; **C.2** pTK05 close-up

pTK05 shows a strong blue staining, almost as strong as the positive control. Upon close inspection there might be a stronger staining co-localizing with leaf vasculature. As the promoter-GUS construct appears to be functional in the transient assay, we decided to send it to the transformation core facility at BTI to be transformed into Rio Grande-PtoR tomatoes. Rio Grande-PtoR tomatoes encode a functional copy of the Pto gene and are consequently resistant to bacterial speck disease caused by *P. syringae* pv *tomato*. One reason to use a tomato line with a functional Pto was the possibility of a positive feedback loop of Pto onto its own transcription.

Transgenic prf3 tomatoes from the Core Facility

27 plants delivered form the core facility as potentially transgenic were genotyped to verify the presence of the transgene. Genomic DNA was purified using the CTAB method, and two different PCR reactions were performed amplifying different parts of the insertion. One targeted the kanamycin resistance gene and the second the GUS ORF. The two amplicons are situated on different ends of the tDNA element; hence, the presence of both sequences should indicate a complete insertion of the tDNA. For the first PCR, standard lab primers were used that target the kanamycin cassette (not shown). For the second PCR, oligos were designed for the GUS cassette. As control for DNA extraction quality, we also included an alternative oligo pair for EF1a (not shown).

PCR #	Product	Primer fr.	Primer rev.	Size in bp
1	Kanamycin	Oligo 728	oligo 729	585
2	P_PTO::GUS	oJM67	oJM69	502

Table 24: Oligo used for genotyping the potentially transgenic tomatoes.

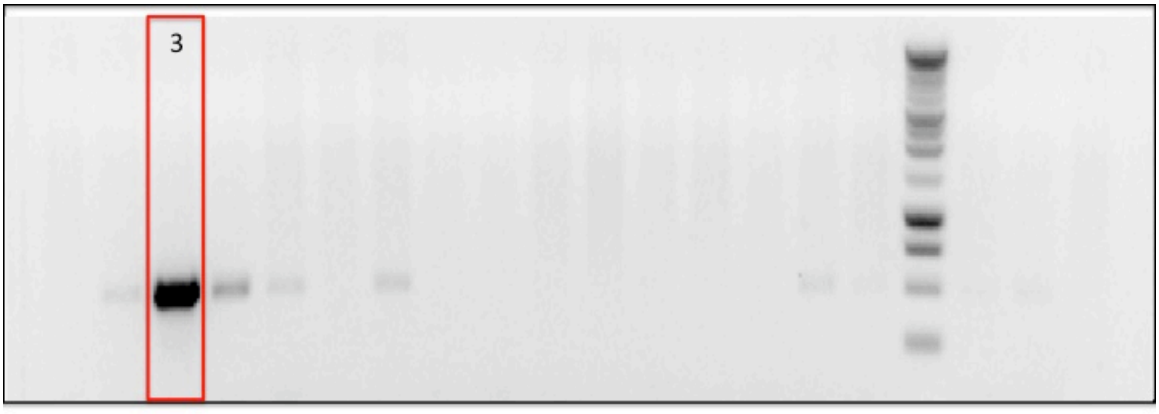

Figure 20: 1% Agarose gel with the product of the PCR for the GUS cassette

Only one of the 27 potentially transgenic tomato lines produced clear PCR products of the correct sizes for both the Kan and the GUS genotypings. This

indicates that the transformation worked for this line and the complete tDNA has been integrated into genome of the plant. The transformation core facility informed us that transformation of Rio Grande tomatoes is generally difficult and that, to have any of the regenerated plants set roots at all, they had to transfer them from selective to non-selective medium after callus formation. This might explain while so many plants not actually containing a transgene had been carried along as potential transgenics.

Next, the positive line 3 was tested for GUS activity. As samples, whole young leaves were taken, placed in tubes containing GUS staining solution and vacuum infiltrated. Pto is part of the tomato immune system, and it is well known that many defense related genes are upregulated after pathogen detection. To test this, leaves of the transgenic tomato were syringe infiltrated with 2 µM flg22 or with *Agrobacterium tumefaciens* at an OD of 0.1 to mimic a bacterial infection. Samples were taken 8, 18 and 24 hours post inoculation and also included in the staining. A stable transgenic plant, expressing an auxin inducible DR5::GUS reporter from the Benková lab [73], was used as a positive control for this test. The expression of these constructs was induced prior to staining by incubation in a tube containing the IAA solution. The histochemical staining with X-Gluc and its destaining were performed exactly as described earlier for the BTI_GUS constructs.

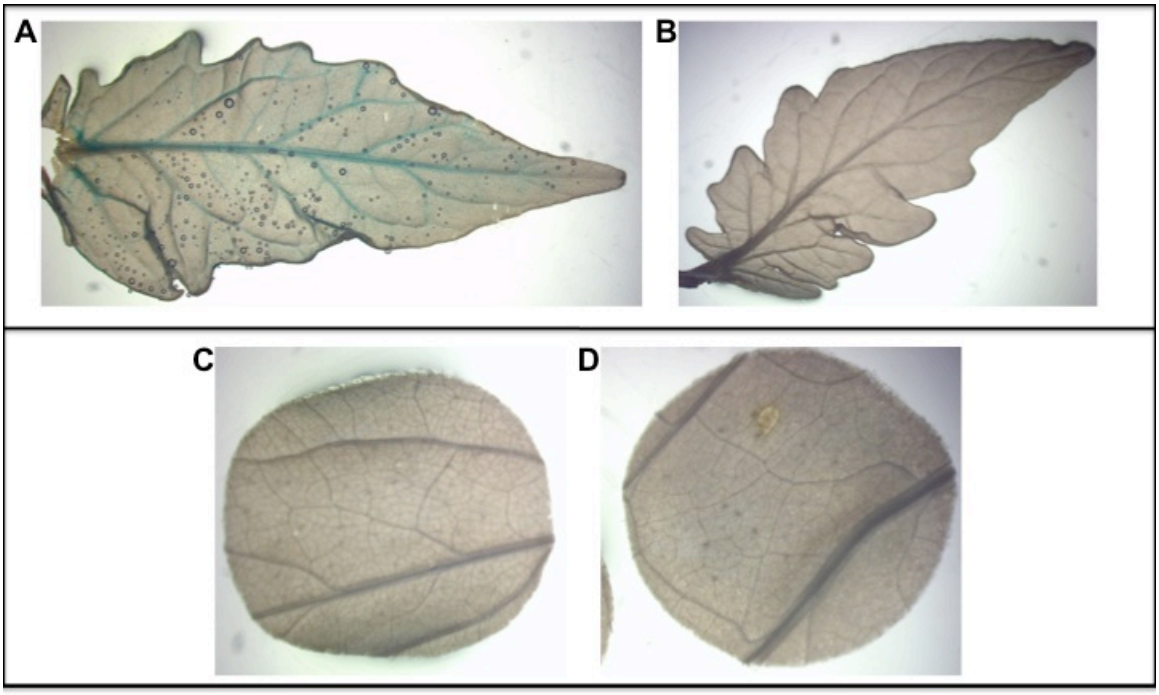

Figure 21: GUS staining of P_PTO::GUS transgenic tomato line 3. A. Induced DR5::GUS transgenic tomato line as positive control; **B)** P_PTO::GUS line 3 untreated; **C)** P_PTO::GUS line 3 + 2 µM flg22; **D)** P_PTO::GUS line 3 + O.D 0.1 *A. tumefaciens*

While positive and negative control show the expected phenotypes (vascular staining respectively no staining), the P_PTO::GUS line showed no discernible staining either untreated or as response to the potentially inductive treatments. The two most likely explanations are either silencing of the transgene or a basal transcription level of Pto that is too low to result in visible staining using GUS. To distinguish in between these possibilities, we used RT-PCR (reverse transcription-polymerase chain reaction) to detect low-level transcription of the P_PTO::GUS transgene and compare it to the levels of the endogenous Pto allele. Towards that end, RNA was isolated from the positive line 3, 3 other lines that appeared to be negative according to the genotyping (6, 8, 16) and from wild type Rio Grande prf3 tomato as negative control. RNA was then reversely transcribed into cDNA and used as a template for 3 parallel PCRs: one specific for GUS expression from the transgene, one to detect endogenous expression of Pto and one for Ef1a as quality control for the generated cDNA. The primers amplifying the P_PTO::GUS mRNA were designed such that the forward oligo would align to the Pto 5’ UTR and the

reverse oligo within the GUS ORF. The generated primers that detect the endogenous Pto mRNA were placed so that the forward oligo aligns with different sequence in the Pto 5' UTR and the reverse oligo with the Pto kinase domain (R. Abramovitch, unpublished). The oligo-combinations for the amplification of the P_Pto::GUS transgene were tested in PCRs using plasmid DNA as template. All Rio Grande prf3 tomatoes contain the Pto gene; detecting its mRNA, which should be transcribed at the same strength as our GUS construct since they have the same promoter, should be a fairly good indicator as to how reliably the cloned promoter fragment reflects the activity of the endogenous promoter. As control for DNA extraction quality, we also included oligos for EF1α.

PCR #	Product	Primer fr.	Primer rev.	Size in bp
1	Pto	oTK105	oTK101	433
2	P_Pto::GUS	oTK104	oTK108	663
3	EF1α	oTK68	oTK69	454

Table 25: Primers used for RT-PCR profiling of Pto and P_Pto::GUS and the expected size of the product.

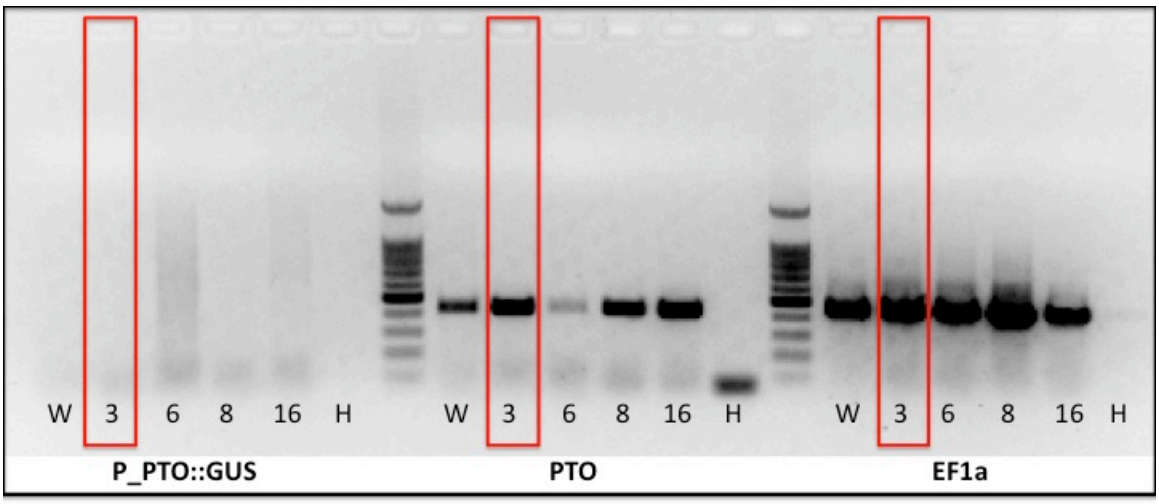

Figure 22: 1% Agarose gel with the product of the RT PCR for P_Pto::GUS, Pto and EF1α. W: wild type Rio Grande prf3; H: water. The line carrying the transgene according to genotyping (3) is marked with red boxes.

All 5 samples produced a clear PCR product of the right size for Pto and Ef1α, indicating that RNA isolation, reverse transcription and PCR worked well. Also, endogenous Pto is transcribed sufficiently to detect the mRNA by RT-PCR. However, no product is visible for transcripts from P_Pto::GUS construct. As the oligos had been demonstrated to work by regular PCR using plasmid as template, the reason for this is not a general non-functionality of the used primer combination. The most likely explanation for being able to detect transcription of Pto, but not for Pto::GUS from the construct is silencing of the transgene in plant line 3. Alternatively, the cloned promoter might not be sufficient to drive transcription of the following gene.

Discussion

The main objective of this diploma thesis was to develop means to address the question whether a recently discovered tomato protein, Bti9, might act as a PRR in plant PAMP triggered immunity. Additionally, the method of transgenic promoter-GUS plants that was part of the attempts to elucidate the potential function of Bti9, was also applied to Pto, a R-gene with a well established function in plant immunity, which is the focus of much work in the lab.

Bti9 is the closest known homologue to CERK1, a well-studied chitin receptor in Arabidopsis. In contrast to CERK1, two different splice forms are being generated from the Bti9 locus in tomato, which differ only in the first of 12 exons. While somewhat different chitin receptors with potentially different functional mechanisms have been described in different plant species (*O. sativa* vs. *A. thaliana*), we postulated based on its homology to AtCERK1 that one or both Bti9 splice forms might function as PRRs in tomato, potentially sensing chitin. The main part of this thesis focused on the characterization of the two Bti9 splice forms and their expression patterns, transcriptional regulation and potential function. Additionally, we aimed to find out more about the expression pattern of Pto.

Expression pattern and transcriptional regulation of the two Bti9 spliceforms in tomato

Recent studies have demonstrated that in plants, alternative splicing (AS) is a common occurrence [74] [75] [76] [77] [78]. These alternatively spliced transcripts are involved in different stages of plants development and physiology, as for example flowering regulation [79] and disease resistance [80]. It was even hypothesized that more then one fifth of all plant genes are

alternatively spliced, although not all splicing variants will be biological active [81]. There have also been advances in studies regarding the relevance of AS in plant defense. Data available to date suggests that R genes are regulated by AS either by attenuation of the amount of expressed R protein or by the synthesis of alternative R protein variants [82] [83]. This all gives us indication that the two splice forms of Bti9 might have a function in the tomato immune system.

Expression levels of the two Bti9 splice forms were tested in tomato plants of different ages and in different tissue types, but no differences in expression levels across those samples are apparent. Bti9 splice form 1b is robustly expressed; expression levels are not significantly changing across plants ages or in between different tissue types as determined by qRT-PCR. The same holds true for the transcripts of splice form 1a; however, compared to splice form 1b, it is expressed only at very marginal expression levels.

To further investigate the role of the two splice forms in the plant immune system, we syringe infiltrated different PAMPs and living bacteria into leaves of 4 week old tomato plants. Although splice form 1b levels are still significantly higher than those of exon 1a, a clear up-regulation of both splice forms was visible in response to the injections. However, both splice forms are also strongly induced after syringe infiltration of water, which was included as negative control. This indicates that the up-regulation is more in response to the wounding inflicted during the process of infiltration and not a specific response to the applied PAMPs or bacteria. In future experiments, it might useful to test this possibility by changing from syringe infiltration to spray application or vacuum infiltration of the PAMPs and bacteria, to minimize physical damage to the leaves. It has been shown that fls2 Arabidopsis exhibit enhanced sensitivity to Pst when the bacteria are applied by spraying, but not after syringe infiltration into the leaf apoplast [16] [17] suggesting that FLS2, a PRR like CERK1 act early against pathogen invasion. Also, it will be interesting to repeat this experiment using chito-octaose as the elicitor, since in further experiments we were not able to detect any production of ROS in response to the chitin available in lab. One reason for this might be the fact that chitin is not very soluble in water, making it difficult to deliver it into plant

tissue. Also, this low solubility means that most of the chitin applied is probably present in the form of small crystals, and it is unclear whether those or the fraction of chitin that goes into solution are sufficient to elicit a response. Furthermore, there might be differences in the acetylation status of the different chitin preparations; this is potentially important, as it has been shown that the acyl groups can be crucial for the elicitation of PTI by chitin. The lack of acyl is also most likely the reason why we were unable to detect any response to chitosan, even though chitosan is soluble in water. It is interesting to note that there are conflicting reports in the literature regarding the potential of chitin to elicit PTI, even though most groups obtain the same product, chitin distributed by Sigma Aldrich. This chitin is produced from crab shells, and variations between different batches might lead to the situation where some preparations contain enough of the active compound to elicit a response while others do not, explaining why some groups are able to elicit a response with this reagent while others are not. Using a well-defined elicitor-prep, purified and reactylated Chito-octaose, we were able to detect the production of ROS with our assay; also, flg22 elicitor included as positive control always produced the expected results. This clearly demonstrates that neither the experiment itself nor our technique is at the base of the problem, but that the chitin used was in fact unable to elicit PTI.

Whether the changes in the transcriptional regulation of the splice forms are in response to PAMPs or in response to wounding, it is interesting to note that the induction profile is substantially different between the two splice forms; while Bti9 exon 1b is upregulated quickly, peaking after one hour and dropping back to basal levels by 6 hrs post infiltration, exon 1a is induced much later, reaching peak levels sometime between 6 to 8 hrs post infiltration. This strongly suggests that the lower expressed splice form 1a is not simply a byproduct resulting from a mis-splicing of splice form 1b; if this was the case, both forms should peak at the same time. Instead, the two forms seem to be differentially regulated. This differential regulation might be reflective of different functions or specificities of the two splice forms. As the LysM domains, which would be responsible for substrate binding and specificity, are encoded in the first exon, it is tempting to speculate that the two splice forms

might have different affinities or are binding to different ligands.

We also created transgenic tomato lines carrying promoter GUS constructs in which the first exons have individually been replaced by GUS. These will hopefully allow visualization of the expression and localization of the two Bti9 splice forms *in vivo.* The upstream region until the next gene and the coding region down to exon 7 were subcloned, followed by individual exchange of the first exon of Bti9 with GUS. We were able to get a clear staining in transiently transformed *N. benthamiana* with the construct in which exon 1b has been exchanged by GUS. On the other hand, the construct in which exon 1a has been exchanged did not produce any significant staining *N. benthamiana* cells. This could be explained in two ways: The construct does not work or the basal levels of Bti9 exon 1a are so low that not enough transcript accumulates to allow visualization of blue color with the stereomicroscope. Both the robust expression of splice form 1b and the marginal expression of 1a would be consistent with our qRT-PCR results.

Complementation of *A. thaliana cerk1-2* with Bti9 splice forms

Bti9 is the tomato gene that is most closely related to *AtCERK1*, the Arabidopsis chitin receptor; consequently, a realistic chance exists that it acts as functional homolog of this PRR in tomato, i.e. directly interacts with and recognizes chitin as a PAMP to initiate PTI. Given the lack of an insertion line collection in tomato, direct genetic testing of this hypothesis by showing that a Bti9 loss of function mutant is suppressed in its response to chitin in tomato is difficult, as it is not possible to easily obtain a knockout line. However, it has been demonstrated that the signaling pathways downstream of PRRs can be highly conserved, as transient expression of EFR in *N. benthamiana* which is normally unable to detect EF1a, renders this plant capable to detect the PAMP and initiate signaling [19]. As in this case a PRR from Arabidopsis retains functionality when expressed in a Solanaceaeous plant, we decided to test the functionality of Bti9 by expressing it in Arabidopsis, hoping that functionality would also be retained in the opposite direction (from Solanaceae to Brassicaceae). This would allow us to use the established cerk1-2 line in Arabidopsis, which represents a functional null for CERK1 and is completely unresponsive to chitin, for a complementation assay. If expression of SlBti9 in Arabidopsis cerk1-2 reconstitutes responsiveness to chitin, this would directly demonstrate the ability of Bti9 to act as a PRR for chitin.

In the course of this work, multiple constructs containing the AtCERK1 promoter followed by one of the splice forms of Bti9 were produced. As a promoter element comprising approximately 1.2 kb sequence upstream of AtCERK1 had previously been described to complement cerk1 plants, we decided to use this fragment. However, a close examination of the genomic sequence using TAIR revealed that this fragment also contains a significant portion of the neighboring upstream gene, including several exons. Therefore, we additionally generated a second set of destination vectors containing only the sequence upstream of CERK1 to the end of the 3’ UTR of the preceding gene. Also, we generated two different versions of each splice form, one

containing a stop codon to allow the expression of an untagged protein and one without STOP to allow fusion of C-terminal tags. We decided to express an untagged version, even though it will not be possible to verify its expression on protein level, as previous work in the lab expressing a C-terminal GFP fusion to Bti9 did not produce functional protein, most likely because the GFP fusion part interferes with kinase domain functionality. Even though the protein tags used in this work are much smaller than GFP, we wanted to maximize our chances to express functional protein, even in case any fusion to the C-terminus of Bti9 might prove detrimental. All constructs were generated as Gateway binary vectors based on a modified pGreenII with and without N-terminal protein fusion tags (Flag, His or AcV5).

To test whether expression of Bti9 is actually possible in Arabidopsis cells, protoplasts were transiently transformed with those of the constructs that contain the two splice forms followed by Flag tags. We additionally recombined the Bti9 noSTOP cDNAs into a Haemagglutinin (HA) tagged vector under the control of a cauliflower mosaic virus 35S constitutively active promoter. Protein expression was then visualized by Western Blot. As positive control we included a vector containing MPK6 fused to an HA tag, a protein that is very highly expressed in protoplasts (K. Munkvold, personal communication). Arabidopsis lines in which MPK6 has been silenced demonstrated that MPK6 plays an important role in plant immune response. Its function is to maintain basal resistance and to activate local disease resistance regulated by specific R genes [84].

We were able to detect strong signals for MPK6- and Bti9 exon 1a- HA fusions, whereas BTI9 exon 1b showed no visible expression. This can be explained in two different ways: either 1b splice form cannot be expressed in Arabidopsis under these conditions, or the transformation did not work. The fact that the quality of the vector preparation for exon 1b used in this experiment was much worse than that of exon1a would suggest that the latter is the case, especially as expression of an – admittedly non-functional – Bti9 exon1b-GFP-fusion in protoplasts was successful. None of the Flag-tagged versions under control of the CERK1 promoter showed expression. Since we

did not include a positive control, the reason for this remains unclear. To test whether this is due to the protein not being expressed or expression levels being too low to detect by Western Blotting (the HA tagged vectors were driven by the over-expression promoter 35S) or because the transformation or detection of the Flag tag did not work, this experiment has to be repeated together with a positive control, possibly MPK6 recombined into the same Flag-tag destination vector. However, the results demonstrating expression of splice form 1a, the less expressed splice form in tomato, can be taken as indication that it is actually possible to express Bti9 in Arabidopsis cells and consequently the complementation approach in Arabidopsis might work.

Transgenic cerk1-2 Arabidopsis containing the different Bti9 constructs were generated. Initially we were having problems with high lethality during selection of the F1 transgenic seedlings, resulting in none or only very few transgenic lines per construct. This was a consequence of the temperature on the lab light shelf peaking to more than 30ºC, resulting in heat stress that, in combination with the stress from growing on the selective medium, was killing even transgenic seedlings. While we determined this problem and, by changing to another light setup were able to get better survival rates of transgenic plants, this inconvenience made us lose time, so that at the end of this thesis only F2 individuals were growing on soil. Each of these lines originates from an individual insertion event; by assessing the segregation ratios, their insertion status (single vs. multiple insertions) will be determined. Once single insertion lines have been identified, the expression of the transgene (the plant being transgenic does not mean that the construct is not silenced) will be tested by either Western Blotting (constructs with C-terminal tags) or RT-PCR (no tag) and homozygous single insertion lines can be established. Subsequently, the tested lines will be used for complementation assays. We will test their ability to suppress bacterial growth and to produce ROS in response to different PAMPs, particularly chitin and chito-octaose. Reconstitution of a ROS response to chitin or chito-octaose would demonstrate that Bti9 is indeed a functional homolog of CERK1 and is directly involved in the binding of these PAMPs. Restoration of the suppression of bacterial growth in *cerk1-2* plants by Bti9 would indicate that it is involved in

this defense pathway elicited by an unknown PAMP from *Pseudomonas syringae*.

Expression pattern of Pto in tomato

Despite Pto being one of the best-described proteins involved in ETI, not much is known about its exact expression pattern and transcriptional regulation *in vivo.* Here, we aim at generating transgenic tomato lines expressing GUS under the control of the Pto promoter to address those questions. The advantage of this approach in comparison to qRT-PCR based analyses is that it will allow visualization of expression at a resolution below tissue types.

The complete upstream region until to the preceding gene (PtoC) was utilized as promoter, and as Pto is a single exon gene, we hypothesized that most or all relevant regulatory elements might be contained in this region. Initially, the generated promoter-GUS construct was tested transiently in *N. benthamiana*. Since the construct gave us an almost as strong coloration as our positive control (GUS cassette driven by a 35S promoter) we preceded to send it to the transformation core facility at BTI, where it was used for transformation of Rio Grande-PtoR tomatoes. Unfortunately, from the 27 potentially transgenic tomatoes produced only one was actually carrying the transgene according to genotyping. The reason for this is that Rio Grande tomatoes are generally difficult to transform, and to have any of the regenerated plants set roots at all, the regenerated calli had to be transferred from selective to non-selective medium. This might explain while so many plants not actually harboring a transgene had been carried along. Next, we tested the transgene tomato plant carrying the construct for GUS activity. To be sure that the histochemical staining would work, we included a stable line expressing an auxin inducible DR5::GUS construct from the Benková lab as positive control. While the positive control produced the expected staining pattern, no staining was

visible with the P_Pto::GUS line. As it was unclear whether this was because the transgene was not expressing correctly or whether expression levels of Pto are too low to generate detectable GUS staining, we compared the expression levels of endogenous Pto and GUS; if the promoter works as expected, the two expression levels should be comparable. While the RT-PCR confirmed expression of Pto, no signal was detectable for GUS. This shows that in this line the reporter construct is not working as expected, but the reason for this remains unclear: either the genomic sequence subcloned is not sufficient to drive gene expression, or the transgene inserted in this one transgenic line has become silenced. However, the fact that the promoter-GUS construct was sufficient to produce staining when transiently expressed in *N. benthamiana* indicates that the used promoter sequence is in principle sufficient to drive expression of the following gene in plant cells. The tomato transformation facility is currently transforming another tomato variety, Moneymaker (MM), with the P_GUS construct. As MM is easier to transform and sets roots directly on selective medium, this will hopefully result in more transgenic lines and less false positives, increasing the chances to find stable transgenic lines that are not silenced.

Summary

The tomato protein Bti9, a putative PRR with three extracellular LysM motifs, a transmembrane domain and an intercellular serine / threonine kinase domain, was discovered in a yeast two hybrid screen using the type III effector protein AvrPtoB from Pst as a bait. Bti9 is the closest known tomato homologue to *CERK1*, the chitin receptor in *Arabidopsis thaliana*. Activation of CERK1 leads to elicitation of PTI; disruption of CERK1 abolishes recognition of chitin as a PAMP in Arabidopsis. In contrast to CERK1, Bti9 is expressed in two forms, which differ only in the first exon. The alternative first exon (exon 1a) resides approx. 2.5kb upstream of the initially described transcriptional initiation site (exon 1b). The two first exons encode all 3 LysM motifs, which in CERK1 form the ligand binding site; this might mean that the splice forms have different binding characteristics. ESTs have been found for both in public databases, indicating their expression *in planta*. Due to the sequence similarity to *CERK1*, we hypothesized that Bti9 might function as a pattern recognition receptor (PRR) in tomato, potentially as chitin receptor. This thesis focuses primarily on the characterization of the Bti9 splice forms and their expression patterns, transcriptional regulation and potential function as a PRR. qRT-PCRs demonstrate that both splice forms are expressed throughout the plant, but in very different quantities: while Bti9 exon 1b is robustly expressed, expression levels of Bti9 exon 1a are very low. After syringe infiltration of PAMPs or living bacteria, both transcripts are strongly upregulated; however, as this induction is also induced by mock infiltration, it most likely results at least in part from a wounding response. The fact that the two splice forms peak at significantly different time points after induction indicates differential regulation, which might reflect functional diversification. Transgenic tomato lines carrying promoter GUS fusions for both splice forms have been generated for a more detailed analysis of expression patterns. Furthermore, Arabidopsis *cerk1-2* lines have been transformed with both Bti9 splice forms and will allow complementation assays to test a potential function as chitin receptors.

Pto is an extensively investigated R-gene from tomato conferring resistance to Pst strains expressing the effectors AvrPto and / or AvrPtoB. Still it is little known about its expression pattern *in planta*. To address this, a Pto promoter GUS construct was generated to be transformed into tomato. Expression of the reporter was confirmed by transient Agrobacterium mediated transformation of *N. benthamiana*. Because of the low transformation efficiency in Rio Grande, only one transgenic line resulted from the tomato transformation, in which the transgene was apparently silenced. The reporter construct is currently being transformed into Moneymaker tomatoes, as this strain is known to be more easily transformable.

Zusammenfassung

Bti9, ein putativer PAMP-Rezeptor aus Tomaten, besteht aus 3 extrazellulären LysM-Domänen, einer Transmembrandomäne und einer intrazellulären Serin / Threonin Kinase. Er wurde in einem “yeast two hybrid” Experiment gefunden, bei dem der durch das Typ 3 Sekretionssystem injizierte bakterielle Effektor AvrPtoB aus *Pseudomonas syringae* als Köder eingesetzt wurde. Das am nächsten verwandte Homolog in Arabidopsis stellt *CERK1* dar, welches den Chitinrezeptor kodiert. Aktivierung von CERK1 löst PAMP-induzierte Immunität aus; bei nichtfunktionalem CERK1 wird Chitin nicht mehr als Reiz wahrgenommen. Im Gegensatz zu CERK1 wird Bti9 in zwei verschiedenen Formen exprimiert, die sich lediglich in ihrem ersten Exon unterscheiden. Das alternative erste Exon (Exon 1a) liegt ca. 2.5 kb oberhalb des zuerst beschriebenen (Exon 1b). Die beiden ersten Exons beinhalten jeweils die kompletten 3 LysM Motive; in CERK1 bilden diese die Ligandenbindestelle. Dies könnte darauf hindeuten, dass sich die beiden Spliceformen in ihrer Bindungsspezifität unterscheiden. Für beide Spliceformen wurden ESTs in öffentlich zugänglichen Datenbanken gefunden, was darauf hindeutet, das beide in der Pflanze exprimiert werden. Aufgrund der Sequenzähnlichkeit zu *CERK1* stellen wir die Hypothese auf, dass es sich bei Bti9 um einen PAMP-Rezeptor handeln könnte, möglicherweise einen Chitin-Rezeptor. Diese Arbeit konzentriert sich hauptsächlich auf die Charakterisierung der beiden Bti9 Spliceformen sowie ihrer Expressionsmuster, transkriptionellen Regulation und möglichen Funktion als PAMP-Rezeptor. qRT-PCRs zeigen, dass beide Formen in der gesamten Pflanze exprimiert werden, sich aber in ihrer Expressionsstärke unterscheiden: während Bti9-Exon 1b deutlich exprimiert ist, sind die Mengen an Bti9-Exon 1a sehr gering. Die Transkripte beider Spliceformen werden in Reaktion auf die Injektion von PAMPs oder lebenden Bakterien mit Hilfe von Spritzen hochreguliert. Allerdings ist auch als Antwort auf eine Schein-Infiltration mit Wasser eine Hochregulierung feststellbar, was darauf hindeutet, das es sich zumindest teilweise um eine Wundantwort handelt. Die

Tatsache, dass die beiden Spliceformen zu sehr unterschiedlichen Zeiten ihre maximalen Expressionen erreichen, deutet auf eine unterschiedliche Regulation hin, die möglicherweise in einer funktionalen Diversifikation begründet liegt. Transgene Tomaten, die Promotor-GUS Fusionen der beiden Spliceformen enthalten, wurden geschaffen, um eine detailliertere Analyse der Expressionsmuster zu ermöglichen. Darüber hinaus wurden *cerk1-2* Arabidopsis Linien generiert, die Proteine der beiden Spliceformen aus Tomaten herstellen; diese erlauben Komplementationsanalysen zur experimentellen Überprüfung einer möglichen Funktion als Chitin Rezeptor.

Pto ist ein intensiv untersuchtes Resistenz-Gen aus Tomaten, das Resistenz gegen Pseudomonas Stämme vermittelt, die mindestens einen der beiden Effektoren AvrPto oder AvrPtoB herstellen. Bis jetzt ist noch wenig über sein Expressionsmuster in der Pflanze bekannt. Um dies zu untersuchen, wurden Konstrukte hergestellt, die den Pto Promotor zusammen mit einem GUS Reporter Gen enthalten, um in Tomaten transformiert werden zu können. Die Funktionalität dieser Reporter wurde mit Hilfe transienter Proteinexpression in *N. benthamiana* bestätigt. Aufgrund der niedrigen Transformationseffizienz des Tomatenstamms Rio Grande ergab die Transformation lediglich eine transgene Linie, in der das Transgen offenbar inaktiv war. Zur Zeit wird das Reporter-Konstrukt in Moneymaker Tomaten transformiert, da diese bekanntermaßen leichter zu transformieren sind.

Abbreviation

TLR5	Toll-like receptor
%	Percent
°C	Celsius
A-site	Aminoacyl site
APS	Ammoniumpersulfate
At	*Arabidopsis thaliana*
ATP	Adenosine triphosphate
avr	avirulence
AvrB	Avirulence B protein
AvrRpm1	Avirulence resistance to P. syringae pv Maculicola protein
AvrRpt2	Avirulence resitance to P.syringae 2 protein
cDNA	Complementation DNA
dATP	Deoxyadenosine triphosphate
ddH_2O	Double-distilled water
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
Ds-transposon	Dissociation transposon
ECL solution	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetate
EFR	EF-Tu receptor
EST	Expressed sequence tag
Fen	Fenthion
g	Gravity force
GTP	Guanosine triphosphate
H_2O	Water
HCl	Hydrochloric acid
hr(s)	Hour(s)

HRP	Horseradish peroxidase
kb	kilobase
l	Liter
M	Molar
MAPKKK	Mitogen associated protein kinase kinase kinase
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
NaCl	Natrium chloride
ng	Nanogram
OD	Optical density
ORF	Open reading frame
Os	*Oryza sativa*
OsFLS2	*Oryza* sativa FLAGELLIN-SENSING 2
P-site	Peptidyl-site
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
P_{for}	Pimer forward
pMol	Picomole
P_{rev}	Primer reverse
Prf	
Pst	*Pseudomonas syringae pv tomato*
pv	pathovar
PVDV-membrane	Polyvinylidene fluoride membrane
rcf	Relative centrifugal force
RIN4	RPM1 INTERACTING PROTEIN 4
RNA	Ribonucleic acid
RNAi	RNA interference
RPM	Revolutions per minute
RPS2	Resistance to P. syringae 2
RPT2	Root phototropism mutants

RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
s	second
SDS	Sodium dodecyl sulfate
Ser	Serin
T-DNA	Transfer DNA
TAE	Tris-acetate-EDTA
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
Thr	Threonine
tRNA	Transfer RNA
UTR	Untranslated region
UV	Ultraviolet
V	Volt
yEGFP	Yeast enhanced green fluorescent protein
µl	Microlitre
µM	Micromolar

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Dates:	1.12.2008 – 30.02.2009
Occupation:	Rotation Student
Name and address of employer:	Jan Kreuze PhD International Potato Center Germplasm Enhancement and Crop Improvement Av. La Molina 1895, La Molina 1558 Lima, Peru

Dates:	02.07.2008 – 31.08.2008
Occupation:	Rotation Student
Name and address of employer:	Peter Schlögelhofer PhD Max F. Perutz Laboratories Department of Chromosome Biology University of Vienna Dr. -Bohr-Gasse 1 1030 Vienna, Austria

Dates:	01.07.2006 – 31.08.2006
Occupation:	Internship
Name and address of employer:	Norbert Schweifer PhD Boehringer Ingelheim Department of Cellular Biology Doktor-Böhringer-Gasse 5-11 1120 Vienna, Austria

Dates:	06.07.2005 – 31.09.2005
Occupation:	Rotation Student
Name and address of employer:	Roland Schafleitner PhD International Potato Center Germplasm Enhancement and Crop Improvement Av. La Molina 1895, La Molina 1558 Lima, Peru

Education and training

Dates:	October 2004 –
University:	University of Vienna
Studies	Molecular Biology
Dates:	1990 – 2003
School	Alexander von Humboldt Elementary and High School in Lima – Peru
A – levels:	German, Spanish, English, French, Mathematics, Biology and German History

Personal skills and competences

Languages:	German (mother tongue), Spanish (mother tongue), English (fluent), French (basic levels)
Computer skills:	Mac OS X, iWork, Windows XP, Microsoft Prism Graph