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Domain shuffling between the Tm-2² and the Tm-2 proteins suggests that the leucine rich repeats determine resistance specificity

Chapter 2

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ABSTRACT

During the last 20 years different natural resistance genes against TMV have been characterized and transgenically expressed in tomato as well as in tobacco. The *Tm-2^2* and *Tm-2* gene products from tomato belong to the category of CNL resistance proteins and the genes are allelic. Seven nucleotide differences between the two alleles result in four amino acid changes between their proteins. Two of those amino acid differences are in the NBS domain Tm-2^2 ≈ Tm-2 at positions 257 (Ile/Phe) and 286 (Met/Ile) while the other two are in the LRR domain at positions 767 (Tyr/Asn) and 769 (Ser/Thr). Despite this small number of differences the *Tm-2^2* gene gives a durable resistance against Tomato Mosaic Virus (ToMV) contrary to *Tm-2*. Two chimeric genes have been generated: one which gives a gene product with the CC and NBS of Tm-2^2 and the LRR domain of Tm-2 and another which gives a gene product with the CC and NBS of Tm-2 and the LRR domain of Tm-2^2. The specificity of those genes has been assessed by transient gene expression as well as in transgenic *Nicotiana tabacum* plants using ToMV variants. The results show that the specificity of resistance is determined by the LRR region. The response of the chimera with the Tm-2^2 LRR domain is similar as the *Tm-2^2* gene while the other chimera shows a *Tm-2* like response in both experiments.

Keywords: *Tm-2*, *Tm-2^2* gene, ToMV, tomato, tobacco, domain shuffling, resistance proteins

1. INTRODUCTION

Like all organisms tomato and tobacco are confronted with pathogens like viruses, bacteria, fungi and insects during their life cycle. The innate immune system of plants, which is one of their defenses against these pathogens, needs the host to perceive a pathogen attack. This relies on two types of receptors: pathogen recognition receptors (PRRs) and resistance (*R*) proteins (van Ooijen *et al.*, 2007). While the PRRs generally recognize commonalities, R-proteins are considered to be more pathogen specific. The concept of the recognition of a single avirulence (Avr) protein of the pathogen by a single specific R protein from the plant, the gene for gene hypothesis, directed plant scientists to *R* gene studies (Flor, 1971). In different plant systems the direct or indirect interactions between *R* gene products and *Avr* gene products have been studied (Ellis *et al.*, 2007; Krasileva *et al.*, 2010; Takahashi *et al.*, 2010; Tameling & Takken, 2008). The R proteins mediate the effector triggered immunity (ETI) which is faster and stronger than the basal plant defense like PAMPs (pathogen associated molecular patterns) triggered immunity (PTI) and is mostly characterized by localized cell death around the site of infection (Tameling & Takken, 2008).

The response of tomato to Tomato Mosaic Virus (ToMV) depends on the interactions of the tomato genotype, the virus genotype and the pre- and post-inoculation environment (Pelham, 1966). During the last few years different resistance genes (*N*, *Tm-1*, *Tm-2* and $Tm\text{-}2^2$) against tobamo-viruses have been isolated from Solanaceous species, characterized and transgenically expressed in tomato and tobacco plants (Ishibashi *et al.*, 2007; Lanfermeijer *et al.*, 2003, 2005; Whitham *et al.*, 1994). These resistances together target the major viral proteins which are encoded in the viral RNA (Lanfermeijer *et al.*, 2010). The *N* resistance gene was introduced from *Nicotiana glutinosa* into *N. tabacum* and the *Tm* resistance genes: *Tm-1, Tm-2* and $Tm\text{-}2^2$ were introgressed from *Solanum hirsutum*, *S. peruvianum* and *S. peruvianum*, respectively, into *S. lycopersicum*. The $Tm\text{-}2^2$ and *Tm-2* resistances are allelic and their R proteins belong to the CNL (CC-NBS-LRR) class of resistance proteins (Lanfermeijer *et al.*, 2010). Analysis of the nucleotide sequence of ToMV strains, which were capable of breaking either $Tm\text{-}2^2$ or *Tm-2*

resistance, showed that in both cases, the movement protein (MP) is the matching *Avr* gene product (Lanfermeijer *et al.*, 2005).

The *Tm-2^2* and *Tm-2* genes were isolated from tomato and were successfully transferred into tobacco without loss in function and specificity of the resistance to the various ToMV strains (Lanfermeijer *et al.*, 2004, 2005). The resistance of the *Tm-2* gene has been broken while until now the *Tm-2^2* gene still confers a durable resistance against ToMV isolates even after more than four decades (Lanfermeijer *et al.*, 2004). The *Tm-2^2* and *Tm-2* genes have seven differences at the nucleotide level which result in only four amino acid changes at the protein level. Two amino acid differences are located in the NBS (nucleotide binding site) region and the other two are in the LRR (leucine-rich repeats) region of the *Tm-2^2* protein (Lanfermeijer *et al.*, 2005).

Domain swapping has been used in Xa3/Xa26 and Xa21 receptor-like kinase (RLK) of rice (Zhao *et al.*, 2009), and Cf-4 and Cf-9 receptor-like proteins of tomato (Van der Hoorn *et al.*, 2001; Wulff *et al.*, 2001) to study their extra-cellularly located LRR domains. Domain swapping has also been studied in the L-6 and L-11 R proteins of the flax *L* locus to identify regions involved in rust resistance specificity (Ellis *et al.*, 2007). In CNL and TNL (TIR-NBS-LRR) R proteins which are intra-cellular proteins, the LRR domain is regarded as a regulator of response specificity and NBS is the carrier of the activation mechanism through the hydrolysis of ATP (Belkhadir *et al.*, 2004; Lukasik & Takken, 2009; van Ooijen *et al.*, 2007; Takken *et al.*, 2006; Tameling *et al.*, 2002, 2006; Ueda *et al.*, 2006). In this study, we exchanged the LRR domains of the Tm-2^2 and the Tm-2 proteins with their counterparts while leaving the remainder of the protein intact to study the role of the NBS and LRR domains in the specificity of resistance.

2. MATERIALS AND METHODS

2.1. Polymerase Chain Reaction (PCR) studies to discriminate the *Tm-2* alleles

Three genotypes of tomato, GCR26, GCR236 and ATV847 which carry the *tm-2*, *Tm-2* and *Tm-2^2* alleles, respectively, were used. PCR was performed on genomic

DNA isolated with the CTAB method (van der Biezen *et al.*, 1996). Diagnostic PCR was carried out using CAPS markers (Lanfermeijer *et al.*, 2005). The primers PrRuG151 (5´GAGTTCTTCCGTTCAAATCCTAAGCTTGAGAAG3´) and PrRuG086 (5´CTACTACACTCACGTTGCTGT GATGCAC 3´) designed from the known sequence of the *Tm-2*^{2} gene (Lanfermeijer *et al.*, 2005) were used. The PCR protocol used was: 5 min at 94°C, followed by 42 cycles that consisted of 45 s at 94°C, 30 s at 55°C and 90 s at 72°C. The final extension was 5 min at 72°C. 10 µl PCR products were digested with the Bfr-1 restriction enzyme.

2.2. Virus resistance assays on tomato genotypes

Two tomato mosaic virus isolates ToMV-GeRo (*Tm-2* breaker) and ToMV-GM65 (*Tm-2*^{2} breaker) (Lanfermeijer *et al.*, 2005) were used to infect four genotypes of tomato. The tomato genotypes used were GCR236 (*Tm-2*), ATV847 (*Tm-2*^{2}), GCR267 (*Tm-2*^{2}) and Money maker VIR (*Tm-2*^{2}). The infected tomato plants were monitored for mosaic symptoms regularly. After 21 dpi (days post inoculation) a leaf homogenate of a systemic mosaic showing tomato plant was used to infect a *N. glutinosa* leaf (an indicator of ToMV). The lesions were scored on *N. glutinosa* 3-5 dpi. The details about the infection process are given in section 2.7.

2.3. Domain shuffling of the *Tm-2*^{2} and *Tm-2* carrying binary vectors

Two 9833 base pairs (bp) long binary vectors, pTM42 and pTM90 (Lanfermeijer *et al.*, 2005) carry the *Tm-2*^{2} and *Tm-2* genes, respectively. Both genes are under the control of the cauliflower mosaic virus (CaMV) 35S promoter and contain the NOS polyadenylation signal. pTM42 and pTM90 share one restriction site of AatII and SacI each at the positions 768 bp and 2628 bp of the coding sequence, respectively. Thus both vectors were digested with AatII and SacI for overnight at 37°C. The restriction products run on an agarose gel showed two fragments of 1860 and 7973 bp, respectively. For domain shuffling of gene products, the 1860 bp fragment of pTM42 was ligated into the 7973 bp fragment of pTM90 to get pTM120 and vice versa to get pTM340. The ligation mixtures were kept for 2 hrs at room temperature (Sambrook *et al.*, 1989) and transformed into *Escherichia coli* (*E. coli*) cells (strain

MM294). The transformation reactions were spread over L-Broth agar media with spectinomycin (75 µg/ml) as selection marker and kept at 37°C overnight. The transformed *E. coli* cultures were sub-cultured in L-Broth media with spectinomycin at 37°C overnight. The plasmid DNA of transformed bacterial cells was isolated with a Miniprep kit (Qiagen) and checked for integrity and size using restriction tests. In this way two recombinant binary vectors, pTM120 and pTM340 were constructed.

An empty plant vector pTM905 (negative control) was constructed by digestion of pTM90 at the SwaI and AleI restriction sites followed by the removal of the 3123 bp fragment. This fragment carries the promoter and the ORF of the *Tm-2* gene. The self-ligation of the 6710 bp fragment of pTM90 resulted in the formation of pTM905.

2.4. Transformation of the gene constructs to *Agrobacterium tumefaciens*

The plasmids pTM42 (*Tm-2*^{2}/*Tm-2*^{2}, referring to the make-up of the gene: NBS/LRR), pTM90 (*Tm-2*/*Tm-2*), pTM340 (*Tm-2*/*Tm-2*^{2}), pTM120 (*Tm-2*^{2}/*Tm-2*) and pTM905 (-/-) were introduced into *A. tumefaciens* strain LBA1119 by electroporation (Lanfermeijer *et al.*, 2004, 2005). In each case, 1 µg plasmid DNA was added to 40 µl electro competent cells of *A. tumefaciens*. In addition 1 µl pSoup (pMBP41, which serves as a helper plasmid) for each transformation reaction was also used. Transformed cells were resuspended in 1 ml L-Broth and incubated in a shaker (200 rpm) for 2 hrs at 28°C. The cultures were then centrifuged at 5100 g for 1 minute to get cell precipitates. Cells were resuspended in 100 µl L-Broth and spread over the L-Broth agar plates containing rifampicin (50 µg/ml) and spectinomycin (75 µg/ml). The plates were kept at 28°C for 48 hrs. Transformants were checked for integrity of the gene constructs by restriction analysis after isolation of the plasmid and transformation into *E. coli*.

2.5. Agro-infiltration of the transformed *A. tumefaciens* in tobacco which express the ToMV movement protein

The transformed *Agrobacterium* cells carrying gene constructs were used to inoculate 100 ml YEB_i medium containing 100 ml YEB (Yeast Extract Broth), 200 mM acetosyringone and 1 ml 1M MES (Methyl Ester Sulphonate) supplemented with 75 µg/ml spectinomycin and grown overnight at 28°C until an OD_{600} of 0.8. Cells were precipitated and resuspended to a final concentration of OD_{600} 2 in induction medium MMAi. (5 g/l Murashige and Skoog's medium, 1.95 g/l MES, 20 g/l sucrose at pH 5.6 and 200 mM acetosyringone). Cultures were incubated at room temperature for 2 hrs before agro-infiltration. Infiltration of the cultures (200 µl) was done on the under surface of the leaves of tobacco plants carrying the ToMV-MP as transgene (Lanfermeijer *et al.*, 2004) using a 2 ml syringe (without needle) (Wulff *et al.*, 2001). After 3-5 dpi localized cell death in agro-infiltrated tobacco leaves were recorded.

2.6. *A. tumefaciens* mediated transformation (AMT) *of N. tabacum*

N. tabacum SRI wild type, which is susceptible to TMV (Tobacco Mosaic Virus) and ToMV isolates, was used in this experiment. Leaf explants (1x1 cm) were dipped for 2 minutes in the *A. tumefaciens* suspensions carrying the chimeric gene constructs and dried for 1 minute before they were placed on a medium in petri dishes containing 4.41 g/l Murashige and Skoog salts including vitamins, 30 g/l sucrose, 8 g/l plant agar, 0.1 mg/l naphthalene acetic acid (NAA) and 1 mg/l benzylaminopurine (BAP) at pH 5.7 for 2 days at 22°C. In order to select the transgenic shoots and to get rid of *A. tumefaciens,* the leaf explants were transferred to the same medium supplemented with 100 µg/ml kanamycin and 250 µg/ml carbenicillin. After four weeks shoots were transferred to the same medium without NAA and BAP, supplemented with 100 µg/ml kanamycin and 250 µg/ml carbenicillin for root development (Horsch *et al.*, 1985). Subsequently plantlets which had developed a proper root and shoot were transplanted to soil in the green house and allowed to self-pollinate to get the first generation of transgenic (T-1) seeds. The T-1 seeds of ten independently transformed plants were grown for the T-

2 generation on MS_{10} agar plates with kanamycin (50 µg/ml). After three weeks, kanamycin resistant plants were transplanted to soil and grown in the greenhouse for the virus resistance studies.

2.7. Virus resistance assay

Two types of virus strains ToMV-GeRo (a *Tm-2* breaking isolate) and ToMV-GM65 (a *Tm-2^2* breaking isolate) were used to infect the transgenic tobacco plants (T-2) (Lanfermeijer *et al.*, 2005). The leaf extract of *N. tabacum* SRI wild type plants infected separately with both isolates of ToMV was used for the infection of tobacco plants after a dilution in 50 mM potassium phosphate buffer pH 7. Ten plants were used for each of the two virus homogenates while 10 plants were kept as control without inoculation. The infected tobacco plants were checked regularly to monitor the systemic spread of the virus. *N. glutinosa,* a non-compatible host for tobamo-viruses, and exhibiting local lesions upon infection, was used as an indicator plant. After 3 weeks, a homogenate of a ToMV infected systemic leaf of a transgenic *N. tabacum* plant was inoculated onto *N. glutinosa* and the lesions were scored after 3-5 days.

3. RESULTS

3.1. Discrimination between the *Tm-2* and *Tm-2^2* alleles using CAPS markers

To discriminate the *Tm-2* and *Tm-2^2* alleles carried by the tomato genotypes GCR236 and ATV847, respectively CAPS markers (Lanfermeijer *et al.*, 2005) were used. The diagnostic PCR for these CAPS markers resulted in a 1084 bp product (Lanfermeijer *et al.*, 2005). Discrimination between the alleles is based on the fact that the *Tm-2* allele has one while the *Tm-2^2* allele has two restriction sites for the Bfr-1 enzyme in the amplified region (Fig. 1). The markers were also used to verify the *Tm-2^2* and *Tm-2* genes in the plasmids pTM42 and pTM90, respectively.

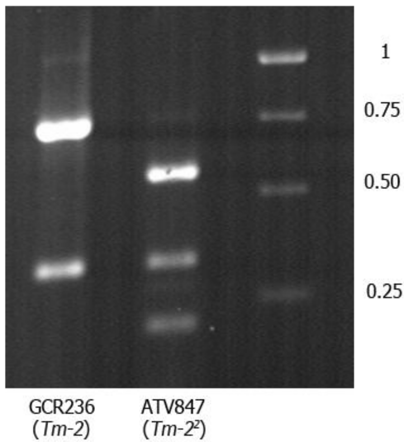

Figure 1. Discrimination of the *Tm-2* and *Tm-2*^{2} alleles using Bfr-I dependent CAPS markers. Two representative genotypes of tomato, GCR236 (*Tm-2*) and ATV847 (*Tm-2*^{2}) have been used. A 1084 bp product was amplified by PCR using primers PrRuG151 and PrRuG086 (see details in materials and methods). The fragment from the *Tm-2* gene bears one Bfr-I restriction site and this result in two fragments. The fragment from the *Tm-2*^{2} gene contains two restriction sites and this result in three fragments.
On gel Gene Ruler^{TM} 1Kb DNA ladder was used as a size marker.

The specificity of tomato mosaic virus isolates, ToMV-GeRo and ToMV-GM65, was studied by infecting those tomato genotypes. In addition GCR267 (*Tm-2*^{2}) and MM (Money maker) VIR (*Tm-2*^{2}) were also used (Table 1). This experiment confirmed the genotypes of the used tomato varieties and the specificity of the virus isolates.

Table 1. Establishment of different tomato genotypes and the tobamo-viruses by virus resistance assays

	Tomato genotypes			
ToMV isolates	**GCR236 (*Tm-2*)**	**ATV847 (*Tm-2*^{2})**	**GCR267 (*Tm-2*^{2})**	**MM VIR (*Tm-2*^{2})**
ToMV-GeRo[a]	+[c]	-[d]	-	-
ToMV-GM65[b]	-	+	+	+

[a]: *Tm-2* breaker; [b]: *Tm-2*^{2} breaker; [c]: Susceptibility; [d]: Resistance

3.2. Construction and transfer of the chimeric *Tm-2^2* genes into *A. tumefaciens*

The two binary vectors pTM42 (*Tm-2^2*) and pTM90 (*Tm-2*) were used to generate the two variants pTM340 (*Tm-2*/*Tm-2^2*) and pTM120 (*Tm-2^2*/*Tm-2*). In addition an empty plant vector pTM905 was constructed. The vector pTM340 carries a recombinant gene whose product contains the Tm-2 residues (Phe and Ile, respectively) at positions 257 and 286 and the Tm-2^2 residues (Tyr and Ser, respectively) at positions 767 and 769. The vector pTM120 contains a recombinant gene whose product contains the Tm-2^2 residues (Ile and Met, respectively) at positions 257 and 286 and the Tm-2 residues (Asn and Thr, respectively) at positions 767 and 769 (Fig. 2).

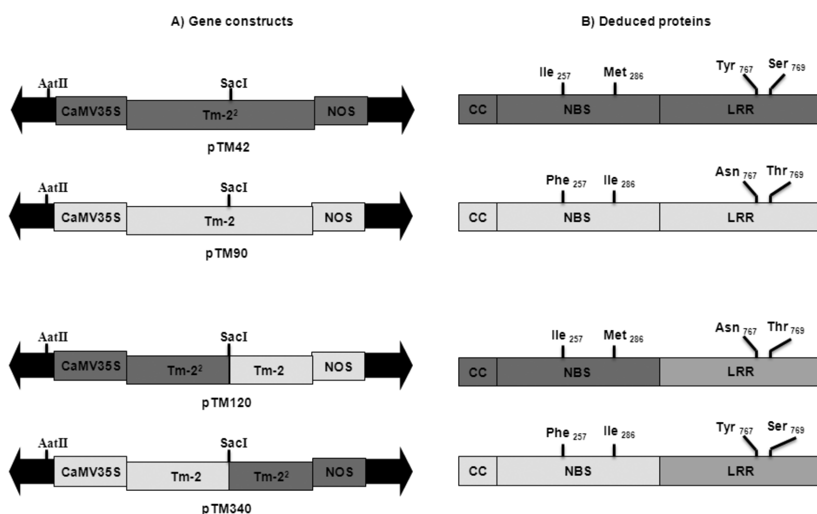

Figure 2. Schematic representation of constructs with the genes and their products made by domain swapping. Two binary vectors pTM42 and pTM90 carrying the *Tm-2^2* (dark grey) and *Tm-2* (light grey) genes, respectively have been used to construct two recombinant vectors: pTM340 (*Tm-2*/*Tm-2^2*) and pTM120 (*Tm-2^2*/*Tm-2*). AatII and SacI restriction sites were used to cut a fragment of pTM42 and this was subsequently cloned into pTM90 to get pTM120 and vice versa to get pTM340. The relevant amino acid changes in the different deduced proteins are shown with their respective positions as subscript.
CaMV35S: Cauliflower mosaic virus 35S promoter, NOS: NOS-polyadenylation signal.

The verification of pTM340 and pTM120 was done using a restriction analysis with the AflII and PsiI enzymes. The plasmid pTM340 digested with both enzymes showed the expected six fragments of 6976, 1277, 565, 513, 304 and 198 base pairs while pTM120 showed the four expected fragments of 6976, 2040, 513 and 304 base pairs (Fig. 3A and 3B).

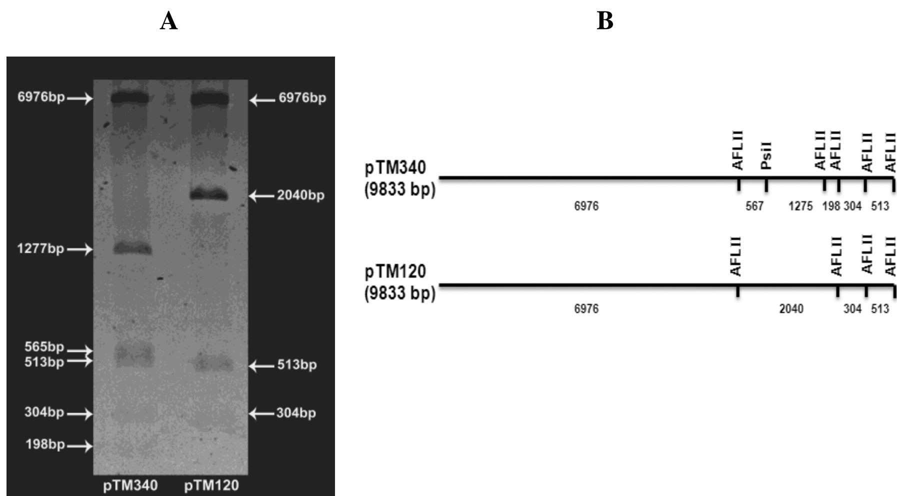

Figure 3. Restriction enzyme analysis of the pTM340 and pTM120 constructs.
A: Two recombinant vectors pTM340 and pTM120 were digested with the enzymes AflII and PsiI. pTM340 and pTM120 can be distinguished on gel electrophoretic separation by six and four fragments, respectively.
B: A restriction map of pTM340 and pTM120 digested with the AflII and PsiI enzymes shows differences between both chimeras.

3.3. Transient gene expression of the chimeric *Tm-2*^{2} constructs in transgenic tobacco plants harboring the movement protein of ToMV

Transgenic tobacco plants harboring the ToMV-MP (Lanfermeijer *et al.*, 2003) were used to study transient expression using *A. tumefaciens* carrying pTM42 (*Tm-2*^{2}*/Tm-2*^{2}), pTM90 (*Tm-2/Tm-2*), pTM340 (*Tm-2/Tm-2*^{2}), pTM120 (*Tm-2*^{2}*/Tm-2*) and pTM905 (-/-). The leaves of transgenic tobacco plants (ToMV-MP) were infiltrated with *Agrobacterium* cultures carrying the chimeric *Tm-2*^{2} genes. This showed localized cell death (hypersensitive response (HR)) around the site of *Agrobacterium*

infiltration in response to pTM42 and pTM340 constructs only. Cell death was not observed in case of infiltration with *Agrobacterium* harboring the pTM90, pTM120 and pTM905 plasmids (Table 2) (Fig. 4). These results indicated that only the constructs harboring the coding sequence for the Tm-2^2 LRR show response and that the pTM340 construct has the same response activity as the *Tm-2^2* construct.

Table 2. Transient gene expression in transgenic tobacco plants harboring the movement protein of ToMV

Construct	Protein structure (NBS/LRR)[a]	HR[b]
pTM42[c]	*Tm-2^2/Tm-2^2*	+[d]
pTM340	*Tm-2/Tm-2^2*	+
pTM120	*Tm-2^2/Tm-2*	-
pTM90	*Tm-2/Tm-2*	-
pTM905	empty plasmid	-

Each construct was assessed 30 times independently.
[a]: Arrangement of NBS and LRR domains in the deduced protein of the genes ; [b]: Hypersensitive response; [c]: Binary vectors; [d]: + indicates reaction, - indicates no reaction

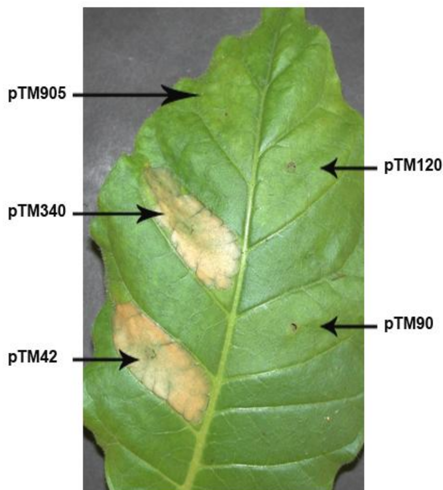

Figure 4. Co-expression of the ToMV-MP and the chimeric *Tm-2^2* genes in tobacco. The five constructs were agro-infiltrated at different positions within the same leaf of a tobacco plant harboring the ToMV-MP. The lesions were scored five days after *Agrobacterium* infiltration.

3.4. Virus specificity of the transgenic tobacco plants containing chimeric *Tm-2*^{2} genes against different tobamo-viruses

The T-DNAs of five binary vectors were transferred to *N. tabacum* separately using *A. tumefaciens* mediated transformation. The first generation of transgenic plants (T-1) was self-pollinated to get the T-2 progeny. Ten independent plant progenies of each construct (T-2) were used to check their resistance against tobamo-viruses. Ten plants of each progeny were taken for each virus isolate as well as for the control (non-infected).

The response of tobacco plants carrying the chimeric R proteins was checked against the external Avr factor (ToMV isolates) (Fig. 5).

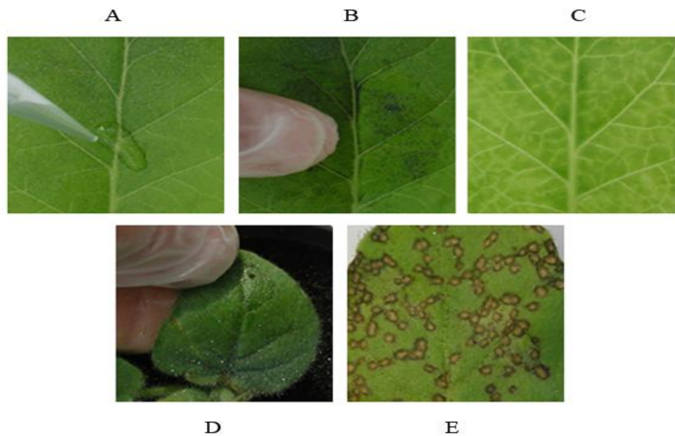

Figure 5. ToMV infection of tobacco. **A:** The upper surface of the transgenic *N. tabacum* (T-2) leaves was wounded using carborundum. A tobamo-virus suspension diluted in phosphate buffer was used for the infection. **B:** The infection was done by gently rubbing the virus suspensions on the upper surface of the leaf. **C:** Mosaic symptoms were visible on a systemic leaf of the infected *N. tabacum* plant three weeks after infection. **D:** Infection of an *N. glutinosa* leaf. A systemic leaf of the infected tobacco plant showing mosaic symptoms was cut and its extract was used to infect (an indicator of ToMV that showed localized cell death on infection). **E:** Local lesions were visible on an *N. glutinosa* leaf 3-5 days after inoculation.

The transgenic tobacco plants contained the T-DNA of pTM42 (*Tm*-2^2/*Tm*-2^2), pTM90 (*Tm-2/Tm-2*), pTM340 (*Tm-2/Tm*-2^2), pTM120 (*Tm*-2^2/*Tm-2*) and pTM905 (-/-). Those transgenic plants were infected with ToMV-GeRo (*Tm-2* breaker) and ToMV-GM65 (*Tm*-2^2 breaker). The plants carrying the constructs, pTM42 (*Tm*-2^2/*Tm*-2^2) and pTM340 (*Tm-2/Tm*-2^2) showed no resistance against the *Tm*-2^2 breaker but were resistant to the *Tm-2* breaker isolates. In contrast the transgenic tobacco plants bearing the pTM90 (*Tm-2/Tm-2*) and pTM120 (*Tm*-2^2/*Tm-2*) constructs could only be infected with the *Tm-2* breaker. The transgenic plants with the empty plant vector, pTM905 did not show any resistance against the tobamo-viruses (Table 3). These results show that the *Tm*-2^2 resistance specificity is present in the pTM340 recombinant while the *Tm-2* resistance specificity is present in the pTM120 recombinant.

Table 3. Virus specificity of the transgenic tobacco plants harboring the chimeric *Tm*-2^2 genes against different tobamo-viruses

Construct[a]	**Protein structure**[b] **(NBS/LRR)**	**ToMV-GeRo**[c]	**ToMV-GM65**[c]
pTM42	*Tm*-2^2/*Tm*-2^2	-[d]	+
pTM340	*Tm-2*/*Tm*-2^2	-	+
pTM120	*Tm*-2^2/*Tm-2*	+	-
pTM90	*Tm-2*/*Tm-2*	+	-
pTM905	empty plasmid	+	+

10 plants of 10 independent progenies of each construct were used for each ToMV isolate infection as well as kept without infection as control.

[a]: Binary vectors; [b]: Arrangement of NBS and LRR domains in the deduced protein of the genes; [c]: Virus isolates: ToMV-GeRo (*Tm-2* breaking isolate); ToMV-GM65 (*Tm*-2^2 breaking isolate); [d]: + indicates broken resistance, - indicates resistance

4. DISCUSSION

The two alleles *Tm-2*^{2} and *Tm-2* from the *Tm-2*^{2} gene locus confer resistance against ToMV with different specificities and characteristics. The resistance of the *Tm-2* gene has been broken while until now the *Tm-2*^{2} gene still confers a durable resistance against most of the Tomato Mosaic Virus isolates even after more than four decades. Two ToMV isolates: namely ToMV L11A and ToMV-2^2 can overcome *Tm-2*^{2} resistance but these viruses are crippled (Weber & Pfitzner, 1998).

The differences in resistance specificity between the two alleles reside in four changes at the amino acid level. In a comparison of proteins (*Tm-2*^{2} to *Tm-2*), the two amino acid changes, Ile^{257}Phe and Met^{286}Ile, are located in the NBS region. More specifically, amino acid position 257 is part of the Walker B motif (Lukasik & Takken, 2009) while position 286 lies in the RNBS-B (Resistance Nucleotide Binding Site-B) motif (van Ooijen *et al.*, 2007). The two other variable amino acid positions (Tyr^{767}Asn and Ser^{769}Thr) are in the second half of the LRR domain (Fig. 6).

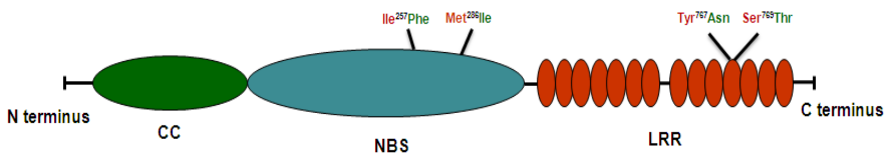

Figure 6. Diagrammatic representation of the Tm-2^2 and Tm-2 proteins. The Tm-2^2 and Tm-2 proteins have a size of 861 amino acids each. These proteins consist of several domains. Indicated are the different domains of the Tm-2^2 and Tm-2 proteins (drawn without scale): Coiled-coil domain (CC), Nucleotide binding domain (NBS), and Leucine rich repeats (LRR). The amino acids changes between the gene products of the *Tm-2*^{2} and *Tm-2* alleles are shown at positions Ile^{257}Phe (Walker B motif) and Met^{286}Ile (RNBS-B) in the NBS domain, and Tyr^{767}Asn and Ser^{769}Thr are in the C-terminal region of the LRR, respectively. The amino acids in red and green colors belong to the Tm-2^2 and the Tm-2 proteins, respectively.

The predominant view is that the LRR domain determines the specificity of the R proteins and that the NBS domain is involved in downstream signal transduction (Belkhadir *et al.*, 2004; Bittner-Eddy *et al.*, 2000; Jones & Jones, 1997; Tameling & Takken, 2008; Tameling *et al.*, 2002). More specifically even a single amino acid

change in the C-terminal region of LRR of potato Rx protein altered the specific recognition of the potato virus X (PVX)(Farnham & Baulcombe, 2006). Due to the variable nature and being under divergent selection, the LRR region is supposed as initial recognition domain (Tameling *et al.*, 2006). Our current study shows that the chimeric gene construct pTM340 exhibited the same response for the activation of cell death as pTM42 with the complete *Tm-2*^{2} gene in transient expression in tobacco plants harboring the movement protein. Similar results were also observed in the virus resistance assays with transgenic tobacco plants carrying the chimeric *Tm-2*^{2} genes. Thus a reproducible response was observed when an external R protein was introduced in the presence of a native Avr factor (Agro-infiltration) and vice versa (virus resistance assays).

Thus domain shuffling experiments between the NBS and the LRR domains of the Tm-2^2 and the Tm-2 proteins showed the significance of LRR in the response activation. We report that the two amino acid changes (Tyr^{767}Asn and Ser^{769}Thr) in the LRR domain of the Tm-2^2 protein are responsible for the specificity of the resistance. This supports the hypothesis that the resistance specificity of the Tm-2^2 protein mainly functions at the level of recognition of the viral protein (Wulff *et al.*, 2001; Zhao *et al.*, 2009). This is consistent with a presumed role of the LRR domain as a response regulator to activate effector triggered immunity (ETI). It was reported earlier that the LRR is required for the expression of an N-terminal half of a CNL R protein which contains auto-activation mutations in the NBS region but lacks the LRR domain. That could not confer the activation of ETI unless the LRR domain was co-expressed (Rairdan & Moffett, 2006; Tameling & Takken, 2008).

In response to the perception of the viral movement protein, the R protein of the host plant gives rise to a mechanism known as activation. That phenomenon causes the initiation of ETI followed by cell death. In fact the cell death takes place at the expense of the plant. That’s why loss of control over activation might lead to an uncontrolled cell death. This culminates in serious damage to the photosynthetic efficiency of plants (Tameling & Takken, 2008). To regulate activation a complementary phenomenon exists in plants called auto-inhibition. That is caused by various intra-molecular interactions between the ARC-2, a sub-domain of the

NBS domain and LRR domains in the absence as well as presence of an elicitor (Rairdan & Moffett, 2006).
pTM120 which expresses the Tm-2 protein with the two amino acids of the Tm-2^2 in the NBS domain could not confer the *Tm-2^2* specificity. This implicates that the mutations in the NBS domain of Tm-2^2, are not the reason of the *Tm-2^2* resistance response. Those might be involved in the downstream signal transduction (Tameling & Takken, 2008) but not in the recognition of the viral protein.
The recognition of the elicitor leads to an ATP-dependent conformational change in the R protein. That mechanism is important for the compatibility of interactions between the domains. Compatible interactions between the domains would activate ETI followed by cell death to restrict the pathogen. In contrast the incompatible interactions result in a limited or complete loss of activation. That might also be one of the reasons for the absence of HR by the pTM120-carrying transgenic tobacco plants. There might be limited activation that could not be expressed significantly.
For the domain exchange in pTM340, the interaction between the LRR and the ARC domains appears to be undisrupted. The transfer of signals between the domains functions properly. The chimeric R protein of the host plant recognizes the Avr protein of the pathogen and exhibits an HR to restrict the pathogen. This resistance specificity was similar to the complete *Tm-2^2* gene. The exact mechanisms by which the domains of the R proteins perceive the viral pathogen and activate defense, are still not completely understood. The results presented here support the well-known model of plant defense in which the LRR domain is thought to be involved in the elicitation of the viral pathogen and the NBS domain transfers the signal by ATP hydrolysis-mediated conformational changes.
Our studies show that the resistance specificity of the Tm-2^2 protein over the Tm-2 protein relies on two amino acids (Tyrosine^{767}and Serine^{769}) located in C-terminal of the LRR domain. Independent expression of both differential amino acids of the LRR domain of the *Tm-2^2* gene product might explain the resistance specificity in more detail.

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