Resistance specificity of the Tm-2^2 protein against ToMV is determined by one amino acid change compared to Tm-2

Chapter 3

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ABSTRACT

During their life cycle Solanaceae plants like tomato and tobacco are under continuous threat of tobamo-virus infection. Tobacco Mosaic Virus (TMV) was the first characterized tobamo-virus causing disease in both plant species. Two ToMV resistance genes from tomato, *Tm-2* and *Tm-2^2* have been characterized and expressed in tomato as well as tobacco. The *Tm-2^2* gene encodes a more durable resistance against ToMV isolates compared to the *Tm-2* allele. Surprisingly their gene products differ in only four amino acids. Of those, two amino acids are present in the NBS domain while the other two are in the LRR domain. In the present study, all possible combinations of four differential amino acids that distinguish the *Tm-2* and *Tm-2^2* alleles were made by gene shuffling. The constructs thus obtained were transferred to and expressed in tobacco. These chimeric constructs were also transiently co-expressed with ToMV-MP in tobacco. Both experiments indicate that the amino acid at position 767 determines the response specificity.

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

1. INTRODUCTION

The Solanaceae plant family contains many plant species of agricultural importance like tomato and tobacco. The virus-plant interactions have gained significant emphasis over the last few decades and tobamo-viruses have come up as a model for unfolding these interactions. Tobacco Mosaic Virus (TMV) was the first characterized tobamo-virus among viruses that cause infection to both plant species. Tomato Mosaic Virus (ToMV) belongs like TMV to the tobamo-virus group and is infectious on tomato. TMV and ToMV are closely related plus stranded RNA viruses with a rod shaped structure. ToMV has a single stranded linear positive sense 6395 nucleotides long RNA genome. This genome on translation gives 126 kDa and 183 kDa proteins along with a 30 kDa movement protein (MP) and a 17.5 kDa coat protein (CP). 2140 copies of CP are stacked helically around the RNA genome. Movement protein (MP) is responsible for cell to cell transfer of ToMV RNA through plasmodesmata and CP plays a role in systemic spread of the virus through the vascular system of the plant (Lanfermeijer *et al.*, 2003, 2004).

In tomato and tobacco, the *R* genes (*N*, *Tm-1*, *Tm-2* and *Tm-2*^{2}) against ToMV/TMV play a significant role in the study of the resistance of plants against viruses (Chapter 2; Lanfermeijer *et al.*, 2010). The *Tm-2*^{2} locus in tomato carries a single *Tm-2*^{2} gene which was isolated by transposon tagging and encodes an 861 amino acids long product. Later on it was observed that the *Tm-2*^{2} and *Tm-2* resistances are allelic and their *R* proteins belong to the CNL class. Most of the intra-cellular *R* proteins belong to the NBS-LRR class which differentiates by the amino-terminal domain known as TNL (TIR-NBS-LRRs) and CNL (CC-NBS-LRRs), respectively.

The NBS domain of tomato R proteins I-2 and Mi-1 were shown to bind and hydrolyze ATP *in vitro*. This implies a role of the NBS domain as a molecular switch that regulates signal transduction by conformational changes, ultimately leading to R protein activation (Krasileva *et al.*, 2010; Tameling *et al.*, 2002). Moreover, mutations in the conserved P-loop motif caused a loss of nucleotide binding *in vitro* and the corresponding loss of cell death *in planta* (Wirthmueller *et al.*, 2007). The intact P-loop motif is needed for ligand-induced oligomerization of the N resistance protein of tobacco (Krasileva *et al.*, 2010). According to

evolutionary studies the association of many R proteins and their respective avirulence gene products has been under selection pressure (Win *et al.*, 2007). This resulted into duplications and re-arrangements at the *R* gene loci as well as a great deal of polymorphism in the LRR region (Ellis *et al.*, 1999). That diversity of the LRR domains allows delivering a recognition function against a range of effectors.

The resistances of the *Tm-2^2* and *Tm-2* gene products have been successfully established in both tomato and tobacco. The *Tm-2^2* gene encodes a durable resistance compared to the *Tm-2* gene. Surprisingly the *Tm-2^2* and *Tm-2* gene products differ only in four amino acids. Compared to the Tm-2 protein, two amino acid differences (Phe^{257}Ile and Ile^{286}Met) are located in the nucleotide binding site (NBS) domain and the other two (Asn^{767}Tyr and Thr^{769}Ser) are in the leucine rich repeats (LRRs) domain of the Tm-2^2 protein (Chapter 2; Lanfermeijer *et al.*, 2005).

Analysis of the nucleotide sequence of ToMV strains, which were capable of breaking either *Tm-2^2* or *Tm-2* resistance, showed that in both cases, MP is the matching avirulence (Avr) gene product. This suggests that the interaction of the Tm-2^2 and the Tm-2 proteins with the movement protein (MP) of the virus could be highly similar. Mutations at different positions in the viral MP are required to combat the resistance of the R proteins (Lanfermeijer *et al.*, 2005).

Our previous study (Chapter 2) showed that two mutations in the LRR domain of the *Tm-2^2* gene product were sufficient to encode a complete *Tm-2^2* like resistance. In the present study the role of the four differential amino acids (daa's) Ile^{257}, Met^{286}, Tyr^{767} and Ser^{769} to confer a durable resistance, will be assessed separately. All possible combinations among those four amino acids in both domains will be made to study the significance of the domains as well as amino acids for resistance specificity. *Nicotiana tabacum* will be used for the transient as well as transgenic expression of those chimeric Tm-2^2 proteins.

2. MATERIALS AND METHODS

2.1. Domain exchanges between binary vectors carrying the *Tm-2²* and the *Tm-2* genes

Two 9833 bp (base pairs) long binary vectors, pTM42 and pTM90 carry the *Tm-2²* and *Tm-2* genes, respectively. In Chapter 2, these two vectors and two chimeric gene constructs, pTM340 and pTM120 are described.

2.2. Construction of *Tm-2²* variants using synthetic oligonucleotides

In the present study, four synthetic *Tm-2²* gene variants were synthesized each of which carries one of the four codons that differ between the *Tm-2* and *Tm-2²* (GeneArt AG, Regensburg, Germany).

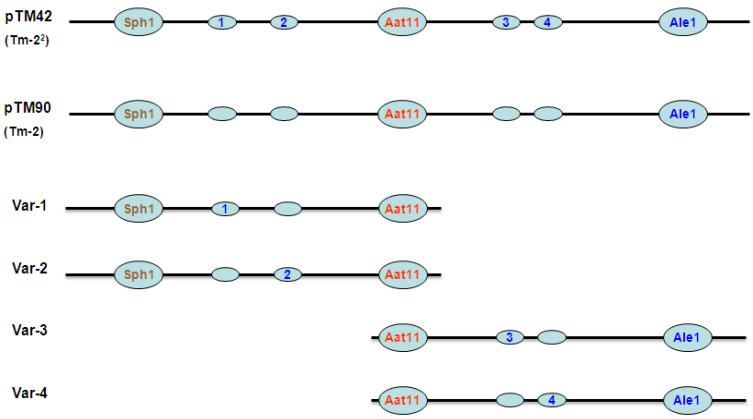

Figure 1. Four variants of *Tm-2²* fragments. Two binary vectors, pTM42 and pTM90 carry the *Tm-2²* and *Tm-2* genes, which are only shown. Four variants of *Tm-2²* fragments were synthesized each carry the codons for one of the four differential amino acids of the Tm-2² protein, respectively. Used restriction enzyme sites are also indicated. An ellipse with a number represents the Tm-2² amino acid encoding codon while an empty ellipse represents the respective Tm-2 amino acid encoding codon.

Variant 1 corresponds to an 800 bp SphI and AleI fragment, and is similar to the *Tm-2* gene except the codon for the first daa, this codon encodes the Tm-2² amino

acid variant (Ile at position 257). Variant 2 also corresponds to an 800 bp SphI and AleI fragment similar to *Tm-2* except the codon for the second daa, this codon encodes the Tm-2^2 amino acid variant (Met at position 286). Contrary to that, variant 3 contains a 1234 bp AatII and AleI region of the *Tm-2* gene except that nucleotide polymorphism which encodes for the third daa, the Tm-2^2 amino acid variant (Tyr at position 767). Variant 4 also carries a 1234 bp AatII and AleI fragment of the *Tm-2* gene except the codon for the fourth daa, the Tm-2^2 amino acid variant (Ser at position 769)(Fig. 1). Those variants were cloned in a pMA (ampR) vector (2380 bp) separately using KpnI and SacI sites (GeneArt). Hence, a set of building blocks were obtained which allowed the construction of all sixteen combinations of the four daa's.

2.3. Coding of the plasmids

Plasmids were named according to the daa encoding codons they contain except pTM90 and pTM42 which were the original *Tm-2* and *Tm-2^2* containing plasmids. The digits in the plasmid names indicate which daa-encoding codon of the *Tm-2^2* variant is present. For example: in pTM100 the first daa-codon for the Tm-2^2 variant is present, in pTM200 the second daa-codon the Tm-2^2 variant is present, in pTM120 the first and second daa-codon for the Tm-2^2 variant are present, and so on.

2.4. Cloning for construction of triple mutants

The pTM42 binary vector that carries the complete *Tm-2^2* gene and the four pMA vectors carrying the synthetic *Tm-2^2* fragments (No. 1-4) were employed to generate the four variants with three Tm-2^2 daa's. pTM42 was digested overnight at 37^{o}C with the restriction enzymes AatII and AleI resulting in a 1234 bp and an 8599 bp long fragment on a 0.8% agarose gel upon electrophoretic separation. The digestion of pMA vectors comprising the variant 3 and 4, respectively, was done with the same enzymes. This showed 1234 bp and 2380 bp fragments after gel electrophoresis. The 1234 bp long fragments of variant 3 and 4 were cloned into the 8599 bp fragment of pTM42, separately (Sambrook *et al*., 1989). This resulted into two chimeric plasmids pTM123 and pTM124, respectively (Fig. 2).

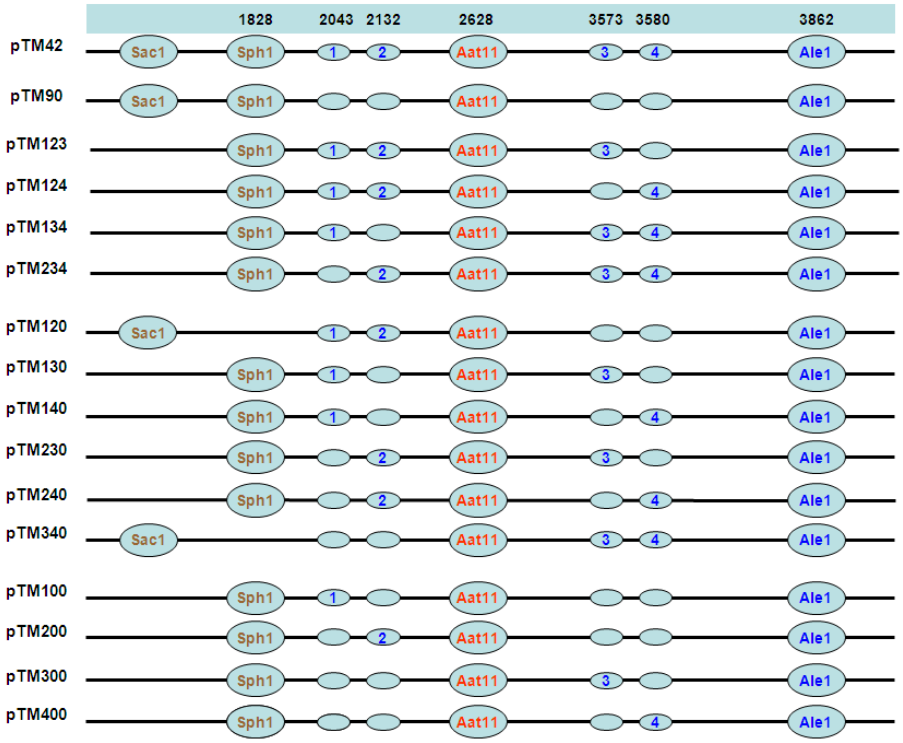

Figure 2. Schematic representation of the *Tm-2²* gene constructs. pTM42 (*Tm-2²*) and pTM90 (*Tm-2*) were used as vectors along with the four *Tm-2²* variants. In case of the construction of pTM120 and pTM340, SacI and AatII, and AatII and AleI restriction sites were used, respectively, to exchange the counter parts of pTM42 and pTM90. For the construction of the other *Tm-2²* chimeric gene constructs, a combination of SphI and AatII, or AatII and AleI restriction sites were used to exchange the counterparts of pTM42 and pTM90. For the construction of pTM100, pTM200, pTM134 and pTM234, some intermediate steps of sub-cloning were used (details are in the materials and methods). An ellipse with a number represents the Tm-2^2 amino acid encoding codon while an empty ellipse represents the respective Tm-2 amino acid encoding codon.

For the cloning of Variant 1 and Variant 2 into pTM42 both variant plasmids were digested with SphI and AatII which resulted in an 800 bp and a 2376 bp fragment. At first a 3432 bp SacI and XhoI fragment of pTM42 was cloned into pBlueScript to raise pTM906. Then the SphI and AatII fragment (5509 bp) of pTM906 was replaced with the SphI and AatII 800 bp fragments of variant 1 and 2 separately.

That resulted into two chimeric vectors, pTM908a and pTM908b. Finally the SacI and XhoI fragments of pTM908a and pTM908b were exchanged with the same counterpart of pTM42. That sub-cloning resulted into the generation of two chimeric binary vectors, pTM134 and pTM234, respectively (Fig. 2).

2.5. Cloning for the construction of single mutants

The *Tm-2* harboring pTM90 binary vector and two variants (3 and 4) cloned in the pMA vector were digested with AatII and AleI. pTM90 after digestion resulted in a 1234 bp and an 8599 bp fragment. The digested variant plasmids showed 1234 bp and 2380 bp fragments, respectively. The 1234 bp fragments were used to replace with the counterpart in the pTM90. This resulted into two plasmids in which either daa 3 or 4 were replaced by the Tm-2^2 amino acid encoding codons, pTM300 and pTM400, respectively.

The 800 bp SphI and AatII fragments of variant 1 and 2 with the *Tm-2* background carry coding regions that encode daa 1 and 2, (Ile and Met at positions 257 and 286), of the Tm-2^2 protein, respectively. That fragment of both variants was replaced separately with the counterpart of pTM90 using pBlueScript as an intermediate vector. At first the SacI and XhoI fragments of pTM90 and pBlueScript were exchanged to get pTM907. Then the 800 bp SphI and AatII fragments of Variant 1 and 2 were cloned into pTM907 to get pTM907/V1 and pTM907/V2, respectively. Finally the 6405 bp SacI and XhoI fragment of pTM90 was exchanged separately with the counterparts of pTM907/V1 and pTM907/V2 to obtain plasmids pTM100 and pTM200, respectively (Fig. 2).

2.6. Cloning for the construction of double mutants

Four single mutants' pTM100, pTM200, pTM300 and pTM400 were employed to raise four double mutants (pTM130, pTM140, pTM230 and pTM240). All single mutants carry the background of *Tm-2* except one of the differential amino acid change of *Tm-*2^2 in each case. pTM100, pTM300 and pTM400 were digested with AatII and AleI. In each case two fragments of sizes 1234 bp and 8599 bp were obtained. In case of pTM100, the 8599 bp fragment bears the coding region of *Tm*2^2

for the daa 1 (Ile at position 257). The smaller (1234 bp) fragments of pTM300 and pTM400 were cloned into the 8599 bp fragment of pTM100 separately, to get two double mutants, pTM130 and pTM140, respectively (Figure 2). Furthermore, pTM200 was also digested with AatII and AleI. In the same way as pTM100, pTM200 showed 1234 and 8599 bp fragments. The bigger fragment contained the coding region for the daa 2 (Met at position 286) of the *Tm-2²* gene product. The same 1234 bp fragments of pTM300 and pTM400 carrying the codons for daa's 3 and 4 (Tyr and Ser, respectively) of the Tm-2^2 protein, were sub-cloned into pTM200. That resulted into double mutants, pTM230 and pTM240, respectively (Fig. 2).

2.7. Transfer of the constructs to *Agrobacterium tumefaciens*

The expression vectors with the fourteen *Tm-2²* chimeric gene constructs, pTM42 with the *Tm-2²* gene, pTM90 with the *Tm-2* gene and pTM905 (an empty plant vector) were introduced into *A. tumefaciens*. Transfers were done by electroporation of the LBA1119 strain and transformants were selected as mentioned (Chapter 2).

2.8. Transient gene expression of the *A. tumefaciens* constructs

Transformed *Agrobacterium* cells bearing the seventeen gene constructs were grown overnight in 100 ml YEBi medium. Cell precipitates were resuspended in an induction medium containing MMAi for 2 hrs. at room temperature. Transgenic tobacco plants, bearing the ToMV Movement Protein (ToMV-MP) as transgene, were used for agro-infiltration of the chimeric gene constructs. Suspensions of the transformed *Agrobacterium* constructs harboring seventeen types of chimeric genes were infiltrated into the ToMV-MP expressing tobacco leaves. Infiltrations were done on the under surface of the leaf using a 2 ml syringe without a needle. The data for hypersensitive response (HR) were recorded 3-5 dpi (days post inoculation).

2.9. *A. tumefaciens* mediated transformation (AMT) of tobacco plants

TMV susceptible *N. tabacum* SRI plants were used to transfer the seventeen expression vectors using *A. tumefaciens* (Horsch *et al.*, 1985). Leaf explants were

suspended for 2 minutes into a freshly grown *Agrobacterium* culture separately for each gene construct. After drying for 1 min on a sterile filter paper, the leaf explants were put on a shooting medium consisting of Murashige and Skoog' medium, sucrose, naphthalene acetic acid (NAA) and benzyl amino purine (BAP) (Chapter 2). Two days later the explants were transferred to new plates with the same medium supplemented with 100 µg/ml kanamycin (to select transgenics) and 250 µg/ml carbenicillin (to get rid of *Agrobacterium*). Four weeks after that, the well grown shoots were transferred to the same medium except NAA and BAP (rooting medium). After a few weeks the successfully grown plantlets with good shoot and root size were transplanted to the soil in the green house. They were kept there for one life cycle of self-pollination to get the first transgenic seed progeny (T-1). The T-1 progeny of ten independently transformed plants were grown on a medium (4.41 g/l Murashige and Skoog's medium, 10 g/l sucrose and 8 g/l agar) supplemented with 50 µg/ml kanamycin. The successfully grown kanamycin resistant transgenic plantlets (T-2) were transplanted to the soil in the green house.

2.10. Infections of the T-2 tobacco plants with the ToMV isolates

ToMV-GeRo (a *Tm-2* breaking isolate) was preserved in the form of systemically infected *N. tabacum* leaves at -80^{o}C. The T-2 generations of tobacco plants were checked for their resistance against ToMV-GeRo (Lanfermeijer *et al.*, 2005). The leaf extracts of the ToMV-GeRo isolate were ground in liquid Nitrogen and diluted in a 50 mM phosphate buffer (pH 7). The diluted suspensions from ground leaf extracts were used to infect 5 independent progenies of each T-2 plant for each gene construct. Ten plants of each progeny were used for infection as well as for the control (without inoculation). The young leaves of the infected plants were monitored regularly for the systemic spread of the virus. Three weeks after infection, a homogenate of a young systemic leaf of the T-2 tobacco plant was used to infect *N. glutinosa* leaves. This plant is an indicator of ToMV and shows local lesions on infection. The data for lesions were recorded 3-5 dpi.

3. RESULTS

3.1. Shuffling of the *Tm-2²* and *Tm-2* genes to generate all possible combinations

Domain swapping between the pTM42 (*Tm-2²*) and pTM90 (*Tm-2*) binary vectors resulted in two chimeric gene constructs; pTM120 and pTM340 (double mutants, Chapter 2 and Fig. 2).

Four synthetic *Tm-2²* variants bearing the complete *Tm-2* coding region except nucleotide changes which code for one of the four differential amino acids of the *Tm-2²* gene product, respectively, were synthesised (Fig. 1). Those variants were exchanged with the counterpart of pTM42 separately to generate four *Tm-2²* chimeric gene constructs (triple mutants, Fig. 2).

The chimeric gene of pTM123 confers a similar gene product as pTM42 gene construct except Ser at position 769. Similarly the chimeric gene of pTM124 confers a gene product similar to the pTM42 except Tyr at position 767. The chimeric gene of pTM134 confers a similar gene product as pTM42 gene construct except Met at position 286. Similarly the chimeric gene of pTM234 confers a gene product similar to the pTM42 except Ile at position 257. Single mutants, pTM100, pTM200, pTM300 and pTM400 confer similar gene products as the *Tm-2* except Ile, Met, Tyr and Ser of the *Tm-2²* gene product at positions 257, 286, 767 and 769, respectively. Double mutants, pTM130, pTM140, pTM230 and pTM240 confer a same protein as the Tm-2 except Met and Ser, Met and Tyr, Ile and Ser, and Ile and Tyr of the *Tm-2²* gene product at positions 286 and 769, 286 and 767, 257 and 769, 257 and 767, respectively. An empty plant vector, pTM905 was constructed as a negative control to be used in the experiments (Chapter 2).

3.2. Transient co-expression of the *Tm-2*, the *Tm-2²* and the chimeric genes in ToMV-MP expressing tobacco via agro-infiltration

Expression of the R protein in the presence of its cognate Avr protein helps to elucidate the specific recognition model. Transgenic tobacco plants harboring the ToMV-MP were used to study the transient expression mediated by the *Tm-2*, *Tm-2²*

gene constructs and the chimeras. Altogether sixteen chimeric *Tm-2^2* gene constructs along with an empty plant vector were separately transferred into *A. tumefaciens* and were used for agro-infiltrations. The data were recorded 3-5 dpi. The infiltrated leaves of the MP harboring tobacco plants exhibited hypersensitive response (HR) in response to infiltration with the pTM42, pTM300, pTM340, pTM130, pTM230, pTM123, pTM134 and pTM234 chimeric gene constructs. In case of the pTM400, pTM140, pTM240 and pTM124 chimeric gene constructs, a limited or no reaction of localized cell death around the site of infiltration was observed. Infiltration with the gene constructs pTM90, pTM100, pTM200, pTM400, pTM120, pTM140, pTM124 and pTM905 did not show any signs of HR on the infiltrated leaves (Table 1) (Fig. 3).

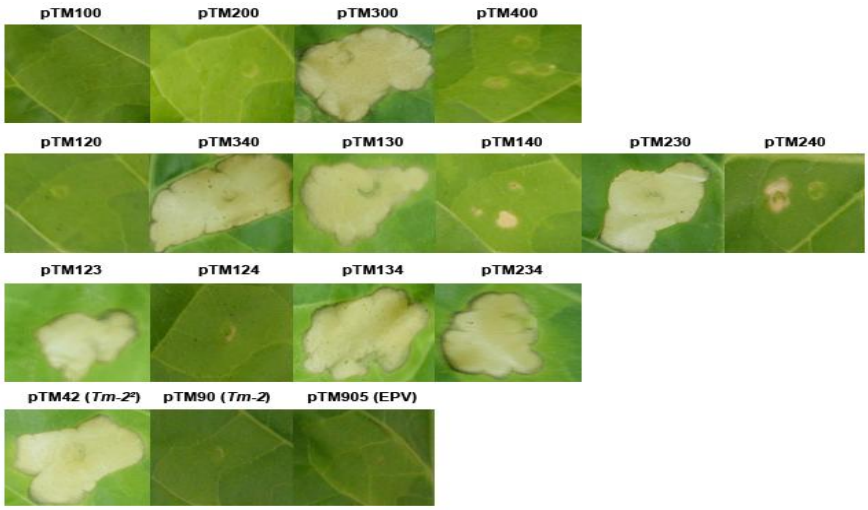

Figure 3. **Transient gene expression by co-expressing ToMV-MP and the *Tm-2^2* chimeras in tobacco**. *Agrobacterium* cells separately containing the sixteen *Tm-2^2* chimeras along with an empty plant vector (pTM905) were infiltrated into transgenic tobacco leaves expressing the MP of ToMV.

Table 1. Co-expression of the viral movement protein and the chimeric *Tm-2*^{2} gene constructs in tobacco

Construct	Arrangement of amino acids[a]	*Tm-2*^{2} specificity	
		Transient gene expression[b]	Transgenic expression[c]
pTM42[d]	1/2/3/4	+[e]	+
pTM100	1/-/-/-	-	-
pTM200	- /2/-/-	-	-
pTM300	-/-/3/-	+	+
pTM400	-/-/-/4	-*[f]	-
pTM120	1/2/-/-	-	-
pTM340	-/-/3/4	+	+
pTM130	1/-/3/-	+	+
pTM140	1/-/-/4	-*	-
pTM230	-/2/3/-	+	+
pTM240	-/2/-/4	-*	-
pTM123	1/2/3/-	+	+
pTM124	1/2/-/4	-*	-
pTM134	1/-/3/4	+	+
pTM234	-/2/3/4	+	+
pTM90	-/-/-/-	-	-
pTM905	empty plant vector	-	-

Infiltrations were done at 30 independent spots on tobacco leaves for each construct in the transient gene expression experiment.
10 plants per independent progeny of each construct were used for the ToMV-GeRo infection as well as for the control in transgenic expression studies.

[a]: Arrangement of the four differential amino acids of the Tm-2^2 protein in the deduced protein of the genes (1-4) (A number indicates the presence of the Tm-2^2 amino acid, a dash indicates the presence of the Tm-2 amino acid); [b]: Agro-infiltration of the chimeric gene constructs into ToMV-MP expressing tobacco leaves; [c]: Infections of the transgenic tobacco plants harboring the chimeric gene

Table 1. Legends continued
constructs with ToMV-GeRo (the *Tm-2* breaker); [d]: Binary vectors; [e]: + /- indicate the presence and absence of *Tm-2^2* resistance specificity, respectively; [f]: *Indicates random and less than 10% *Tm-2^2* like response.

3.3. Virus specificity of transgenic tobacco plants carrying the *Tm-2* and the *Tm-2^2* genes and their chimeras

In this study the tobacco plants carrying the *R* gene were assessed for their resistance to a tomato mosaic virus *Tm-2* breaker (ToMV-GeRo). The rational was that when the *Tm-2* breaker could infect, the plant displays a resistance with *Tm-2* specificity and when it is not the plant displays a *Tm-2^2* resistance. *Agrobacterium* constructs with the *Tm-2* and *Tm-2^2* genes and their shuffled genes were transferred to *N. tabacum* using *A. tumefaciens* mediated transformation. The kanamycin resistant plantlets were transplanted to the soil and allowed to self-pollinate to get T-1 and later T-2 generations. Those plants were used for the virus tests. The plants containing the chimeric gene constructs of pTM300, pTM340, pTM130, pTM230, pTM123, pTM134 and pTM234 expressed resistance to the *Tm-2* breaker. Hence, these plants showed the *Tm-2^2* (pTM42) like response. Contrary to that, the plants carrying the chimeras: pTM100, pTM200, pTM120, pTM140, pTM240, and pTM905 could not resist the virus isolate (Table 1). These tobacco plants exhibited mosaics and caused lesions when *N. glutinosa* leaves were infected with their systemically infected leaves. The response of these plants was like *Tm-2* (pTM90) (Table 1).

An exception to that, transgenic tobacco plants containing gene constructs pTM400 and pTM124 showed a *Tm-2^2* like resistance against the *Tm-2* breaker but not on all events. That was only for few plants of some independent progenies (data not shown).

4. DISCUSSION

The *Tm-2^2* and *Tm-2* alleles have been isolated from tomato as *R* genes against ToMV. Both alleles encode resistances belonging to the most common CNL class of R proteins in plants. In homozygous state both *R* genes confer absolute resistance

with little or no induction of HR. Relatively moderate resistance has been observed in heterozygous or even homozygous state at high temperature (>28^{o}C) (Hall, 1980; Kobayashi *et al.*, 2011). This shows their potential to induce an HR reaction to ToMV infections. Both alleles encode R proteins that exhibit four amino acid differences. Two differential amino acids (daa's 1 and 2) are present in the NBS domain of the Tm-2^2 gene product. While the other two (daa's 3 and 4) are located in the LRR domain of the Tm-2^2 protein. Four differential amino acid coding motifs that distinguish both alleles can change the resistance specificity (Lanfermeijer *et al.*, 2005). Compared to *Tm-2*, the *Tm-2^2* gene confers a durable resistance against most of the ToMV isolates (Weber & Pfitzner, 1998).

Co-expression of ToMV-MP and chimeric Tm-2^2 R proteins exhibited a specific resistance behavior against the Avr factor. The response was similar in both assays: whether an R protein was introduced in the presence of its cognate movement protein (agro-infiltration) or vice versa (virus resistance assay). The resistance behavior of the *Tm-2^2* chimeric gene constructs could be categorized based on two criteria. First the domain position of differentially displayed amino acids: the LRR or the NBS. Secondly two types of resistance specificities: the *Tm-2^2* or the *Tm-2* encoded.

Two recombinant binary vectors, pTM120 and pTM340 were constructed that differ by encoding genes with codons for daa 1 and 2, and 3 and 4, respectively. pTM120 with the *Tm-2* background but two NBS located *Tm-2^2* amino acid mutations at the protein level, conferred *Tm-2* like resistance. Contrary to that pTM340 also encodes a Tm-2 like gene product except two LRR located amino acid mutations of the Tm-2^2 protein. In tobacco this showed a complete *Tm-2^2* like response (Chapter 2). This put forward the assumption that the LRR domain is regulating the resistance specificity of the Tm-2^2 gene product which is in accordance with the general view (Belkhadir *et al.*, 2004; Tameling & Takken, 2008; Tameling *et al.*, 2002).

In this study the construction of all possible chimeras between both alleles was done to analyze the four differential amino acids between the proteins of the *Tm-2^2* and the *Tm-2* genes separately. Sixteen chimeras along with an empty plant vector

without *R* gene were co-expressed with the ToMV-MP in tobacco. This showed the same response as for pTM340 and pTM120.

Transient gene expression studies implicate that the pTM300, pTM340, pTM130, pTM230, pTM123, pTM134 and pTM234 gene constructs conferred a *Tm-2^2* like resistance response. Similar to transient gene expression, in the virus resistance assay, the same gene constructs again exhibited the *Tm-2^2* like resistance specificity. This shows that in both cases whether an *R* gene is expressed transiently or in transgenic tobacco plants, the results were the same. In fact in all gene constructs showing the *Tm-2^2* like response, one of the two LRR-located differential amino acids was present. These seven gene constructs share a similar (third) differential amino acid (Tyr at position 767) of the *Tm-2^2* gene product. This amino acid is present in the LRR domain. That depicts the significance of the LRR domain to differentiate the recognition pattern of the chimeric R proteins (Ellis *et al.*, 1999; Jung & Hwang, 2007; Takahashi *et al.*, 2010; Tameling & Takken, 2008; Tameling *et al.*, 2002).

Contrary to that, three gene constructs: pTM100, pTM200 and pTM120 showed the *Tm-2* like resistance specificity in both assays. This might be due to the fact that these mutations were in the NBS domain. That domain is supposed to be involved in signal transfer in the signal transduction pathway rather than recognition (Rairdan & Moffett, 2007). According to this view those mutations could not change the specificity and thus retained the *Tm-2* specificity in the agro-infiltration assay.

In contrast to this, the resistance behavior of four gene constructs, pTM400, pTM140, pTM240 and pTM124 was not very obvious. They mainly showed a *Tm-2* like response but in some cases they also exhibited a *Tm-2^2* like reaction. Interestingly the intensity of their *Tm-2^2* response was much less, almost 10% compared to that conferred by pTM42. These constructs share daa 4, Serine of the Tm-2^2 protein. The reason might be that this differential amino acid alone as well as in combination with daa 1 and 2 of the NBS domain causes a residual effect for recognition. This indicates a limited role of that amino acid in the recognition of the movement protein.

Generally the NBS-LRR proteins are in an auto-inhibition state due to various intra-molecular interactions (Lukasik & Takken, 2009). That is needed to avoid damage by excessive HR due to spontaneous inappropriate activation. Any manipulation or disturbance in those arrangements or interactions would release the auto-inhibition and start activation of the protein (Lukasik & Takken, 2009; Tameling & Takken, 2008). This might also be due to the fact that sometimes mutations in the NBS domain cause auto-activation. For example in the protein of the *I-2* gene of tomato, two mutations in the NBS region resulted in an auto-activating protein that induced elicitor-independent HR (Tameling *et al.*, 2006).

Based on these findings two conclusions can be formulated. First the resistance specificity of *Tm-2*^{2} compared to *Tm-2* is due the LRR domain and not the NBS domain. That is similar to findings of domain swap experiments in the *Arabidopsis RPS2* gene to determine its interaction with other host factors to perceive *Pseudomonas syringae*. Six amino acid polymorphisms in the LRR domain were reported to be responsible for its allele-specific characteristic to function with other host proteins (Banerjee *et al.*, 2001). Similarly the LRR domain of the RPP1 R protein of *Arabidopsis* was found to be involved in the interactions with its cognate ATR1 (Krasileva *et al.*, 2010). That is also in accordance with the significant role of the LRR in recognition specificity when the *Cf-4* and *Cf-9* homologs were co-expressed with their respective cognate *Avr*4 and *Avr*9 polypeptides in tomato (Wulff *et al.*, 2001). Another study using different Receptor-like kinases (*Xa3, Xa21* and Xa26) and their chimeras showed that the LRR domains of XA21 and XA3/XA26 were important race-specific determinants (Zhao *et al.*, 2009).

The significance of the LRR region might also be supported by evolutionary studies (Pfitzner, 2006). The NBS region of the Tm-2^2 protein is similar to the tm-2 and both differ from the Tm-2. Contrary to that the LRR mutations are different among the tm-2, Tm-2 and Tm-2^2 proteins (Pfitzner, 2006). An assumption might be that the uniqueness of LRR mutations causes the differences in the resistances of the *Tm-2*^{2} locus. This LRR region has been under continuous positive selection pressure from the TMV resistance gene pool. Therefore, the difference between the *Tm-2* and the *Tm-2*^{2} resistances might be concentrated in the LRR region.

The NBS domain might be involved in transfer of signals to activate the plant defense system. This is similar to the CNL type Rx protein of potato in which perception of the effector causes conformational changes in the ATPase NBS domain. That in turn allows the nucleotide exchange by the NBS domain. This results in conformational changes in the NBS and the N terminus regions, helping recognition to convert into signaling and to activate plant defenses (Rairdan & Moffett, 2007, 2006; Tameling & Takken, 2008).

The second hypothesis presumes that only one of the four differential amino acids of the Tm-2^2 protein compared to the Tm-2 protein could restore the resistance specificity. The replacement of the Asparagine of the Tm-2 protein by the Tyrosine at position 767 has shown that only one amino acid is responsible for the specific resistance response of the Tm-2^2 gene product. A single amino acid substitution in the LRR domain of Pi-ta, a rice NBS-LRR protein that confers resistance to the blast fungus altered its recognition specificity (Bryan *et al.*, 2000) and in another case has been shown to cause loss of function in yeast and *in vitro* (Jia *et al.*, 2000). Random *in vitro* mutation analysis was also done to study the effects of changes in the LRR domain of the potato Rx protein against potato virus X (PVX). The results showed that a single amino acid change in the C-terminal region of the LRR alters the specific recognition of the Avr factor (Farnham & Baulcombe, 2006). Recently the *Tm-2* and *Tm-2^2* genes and their chimeras were co-expressed with ToMV-B7 (a *Tm-2* breaker virus) by *Agrobacterium*-mediated transient expression in *N. benthamiana*. The site-directed amino acid mutagenesis showed that only Tyr^{767} was required for the *Tm-2^2* resistance specificity (Kobayashi *et al.*, 2011). Moreover transient and transgenic expression in our studies show that despite the major role of Tyr^{767} in determining the *Tm-2^2* specificity, Ser^{769} (daa 4) also appears to be involved in the recognition but with a limited role.

In general a similar response by the *Tm-2^2* chimeric gene constructs in both assays supports the assumption that the resistance specificity of the Tm-2^2 R protein mainly functions at the level of recognition of the viral protein by the LRR domain (Takahashi *et al.*, 2010). These findings support the role of the LRR domain as a response regulator in determining the resistance specificity of the Tm-2^2 R protein.

The *Tm-2*^{2} chimeric gene constructs could not restore their *Tm-2*^{2} like resistance specificity until and unless the LRR domain with the concentrated differential amino acids were co-expressed (Chakrabarti *et al.*, 2009; Tameling & Takken, 2008). The NBS domain is mainly responsible for the transduction of the downstream signals (Krasileva *et al.*, 2010; Zhao *et al.*, 2009). This is similar to a well-known mechanism of immunity followed in plant defenses. Earlier we showed that two differential amino acids in the LRR region of the Tm-2^2 R protein are required for the specific durable resistance of the gene (Chapter 2). This study shows that of these two amino acids, only one amino acid substitution is sufficient to show the typical *Tm-2*^{2} response. The replacement of Asparagine by Tyrosine at position 767 in the LRR region of the *Tm-2* protein is responsible for the deduced resistance specificity of the Tm-2^2 protein.

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