Chapter B4

Resistance to *Tobacco Mosaic Virus* and *Tomato Mosaic Virus* in Tomato

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Introduction

Tobacco mosaic virus (TMV) and *Tomato mosaic virus* (ToMV) cause a serious disease in tomato, with systemic mosaic symptoms and losses in fruit yield and quality. Both viruses are closely related tobamoviruses, plus stranded RNA viruses with a rod like particle structure. The genomic structure of TMV and ToMV has been well characterized, as a positive-sense single-stranded RNA genome that encodes at least four proteins (Goelet et al. 1982; Ohno et al. 1984; Canto et al. 2004). The 130 kDa methyltransferase/helicase and the 180 kDa RNA dependent RNA polymerase are translated directly from the genomic RNA using the same first initiation codon, the latter is synthesised by the read-through of the amber termination codon of the 130 kDa protein gene. The movement protein (MP) and the coat protein (CP) are translated from their respective subgenomic mRNAs, which are synthesised during the replication cycle. Involvement of the 130 kDa and 180 kDa proteins in intracellular replication has been demonstrated by deletion or substitution mutants of each protein (Ishikawa et al. 1986). It has also been shown that the MP is involved in cell-to-cell transport (Meshi et al. 1987), and that the CP is involved in long-distance movement (Saito et al. 1990; Hilf and Dawson, 1993). In tomato, TMV infection is more or less a rare event because the virus is soon competed out in tomato populations by ToMV, which is more adapted to

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this host plant. Both viruses are readily sap transmitted but can also be transmitted through root infection from contaminated soil. ToMV is present in the external mucilage, testa and sometimes endosperm of tomato seeds, but was not proved to be within the embryo (Broadbent, 1976). The percentage of contaminated seeds varies greatly in different fruits; up to 94% of the seeds may contain the virus (Broadbent, 1976). Because ToMV infection is aggressive and highly contagious, many breeding programs were started to find sources of resistance against this virus. So far, three dominant resistance genes have been found in wild *Lycopersicon* species and introduced into commercial tomato lines: ***Tm-1*** (from *Lycopersicon hirsutum*), ***Tm-2*** and ***Tm-2^2*** (both from *Lycopersicon peruvianum*) (Pelham, 1966; Pelham, 1972).

Tm-1

Breeding of ToMV resistance based on the Tm-1 gene

The first report of any form of resistance to ToMV in the genus *Lycopersicon* came from infection experiments with plants of *L. hirsutum* grown from seeds collected in South America. Infected plants were symptomless even though low levels of virus were detected in their tissues (Porte et al. 1939). In the following decades many plant breeders tried to transfer this resistance gene from *L. hirsutum* into *L. esculentum* (for a review see Pelham, 1966). A work of major importance was started in 1941 in Hawaii (Frazier et al. 1946) with the aim to transfer resistance from *L. chilense* and *L. hirusutum* to *L. esculentum*. This breeding program yielded useful breeding stock for a number of different attempts to obtain new resistance sources. Holmes (1954) used the back cross method for transferring resistance from the Hawaii material into a susceptible variety of *L. esculentum*. The gene was later given the symbol *Tm-1*. In 1960 a back cross breeding programme was started at the Glasshouse Crops Research Institute in Sussex, England aiming to incorporate ToMV resistance into commercial tomato varieties. *Tm-1* was included in this breeding programme. A homozygous line was generated and the resistance gene was mapped on chromosome 5 (Pelham, 1972).

Characterisation of the resistance mechanism of Tm-1

Major contributions concerning the understanding of the resistance mechanism of *Tm-1* were made by Motoyoshi and Oshima (1977, 1979) and Fraser and coworkers (Fraser and Loughlin, 1980; Fraser et al. 1980). Motoyoshi and Oshima isolated protoplasts from Pelham's breeding lines, which contained the *Tm-1* resistance gene in a homozygous form in the genetic background of the tomato line "Craigella". By infecting the protoplasts with ToMV they showed, that the *Tm-1* resistance is not only found in differentiated tomato plants but also in isolated protoplasts. This was surprising, because many classical resistance genes like N' are not active in protoplasts but rather depend on differentiated tissue to induce a defence reaction (Pfitzner and Pfitzner, 1990). Furthermore, Motoyoshi and Oshima could show by inoculation of tomato plants homozygous for *Tm-1* with ToMV RNA, that this resistance is also effective against infection with RNA inocula. This indicated that the *Tm-1* resistance somehow interferes with ToMV RNA replication rather than virus uncoating. These experiments were confirmed by Fraser and co-workers (1980) who performed infection experiments with tomato plants without any resistance gene (GCR 26, +/+), plants heterozygous for *Tm-1* (J484, *Tm-1*/+), or tomato plants homozygous for *Tm-1* (GCR 237, *Tm-1*/*Tm-1*) with ToMV. Furthermore, they could show that inhibition of virus replication by the *Tm-1* gene is gene dose dependent. Thus, while in homozygous *Tm-1* tomato plants, virus RNA accumulation was reduced down to 1% in comparison to susceptible tomato lines, heterozygous plants only show a reduction to 10%. This is in good agreement with the data from tomato protoplast infections obtained by Okada and co-workers (Yamafuji et al. 1991). In addition, Fraser and co-workers (1980) showed, that the virus responsible for the low level ToMV replication in *Tm-1* plants is not a resistance breaking virus strain, but that the low background replication rate is still possible in the presence of the *Tm-1* gene. Furthermore, they demonstrated that the *Tm-1* resistance also suppresses symptoms.. *Tm-1*/*Tm-1* and *Tm-1*/+ tomato plants show no viral symptoms like leaf mosaic or malformation of tomato fruits, although a considerable amount of ToMV (up to 10 % of the wild type rate) was found in the heterozygous plants. This feature made *Tm-1* even more attractive for plant breeders.

Tm-1 resistance breaking ToMV strains

Soon after the introduction of *Tm-1* into commercial breeding lines ToMV strains were observed which were able to overcome the *Tm-1* gene. In fact, it turned out that *Tm-1* breaking ToMV strains occur quite frequently and could be isolated from many different locations where tomato plants are

grown (for a review see Pelham, 1972). These ToMV strains caused severe mosaic symptoms on *Tm-1*/*Tm-1* plants (Pecaut, 1966), and could replicate in protoplasts of tomato plants homozygous for the *Tm-1* gene to even higher levels as in wild type tomato plants (Motoyoshi and Oshima, 1979). After the cDNA cloning and sequencing of the RNA genome of TMV (Goelet et al. 1982) and ToMV (Ohno et al. 1984), the tools were available to determine the molecular basis of the *Tm-1* resistance-breaking phenotype. Several *Tm-1* breaking ToMV strains were sequenced by Okada and co-workers (Meshi et al. 1988) and Pfitzner and co-workers (Strasser, 2002). Comparison of the nucleotide sequence of all these virus strains and also of the deduced amino acid sequence of the respective viral proteins revealed that all *Tm-1* breaking ToMV strains contained amino acid exchanges in the overlapping open reading frames of the 130 kDa /180 kDa replication proteins (Fig. 1). In fact, all amino acid exchanges are found in a small region of about 150 amino acids at the C-terminus of the 130 kDa methyltransferase/helicase protein. Mutation analysis of these ToMV strains revealed that at least two amino acid (aa) exchanges (aa 979 Gln > Glu and aa 984 His > Tyr) are necessary to overcome the *Tm-1* resistance. Single substitutions resulted in secondary, compensatory amino acid exchanges in this area of the 130 kDa / 180 kDa proteins (Meshi et al., 1988). However, amino acid 979 (Gln) can not only be replaced by glutamic acid, but also by lysine or aspartic acid and to a minor extent by arginine and asparagine (Hamamoto et al. 1997). The resulting virus is a viable ToMV strain, which is able to overcome the *Tm-1* resistance gene. These results indicate that the region between amino acid 900 – 1100 is not important for the function of these proteins in the replication complex but is important for the interaction with the putative *Tm-1* gene product. However, the *Tm-1* gene product must be an integral part of each replication complex, because a *trans*-complementation is not possible. *Tm-1* breaking ToMV strains are not able to rescue the replication of wild type ToMV in protoplasts from tomato plants containing the *Tm-1* gene (Yamafuji et al. 1991).

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aa-Pos.   940               970 971               990
130.0     INRVTGFPYPA--//--RCP ADVTHFLNQRYEGHVMCTSS
130.1     INRVIGSPYPA--//--RCP ADVTHFLNERYEGYVMCTSS
130.Lta1  INRVTGFPYPA--//--RCP ADVTHFLNERYEGYVMCTSS
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Fig. 1. Amino acid sequence of the C-terminus of the 130 kDa protien of wild type ToMV (130.0, Ohno et al.1984) , and of two *Tm-1* breaking ToMV strains (130.1, Strasser, 2002 and 130.Lta1, Meshi et al. 1988). Amino acid exchanges are depicted in bold letters.

Tm-2 and Tm-2²

Breeding of ToMV resistance based on Tm-2 and Tm-2²

A second dominant gene for resistance to ToMV was isolated from the Hawaiian breeding lines by Soost (1958, 1959, 1963). It was reported to confer a higher level of ToMV resistance than *Tm-1* and was allocated to chromosome 9. It was given the symbol *Tm-2*. Unfortunately, an undesirable recessive gene, "netted-virescent" (*nv*), which caused stunting and yellowing in the homozygous condition was found to be tightly linked to it (Clayberg, 1959). Attempts to break this linkage were unsuccessful but a new source of *Tm-2* was located in *L. peruvianum* by Laterrot and Pecaut (1969), which did not contain the *nv* gene.

An additional gene for resistance to ToMV infection was selected from a cross with *L. peruvianum* P.I. 128650 (Alexander, 1963). The resistance was found to be due to a single dominant gene. Pecaut (1965, 1966) and Schroeder et al. (1967) studied the allelic relationship of the gene *Tm-2* and the new resistance factor. Both were shown to be on chromosome 9, either at the same locus or extremely closely linked. Because of the apparent allelism with *Tm-2,* the gene symbol *Tm-2²* was proposed.

Characterisation of the mechanism of Tm-2 and Tm-2²

The first information on the possible resistance mechanism of *Tm-2* and *Tm-2²* came from infection experiments done by Pelham (1964). He found that both resistance genes are effective against ToMV. Sometimes, however, a necrotic reaction occurred in both genotypes in response to infection by common strains of the virus. This reaction was of two types, either local necrotic lesions or systemic necrosis. The former usually appeared on inoculated leaves within four days of inoculation and is regarded by most breeders as a hypersensitive reaction - an expression of resistance. The other form of necrosis, the systemic reaction, is considered to be a consequence of incomplete dominance. It is shown slightly by *Tm-2* and particularly by *Tm-2²* genotypes at higher temperatures. Many plant breeders also refer to this type of necrotic reaction as systemic hypersensitivity, even though the term hypersensitivity normally refers to systems where pathogens are localised in necrotic lesions. The development of the necrotic phenotype also depends on the gene dose. ToMV produces no necrosis at any temperature on *Tm-2/Tm-2* plants but it does on *Tm-2*/+ plants at 30°C. Systemic necrosis appears on *Tm-2²* /+ plants at 25°C and on *Tm-2²*/*Tm-2²* tomatoes at 30°C.

Another line of information concerning the mode of action of the *Tm-2* alleles came from protoplast infection experiments. Motoyoshi and Oshima

(1975, 1977) found that leaf mesophyll protoplasts from isogenic lines of *Lycopersicon esculentum* cv. Craigella carrying *Tm-2/Tm-2* or *Tm-2²/Tm-2²* were as readily infected by ToMV as those without any *Tm* gene. ToMV growth and final yield of the virus between the three types were not significantly different. However, virus multiplication in leaf discs was limited by the presence of *Tm-2* and *Tm-2²*. These results suggested that the genes might be acting to prevent cell-to-cell movement of virus.

Resistance breaking ToMV strains for Tm-2 and Tm-2²

McRitchie and Alexander (1963) in Ohio, USA identified four tomato strains of TMV (ToMV), designated I, II, III and IV, by the reactions produced by one resistant line of cultivated tomato and three accessions of *L. peruvianum*. Further characterisation of these naturally occurring ToMV strains by Pelham (1972) revealed that Alexander strains I and II cannot overcome *Tm-1* or *Tm-2* and were classified as ToMV-0. Alexander strain III is able to overcome *Tm-1* and was named ToMV-1. Alexander strain IV was able to infect T*m-2* containing tomato plants and was designated ToMV-2. Several other *Tm-2* breaking ToMV strains were recognized and characterized in detail (Pelham 1972; Hall, 1980).

To analyse the molecular interaction between ToMV and the *Tm-2* resistance genes, different *Tm-2* breaking viruses from Italy (Strasser, 2002), from Japan (Meshi et al. 1989) and from the Netherlands (Calder and Palukaitis, 1992) were sequenced. All these ToMV strains contained nucleotide exchanges in the ORF coding for the 30 kDa MP in comparison to ToMV-0, which resulted in amino acid substitutions (Fig. 2). Interestingly, there is one amino acid exchange (aa 133 Glu > Lys) found in all *Tm-2* breaking virus strains, indicating that this region of the 30 kDa MP is important for the recognition of the *Tm-2* gene. As observed for the *Tm-1* overcoming virus isolates, there is always a second amino acid substitution in the *Tm-2* breaking MP, probably to compensate for the structural changes caused by the first amino acid substitution.

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aa-Pos.  36                55 56                75
30.0     VSKVDKIMVHENESLSEVNL LKGVKLIEGGYVCLVGLVVS
30.2     VSKVDKIMVHENESLSEVNL LKGVKLIEGGYVCLVGLVVS
30.Ltb1  VSKVDKIMVHENESLSEVNL LKGVKLIEGGYVFLVGLVVS
         76               126 127              146
30.0     GEWNL----//----VPNYG ITTKDAEKNIWQVLVNIKNV
30.2     GEWNL----//----VPNYG ITTKDAKKSIWQVLVNIKNV
30.Ltb1  GEWNL----//----VPNYG ITTKDAKKNIWQVLVNIKNV
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Fig. 2. Partial amino acid sequence of the 30 kDa protein of wild type ToMV (30.0, Ohno et al. 1984), and of two *Tm-2* breaking ToMV strains (30.2, Strasser, 2002 and 30.Ltb1, Meshi et al. 1989). Amino acid exchanges are depicted in bold letters.

In contrast to *Tm-1* and *Tm-2*, *Tm-2²* remained in commercial use for several years. Only two isolations of *Tm-2²* overcoming virus strains have been reported (Hall, 1980; Rast, 1975). These ToMV strains were less virulent than wild type isolates (Fraser et al. 1989) and were therefore not capable of becoming a threat to the durability of the *Tm-2²* resistance. Two ToMV-*2²* strains have been cloned and sequenced (Calder and Palukaitis, 1992; Weber et al. 1993). Both strains contain amino acid exchanges in the 30 kDa MP. This was not unexpected because, as an allele of *Tm-2*, the *Tm-2²* gene was supposed to interact with the same viral gene product. However, the amino acid exchanges are different from the substitution of the *Tm-2* resistance breaking virus strains. Four amino acid alterations were found for both ToMV-*2²* movement proteins, three of which are identical (aa 130 Lys > Glu , aa 238 Ser > Arg, aa 244 Lys > Glu). These results already indicate that the evolution of *Tm-2²* resistance breaking viruses requires much more drastic changes in the viral sequence than are required for breakage of *Tm-2* or *Tm-1* resistance.

Molecular analysis of the interaction between the 30 kDa movement protein of ToMV and the resistance genes Tm-2 and Tm-2²

From the sequence analysis of *Tm-2* and *Tm-2²* breaking ToMV strains it was clear that both resistance genes somehow interact with the 30 kDa movement protein. On the other hand, although being alleles, both resistance genes behave quite differently. *Tm-2* can be overcome by naturally occurring virus strains, while *Tm-2²* has been stable for decades and so far only few, very ineffective *Tm-2²* breaking virus strains have been isolated. The first series of experiments to study this phenomenon was conducted by generating recombinant viruses with different numbers of the amino acid substitutions originally found in the resistance breaking movement protein. Meshi et al. (1989) showed that a single amino acid exchange at position 133 (Glu > Lys) or at position 68 (Cys > Phe) yielded virus mutants which could partially overcome the *Tm-2* resistance. Both substitutions are needed for a full virulent phenotype in *Tm-2* tomatoes. Weber et al. (1998) showed that *Tm-2²* recognition requires the C-terminus of the ToMV MP and that both amino acid substitutions in this region (aa 238 Ser > Arg, aa 244 Lys > Glu) are needed for overcoming the resistance gene.

Virus mutants can give only limited information on the molecular interaction between a viral gene product and a plant resistance gene because the number of mutants that can be generated is restricted by the functional requirements of the particular gene product. In addition, the MP mutants analysed could not distinguish between the two potential modes of action for the resistance genes: restriction of virus movement mediated directly by *Tm-*

2 and *Tm-2²* , or interaction of *Tm-2* and *Tm-2²* with the ToMV MP eliciting a hypersensitive defence reaction. To distinguish between these possibilities, Weber et al. (2004) generated transgenic tomato lines with constructs comprising the MPs of wild type or resistance breaking ToMV strains in the cultivar *Moneymaker*, which does not contain a resistance gene against ToMV. To investigate the effect of different domains of the movement protein in the resistance reaction, deletion mutants of the ToMV movement protein as well as N- and C- terminal fusions of the 30 kDa protein to the β-glucuronidase (GUS) coding region were constructed and transferred to *Moneymaker* tomatoes. The transgenic lines were crossed to isogenic lines of the tomato cultivar *Craigella,* containing either *Tm-2*, *Tm-2²* or no resistance gene. The phenotypes of the germinated progeny were scored and the results are summarised in Figure 3.

In the *Tm-2* genetic background the MPs of ToMV-0 (pTA.30-L) and ToMV-2² (pTA30. 2²), but not ToMV-2 (pTA30. 2), were able to elicit a necrotic reaction, indicating that *Tm-2* resistance involves a hypersensitive reaction. Fusion of the 30 kDa protein to β-glucuronidase (pTA30G, pTAG.30) and C-terminal deletions (pTA30.5) did not abolish the necrotic response. These results indicate that *Tm-2* recognizes a well-defined domain in the N-terminal part of the movement protein.

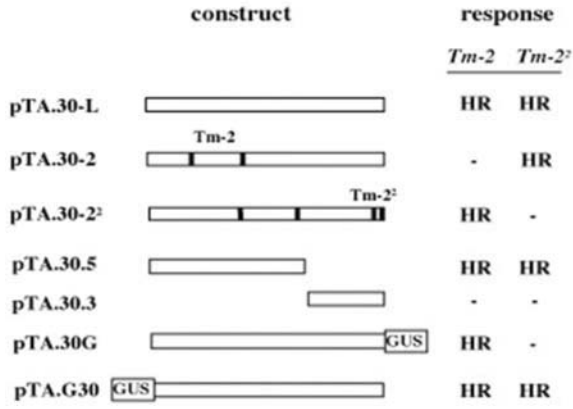

Fig. 3. Responses of tomato plants containing the *Tm-2* or the *Tm-2²* gene upon expression of different ToMV movement protein constructs. Expression of construct TA.30G, although containing resistance inducing sequences for both resistance genes, elicits differential reactions in tomato plants carrying *Tm-2* or *Tm-2²*, thus suggesting that the native three dimensional structure of the ToMV movement protein is crucial for interaction with the *Tm-2²* gene. HR = hypersensitive reaction.

Similar results were obtained for the full length ToMV MP in the *Tm-2*^{2} genetic background. The MP of ToMV-0 (pTA.30-L)and ToMV-2 (pTA30.2), but not the resistance breaking ToMV-2^2 (pTA30. 2^2), induced a necrotic reaction in the progeny seedlings. These results support the hypothesis that the *Tm-2*^{2} resistance gene also induces a hypersensitive reaction. However, the deletion mutants and the fusion constructs gave a completely different picture of the interaction of *Tm-2*^{2} with the 30 kDa protein in comparison to *Tm-2*. Modification of the C-terminus of the 30 kDa protein (pTA30.G) completely abolishes the elicitor activity of the protein. On the other hand, expression of the 78 amino acid C-terminal peptide (pTA30.3) was not sufficient on its own to induce a necrotic reaction. The N-terminal part of the 30 kDa MP (pTA30.5) which is inactive in the context of the full length protein, leads to a delayed necrotic response if expressed together with the *Tm-2*^{2} resistance gene. These results show, that the interaction of *Tm-2*^{2} with the 30 kDa protein is complex. It involves at least two different binding sites, one at the C-terminus and a second one at the amino terminal part of the movement protein (Fig. 4).

Cloning of the Tm-2 and Tm-2^2 *the resistance genes*

Many laboratories have tried to isolate the *Tm-2* genes because of the durability of the *Tm-2*^{2} gene. Molecular cloning of these genes via the map based approach turned out to be difficult, especially due to the lack of recombination in the centromeric region of chromosome 9 (Tanksley et al. 1992; Sobir et al. 2000). Two groups independently designed a transposon tagging approach (Silber, 2001; Lanfermeijer et al. 2003), which resulted in the isolation of the *Tm-2* (Gerhardts and Pfitzner, 2003) and the *Tm-2*^{2} (Lanfermeijer et al. 2003) resistance genes. The observation of Weber and Pfitzner (1998), who showed that the cross between tomato plants containing the *Tm-2*^{2} gene or the *Tm-2* gene and transgenic tomato plants expressing the ToMV MP gene results in a progeny which dies after germination, was used to develop a selection method.

Plants with the lethal combination of the *Tm-2* or the *Tm-2*^{2} gene and the MP will survive if the resistance gene is inactivated by the insertion of a transposable element. For both resistance genes, tagging lines were developed which contained a Ds-element closely linked to the *Tm-2* locus (Knapp et al. 1994). The Ds-elements were activated by an immobilised Ac-element (sAc: Jones et al. 1992) and the mutagenized tomato lines were crossed with the screening lines, which contained the ToMV MP transgene. The progeny were germinated and surviving plants were obtained. However,

further analysis revealed, that most of the surviving tomato plants had either lost the transgene (Silber, 2001) or the resistance gene (Lanfermeijer et al. 2003) by recombination. Finally, plants were identified, which contained a transposon inserted in the respective resistance gene.

These genes were isolated, their sequences analysed and the corresponding alleles were amplified by PCR (Gerhardts and Pfitzner, 2003; Lanfermeijer et al. 2003). The genes for *Tm-2* and *Tm-2²* both contain one open reading frame of 2586 bp, which translates into a protein of 861 amino acids. Alignment of the predicted proteins with the data bases revealed that the *Tm-2* genes belong to the CC-NBS-LRR class of resistance genes. The highest homology was found with the RPP13 gene from *Arabidopsis thaliana*, which confers resistance to infection with *Peronospora parasitica* (Bittner-Eddy et al. 2000). In the first 100 N-terminal amino acids 12 putative leucine zipper motifs (CC) could be recognized. A NB-ARC (nucleotide binding site, apoptosis, resistance gene products, CED4) (Hammond-Kosack and Jones, 1997) region between amino acid 145 – 441 was predicted using the BLAST-P program. The carboxy-terminal part of the *Tm-2* protein contains 16 LRR (leucine rich region) domains, typical for most resistance genes. The *Tm-2* gene product contains 40 amino acid substitution in comparison to the susceptible allele (*tm-2*), two exchanges in the CC domain, 6 exchanges in the NB-ARC domain and 32 in the LRR domain, which is consistent with the hypothesis that the LRR region of the resistance genes is mainly responsible for the recognition of the pathogen (Ellis et al. 2000). Interestingly, *Tm-2* and *Tm-2²* are highly homologous, with only four different amino acids in the putative protein sequence. The differences in the amino acid sequence are in the NB-ARC region at aa 257 Phe (*Tm-2*) > Ile (*Tm-2²*) and aa 286 Ile (*Tm-2*) > Met (*Tm-2²*) and in the LRR region at aa 767 Asn (*Tm-2*) > Tyr (*Tm-2²*) and aa 769 Thr (*Tm-2*) > Ser (*Tm-2²*).

The differences in the amino acid sequence between *Tm-2* and *Tm-2²* are significant for two reasons. Firstly, deletion analysis had revealed that, in contrast to *Tm-2,* there are at least two different sites of interaction between the *Tm-2²* resistance gene and the ToMV 30 kDa protein (Weber et al. 2004). Therefore, alterations at two domains of the respective gene product of *Tm-2²* in comparison to *Tm-2* would have been predicted. This is in good agreement with the results of Baulcombe and co-workers (Moffet et al. 2002), who showed that the Rx resistance gene, a CC-NBS-LRR resistance gene against *Potato virus X*, also requires two protein domains, the NB-ARC region and the LRR region for the recognition of the viral coat protein. The results of the molecular interaction experiments and the sequence comparisons are summarised in Figure 4.

The second, interesting aspect, which comes from the comparison of the sequences of *tm-2, Tm-2* and *Tm-2*^{2}, contributes to the question of the origin of *Tm-2* and *Tm-2*^{2}. Although originally obtained from different sources

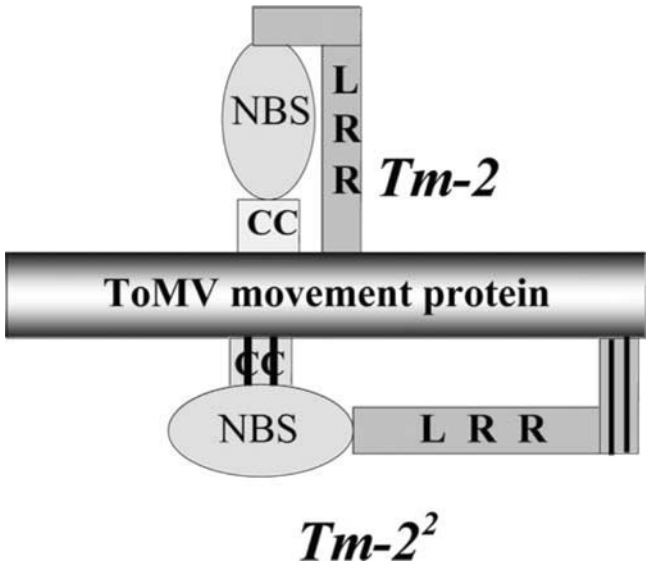

Fig. 4. Hypothetical model for the interaction of the *Tm-2* or the *Tm-2*^{2} gene products with the ToMV movement protein. The four amino acid differences between the *Tm-2* and the *Tm-2*^{2} gene are indicated by black bars.

(Soost, 1958, Alexander, 1963) both genes behave like alleles. Thus, they ended up integrated at the same region of chromosome 9, both give a similar defence response against ToMV, and both show a necrotic reaction if they are heterozygous. Therefore, it was argued by many breeders that *Tm-2*^{2} might have evolved from *Tm-2*. The argument against this common origin was that if *Tm-2*^{2} is only an optimised form of *Tm-2*, all *Tm-2*^{2} breaking virus strains should be able to overcome *Tm-2*. This is not the case. If we compare the amino acid sequence of the *Tm-2* and the *Tm-2*^{2} gene products it turns out, that the two amino acid exchanges in the LRR region between *Tm-2* and *Tm-2*^{2} are different for *tm-2*, *Tm-2* and *Tm-2*^{2}. For the two amino acid exchanges in the NB-ARC region, the situation is different. While *Tm-2* has different amino acids at position 257 and 286 in comparison to the

susceptible allele *tm-2,* the amino acid sequence at this position is identical between *tm-2* and *Tm-2*^{2}. Therefore, *Tm-2*^{2} has only 38 amino acid exchanges compared to *tm-2*. Since it is highly unlikely that the amino acid sequence of the *Tm-2*^{2} protein was reverted to the wild type sequence at two positions during the course of the evolution from *Tm-2* to *Tm-2* ^{2}, it seems more reasonable to assume that *Tm-2* and *Tm-2*^{2} developed independently from a common ancestor gene.

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