Virus resistance specificity of the tomato *Tm-2²* locus in *Arabidopsis thaliana*

Chapter

4

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ABSTRACT

Plant pathogen interactions are often being studied in the model plant *Arabidopsis thaliana* because of the superior genetic and genomic knowledge of this plant species. The model plant was also useful to elaborate plant-virus interactions. In this study, two ecotypes of *Arabidopsis* (Col-0 and UK-4) were infected separately with four TMV isolates (TMV-Cg, TMV U1, the *Tm-2* breaker and the *Tm-2*^{2} breaker). Col-0 was transformed with the tomato derived *Tm-2*^{2} gene and analyzed for functional expression of that gene. The wild type and transgenic *Arabidopsis* plants were studied for resistance as well as for the time needed for systemic infection. TMV-Cg was the most robust and rapidly infecting virus isolate, systemically infecting all tested plants. Col-0/*Tm-2*^{2} transgenic plants showed ten days delayed systemic spread of the *Tm-2* breaker virus whereas the *Tm-2*^{2} breaker needed four extra days to systemically infect the plant which suggests that the Tm-2^2 protein hampers the infection process of the tobamo-viruses. Although the introduction of resistance could not be achieved this report suggests expression of the *Tm-2*^{2} gene in *Arabidopsis*. However, this study also indicates that the interaction of the R-protein with host factors, e.g. the guardee or down-stream signaling elements, is critical for optimal functioning of the R-protein.

Keywords: Tomato, tobacco, *Tm-2*^{2}, *Arabidopsis*, ToMV, resistance

1. INTRODUCTION

Arabidopsis thaliana has a short life cycle, is easy to grow and has an abundant seed production capacity. Hence, this plant species is used as a model for genetic and molecular studies also due to its compact genome that has been sequenced completely (Arabidopsis, 2000). This plant has also been widely used for genetic characterization of disease signaling and identification of different plant defense response loci (Nishimura & Dangl, 2010; Pereda *et al.*, 2000).

Viral infections in *Arabidopsis* were studied and appeared to be useful to unravel the complex processes of viral replication and movement through the host plant (Serrano *et al.*, 2008). Differential responses to specific viral infections by different ecotypes of *Arabidopsis* emphasized the role of various plant loci involved (Cooley *et al.*, 2000; Serrano *et al.*, 2008). The crucifer-infecting tobacco mosaic virus, TMV-Cg infects *Arabidopsis* efficiently without causing severe symptoms. TMV-Cg and TMV-U1 are closely related tobamo-viruses but their mode of infection is different in *Arabidopsis* (Arce-Johnson *et al.*, 2003). Their systemic infections were examined in fourteen ecotypes of *Arabidopsis* using *in vitro* grown plants (Arce-Johnson *et al.*, 2003). Differences were found in the rate of systemic infections in various ecotypes. The *Arabidopsis* Umkirch-4 (UK-4) genotype was infected at the highest while the Columbia-0 (Col-0) was at the lowest systemic infection rate, respectively.

Based on the general resistance model of plant defense, upon recognition of the cognate avirulence elicitor, the resistance (R) protein triggers a series of defense responses (Chapter 1). This mainly involves activation of signal transduction cascades followed by induction of an HR (hypersensitive response) to arrest the virus spread (Liu *et al.*, 2002). The HR is accompanied by a rapid outburst of reactive oxygen species (ROS). This causes restriction of the pathogen at the site of ingress (Liu *et al.*, 2002). In addition, the HR leads to systemic acquired resistance (SAR). The SAR is characterized by an increase in the level of salicylic acid (SA) around the HR lesion and expression of pathogenesis related (PR) defense genes (Liu *et al.*, 2002). This phenomenon may also require additional support of regulatory genes for downstream signaling.

To date, more than 30 resistance (*R)* genes were characterized and have been shown functional. Within Solanaceae plants, *R* genes were introduced into homologous as well as heterologous plant species which conferred functional resistances against their respective pathogens. These inter-generic introgressions were either from tomato to tobacco (*Pto*), from tobacco to tomato (*N*), or from pepper to tomato (*Bs2*) (Rommens *et al.*, 1995; Tai *et al.*, 1999; Thilmony *et al.*, 1995; Whitham *et al.*, 1996). Extra-family gene transfers can also be exemplified but to a limited extent. For instance the *Arabidopsis R* genes *RPW8.1* and *RPW8.2*, conferring resistance to powdery mildew, were introduced into *Nicotiana tabacum*, *N. benthamiana* and *Solanum lycopersicum* (Xiao *et al.*, 2003). Both *R* genes implicated resistance in transgenic tobacco plants but did not function in tomato. Instead an inter-family transfer of the tomato *Ve1* Verticillium resistance gene encoded functional resistance in *Arabidopsis* (Fradin *et al.*, 2011).

The TMV resistance gene, *Tm*-2^2, was characterized and shown functional in tomato and tobacco, plants of the same family, the Solanaceae (Lanfermeijer *et al.*, 2005). To our knowledge, the gene has never been transferred to and shown to be functional in *Arabidopsis*, which would be an extra-family gene transfer. In this study, two *Arabidopsis* ecotypes Col-0 and UK-4 were studied for their response to various TMV virus isolates. In addition, Col-0 was transformed with and checked for functional expression of the *Tm-2*^{2} gene. *N. tabacum* transgenic plants expressing the *Tm-2* and *Tm-2*^{2} genes were used in this study as controls.

2. MATERIALS AND METHODS

2.1. Virus infection assay on wild type *Arabidopsis* ecotypes

Two ecotypes of *Arabidopsis*, Col-0 and UK-4 were grown in the soil in a growth chamber at 22-20^{o}C during the 16-h/8-h day/night period with 70% relative humidity. Three weeks later, at the five leaves rosette stage, plants were infected separately with four tobamo-virus isolates. TMV-Cg, TMV-U1, ToMV-GeRo (the *Tm-2* breaker) and ToMV-GM65 (the *Tm-2*^{2} breaker) with leaf extracts of *N. tabacum* infected with the respective virus isolates (Chapter 2). *N. glutinosa* is an indicator plant for TMV infection (Chapter 2). Nine days post infection (dpi) until

24 dpi with intervals of three days leaf homogenates of non-infected leaves of infected *Arabidopsis* plants were used to infect the leaf of a *N. glutinosa* plant. Lesions on the *N. glutinosa* leaf were scored at 3-5 dpi.

2.2. Transformations of Col-0 plants

The binary T-DNA vector pTM42 carries the *Tm-2*^{2} gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter and contains the NOS polyadenylation signal (Lanfermeijer *et al.*, 2005). Col-0 plants were grown in a growth chamber under conditions described earlier. The binary vector pTM42 was transferred to *Agrobacterium tumefaciens* strain LBA1119 (Chapter 2). The transformed *Agrobacterium* cells supplemented with 75 µg/ml spectinomycin (selection marker) were grown in 250 ml Erlenmeyer flasks overnight at 28^{o}C until an OD_{600} equal to 0.8. Cells were precipitated and resuspended in a 5% sucrose solution. Four weeks old Col-0 plants containing maximum immature flower clusters were selected for transformation using the floral dip method (Clough, 2005). Before dipping, the surfactant Silwet L-77 was added to the *Agrobacterium* solution at a concentration of 0.05%. The above ground parts of the plants including the rosette were dipped into the *Agrobacterium* solution for 5-10 seconds accompanied with gentle agitation. These treated plants were placed in trays in horizontal position, covered with Aluminium foil for maximum humidity and to avoid sunlight, for 16 hrs. Later on, the foil was removed and the plants were placed in vertical position. The next day plants were shifted to the green house and allowed to self-pollinate. Six weeks later, those plants were harvested for seeds and the seeds were stored in 2 ml tubes at 4^{o}C.

2.3. Virus resistance studies on transgenic plants

First generation of floral dip transformed seeds of Col-0 plants were sown on MS_{10} agar plates supplemented with kanamycin (50 µg/ml) to select transgenic plantlets (Chapter 2). The wild type Col-0 plants were also germinated on the same medium without kanamycin. Successfully grown plantlets with a proper root and shoot size were transplanted to soil and placed in the growth chamber. Three weeks later, at the

five leaves rosette stage, plants were infected with the four tobamo-virus isolates as described.

The same procedure of infections was also followed for three weeks old transgenic tobacco plants. Those transgenic tobacco plants were expressing the *Tm-2* (pTM90) and *Tm-2*^{2} (pTM42) genes, along with an empty plant vector (pTM905), separately (Chapter 2). The systemic leaves of infected plants of both *Arabidopsis* and tobacco were checked for systemic spread of virus on *N. glutinosa* indicator plants after 9dpi until 24dpi with intervals of three days.

3. RESULTS

3.1. Virus infections on two *Arabidopsis* ecotypes

Twelve plants of the *Arabidopsis* ecotypes Col-0 and UK-4 were infected with TMV-U1, TMV-Cg, the *Tm-2* breaker and the *Tm-2*^{2} breaker virus isolates. Both ecotypes were susceptible for all tobamo-virus isolates except UK-4, which showed resistance to the *Tm-2* breaker (Table 1). A rapid rate of systemic virus spread was recorded because all the plants were infected systemically at first testing (9dpi). Those susceptible plants did not show mosaic symptoms on apical uninfected leaves. Instead a yellowing of leaves was observed that might be due to the infection. On the basis of these virus infection results Col-0 was selected for transformation with the *Tm-2*^{2} construct.

Table 1. Virus infections of two *Arabidopsis* ecotypes

Virus isolate / Plant genotype	**TMV-Cg**	**TMV U1**	***Tm-2* breaker**	***Tm-2*^{2} breaker**
Col-0	+[a]	+	+	+
UK-4	+	+	-	+

[a]: +/- indicate susceptibility and resistance, respectively

3.2. Transformation of Col-0 with the *Tm-2²* gene using the floral dip method

The binary vector pTM42 harbors the *Tm-2²* gene from tomato. The T-DNA segment of pTM42 contains the *Tm-2²* gene fused to the 35S constitutive promoter and the NOS-terminator (Fig. 1).

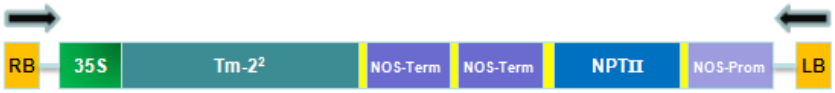

Figure 1. T-DNA of the *Tm-2²* gene construct. The T-DNA segment of the *Tm*-2² gene construct is shown. Between the arrows the right and left borders (RB and LB) shown in yellow, and within that the CaMV-35S promoter (green), the *Tm-2²* gene (light blue), the NOS-terminator (purple), the *NPTII* gene (blue) and the NOS-promoter (light purple) are indicated. The intervening regions are shown in yellow too (not drawn according to scale).

This construct was transferred to *A. tumefaciens*. The *Agrobacterium* strain was used to transform Col-0 plants using the floral dip method. Transgenic plants with the *Tm-2²* gene were selected on the basis of their ability to grow in the presence of kanamycin. The efficiency of transformation was less than 1%, as mentioned previously (Fig. 2)(Davis *et al.*, 2009). The kanamycin test to select transgenic plants (Col-0/*Tm-2²*) resulted into 48 transformants. After two weeks these independent transformants were transplanted to the soil. Compared to the wild types, some of these transgenic plants showed somaclonal variations like narrower leaf shape and early flowering.

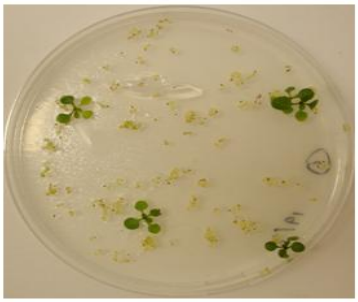

Figure 2. Screening of the Col-0/*Tm-2²* transgenic plantlets. Col-0 plants were transformed with the *Tm-2²* gene construct using the floral dip method. The first self-pollinated seeds were sown on MS_{10} agar plates supplemented with kanamycin. The picture was taken three weeks after germination of the seeds.

For the transformation of tobacco plants with the *Tm-2*^{2} gene construct, the leaf explant transformation method was used as described in Chapter 2. In this case, somaclonal variations were more obvious as short internodes and early flowering were observed. Ten independent transformants were grown in the greenhouse to self-pollinate and to get the T-2 generation.

3.3. Resistance specificity of wild type and transgenic *Arabidopsis* and tobacco plants

The wild type Col-0 and 48 independent transgenic Col-0/*Tm-2*^{2} plants were assayed in duplicate for a resistance response to the four TMV isolates. For infection with each isolate, 12 plants of the wild type as well as the transgenic *Arabidopsis* plants were used. Both types of plants were susceptible in each case. There were no mosaic symptoms found on the systemic leaves of infected plants. Instead some leaves were yellowing compared to uninfected plants. The non-infected leaves were tested for systemic infection using their homogenates on leaves of the indicator plant *N. glutinosa*. All plants showed to be systemically infected.

Transgenic tobacco plants expressing the *Tm-2*^{2} gene were susceptible to the *Tm-2*^{2} breaker only, whereas for *Tm-2* bearing tobacco plants, susceptibility was observed to the tobacco mosaic virus *Tm-2* breaker only (Table 2).

Table 2. Virus resistance assays of transgenic *Arabidopsis* and tobacco plants

Virus isolate / Plant genotype	**TMV-Cg**	**TMV U1**	***Tm-2* breaker**	***Tm-2*^{2} breaker**
Col-0	+[a]	+	+	+
Col-0/*Tm-2*^{2}	+	+	+	+
N. tabacum (*Tm-2*^{2})	-	-	-	+
N. tabacum (*Tm-2*)	-	-	+	-
N. tabacum (EPV)[b]	+	+	+	+

[a]: +/- indicate susceptibility and resistance, respectively; [b]: Transgenic tobacco plants carrying the empty plant vector

3.4. Differential time-period of viral systemic infections in *Arabidopsis* ecotypes

The wild type and transgenic *Arabidopsis* plants were also analyzed for differences in the rate by which the four tobamo-viruses could accomplish systemic infection. The infected plants were tested for local lesions on *N. glutinosa* with intervals of three days beginning at 9dpi until 24 dpi. The data were recorded on basis of number of plants infected as well as their weighted average for each virus isolate. The wild type Col-0 was susceptible to all virus isolates and infected systemically at first testing (9dpi) in all cases (Table 3). The weighted average calculated was 9 days (Supplementary Table 1).

Table 3. Differences in systemic virus spread rates (dpi) of *Arabidopsis* and tobacco plants

Virus	**TMV-Cg**		**TMV U1**			***Tm-2* breaker**				***Tm-2²* breaker**		
DPI	**9**[a]	**12**	**9**	**12**	**15**	**9**	**12**	**18**	**21**	**9**	**12**	**15**
Plant												
Col-0	12[b]	-	12	-	-	12	-	-	-	12	-	-
Col-0/*Tm-2²*	11	12	-	9	12	-	-	8	12	-	8	12
N. tabacum (*Tm-2²*)	-	-	-	-	-	-	-	-	-	8	12	-
N. tabacum (*Tm-2*)	-	-	-	-	-	-	12	-	-	-	-	-
N. tabacum (EPV)	12	-	12	-	-	-	12	-	-	12	-	-

[a]: Days post infection; [b]: Number of plants systemically infected

For transgenic *Arabidopsis* plants, Col-0/*Tm-2²*, the rate of systemic infection varied for each tobamo-virus infection. Upon infection with TMV-Cg, 11 out 12 plants were infected at 9dpi while only the last one was infected at 12dpi. This resulted in a weighted average of 9 days. In case of TMV U1 infections, 9 plants were infected systemically at 12 dpi while all plants were infected at 15dpi with a weighted average of 13 days. Upon infection with the *Tm-2* breaker, 8 out of 12 plants of Col-0/*Tm-2²* were infected systemically at 18dpi while all 12 plants were infected at

21dpi. The weighted average found was the highest (19 days). In response to the infection with the *Tm-2*^{2} breaker, 8 and 12 plants were observed as infected at 12 and 15dpi, respectively, with a weighted average of 13 days.

Transgenic tobacco plants, expressing the *Tm-2*^{2} gene were only infected with the *Tm-2*^{2} breaker. The numbers of plants infected were 8 and 12 at 9 and 12 dpi, respectively, showing a weighted average of 10 days. All 12 transgenic tobacco plants expressing the *Tm-2* gene were infected at 12dpi, with a weighted average of 12 days. While all transgenic tobacco plants carrying the empty plant vector used as negative control, were infected at 9dpi against all isolates except the *Tm-2* breaker at 12dpi. Hence they showed a weighted average of 9 days to three viruses except 12 to the *Tm-2* breaker.

Our results implicate that the movement of virus isolates except TMV-Cg was slowed down in transgenic Col-0/*Tm-2*^{2} plants compared to the wild type. The *Tm-2* breaker showed the slowest systemic infection rate in those plants.

4. DISCUSSION

Systemic viral infection in plants is an intricate process that needs specific interactions between virus and plant proteins (Arce-Johnson *et al.*, 2003). These interactions comprise: entry of virus into the plant cell, its replication in the host cell cytoplasm, cell to cell transfer and systemic movement through the vascular tissues to finally reach the distant fresh leaf tissues (Serrano *et al.*, 2008). Lack of interaction or compatibility at any of these steps might lead to no or incomplete systemic infection (Arce-Johnson *et al.*, 2003; Serrano *et al.*, 2008). One of the several plant strategies to defend themselves against pathogens is the hypersensitive response (HR). This restricts the pathogen to the site of infection (Nurnberger & Scheel, 2001).

Two tobamo-viruses, TMV-Cg and TMV-U1, efficiently infected the Solanaceae plants, tomato and tobacco. However, their response differed in *Arabidopsis* (Serrano *et al.*, 2008). They did infect the *Arabidopsis* (Col-0 and UK-4) plants but the systemic infection rate of TMV-Cg was rapid and more efficient compared to the TMV-U1 (Arce-Johnson *et al.*, 2003; Serrano *et al.*, 2008). In this study, both

ecotypes of *Arabidopsis* showed susceptibility to these virus isolates and the rate of systemic spread of both viruses was the same (data not shown).

By the last decade, more than 30 *R* genes were isolated and characterized in different plant species (Stange *et al.*, 2008; Xiao *et al.*, 2003). In this work, the TMV resistance gene *Tm-2*^{2} was transformed into *Arabidopsis* (Col-0) and its functional expression was tested against four TMV isolates. Col-0/*Tm-2*^{2} transgenic plants showed rapid systemic movement of TMV-Cg compared to the TMV-U1 isolates. In fact the other two isolates (the *Tm-2* and *Tm-2*^{2} breakers) also showed a slower systemic spread compared to TMV-Cg. The *Tm-2* breaker was the slowest. Most likely one assumption might be that the *Tm-2*^{2} resistance gene did not function properly in *Arabidopsis*. This is similar to inter-family gene transfer studies of powdery mildew resistance genes from *Arabidopsis*, *RPW8.1* and *RPW8.2*, into tomato. These genes did not confer functional resistance in transgenic tomato plants (Xiao *et al.*, 2003).

It remains, nevertheless, that Col-0/*Tm-2*^{2} plants showed a reduction of the spread of three of the four virus isolates. The wild type Col-0 plants were systemically infected more rapidly (9dpi) with the same virus isolates (Table 3). Hence the presence of the *Tm-2*^{2} gene affected the virus infections and, thus, apparently the protein is expressed and functions with a reduced efficiency. Several explanations can be given for the reduced efficiency.

Firstly, reduced expression levels of the *R*-gene could result in a less efficient response. It has been suggested that variations in expression levels cause the observed differences in phenotypes of *Tm-2*^{2} containing transgenic plants (Lanfermeijer *et al.*, 2004). In fact the transgenic expression of the *Tm-2*^{2} gene was variable ranging from extreme resistance (like the potato *Rx* gene) to spreading necrosis in transgenic tomato and tobacco plants against ToMV infections (Lanfermeijer *et al.*, 2003, 2004). However, it is generally assumed that genes under the control of the CaMV 35S promoter have high levels of expression, also in *Arabidopsis*; hence, it seems very unlikely that the reduced efficiency observed in *Arabidopsis* results from a reduced expression.

A second explanation might result from the fact that host-factors are involved in the defense mechanism. Two types of host factors can be considered: 1) the guardee and 2) the downstream signaling elements. In order to function correctly the R-protein has to interact with the Avr-protein and the downstream signaling elements. A common hypothesis of R-protein- mediated response is that R-proteins guard host proteins against interaction and hi-jacking by the Avr-proteins of the pathogen. Either the R-protein interacts directly with the Avr-protein or the R-protein interacts with an Avr-guardee complex (Van der Hoorn *et al.*, 2002). In the latter case the guardee is host specific and as such differs between hosts. Hence, variations in the guardee could result in a less efficient recognition by the R-protein and thus a less efficient defense response. On the other hand downstream signaling elements are fine-tuned for the interaction with endogenous R-proteins and therefore will have a reduced affinity for introduced exogenous R-proteins, like Tm-2^2 in *Arabidopsis*. Such a reduced interaction will result in a reduced efficiency of the defense. However, in the case of an impaired defense response one might expect to see some kind of "running necrosis" as observed in tomato plants transformed with the *Tm-2*^{2} gene (Lanfermeijer *et al.*, 2003). In the transgenic *Arabidopsis* plants we still observe infection without necrosis, and thus the most logical explanation is that spreading of the virus is slowed down. This suggests interference of the Tm-2^2 protein with spreading of the virus through the plant or in other words with the cell-to-cell movement of the virus. This hypothesis is in line with the fact that the Avr-protein of the Tm-2^2 protein is the Movement Protein (MP) of the tobamo virus and this protein is involved in cell-to-cell movement of the virus. Interaction of the Tm-2^2 protein with MP might then affect the function of MP. Because host factors are involved in the movement, this mechanism might be less efficient in the heterologous *Arabidopsis* and hence the virus might be able to slip through the defense.

Also in favor of this hypothesis is that MP needs to be changed in order to break resistance but this cannot always be done without a price: the *Tm-2*^{2} breakers are crippled (Lanfermeijer *et al.*, 2010, 2005). Using these observations it is suggested that *Tm-2*^{2} is able to practically stop cell-to-cell movement in Solanaceae and still is

able to slow down movement in *Arabidopsis*. How this is done we do not know but it might be that host-factors are involved (Van der Hoorn *et al.*, 2002). This could either be the guardee (which differs enough between tomato and *Arabidopsis* to hamper recognition of the *Avr-guardee* complex in *Arabidopsis* and slows movement down but does not stop it), or other downstream signaling pathways, either defense response related or some unknown one related to cell-to-cell transport. Based on our studies, it can be concluded that the *Tm-2*^{2} gene is expressed in *Arabidopsis* as it does in tobacco and tomato. However, its function is severely hampered either due the lack of support from host factors which contribute to the signal transduction pathways or due to a reduced interaction with the guardee. It remains interesting that the *Tm-2*^{2} resistance still displays its specificity. The *Tm-2* breaker strain has a lower rate of spreading than the *Tm-2*^{2} breaker. This also suggests expression of the gene in *Arabidopsis*. More investigations are required to assess the reason for the reduced functioning of the Tm-2^2 protein.

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SUPPLEMENTARY TABLE

Table 1. Differences in systemic virus spread of 12 independent transgenic plants based on their weighted averages

Virus isolate / Plant genotype	**TMV-Cg**	**TMV U1**	***Tm-2* breaker**	***Tm-2*^{2} breaker**
Col-0	9[a]	9	9	9
Col-0/*Tm-2*^{2}	9	13	19	13
N. tabacum (*Tm-2*^{2})	-	-	-	10
N. tabacum (*Tm-2*)	-	-	11	-
N. tabacum (EPV)	9	9	9	9

[a]: Weighted average in days of 12 infected plants