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Chapter 15

Evaluation of TMV Lesion Formation and Timing of Signal Transduction during Induction of Systemic Acquired Resistance (SAR) in Tobacco with a Computer-Assisted Method

Zoltán Á. Nagy, György Kátay, Gábor Gullner and Attila L. Ádám

Additional information is available at the end of the chapter

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Abstract

Nicotiana tabacum L. cv. Xanthi nc plants were inoculated with tobacco mosaic virus (TMV) in order to develop a method for evaluation of lesion size and its distribution characteristics during the induction of systemic acquired resistance (SAR). All necrotic lesions were scored with an image analysis software and subjected to statistical analysis. The diminished lesion size and its right-skewed, non-normal distribution seem to be an important feature of SAR response. The results showed that the degree of induced resistance differs according to the position of the leaf on the plant's shoot. In order to detect the timing of signal transduction from TMV infected leaves to distant ones, the infected leaves were removed from the tobacco plants at different time intervals. When the infected leaves were removed after 4 days, the SAR was always induced on the distant leaves indicating complete signal transduction within 4 days.

Keywords: Lesion size and distribution, signal transduction, SAR, TMV, tobacco

1. Introduction

Systemic acquired resistance (SAR) is a defence response that initiates immunity to a wide range of pathogens in distant uninfected leaves after a former localised necrotic infection of plants. It was described after tobacco mosaic virus (TMV) infection in tobacco by Ross [1]. In agricultural practice, SAR has been recognised as a strategy to control plant pathogens because of its evolutionary stability [2], long-lasting effectiveness [3] and putative transgenerational effect [4]. Not only pathogen infections but also a number of chemicals and biotic factors

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including hormonal compounds (salicylic acid and methyl jasmonate), benzothiadiazole, Ningnanmycin (an antiviral agent against TMV) and insect eggs have been shown to induce SAR [5-9]. The signal transduction process and its timing are essential components of SAR induction that moves from induced to distant leaves. Recent studies indicated that the movement of SAR signal(s) is connected to plasmodesmata [10].

Induction of SAR is often validated by the increased expression of marker genes in distant tissues and/or by limited symptom expression/multiplication of the pathogen. However, the exact evaluation of symptom expression often has serious limitations. Visual assessment of leaf spots may lead to false analysis [11]. The number of developing TMV lesions is influenced by many factors (especially by the inoculation method and physiological state of plants) and therefore not well suitable as a single factor for characterization of the development of SAR [1,12].

In the present contribution we analysed local TMV lesion size formation and its distribution after SAR induction by a computer-assisted method. The method can detect nearly all lesions on tobacco leaves including the smallest ones (ca. 0.2 mm) and consequently is suitable for the exact determination and comparison of lesion size distribution of differentially treated plants. It is particularly important if a certain component is not normally distributed and mean of data is masking the fine differences. Sherwood [13] rejected normal distribution of fungal lesions (orchardgrass—*Stagonospora arenaria* interaction) in resistant genotypes, but not in susceptible ones. However, to the best of our knowledge, there is no systematic research on lesion size distribution of viral local necrotic infections.

Therefore, our tasks were as follows:

a. To develop a reliable and well-adaptable method for semi-automated measurement of lesion size and its distribution using an appropriate statistical analysis (see Materials and methods)

b. To evaluate and compare TMV lesion size distribution in control versus SAR plants

c. To determine the timing of signal transduction process(es) by comparing lesion size distributions of distant leaves after removal of TMV-infected signal-inducing leaves at different time intervals

2. Materials and methods

Nicotiana tabacum L. cv. Xanthi nc plants were grown in greenhouse and inoculated with TMV U1 strain at 6 leaf stage (fully expanded leaves) as we have described earlier [12]. The four bottom-most leaves of plants were inoculated for induction of systemic acquired resistance without any further treatments (treatment: SAR) [1]. A subset of these plants was further treated: the inoculated leaves were removed from the plants 2 and 4 days after inoculation (treatments: leaf removal, LR2+TMV and LR4+TMV, respectively). As a control, leaves were removed similarly from uninoculated plants (treatments LR2 and LR4). Seven days after inoculation, the two fully expanded distant leaves (the 5th and 6th leaf levels) were used for challenge inoculation and data analysis. Abrasive (carborundum, 50 mg/100ml) was added to

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the inoculum. Mock inoculation was performed only with abrasive in 10 mM K-phosphate buffer (pH = 7.0).

Four days after challenge inoculation two or three detached leaves (of the same leaf level) per treatment, were directly scanned to obtain high resolution (300 dpi) digital images with a scanner (HP Scanjet G2k710). Data were collected from these images after threefold magnification on the computer screen. All dark, brownish black, round-shaped TMV lesions typical of this infection on tobacco plants were selected visually for analysis. The ImageJ 1.48v image analysis software [14] was used for lesion selection and size calculations. However, due to the low contrast of small TMV spots, lesion selection was done manually using a drawing tablet that outperforms the precision of a standard computer mouse. Lesion size was expressed as the mean of the major and minor axes of the best fitting ellipse having equal area to the lesion. Considering all lesions per leaf resulted in more accurate estimation of lesion size compared to former approaches [7,12,13]. Figure 1 demonstrates this process on TMV-infected tobacco leaves.

All calculations (lesion size distribution, mean and variance of lesion size) were carried out with R [15]. Shapiro-Wilk *w* test for normal distribution and density estimation of data were calculated with functions 'shapiro.test' and 'density', respectively with their default settings.

For comparison of sample means a multiple comparison procedure was used with the R package multcomp [16]. The method allows simultaneous comparisons while the family-wise error rate, used as the standard measure for false positive results in multiple testing, remains well controlled [17]. The method uses the HC3 covariance estimation [18,19]. Furthermore, this method tolerates unequal variances, non-normal distribution of data and unbalanced group sizes, which often occur in biological datasets.

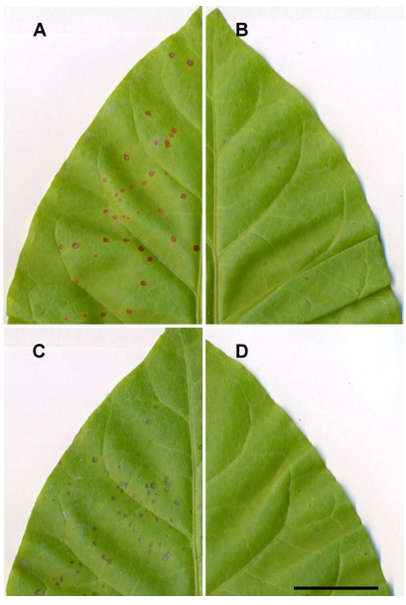

Figure 1. Leaf necrosis on *Nicotiana tabacum* cv. Xanthi nc leaves after challenge inoculation. (A): control, (B): SAR, (C): the plants were not inoculated with TMV, but their leaves were removed after 4 days (LR4), (D): the four inoculated leaves were removed from the plants 4 days after inoculation (LR4+TMV). Bar: 3 cm. Red circles on (A) mark the processed lesions during image analysis.

3. Results and discussion

3.1. Effect of SAR induction on TMV lesion size and its distribution

Data from Figs. 2, 3 and 4 are based on one representative experiment (Table 1) and serve as an example of three separate experiments. Mock inoculation with abrasive had no effect on lesion diameter and its distribution compared to control plants (Figs. 2, 3A, B and 4A, B). Induction of SAR was clearly manifested in differences of lesion development (Fig. 1) and in lesion size (Fig. 2) 4 days after challenge inoculation in all experiments. Although in control plants no significant differences were found between 5th and 6th leaf levels (Fig. 2), induction of SAR caused significant differences in degree of diminishing TMV lesion size between the 5th and 6th leaf levels. The effect of SAR induction often was more pronounced on the 5th leaf level than the 6th one (Fig. 2). Therefore, data for 5th and 6th leaves are presented separately in Figs. 2, 3 and 4. Generally, mean lesion diameter of leaves with SAR (0.528 and 0.659 mm for 5th and 6th leaf, respectively) was about half of the leaves from control plants (1.099 – 1.110 mm) (Fig. 2, Table 1).

Treatments	Leaf No.	n[1]	Lesion diameter (mm)			Normality test	
			Min	Max	Mean	Shapiro-Wilk's *w*	*p*
Control	5	343	0.25	2.01	1.099	0.9906	0.027
LR2	5	656	0.20	2.30	0.793	0.9396	< 0.001
LR2 + TMV	5	471	0.28	2.35	1.019	0.9747	< 0.001
LR4	5	428	0.29	2.27	1.024	0.9897	0.004
LR4 + TMV	5	187	0.22	1.32	0.525	0.8847	< 0.001
Mock	5	311	0.32	2.08	1.169	0.9929	0.150
SAR	5	226	0.23	1.31	0.528	0.8570	< 0.001
Control	6	132	0.43	1.80	1.110	0.9781	0.031
LR2	6	250	0.38	1.66	1.000	0.9835	0.005
LR2 + TMV	6	408	0.41	2.07	1.087	0.9851	< 0.001
LR4	6	145	0.46	1.96	1.182	0.9891	0.320
LR4 + TMV	6	145	0.29	1.35	0.666	0.9159	< 0.001
Mock	6	154	0.32	1.89	1.170	0.9889	0.266
SAR	6	103	0.25	1.55	0.659	0.9195	< 0.001

[1]For abbreviations and explanations of treatments see Fig. 2.

[2]Number of lesions.

Table 1. Test for normal distribution and lesion size data of TMV inoculated *Nicotiana tabacum* cv. Xanthi nc plants.

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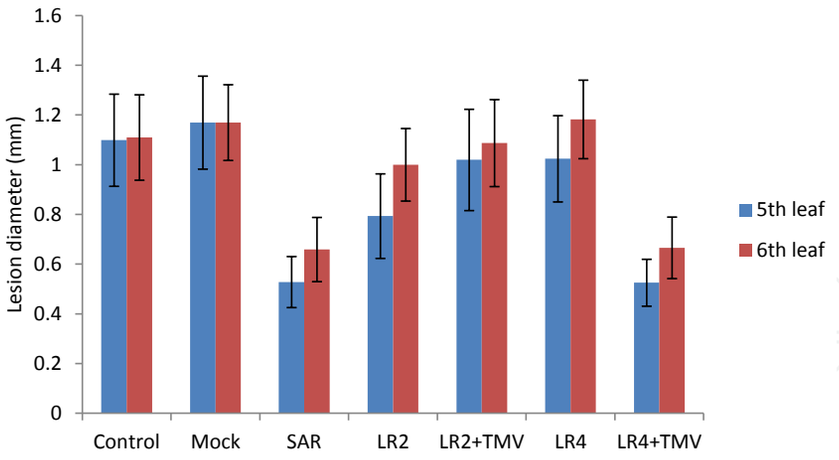

Figure 2. Mean lesion size (and standard deviation) after challenge inoculation with TMV on *Nicotiana tabacum* cv. Xanthi nc plants at leaf level 5 and 6. The four bottom-most leaves of plants were first inoculated with TMV and challenged on the 7th day. The four inoculated leaves were removed from the plants 2 and 4 days after inoculation (LR2+TMV and LR4+TMV, respectively). LR2 and LR4: the plants were not inoculated with TMV, but their leaves were removed at similar time intervals. Control: untreated plants; mock inoculated plants were treated with abrasive only; SAR: inoculated with TMV and challenged 7 days later without further treatments [1,12].

The distribution of TMV lesion size in most cases did not follow a normal distribution neither in control nor in leaves with SAR and other treatments as indicated by the results of Shapiro-Wilk *w* test (Table 1). Therefore, we used a statistical method suitable for comparison of non-normally distributed data. Comparison of multiple sample means under heteroscedasticity also showed highly significant differences ($P < 0.001$) between control leaves and leaves with SAR both in the 5th and 6th leaf levels (Fig. 4A, B).

Supporting the above data, the distribution of lesion sizes in control leaves and in leaves with SAR was massively different both in 5th and 6th leaves (Fig. 3A, B). The lesion sizes in control plants showed a plateau-like distribution and covered a wide range from 0.25 to 2.01 mm and 0.43 to 1.80 mm on leaves 5 and 6, respectively (Table 1). The distribution of lesions in resistant leaves with SAR was completely different showing a peak at 0.3—0.8 mm range (about 70-80% of total number of lesions) and above 1.5 mm size, practically no lesions were detected (Fig. 3A, B, Table 1).

TMV causes local hypersensitive necrotic lesions in tobacco plants carrying *N* gene from *Nicotiana glutinosa* L. [20], accompanied by programmed cell death and development of symptoms within 2 days after inoculation. This resistant response is further strengthened during SAR induction as indicated by limited lesion size and a different type of lesion size distribution as compared to control plants. Similar to our results, a non-normal distribution of necrotic spots was reported in a resistant plant—fungus interaction [13]. This shift in lesion size distribution is probably due to biochemical responses that are manifested in more effective restriction of lesion development, multiplication/growth and/or movement of the pathogen in resistant genotypes. It has been reported recently that an antiviral agent against TMV can induce SAR in tobacco [9].

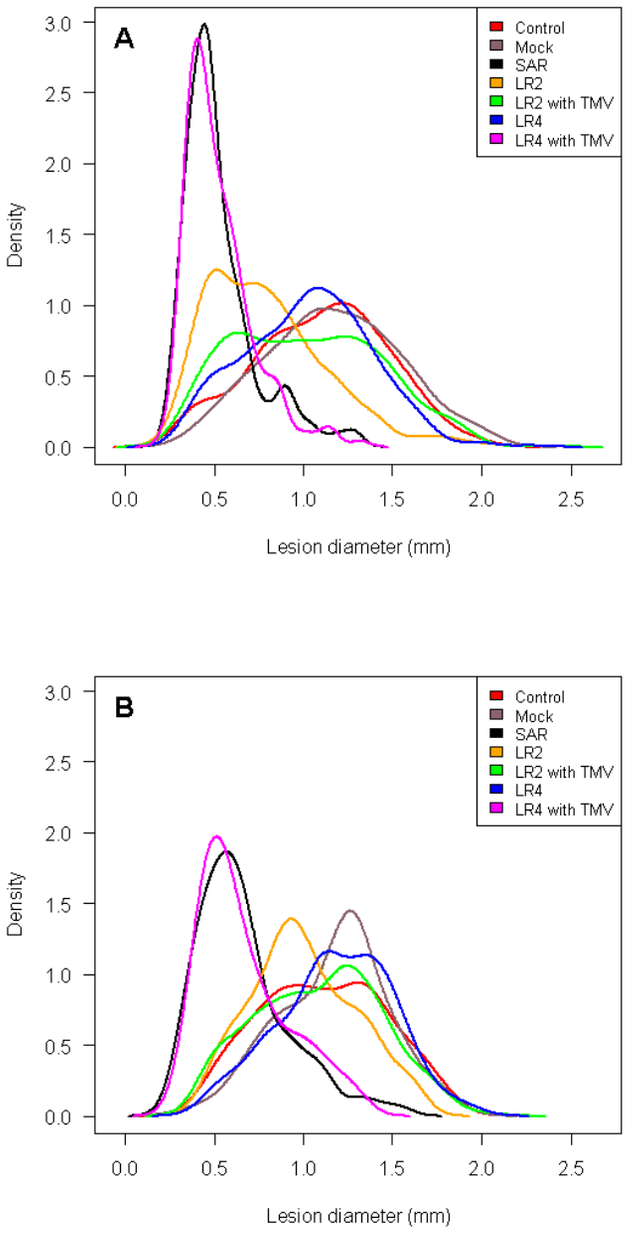

Figure 3. Kernel density estimation of TMV lesion size distribution on *Nicotiana tabacum* cv. Xanthi nc leaves at leaf level 5 (upper panel, A) and 6 (lower panel, B) after induction of systemic acquired resistance (SAR). For abbreviations and explanations of treatments see Fig. 2.

3.2. Effect of sequential removal of inducing leaves on SAR development

In order to detect the timing of signal transduction process from infected leaves to distant leaves, four bottom-most infected leaves were removed from tobacco plants at different time intervals, 2 (leaf removal, LR2) or 4 (LR4) days after TMV infection (Figs. 2, 3 and 4). The leaf removal without TMV infection after 4 days did not result in a significant shift in lesion

development (LR4, Figs. 1 and 2, Table 1). Moreover, distribution of lesion sizes showed a plateau-like picture, comparable to control leaves (Fig. 3). Simultaneous comparison of treatments also showed that LR4 treated plants did not significantly differ from control plants at least on the 6th leaf level ($p = 0.3500$ with confidence interval: [-0.0353; 0.1805], Fig. 4B). On leaf level 5 the effect was almost the same as on level 6 indicating a limited effect ($p = 0.0346$ with confidence interval: [-0.146; -0.003], Fig.4A). On the contrary, removal of TMV-infected leaves after 4 days (LR4+TMV) mimicked the development of SAR in all characteristics in all three experiments. Lesion development was considerably, about 50% inhibited (Figs. 1, 2 and Table 1). The statistical analysis of data clearly showed highly significant differences between LR4+TMV and control plants ($p < 0.001$ for both leaf levels) but no significant differences between LR4+TMV and SAR treatments at both leaf levels (Fig. 4A, B). Not surprisingly, the distribution of lesion development of LR4+TMV plants was nearly the same as in leaves with SAR showing a characteristic peak at about 0.5 mm of lesion diameter. (Fig. 3A, B). These results clearly indicate that a 4-day period after the inducing infection of lower 4 leaves is enough for complete signal transduction of SAR in distant leaves. Consequently, the movement of signal molecule(s) should be detectable before this time point. These results also indicate that lesion size distribution as a resistance marker is a suitable tool for prediction of signalling events. Similar experiments with removal of leaves after a 2-day interval (LR2 and LR2+TMV) showed less clear evidences. Leaf removal without TMV infection (LR2) considerably influenced lesion development in all experiments (Fig. 2, Table 1) and somewhat shifted distribution of lesion size at both leaf levels (Fig. 3A, B). LR2 plants showed significant differences as compared either to control or SAR treated plants (Fig. 4A, B). This fact could be related to a different mechanism as compared to SAR induction, for example differences in hormone balance of distant leaves during longer incubation period after leaf removal. In LR2+TMV plants, the development of SAR was not detected on the basis of lesion size and its distribution characteristics (Figs. 2, 3A, B). Family-wise comparison of data rather suggests that LR2+TMV plants did not significantly differ from control ones but differed from the SAR treatment ($p = 0.0316$, confidence interval: [-0.1543; -0.0044]), Fig. 4A, B).

Altogether, these results indicate that the signal transduction starts probably only after visual appearance of local TMV symptoms (40—48 h post inoculation) and it is completed within the next 2 days. The identification of the exact timing of signal transduction from induced leaves is necessary for the further characterization of signal molecule(s) in phloem sap-enriched petiolar exudates.

In conclusion, we developed an easily applicable semi-automated method for the detection of the size of necrotic lesions and its distribution in tobacco leaves after TMV inoculation using appropriate statistical analysis. Decreased lesion size diameter and its characteristic non-normal, right-skewed distribution seem to be an accurate and important feature of the resistant response in distant leaves with SAR. Application of this method during SAR induction indicated that signal transduction is completed in distant leaves by the 4th day after inducing TMV inoculation. Further experiments are in progress to characterize the chemical nature of this signal.

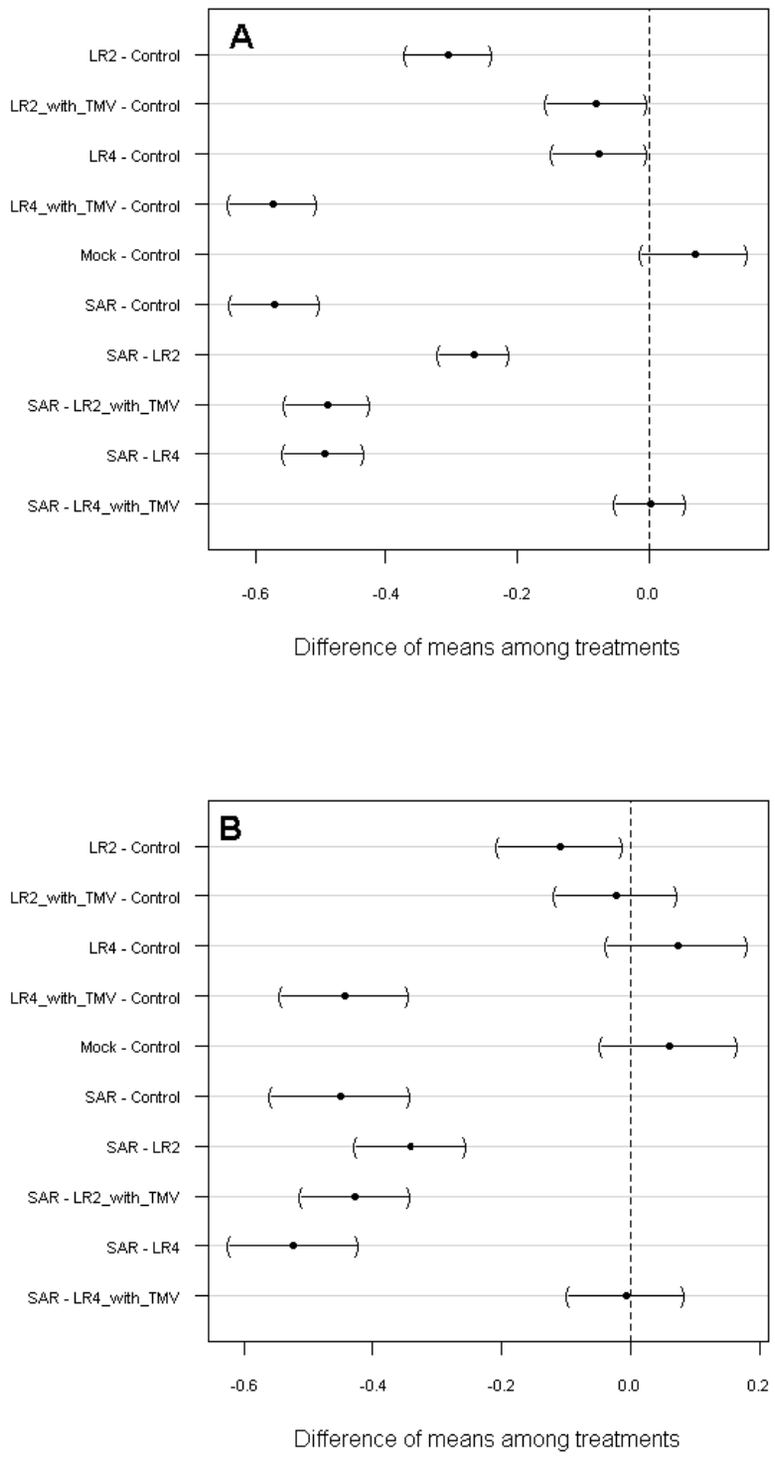

Figure 4. Multiple comparisons of group means of selected treatments on leaf level 5 (A) and 6 (B). Dots represent the difference of the estimated means between treatments. Brackets flank the 95% confidence intervals. The difference is considered significant if the confidence interval does not contain the 0, represented by a vertical dashed line. Abbreviations and explanations are the same as on Fig. 2.

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