*Proceedings of the 8th Hungarian Congress on Plant Physiology and the 6th Hungarian Conference on Photosynthesis, 2005*

**Volume 49(1-2):85-87, 2005**
**Acta Biologica Szegediensis**
http://www.sci.u-szeged.hu/ABS

# Induction of hypersensitive necrosis at high temperatures by generation of reactive oxygen forms in virus resistant tobacco

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**ABSTRACT** Tobacco (*Nicotiana tabacum* cv. Xanthi nc) resistant to *Tobacco mosaic virus* (TMV) displays a hypersensitive response (HR) following virus infection, characterized by localized necrotic lesions around infection sites at ambient temperatures (e.g. 20°C). We have demonstrated that application of chemical compounds that generate reactive oxygen species (ROS), such as the riboflavin/methionine and glucose/glucose oxidase systems or $H_2O_2$ treatment induce HR-type necroses in leaves of Xanthi-nc tobacco infected with TMV even at high temperatures (30°C), when both necrosis and virus resistance are impaired. It was possible to suppress chemically induced HR-type necrotization at 30°C by application of antioxidants like superoxide dismutase (SOD) and catalase (CAT). Importantly, high TMV levels at 30°C did not differ in infected plants, regardless of the presence or absence of HR-type necrotization. Levels of one of the ROS, superoxide ($O_2^{\cdot-}$), activity of NADPH-oxidase and expression of a tobacco NADPH-oxidase gene responsible for $O_2^{\cdot-}$-production were significantly lower in leaves of infected and healthy Xanthi-nc tobacco at 30°C, as compared to 20°C.
It is concluded that development of HR-type necroses caused by TMV infection depends on a certain level of superoxide and other ROS, while suppression of virus multiplication in resistant tobacco is associated with low temperature but seems to be independent of HR-type necrotization.
**Acta Biol Szeged 49(1-2):85-87 (2005)**

**KEY WORDS**

TMV
virus resistance
necrosis
HR
ROS
antioxidants

Probably one of the best characterized plant-pathogen interactions is the hypersensitive type of resistance (hypersensitive response, HR) elicited by *Tobacco mosaic virus* (TMV) in tobacco (*Nicotiana tabacum*). It is known that in case of the tobacco-TMV interaction, the HR is governed by interaction of proteins encoded by the tobacco *N* (necrosis) gene (Holmes 1938) and the replicase gene of TMV (Padgett and Beachy 1993; Padgett et al. 1997). A typical HR is characterized by localized necrotic lesions around infection sites. In other words, tissue necrosis and resistance (localization of the virus) occurs together. However, in the past years several investigators have demonstrated that the resistance and tissue necrosis that comprise an HR can be separate phenomena in case of resistance to certain fungal (Király et al. 1972), bacterial (Yu et al. 1998) and viral (Bendahmane et al. 1999; Cole et al. 2001) diseases. It is also known that tissue necrosis in plants exposed to disease or abiotic stress is related to the formation of reactive oxygen species (ROS; e.g. Király et al. 1993; Baker and Orlandi 1995; Fodor et al. 2001). In fact, it is assumed that ROS accumulation could be responsible for both HR-type necrotization and resistance.

Interestingly, it has been noticed several decades ago that HR-type necroses caused by TMV do not develop at temperatures above 28°C and the virus replicates and moves systemically in the originally resistant *N* gene expressing plants (Samuel 1931). In order to demonstrate that ROS are indeed responsible for necrotization, we have applied $H_2O_2$ and different chemical compounds that generate ROS to virus-infected leaves kept above 28°C. In order to investigate the role of ROS in resistance, we have monitored TMV concentration in infected leaves kept above 28°C when necrosis either did not develop or was chemically induced.

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## Materials and Methods

Tobacco (*Nicotiana tabacum* cv. Xanthi nc) was grown under greenhouse conditions. For mechanical virus inoculation the U1 strain of TMV was used. Inoculated plants were kept in growth chambers set at temperatures of 20°C and 30°C.

Tissue necrosis at 30°C was induced by $H_2O_2$ treatment and the riboflavine/methionine and glucose/glucose oxidase photochemical systems that generate the ROS such as $O_2^{\cdot-}$ and $H_2O_2$, while antioxidants (SOD and CAT) to suppress necroses were employed as described (Király et al. 2003). Levels of $O_2^{\cdot-}$ were detected by the nitroblue tetrazolium (NBT) method (see Király et al. 2002). NADPH oxidase activity was determined by a method described in Ott et al. (2000). mRNA level gene expression was accomplished by reverse transcription polymerase chain reaction (RT-PCR) with a kit by Fermentas (Burlington, Canada). Oligonucleotide primers based on a tobacco NADPH oxidase (Genbank AJ309006 and AF506374) and actin (Genbank X69885) sequences were

synthesized by Metabion (Martinsried, Germany).

TMV concentration was determined by enzyme-linked immunosorbent assays (ELISA) according to Tóbiás et al. (1982), using a Bioreba kit (Reinach, Switzerland).

## Results

Application of chemical compounds that generate reactive oxygen species (ROS), such as the riboflavin/methionine and glucose/glucose oxidase systems or $H_2O_2$ treatment induced HR-type necroses in leaves of a resistant local lesion host, Xanthi-nc tobacco infected with TMV even at high temperatures (30°C). It is important to stress, however, that in control leaves (virus-infected and untreated; non-infected and treated with ROS-generating compounds or $H_2O_2$) tissue necrotization did not develop at 30°C.

HR-type tissue necroses caused by TMV infection at ambient temperatures (20°C) or TMV infection + artificial ROS-generation/$H_2O_2$ treatment at 30°C could be almost completely suppressed by application of two antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT). These results underline the pivotal role of prooxidants (*i.e.* ROS) in the development of tissue necroses.

Another proof for the role of ROS in elicitation of HR-type necroses by TMV is the fact that the levels of one of the ROS, $O_2^{\cdot-}$ (superoxide), were substantially (ca. 60 %) reduced at 30°C, as compared to 20°C both in healthy and virus infected leaves. In addition, activity of NADPH oxidases, enzymes that play a key role in plant $O_2^{\cdot-}$ synthesis (Levine et al. 1994; Jabs et al. 1997) showed at least a 50% reduction at high temperatures (30°C), in line with a significant decrease in expression of a NADPH oxidase gene (Simon-Plas et al. 2002).

It is known from previous research (Samuel 1931; Holmes 1938; Kassanis 1952; Da Graca et al. 1976) that TMV does not spread in a local lesion host which produces HR-type necroses in leaves. Consequently, the virus content is also reduced, as compared to a systemic host. In our experiments, concentration of TMV in necrotized leaves of Xanthi-nc tobacco held at 20°C was significantly (ca. 60-70%) lower than in virus infected leaves at 30°C where either necroses were induced by $H_2O_2$ or ROS-producing chemicals or necroses were not present. Therefore, virus concentration seems to depend on temperature, rather than on HR-type necrotization.

## Discussion

Our results demonstrate that HR-type leaf necrotization caused by TMV infection in a local lesion host depends on the presence of reactive oxygen species (ROS), such as $O_2^{\cdot-}$ (superoxide), $H_2O_2$ (hydrogen peroxide) etc. We were able to induce HR-type necroses in virus inoculated Xanthi-nc tobacco leaves even at high temperatures (30°C), where necrotic lesions developed following treatment of leaves with ROS or ROS-generating compounds, in addition to inoculation with TMV. ROS-treatments alone or virus-inoculation alone were not effective in inducing HR-type leaf necroses at 30°C.

The instrumental role of ROS in HR-type necrosis is also demonstrated by the fact that two antioxidant enzymes (SOD and CAT) were able to suppress the necrosis-inducing effects of ROS in virus infected tobacco leaves. In addition, the level of $O_2^{\cdot-}$ significantly decreased at 30°C, as compared to 20°C either in TMV-infected or control leaves, further underlining the role of ROS, namely $O_2^{\cdot-}$, in the elicitation of HR-type necrosis. In fact, this result supports the hypothesis of Samuel (1931) stating that at 30°C necroses are overcome but the virus is able to multiply and spread systemically. Activity of NADPH oxidases, enzymes that play a key role in plant $O_2^{\cdot-}$ synthesis (Levine et al. 1994; Jabs et al. 1997) and expression of a NADPH oxidase gene that contributes to plant disease resistance (Yoshioka et al. 2003) were also significantly reduced at high temperatures (30°C). These results suggest that during TMV infection $O_2^{\cdot-}$ production by tobacco NADPH oxidases is regulated on the mRNA level. The pivotal role of NADPH oxidases in elicitation of HR-type necrotization is supported by our earlier results that inhibition of NADPH oxidase activity by diphenilene iodonium (DPI) reduces both TMV-induced tissue necroses and accumulation of $O_2^{\cdot-}$ (Király et al. 2002).

According to a generally accepted hypothesis the cause of virus resistance during HR is the appearance of tissue necroses. However, this hypothesis was recently criticized on the basis of new experiments (Bendahmane et al. 1999; Cole et al. 2001), showing that resistance can be independent of tissue necrotization during a virus-induced HR. This idea is supported by our results showing that TMV concentration is significantly higher in virus infected leaves at 30°C than at 20°C, whether or not necroses were induced at 30°C by $H_2O_2$ or ROS-producing chemicals. Therefore, suppression of virus multiplication in resistant tobacco seems to be independent of HR-type necrotization but is rather associated with low temperature.

## Acknowledgements

This research was supported by grants from the Hungarian Scientific Research Fund, OTKA T 043431, T 042801 and Ts 040835.

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