Suppression of tobacco mosaic virus-induced hypersensitive-type necrotization in tobacco at high temperature is associated with downregulation of NADPH oxidase and superoxide and stimulation of dehydroascorbate reductase

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Tissue necroses and resistance during the hypersensitive response (HR) of tobacco to tobacco mosaic virus (TMV) are overcome at temperatures above 28 °C and the virus multiplies to high levels in the originally resistant N-gene expressing plants. We have demonstrated that chemical compounds that generate reactive oxygen species (ROS) or directly applied hydrogen peroxide (H2O2) are able to induce HR-type necroses in TMV-inoculated Xanthi-nc tobacco even at high temperatures (e.g. 30 °C). The amount of superoxide (O2$^{•−}$) decreased, while H2O2 slightly increased in TMV- and mock-inoculated leaves at 30 °C, as compared with 20 °C. Activity of NADPH oxidase and mRNA levels of genes that encode NADPH oxidase and an alternative oxidase, respectively, were significantly lower, while activity of dehydroascorbate reductase was significantly higher at 30 °C, as compared with 20 °C. It was possible to reverse or suppress the chemically induced HR-type necrotization at 30 °C by the application of antioxidants, such as superoxide dismutase and catalase, demonstrating that the development of HR-type necroses indeed depends on a certain level of superoxide and other ROS. Importantly, high TMV levels at 30 °C were similar in infected plants, whether the HR-type necrotization developed or not. Suppression of virus multiplication in resistant, HR-producing tobacco at lower temperatures seems to be independent of the appearance of necroses but is associated with temperatures below 28 °C.

INTRODUCTION

One of the best characterized plant–pathogen interactions is the hypersensitive type of resistance (HR) elicited by tobacco mosaic virus (TMV) in tobacco (Nicotiana tabacum). It is known that in the case of the tobacco—TMV interaction, the HR is governed by the interaction of proteins encoded by the tobacco N (necrosis) gene (Holmes, 1938) and the replicase gene of TMV (Padgett & Beachy, 1993; Padgett et al., 1997). A typical HR is characterized by localized necrotic lesions around the infection sites. It was shown as early as 1931 that tissue necroses associated with the HR of Xanthi-nc tobacco to TMV do not develop at temperatures above 28 °C and the virus multiplies to high levels in the originally resistant N-gene expressing plants (Samuel, 1931).

Doke & Ohashi (1988) showed that in TMV-infected N-gene expressing local lesion tobacco hosts, a superoxide (O2$^{•−}$) generating system is induced. However, this is not the case with systemic hosts lacking the N resistance gene, where virus multiplication and spread are accompanied by non-necrotic symptoms. Furthermore, in a transgenic local lesion host (NahG tobacco) that produces large necrotic lesions upon TMV infection, an increased level of O2$^{•−}$ and hydrogen peroxide (H2O2) production can be detected at the edge of necrotic spots, as compared with the control non-transgenic tobacco that displays normal-sized lesions (Király et al., 2002). Some investigators claimed that reactive oxygen species (ROS) like O2$^{•−}$ and H2O2 could be responsible not only for host cell necrotization but also for cell wall strengthening, signalling for defence genes and even killing pathogen cells. However, it remained unclear whether ROS can indeed exert a dual role in executing both pathogen and host cells (Doke & Ohashi, 1988; Király et al., 1993; Tzeng & DeVay, 1993; Hafez & Király, 2003;
El-Zahaby et al., 2004; Torres et al., 2006). In other words, ROS may arrest pathogen growth (resistance) and induce necrotic host symptoms (plant cell death).

We raised the question as to whether it is possible to induce HR-type necroses in TMV-infected resistant tobacco even at high temperature (e.g. 30 °C) with the application or generation of ROS. We also investigated whether antioxidants applied to the host plant can modify the HR. In order to study the relationship between virus resistance, tissue necrotization and temperature during an HR induced by TMV, we assayed virus levels in tissues with and without necrotic symptoms at 30 °C in comparison to virus levels at 20 °C.

**METHODS**

**Virus inoculation and quantification of virions.** The U1 strain of TMV was maintained on the Samsun (nm) cultivar of tobacco (Nicotiana tabacum L.). For mechanical virus inoculation, the sap of TMV-infected leaves, homogenized in tap water, was rubbed onto healthy leaves of 60–70-day-old Xanthi-nc tobacco, a local lesion host of TMV. Carborundum was used as an abrasive for both virus and mock inoculations. Inoculated plants were grown at 20 or 30 °C in growth chambers (150 μE m−2 s−1 light intensity for 16 h per day). Two days after inoculation with TMV, visible necrotic lesions (HR) developed at 20 °C, however, at 30 °C no HR had appeared.

To determine TMV concentration, ELISA was performed according to Clark & Adams (1977) and Tobías et al. (1982) using a TMV-ELISA kit from Bioreba.

**Generation of ROS.** Inoculated leaves were detached 3 days after inoculation with TMV and placed in Petri dishes on filter paper soaked with riboflavin/methionine solution. A volume of 6 ml of a mixture containing 266 μM riboflavin and 10 mM l-methionine (Reanal Rt.) was used to produce superoxide according to Jordan et al. (1992). The Petri dishes were illuminated (150 μE m−2 s−1) and incubated at 30 °C for an additional 3 days. TMV-inoculated leaves (without riboflavin treatment) and riboflavin treated mock-inoculated leaves were used as controls.

Glucose oxidase acts aerobically upon glucose and generates H2O2 (Wu et al., 1997). A volume of 6 ml solution containing 200 U glucose oxidase ml−1 and 2 mM glucose (Reanal Rt.) was poured onto filter paper in Petri dishes containing detached leaves. TMV-inoculated leaves were treated 3 days after inoculation and incubated at 30 °C for an additional 3 days following treatment. TMV-inoculated leaves (without glucose–glucose oxidase) and glucose–glucose oxidase treated mock-inoculated leaves were used as controls.

Direct application of 25 mM H2O2 was carried out by treating the detached leaves in Petri dishes as described above.

The action of ROS at 30 °C was reversed by treatment with 4000 U horseradish peroxidase superoxide dismutase (SOD) (E.C.1.15.1.1) per Petri dish and 5000 U bovine liver catalase (E.C.1.11.1.6) (Sigma Aldrich) per Petri dish. SOD and catalase were directly incorporated into the riboflavin/methionine solution 3 days after inoculation with TMV. Similarly, catalase was applied to leaves during treatment with glucose–glucose oxidase or H2O2.

**Histochemical analysis of ROS.** Histochemical staining for superoxide production was conducted in leaf tissues using 10 mM potassium phosphate buffer (pH 7.8) containing 0.1 % nitro blue tetrazolium (NBT; Sigma Aldrich) according to the procedure of Adám et al. (1989). NBT-treated samples were incubated under daylight for 20 min and subsequently cleared in 0.15 % trichloroacetic acid (w/v) in ethanol:chloroform 4:1 (v/v) as described by Hückelhoven et al. (1999). Subsequently, leaves were stored in 50 % glycerol prior to evaluation. Discolouration of leaf discs was quantified using a ChemiImager 4000 digital imaging system (Alpha Innotech Corp.).

H2O2 was visualized as a reddish-brown coloration of 3,3-diaminobenzidine (DAB). Detection of H2O2 was performed using the DAB-uptake method as described by Thordal-Christensen et al. (1997). DAB-films were vacuum infiltrated with 10 mM potassium phosphate buffer (pH 7.8) containing 0.1 % DAB (Fluka). DAB-treated samples were incubated under daylight for 2 h, and subsequently cleared and analysed as described above.

**Biochemical assays of enzymic activities.** For enzymic activity assays of catalase and dehydroascorbate reductase (DHAR), 0.5 g tobacco leaf material was homogenized on ice in 3 ml 50 mM Tris buffer (pH 7.8), containing 1 mM EDTA-Na2 and 7.5 % polyvinylpyrrolidone K-25. Homogenates were centrifuged (12 000 g, 20 min, 4 °C) and total soluble enzymic activities in the supernatant were measured spectrophotometrically. Assays were carried out at 25 °C, using a model UV-160A spectrophotometer (Shimadzu). Activities of catalase (E.C. 1.11.1.6) and DHAR (E.C. 1.8.5.1) were determined according to Aebi (1984) and Klapeck et al. (1990), respectively.

NADPH oxidase activity was assayed based on the method of Adám et al. (1997) with modifications. Leaf material (0.5 g) was homogenized on ice in 3 ml 50 mM Na-phosphate buffer (pH 7.0) containing 0.1 % NaN3O2. Immediately before homogenization, 0.15 g insoluble polyvinylpyrrolidone was added to each sample. The homogenate was centrifuged (12 000 g, 20 min, 4 °C) and total soluble NADPH oxidase activity in the supernatant was determined at 530 nm. The assay buffer included 0.2 mM NADPH, 0.3 mM NBT, 50 mM HEPES (pH 6.8) and 50 μl supernatant in 2 ml volume. In order to detect NADPH oxidase specific activity, horseradish SOD (40 U ml−1) was added to the reaction mixture and the obtained activity was subtracted from that measured without SOD.

**RNA extraction and gene expression analysis.** At least 200 mg fresh leaves/sample were homogenized in liquid nitrogen. For total RNA isolation, 100 mg homogenized plant material was used by applying a total RNA isolation minicolumn kit according to the manufacturer’s instructions (Viogene).

For plant gene expression analysis at the mRNA level, a two-step RT-PCR procedure was applied (MBI Fermentas) following the manufacturer’s instructions. PCR reactions were done with relatively low cycle numbers (22–28 cycles) in order to maintain initial differences in target transcript amounts, as much as possible. Amplified cDNA fragments were separated in 2.0 % agarose gels. Photographs of ethidium bromide-stained gels were used to quantify gene expression by using a ChemiImager 4000 digital imaging system (Alpha Innotech Corp.). Expression of a tobacco actin gene served as a reference control.

Primers used in the RT-PCR assays were as follows: 5′-CGGAAATCCAGGAGACTACATC-3′ [5′ primer (forward)] and 5′-GGGAAGCCAAGATAGCC-3′ [3′ primer (reverse)] for a 230 bp tobacco actin (NiAct) cDNA fragment (GenBank accession no. X69885); 5′-GAAACAGTGGTGACTGAGG-3′ [5′ primer] and 5′-GTGATACCCTGTTG-3′ [3′ primer] (Chivasa et al., 1997) for a 516 bp tobacco alternative oxidase (NiAOX1-2) cDNA fragment (GenBank accession nos S71335 and X79768); 5′-AACACACGGGC-TACAAAT-3′ [5′ primer] and 5′-GACGAGAAGAGATCAC-3′ [3′ primer] for a 681 bp tobacco NADPH oxidase (NiRoboD) cDNA fragment (GenBank accession nos AJ309006 and AF506374).
The cDNA fragments amplified by RT-PCR were sequenced by an automated DNA sequencer (MWG Biotech AG). In order to confirm identity of the obtained sequences, database searches were performed using the BLAST network service at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

**Statistical analysis.** At least three independent parallel experiments were conducted in each case with three to five replicates for each treatment. Data represent the mean ± SD. Student’s *t*-test was used to determine whether significant difference (*P*<0.05) existed between mean values.

**RESULTS**

**Induction of tissue necrotization in TMV-infected tobacco by generation of ROS at high temperature (30 °C)**

TMV infection induces HR-type necrosis in local lesion hosts, such as Xanthi-nc tobacco at temperatures below 28 °C, while no necrotization occurs above 28 °C. However, if we applied ROS-producing chemical systems or H2O2 directly to the inoculated leaves, it was possible to induce HR-type necroses at 30 °C.

In one type of experiments, detached TMV-inoculated tobacco leaves were placed in Petri dishes containing filter paper soaked with a riboflavin/methionine mixture and kept for 3 days under constant illumination (150 μE m⁻² s⁻¹). In similar experiments we applied a glucose–glucose oxidase system. In both cases it was possible to induce HR-type necroses at 30 °C. Necroses also appeared at 30 °C if we applied a solution of 25 mM H2O2. It is important to point out that in controls (TMV-inoculated untreated leaves or mock-inoculated leaves treated with the ROS-producing chemical systems or H2O2) tissue necroses did not appear at 30 °C (Fig. 1).

To demonstrate the roles of superoxide and H2O2 in the induction of necrosis by TMV, detached TMV-inoculated tobacco leaves were treated with the antioxidants SOD and catalase simultaneously with the application of the ROS-generating riboflavin/methionine mixture. Three days after treatment, the necrosis-inducing action of the riboflavin/methionine mixture was almost completely suppressed at 30 °C. In additional experiments, catalase was used simultaneously either with the glucose–glucose oxidase ROS-generating system or with directly applied H2O2. Catalase treatments were able to significantly suppress the necrosis-inducing action of both glucose–glucose oxidase system and H2O2 (Fig. 1).

**TMV levels in tobacco leaves are not influenced by tissue necrotization at high temperature (30 °C)**

It is known from previous research (Samuel, 1931; Holmes, 1938; Kassanis, 1952; Da Graça & Martin, 1976) that TMV does not usually spread in a local lesion host that produces HR-type necroses in inoculated leaves. Consequently, the virus concentration is also reduced, as compared with inoculated leaves of a systemic host. In our experiments, the concentration of TMV in inoculated and necrotized leaves of Xanthi-nc tobacco held at 20 °C was indeed lower (see Fig. 2) than in inoculated leaves at 30 °C (necroses not present). The same difference in virus concentration was experienced when necroses were induced by H2O2 or ROS-producing chemicals at 30 °C. Therefore, virus concentration seems to depend on temperature, rather than on HR-type necrotization.

**Levels of superoxide and H2O2 in leaves of Xanthi-nc tobacco at low (20 °C) and high (30 °C) temperatures**

From the previous experiments one can conclude that the HR-type necroses caused by TMV are in association with the presence or accumulation of ROS. Levels of superoxide in TMV-inoculated tobacco leaves were determined by infiltrating the leaves with NBT at 20 and 30 °C. The amount of O$_2^-$ was substantially reduced at 30 °C, as compared with 20 °C. This occurred in mock-inoculated, as well as in virus-inoculated leaves (Fig. 3a). Levels of
H$_2$O$_2$ were also determined by infiltrating the leaves with DAB at 20 and 30 °C. The amount of H$_2$O$_2$ at 30 °C increased slightly, but not significantly in both mock- and virus-inoculated leaves, as compared to leaves held at 20 °C (Fig. 3b).

Enzymic activities of antioxidants and NADPH oxidase in leaves of Xanthi-nc tobacco at low (20 °C) and high (30 °C) temperatures

Activities of catalase, DHAR and NADPH oxidase were determined spectrophotometrically at 20 and 30 °C. We found that catalase activity was slightly lower at 30 °C in both mock- and TMV-inoculated leaves than at 20 °C (Fig. 4a). However, DHAR activity was significantly higher at 30 °C in mock-inoculated as well as in virus-inoculated leaves than at 20 °C (Fig. 4b). Interestingly, the activity of NADPH oxidase was significantly reduced in mock-inoculated, as well as in TMV-inoculated leaves at 30 °C, as compared with that at 20 °C (Fig. 4c). This is in line with a similar reduction in superoxide levels (compare Fig. 4c and Fig. 3a). Since plant NADPH oxidases are known to be responsible for O$_2^-$ generation, these results suggest that a reduction in NADPH oxidase activity might contribute to the reduction of O$_2^-$ levels in leaves kept at 30 °C.

**Fig. 2.** TMV levels in Xanthi-nc tobacco leaf extracts, as determined by ELISA 4 days after virus inoculation. TMV/30, Leaves inoculated with TMV and held at 30 °C. TMV/20, Leaves inoculated with TMV and held at 20 °C. TMV + GO/30, Leaves inoculated with TMV and treated with the glucose–glucose oxidase system at 30 °C. TMV + Rf/30, Leaves inoculated with TMV and treated with the riboflavin/methionine system at 30 °C. TMV + H$_2$O$_2$/30, Leaves inoculated with TMV and treated with H$_2$O$_2$ at 30 °C. Means of three independent experiments are shown. Error bars ± SD.

**Fig. 3.** Levels of (a) superoxide and (b) H$_2$O$_2$ in TMV- and mock-inoculated Xanthi-nc tobacco leaves at low (20 °C) and high (30 °C) temperatures 24, 36, 48 and 60 h after virus inoculation (hai). Leaves were either mock-inoculated or inoculated with TMV and kept at 20 or 30 °C. Mock/20, Mock-inoculated leaves kept at 20 °C. Mock/30, Mock-inoculated leaves kept at 30 °C. TMV/20, Leaves inoculated with TMV and kept at 20 °C. TMV/30, Leaves inoculated with TMV and kept at 30 °C. Means of three independent experiments are shown. Error bars ± SD.

**Fig. 4.** Activities of (a) catalase, (b) dehydrosorcorbate reductase and (c) NADPH oxidase in TMV- and mock-inoculated leaves of Xanthi-nc tobacco at low (20 °C) and high (30 °C) temperatures 4 days after virus inoculation (dai). Leaves were either mock-inoculated or inoculated with TMV and kept at 20 or 30 °C. Mock/20, Mock-inoculated leaves kept at 20 °C. Mock/30, Mock-inoculated leaves kept at 30 °C. TMV/20, Leaves inoculated with TMV and kept at 20 °C. TMV/30, Leaves inoculated with TMV and kept at 30 °C. Means of three independent experiments are shown. Error bars ± SD.
Changes in expression of genes that encode cell death regulators in leaves of Xanthi-nc tobacco at low (20°C) and high (30°C) temperatures

We found that in TMV-inoculated leaves of Xanthi-nc tobacco the transcript levels of an alternative oxidase (NtAOX1-2) and NADPH oxidase (NtRbohD) genes were suppressed at high temperature (30°C), as compared with lower temperatures (20°C) when assayed by RT-PCR. In the case of NtAOX1-2, two bands of similar size were visible but the lower band (516 bp) should correspond to NtAOX1-2, since the primer pairs (Chivasa et al., 1997) used for RT-PCR in the present study were designed to amplify a 516 bp cDNA in the conserved region of two tobacco alternative oxidase genes (GenBank accession nos S71335 and X79768). Therefore, NtAOX1-2 could represent either or both of these alternative oxidase genes, as verified by sequencing (see below). Interestingly, the suppression of NtAOX1-2 was quite substantial in TMV-infected plants kept at 30°C, 6 and 12 h after virus inoculation. In mock-inoculated plants, such a down-regulation of NtAOX1-2 expression was not detectable at 30°C (Fig. 5).

Expression of a NADPH oxidase gene (NtRbohD) was also highly suppressed in TMV-inoculated plants kept at 30°C, 6 and 12 h after virus inoculation (Fig. 5). In mock-inoculated plants, suppression of NtRbohD transcripts was not detectable at 30°C. The suppression of gene expression of NtRbohD at 30°C in TMV-inoculated plants suggests that this gene might contribute to the decline of NADPH oxidase enzymic activity at these high temperatures.

Identities of the cDNA fragments amplified by RT-PCR were checked by sequencing. As expected, the NtAOX1-2 lower (516 bp) band displayed a high identity to three published tobacco alternative oxidase sequences (99, 97 and 96% identity to GenBank accession nos X79768, AB281425 and S71335, respectively). The NtRbohD sequenced fragment showed the highest identity (96%) to three N. tabacum mRNAs that all encode RbohD (GenBank accession nos EF366670, AF506374 and AJ309006). The NtAct fragment that was used as a reference control of gene expression displayed the highest identity to tobacco actin genes (98 and 97% identity to GenBank accession nos U60490 and X69885, respectively).

**DISCUSSION**

We have demonstrated that downregulation of the ROS superoxide and activation of antioxidant genes/enzymes are associated with suppression of virus-induced necrotization at high temperature (30°C). Tissue necroses that accompany the HR of Xanthi-nc tobacco to TMV do not develop at temperatures above 28°C and the virus multiplies to high levels in the originally resistant N-gene expressing plants. According to our results, the HR-type leaf necrotization caused by TMV infection in a local lesion host depends on the presence of ROS, namely superoxide and H₂O₂. We were able to induce HR-type necroses in virus-inoculated Xanthi-nc tobacco leaves even at high temperature (30°C), where necrotic lesions developed following treatment of leaves with ROS, in addition to inoculation with TMV. ROS-treatments alone or virus-inoculation alone were not effective in inducing HR-type leaf necroses at this high temperature.

In past years, the generally accepted hypothesis regarding suppression of virus spread and multiplication during a macroscopic HR was that the appearance of tissue necroses...
Upon infection is the cause of virus resistance. However, this hypothesis has been recently criticized on the basis of new experiments (Yu et al., 1998; Bendahmane et al., 1999; Cole et al., 2001; Gassmann, 2005; Király & Király, 2006), showing that in certain plant–pathogen interactions resistance is independent of tissue necrotization. In fact, excessive necrotization due to disruption of N gene encoded TMV-elicited resistance is associated with increased virus spread and multiplication (Delaney et al., 1994; Dinesh-Kumar et al., 2000; del Pozo & Lam, 2003; Cole et al., 2004; Liu et al., 2005). In our study, the lack of necrotization in a resistant local lesion host at 30 °C did not result in increased virus multiplication in comparison to plants in which necrotization was induced by ROS. This result demonstrates that TMV resistance is independent of the development of tissue necroses and high temperature itself is in a cause-and-effect relationship with increased virus content. In TMV-infected leaves kept at 30 °C indeed higher virus levels were detected, whether the HR-type necrotization developed or not, than in TMV-infected leaves kept at low temperature (20 °C).

It is well known that plant defences to abiotic stresses and pathogens can be stimulated at lower temperatures. For example, in Arabidopsis thaliana pathogen-induced biosynthesis of salicylic acid (SA), a central component of plant resistance responses like the HR, is dependent on isochorismate synthase. Interestingly, this enzyme was shown to be remarkably active at a very low temperature (4 °C), consistent with an additional role for SA in cold-tolerant plant growth (Strawn et al., 2007). Certain Arabidopsis mutants display constitutively active defence responses only at temperatures not higher than 22 °C. Spontaneous lesion formation or dwarfing is accompanied in these mutants by increased SA production and/or elevated expression of defence-related genes (e.g. EDS1, PAD4, PR1, PR2 and PR5) at these low temperatures (Jambunathan & McNeillis, 2003; Yang & Hua, 2004). In fact, one of the mutants (cpn1-1) also exhibits low temperature-dependent enhanced resistance to infection by avirulent and virulent bacteria (Jambunathan & McNeillis, 2003).

Exposure of TMV-infected N-gene expressing local lesion tobacco hosts to high temperatures like 30 °C results in complete suppression of virus resistance, as compared with plants kept at lower temperatures (e.g. 20 °C). Not only is this indicated by high virus levels and the absence of HR-type necrotization, but also by the suppression of SA levels and defence-related genes encoding PR1, an epoxide hydrolase (EH-1) and a receptor-like protein kinase (WRK) (Malamy et al., 1992; Guo et al., 1998; Ito et al., 2002). The temperature sensitivity of N gene-mediated resistance to TMV could be caused by the effect of high temperatures on interactions between the N protein and other plant and virus-encoded proteins or on N gene transcription (Marathe et al., 2002; Liu et al., 2004; Takabatake et al., 2006). Our study, however, suggests that the N protein might be able to recognize TMV at high temperatures but the impaired expression of downstream signalling elements that are temperature sensitive (e.g. the ROS superoxide) is not sufficient to confer virus resistance.

When we applied antioxidant enzymes (SOD and catalase) to TMV-infected tobacco leaves treated with ROS at 30 °C, necrotization was reversed or suppressed. Our results support the view that antioxidants play a pivotal role in balancing the action of ROS in several host/pathogen combinations (Levine et al., 1994; El-Zahaby et al., 1995; Foyer et al., 1997; Hafez & Király, 2003). Interestingly, when we measured the levels of ROS (O$_2^\cdot$ and H$_2$O$_2$) in tobacco leaves, we found that the level of O$_2^\cdot$ was significantly decreased at 30 °C, as compared with 20 °C either in mock-inoculated control or TMV-infected leaves. However, H$_2$O$_2$ did not change significantly. This result strongly points to the role of ROS, namely O$_2^\cdot$, in the production of HR-type necrosis. Several reports have indicated a preferential association between the accumulation of ROS and the degree of cell death (Levine et al., 1994; Jabs et al., 1996). In accordance with this model, antioxidative mechanisms that scavenge ROS were shown to be suppressed during the early steps of TMV-induced HR-type necrotization in tobacco (Fodor et al., 1997; Dorey et al., 1998; Mittler et al., 1998; Yi et al., 1999).

The significant decrease of O$_2^\cdot$ levels in tobacco leaves at 30 °C could be caused by one or more of several effects that might be accelerated at higher temperatures. The O$_2^\cdot$ anion is very unstable, although it reacts with a few biomolecules (Halliwell, 2006). For example, its rapid reaction with nitric oxide (NO$^\cdot$) generates peroxynitrite (ONOO$^-$). Interestingly, it has been shown that ONOO$^-$ does not mediate bacteria-elicited HR-type plant cell death (Delledonne et al., 2001), a possible explanation for the lack of TMV-elicited necrotic lesions at high temperatures. It cannot be ruled out that at 30 °C elevated spontaneous and/or SOD-catalysed dismutation of O$_2^\cdot$ to H$_2$O$_2$ might also contribute to decreased levels of O$_2^\cdot$. However, we found that similar to H$_2$O$_2$ levels, SOD enzymic activity and gene expression do not change significantly in mock-inoculated and TMV-infected tobacco kept at 30 °C, as compared to 20 °C (data not shown). An additional effect of high temperature could be the downregulation of apoplastic peroxidases, amine oxidases or NADPH oxidases, enzymes known to play a role in O$_2^\cdot$ production in plants following recognition of a variety of pathogens (Lamb & Dixon, 1997; Grant & Loake, 2000; Torres & Dangl, 2005; Møller et al., 2007). We have shown that NADPH oxidase activity was suppressed at 30 °C, as compared with 20 °C in mock-inoculated or TMV-inoculated leaves. Similarly, expression of an NADPH oxidase gene (NrRbohD) was also suppressed at 30 °C in TMV-inoculated leaves. NrRbohD encodes an NADPH oxidase shown to be involved in ROS production in tobacco cells treated with the fungal elicitor cryptogein (Simon-Plas et al., 2002). It is likely that the decrease in levels of O$_2^\cdot$ detected at 30 °C is, at least in part, a consequence of decreased NADPH oxidase gene expression.
and activity. It has been noted that in TMV-inoculated Xanthi-nc tobacco, overexpression of a dominant negative form of the rice Osr31 gene that exerts its effects, most probably, via modulating NADPH oxidase activity (Sagi & Fluhr, 2006), significantly reduces HR-associated production of ROS (Moeder et al., 2005). In comparison with wild-type Xanthi-nc plants, these transgenic plants exhibit a delay in HR formation and produce smaller TMV-induced lesions. Although the authors did not assay TMV titres in virus-infected transgenic plants (Moeder et al., 2005), suppression of HR-type cell death coupled with reduced superoxide levels is in agreement with our results. However, ROS might serve different signalling functions in different types of disease resistance during the HR (Torres & Dangl, 2005). For example, silencing of NbRbohA and NbRbohB in Nicotiana benthamiana plants led to less ROS production and enhanced susceptibility to normally avirulent Phytophthora infestans and suppression of HR (Yoshioka et al., 2003). In contrast, the mutation of AtRbohD in Arabidopsis had only a slight effect on HR caused by the bacterial pathogen Pseudomonas syringae pv. tomato DC3000 (Avr Rpm1) (Torres et al., 2005). Furthermore, there is genetic evidence that plant NADPH oxidases involved in responses to pathogen attack are activated by mitogen-activated protein kinase kinases (Yoshioka et al., 2003; Yamamizo et al., 2006). It remains to be seen whether in TMV-infected plants the decrease in NADPH oxidase activity at 30 °C is a consequence of a lack of mitogen-activated protein kinase kinase activation.

SOD and catalase are key antioxidant enzymes that convert O$_2^-$ to H$_2$O$_2$ and H$_2$O to water, respectively. These two enzymes are ubiquitous in aerobic organisms where they play a major role in defence against oxygen radical-mediated toxicity (Tsang et al., 1991; Mittler, 2002). We did not find any differences in SOD or ascorbate peroxidase (APX) enzyme activities and gene expression in mock-inoculated and TMV-infected plants kept at 30 °C, as compared to 20 °C (data not shown). On the other hand, catalase activity was slightly lower at 30 °C both in mock-inoculated and virus-infected leaves, as compared with that at 20 °C. It is possible that the small decrease in catalase enzymic activity at 30 °C is one of the factors responsible for the slight increase in levels of H$_2$O$_2$ that we observed at this higher temperature. The balance between the activity of SOD- and H$_2$O$_2$-degrading antioxidant enzymes, such as catalase or APX is crucial for controlling the steady-state levels of ROS and the development of plant cell death (Mittler et al., 1998). For example, transgenic antisense tobacco plants with reduced catalase or APX expression display increased sensitivity, ‘hyperresponsiveness’ to cell death during bacteria-induced HR (Mittler et al., 1999). Furthermore, increases in SOD expression are not sufficient to counteract oxidative damage and necrosis: transgenic SOD-overexpressing tobacco that is tolerant to necrosis caused by high light intensity and low temperature, also displays elevated levels of endogenous APX (Gupta et al., 1993). Also, transgenic tobacco or potato that simultaneously overexpress a SOD and an APX gene show enhanced tolerance to necrosis caused by the herbicide paraquat in comparison to transgenic plants that overexpress either SOD or APX (Kwon et al., 2002; Tang et al., 2006). Moreover, simultaneous overexpression of SOD, APX and DHAR in chloroplasts is more effective in inducing tolerance to paraquat and salt treatments, as compared with single or double transformants (Lee et al., 2007). In the present study, DHAR activity was considerably higher at 30 °C, as compared with 20 °C, in both mock-inoculated and TMV-infected plants. Our results suggest that DHAR might contribute to the inhibition of HR-type necroses at 30 °C. This conclusion is supported by research showing that DHAR-overexpressing tobacco and maize plants exhibit elevated levels of reduced ascorbate and glutathione (Chen et al., 2003). The latter antioxidant, glutathione, provides the reducing power for DHAR to recycle ascorbate that plays a pivotal role in the detoxification of ROS (Asada, 1999). An increase in ascorbate levels is a likely consequence of DHAR-induction at 30 °C. We have shown that ascorbate, when exogenously applied, could be directly responsible for the elimination of necrosis during viral HR (Farkas et al., 1960).

Alternative oxidase (AOX) lowers the generation of ROS in the mitochondria by preventing overreduction of the cytochrome respiratory electron transport chain during stresses like ageing and pathogen attack (Maxwell et al., 1999; Chivas & Carr, 1998). Thereby, AOX was shown to contribute to a slight (15%) restriction of the size of necrotized viral lesions during TMV-induced HR-type cell death in tobacco (Ordog et al., 2002). Nicotiana sylvestris mitochondrial mutants with elevated levels of AOX protein and altered expression of antioxidant enzymes also display reduced number and size of lesions following TMV inoculation compared with control plants (Dutululeu et al., 2003). On the other hand, use of a TMV vector to produce extremely high levels of wild-type or mutant AOX protein in tobacco resulted in larger HR lesions than those produced by the empty TMV vector (Murphy et al., 2004). It seems that when AOX levels are several fold higher than normal this could cause perturbation of the mitochondrial redox balance, compromising the host’s ability to limit HR. In our study, an AOX gene (NtAOX1-2) was suppressed at 30 °C, as compared with 20 °C in TMV-inoculated tobacco leaves. The reduced expression of NtAOX1-2 at 30 °C could be, in part at least, a consequence of the absence of TMV-elicited HR-type cell death and resistance. AOX transcript and protein levels increase in tobacco that displays HR during TMV-infection, but do not change during a compatible interaction, where virus localization and HR-necrosis are absent (Lennon et al., 1997; Chivas & Carr, 1998). In addition, transgenic antisense tobacco plants with reduced catalase levels display spontaneous necrotization in response to moderate light coupled with induction of NtAOX1-2 gene expression (Rizhsky et al., 2002). These
results suggest that AOX activity is indeed involved in limiting cell and tissue necrosis during both abiotic stress and virus infection.

The HR type of resistance is overcome in TMV-infected Xanthi-nc tobacco plants kept at high temperature (30 °C). This is manifested by increased multiplication and systemic spread of the virus, concomitantly with elimination of tissue necrotization, which does not seem to have a role in virus resistance. Results of this study provide evidence that at high temperature (30 °C) the downregulation of superoxide and the stimulated activity of antioxidant genes/enzymes are associated with suppression of TMV-induced HR-type necrotization and resistance.

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