

Catalases may Play Different Roles in Influencing Resistance to Virus-induced Hypersensitive Necrosis

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High concentrations of the reactive oxygen species (ROS) superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) contribute to the induction of plant cell and tissue death (necrosis). In an effort to create transgenic plants with high antioxidant capacity that could resist necrotic symptoms we produced two transgenic tobacco (*Nicotiana tabacum* cv. SR1) lines (S1 and S2) overexpressing a tomato chloroplast superoxide dismutase (*SChSOD*). *SOD* genes encode for antioxidant enzymes that dismutate superoxide to hydrogen peroxide. Therefore, SOD-overproducing plants may contain high levels of hydrogen peroxide and are sensitive to stress-related necrosis unless sufficient degradation of hydrogen peroxide is conferred by elevated expression of antioxidants like e.g. catalases and peroxidases. Indeed, line S1 displayed elevated expression of a glutathione peroxidase (*NtGPX*) and a glutathione S-transferase (*NtGSTU1b*), as compared to wild type plants. Interestingly, however, expression of a catalase (*NtCAT1*) was repressed in both SOD-overexpressing lines. This predicts that such plants could be sensitive to localized necrosis (HR) caused by virus infection, since repression of *NtCAT1* has been shown to occur during virus-induced HR (e.g. Dorey et al., 1998; Künstler et al., 2007). To elucidate whether other catalases might play a role in resistance to virus induced HR-type necrotic symptoms, a maize catalase (*ZmCat2*) was transiently overexpressed in *Nicotiana edwardsonii* and *N. edwardsonii* var. Columbia plants by agroinfiltration. Inoculation of agroinfiltrated plants with *Tobacco mosaic virus* (TMV) revealed that *ZmCat2* confers enhanced resistance to HR-type necrosis during TMV infection. It seems that catalases may play different roles in influencing resistance to virus-induced hypersensitive necrosis.

Keywords: reactive oxygen species, antioxidants, superoxide dismutase, catalase, hypersensitive necrosis, *Tobacco mosaic virus*.

In plants, pathogenic infections and abiotic stresses are frequently associated with accumulation of reactive oxygen species (ROS) like superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). High ROS levels induce oxidative stress ultimately resulting in cell and tissue death (necrosis) (e.g. Doke and Ohashi, 1988; Ádám et al., 1989; Levine et al., 1994; Jabs et al., 1996; Karpinski et al., 1999; Mittler, 2002; Hernandez et al., 2004; Torres et al., 2006). Under natural conditions the up-regulation of antioxidant defense systems seems to be a general response to oxidative stress. Often this is the only evidence that oxidative stress has occurred *in vivo* (Halliwell and Gutteridge, 1999). Antioxidant defense includes non-enzymatic scavengers and low molecular weight compounds such as ascorbic acid

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(ASC), glutathione (GSH) and phenols. Enzymatic ROS scavengers include antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and enzymes related to ascorbate and glutathione metabolism (e.g. ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, glutathione reductase, glutathione peroxidase, and glutathione S-transferase) (Noctor and Foyer, 1998; Asada, 1997, 1999; Foyer and Noctor, 2011; Heyneke et al., 2013).

SOD genes encode for antioxidant enzymes that dismutate superoxide to hydrogen peroxide, while degradation of hydrogen peroxide is conferred by elevated expression of e.g. catalases and peroxidases (Tsang et al., 1991; Bowler et al., 1992; Mittler, 2002). The balance between the activity of SOD and H₂O₂-degrading antioxidant enzymes (e.g. CAT or ascorbate peroxidase) is crucial for controlling the steady-state levels of ROS and the development of plant cell death (see e.g. Mittler et al., 1998). Transgenic antisense tobacco plants with reduced CAT or ascorbate peroxidase (APX) expression develop spontaneous necrotic lesions and display increased sensitivity to cell death during bacterial infection (Chamnongpol et al., 1996; Takahashi et al., 1997; Mittler et al., 1999). This implies that increases in SOD expression alone may not be sufficient to counteract oxidative damage and necrosis, due to the accumulation of H₂O₂. Indeed, transgenic SOD-overexpressing tobacco that is tolerant to necrosis caused by high light intensity and low temperature, also displays elevated levels of endogenous APX (Sen Gupta et al., 1993). Transgenic plants that simultaneously overexpress a SOD and an APX gene show enhanced tolerance to necrosis caused by various abiotic stresses and pathogenic infections (Kwon et al., 2002; Tang et al., 2006; Lee et al., 2007; Faize et al., 2011, 2012; Diaz-Vivancos et al., 2013).

In an effort to create transgenic plants with high antioxidant capacity that could resist necrotic symptoms, a preliminary analysis of two transgenic tobacco (*Nicotiana tabacum* cv. SR1) lines overexpressing a tomato chloroplast superoxide dismutase (*S1Ch-SOD*) is presented. Differential expression of genes encoding H₂O₂-degrading antioxidant enzymes occurred in both SOD-overexpressing lines. Notably, expression of a catalase (*NtCAT1*) known to be down-regulated during virus-induced localized necrosis (HR) (Dorey et al., 1998; Yi et al., 1999, 2003; Künstler et al., 2007) was repressed in healthy, SOD-transgenic tobaccos, predicting that such plants could be sensitive to localized necrosis caused by virus infection. To investigate whether other catalases might play a role in resistance to virus induced HR-type necrosis, a maize catalase (*ZmCat2*) was transiently overexpressed in *Nicotiana edwardsonii* and *N. edwardsonii* var. Columbia plants by agroinfiltration.

Materials and Methods

Plants and pathogens

Nicotiana tabacum cv. Samsun nn, cv. SR1, *Nicotiana edwardsonii* and *N. edwardsonii* var. Columbia plants were grown under normal greenhouse conditions (18–23 °C, ca. 160 µmol/m²/s supplemental light for 8 hours/day, 75–80% relative humidity).

The U1 strain of *Tobacco mosaic virus* (TMV) was maintained on *N. tabacum* cv. Samsun nn. For mechanical virus inoculation the sap of TMV-infected leaves homogenized in tap water was rubbed onto leaves of healthy plants with carborundum as an abrasive. 60–70 days old plants were used for all experiments.

Agrobacterium tumefaciens LBA4404 was maintained on solid yeast extract peptone (YEP) medium with 50 µg/ml rifampicin and 50 µg/ml streptomycin. *A. tumefaciens* LBA4404 containing binary vectors pBB31S or pBB30Cat that carry a tomato chloroplast superoxide dismutase (*SlChSOD*) and a maize catalase 2 (*ZmCat2*), respectively, were maintained on the same medium including 50 µg/ml kanamycin as well.

Plant transformation

Transformation of *N. tabacum* cv. SR1 plants with *SlChSOD* was conducted essentially as described by Horsch et al. (1985) with modifications. Sterilized leaf discs one cm in diameter were dipped in a liquid culture of *A. tumefaciens* LBA4404 pBB31S resuspended in Murashige-Skoog (MS) medium and transferred to solid MS medium for two days. Afterwards, leaf discs were washed with sterile distilled water containing 500 µg/ml cefotaxim to eliminate *A. tumefaciens* and then transferred to callus-inducing medium containing 200 µg/ml cefotaxim and 50 µg/ml bialaphos to select for transformant plant calli. Shoot regenerated from calli within ca. three weeks were transferred to a similar medium (containing only 50 µg/ml bialaphos) for rooting. Intact, grown plants were transferred to pots containing sterile soil and later moved to the greenhouse. PCR analysis detected the *SlChSOD* transgene in two such plants (T0 generation, S1 and S2) which were also used for gene expression assays and subsequently grown to maturity to produce T1 seeds and establish lines S1 and S2.

PCR analysis for transgene detection in bacteria and plants

The presence of the *SlChSOD* and *ZmCat2* transgenes in *Agrobacterium tumefaciens* LBA4404 pBB31S and pBB30Cat, respectively, was checked by polymerase chain reaction (PCR). To prepare bacterial template DNA for PCR, 100 µl of 2 ml bacterial cultures grown for two days at 28 °C in liquid YEP medium were briefly centrifuged and resuspended in an equal volume of sample buffer (2.5 mg/ml Na₃N, 2% Triton X-100, 0.1 M Tris, pH 8.1). Samples were then kept at 99 °C for 15 minutes and centrifuged again. 1 µl of each supernatant was used in PCR reactions.

The presence of the *SlChSOD* transgene in bialaphos-resistant transformed and regenerated *N. tabacum* cv. SR1 plants was also verified by PCR analysis. Plant genomic DNA was extracted from ca. 0.1 g leaf material homogenized in liquid nitrogen with a plant DNA extraction minicolumn kit according to instructions of the manufacturer (VioGene, Sunnyvale, CA, USA).

Semiquantitative PCR for detection of the *SlChSOD* and *ZmCat2* transgenes in *A. tumefaciens* and *N. tabacum* cv. SR1 was conducted with reagents supplied by Thermo Fisher Scientific Inc. (Waltham, MA, USA), always using 1 µl of template DNA in a total

reaction volume of 25 µl, according to instructions of the manufacturer. PCR reactions were carried out with a PTC 200 DNA Engine (MJ Research, Waltham, MA, USA).

Oligonucleotide primers used in PCR reactions were the following: 5'-GGGTGGCCATGAACTCAGTCT-3' (5' primer [forward]) corresponding to a tomato chloroplastic SOD (*SlChSOD*) sequence (GenBank X14041) and 5'-CATCGCAAGACCGGCAACAGG-3' (3' primer [reverse]) corresponding to an *A. tumefaciens* nopaline synthase terminator sequence (*t-NOS*) (GenBank AF434753) giving a 200 bp transgene PCR product; 5'-GGGTTCGACGCGCTCTCCGACC-3' (5' primer) corresponding to a maize catalase 2 (*ZmCat2*) sequence (GenBank J02976) and the 3' *t-NOS* primer as above, giving a 260 bp transgene PCR product.

RT-PCR analysis for plant gene expression assays

To prepare samples from wild type and transgenic *N. tabacum* cv. SR1 plants for gene expression analysis, total RNA was extracted from ca. 0.1 g leaf material homogenized in liquid nitrogen with a plant RNA extraction minicolumn kit according to instructions of the manufacturer (Viogene, Sunnyvale, CA, USA). Gene expression was assayed by a two step reverse transcription polymerase chain reaction (RT-PCR) procedure following the manufacturers' instructions (Thermo Fisher Scientific Inc., Waltham, MA, USA). In the RT step (i.e., first-strand cDNA synthesis), an oligo(dT) primer was used. In the PCR step, relatively low cycle numbers (22–28 cycles) were applied to maintain initial differences in target transcript amounts as much as possible. PCR reactions were carried out in a PTC 200 DNA Engine (MJ Research, Waltham, MA, USA). Expression of a tobacco actin gene (GenBank X69885) served as a reference (constitutive control) of gene expression.

Oligonucleotide primers used in the PCR step of RT-PCR reactions were the following: 5'-CGGAATCCACGAGACTACATAC-3' (5' primer) and 5'-GGGAAGCCAA-GATAGAGC-3' (3' primer) for a 230 bp tobacco actin (*NtAct*) cDNA fragment (GenBank X69885); 5'-TGGACTTCACGGGTTTCAT-3' (5' primer) and 5'-CACCCCTTTC-CGAGGTCATCC-3' (3' primer) for a 269 bp tomato chloroplastic SOD (*SlChSOD*) cDNA fragment (GenBank X14041); 5'-GCCGTCCTTAGCAGCAGT-3' (5' primer) and 5'-ACAAGCAACCCTTCCACC-3' (3' primer) for a 420 bp *N. plumbaginifolia* and putative tobacco SOD (*NtSOD*) cDNA fragment (GenBank X55974 and EU123521); 5'-TCCGCTTGATGTGACTAAA-3' (5' primer) and 5'-TCCACCCACCGACGAATA-3' (3' primer) for a 501-bp *N. glutinosa* and tobacco catalase 1 (*NtCAT1*) cDNA fragment (GenBank AF006067, U93244 and HF564632); 5'-TTTAGYCRYAGAGTTGARTGG-3' (5' primer) and 5'-ATTTSCARCMATATCAGC-3' (3' primer) for a 426-bp tobacco tau class glutathione S-transferase (*NtGSTU1b*) cDNA fragment (degenerate primers based on tobacco, *N. benthamiana* and potato sequences, GenBank X56263, X56264, X56265, AY206006 and J03679); 5'-CTCCTCCGATTCCAACAG-3' (5' primer) and 5'-GAG-CAGCATTATCAC CATTC-3' (3' primer) for a 423 bp tobacco glutathione peroxidase (*NtGPX*) cDNA fragment (GenBank AB041518).

Agroinfiltration

A. tumefaciens LBA4404 pBB30Cat that carries a maize catalase 2 (*ZmCat2*) was used for agroinfiltration of *Nicotiana edwardsonii* and *N. edwardsonii* var. Columbia plants two days before TMV inoculation. Prior to agroinfiltration, the presence of the *ZmCat2* transgene in the bacterium was verified by PCR as described above. Agroinfiltration was done essentially as described by Van der Hoorn et al. (2000) with modifications. 5 ml bacterial cultures grown overnight at 28 °C in liquid YEP medium (supplemented with 50 µg/ml rifampicin, 50 µg/ml streptomycin and 50 µg/ml kanamycin) were briefly centrifuged, resuspended in 50 ml YEB (yeast extract beef) medium and grown again overnight at 28 °C. This 50 ml bacterial culture was again briefly centrifuged and resuspended in 25 ml infiltration medium (10 mM Mg SO₄, 10 mM MES, pH 5.6). Acetosyringone (4'-hydroxy-3',5'-dimethoxyacetophenone, Sigma-Aldrich Corp., St. Louis, MO, USA) was added to 200 µM and the culture was shaken (100 rpm) at room temperature for ca. 1 hour. Leaves were infiltrated with the bacterial suspension with a syringe and needle as described by Hagborg (1970).

Results

Transformation of tobacco (*Nicotiana tabacum* cv. SR1) with a tomato chloroplast superoxide dismutase (*SlChSOD*) gene yielded several bialaphos-resistant intact plants. PCR analysis confirmed the presence of the *SlChSOD* transgene in two such plants (T0 generation) designated S1 and S2 with a habit identical to that of wild type (non-transformed) SR1. Although in S2 the size of the PCR product was larger than the expected 200 bp (Fig. 1), gene expression analysis by RT-PCR revealed presence of the same *SlChSOD*-specific mRNA species in both S1 and S2 tobaccos. In addition, transgenic and wild type tobaccos expressed an endogenous *SOD* gene (*NtSOD*) to similar high levels (Fig. 1).

It can be anticipated that SOD-overproducing plants contain high levels of H₂O₂ and are sensitive to stress-related necrosis unless sufficient H₂O₂-degradation is conferred by elevated expression of antioxidants like e.g. catalases and peroxidases. Indeed, S1 tobacco displayed elevated expression of an endogenous glutathione peroxidase (*NtGPX*) and a tau class glutathione S-transferase (*NtGSTU1b*), as compared to wild type plants (Fig. 2). Interestingly, however, expression of a catalase (*NtCAT1*) was repressed in both SOD-overexpressing plants (Fig. 2). Repression of *NtCAT1* has been also shown to occur during virus-induced HR (Dorey et al., 1998; Yi et al., 1999, 2003; Künstler et al., 2007) suggesting that this catalase may not play a role in reducing localized necrosis during virus infections.

In order to investigate whether catalases other than *NtCAT1* might play a role in resistance to virus induced HR-type necrotic symptoms, a maize catalase (*ZmCat2*) was transiently overexpressed in *Nicotiana edwardsonii* and *N. edwardsonii* var. Columbia plants by agroinfiltration. We have chosen these two interspecific hybrid tobaccos because our earlier research showed that var. Columbia displays enhanced resistance to HR-type necrosis induced by e.g. *Tobacco mosaic virus* (TMV), as compared to *N. edward-*

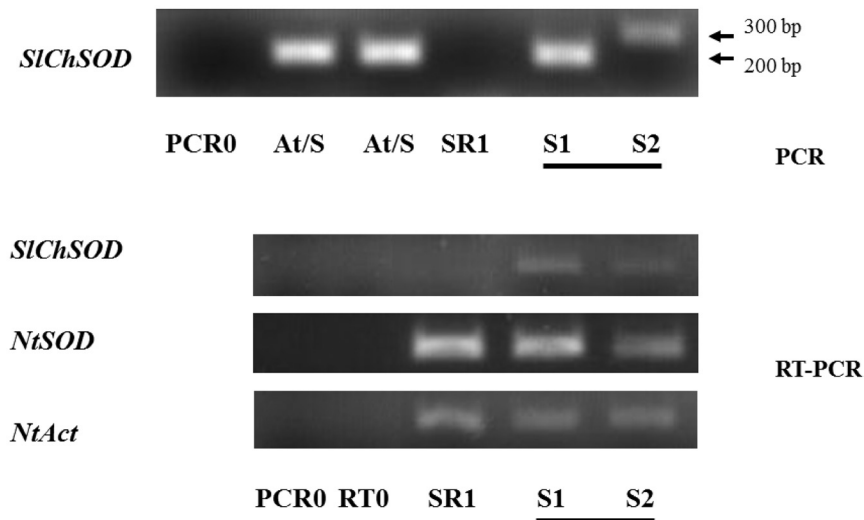


Fig. 1. Incorporation of a tomato chloroplast superoxide dismutase (*SiChSOD*) transgene into *Nicotiana tabacum* cv. SR1 (T0 generation, S1 and S2 lines) and expression of its mRNA as verified by polymerase chain reaction (PCR) and reverse transcription-PCR, respectively. *NtSOD* = expression of an endogenous *SOD* gene in wild type (non-transformed) and transgenic tobaccos. For RT-PCR, a tobacco actin gene (*NtAct*) served as a reference. RT0 = negative control of reverse transcription (no RNA template). PCR0 = negative control of polymerase chain reaction (no DNA template). At/S = presence of *SiChSOD* in *Agrobacterium tumefaciens* LBA4404 pBB31S used for plant transformation. SR1 = absence of *SiChSOD* in non-transformed tobacco. Arrows depict sizes of PCR-amplified fragments of *SiChSOD* (bp = base pairs)

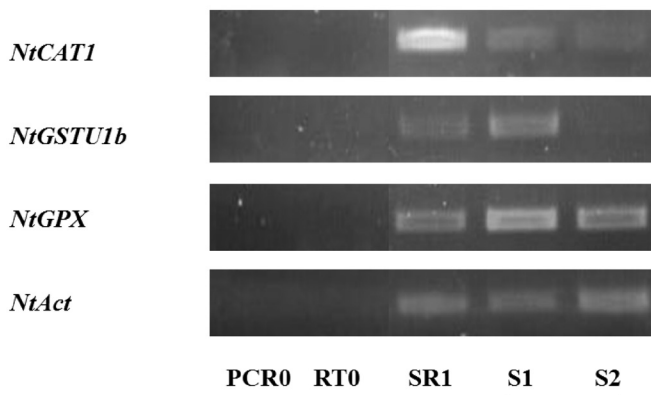
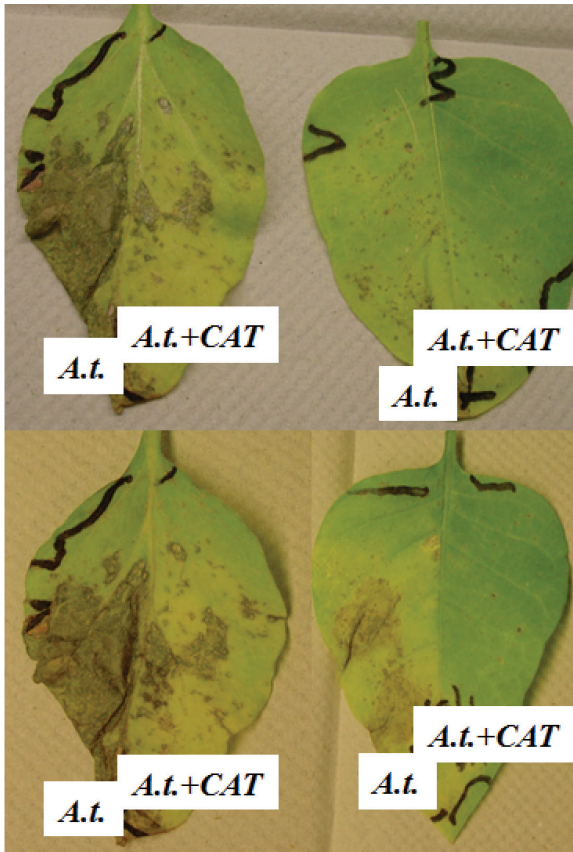


Fig. 2. Changes in expression of genes encoding H_2O_2 -degrading antioxidant enzymes in tobacco plants expressing a tomato chloroplast superoxide dismutase (*SiChSOD*) transgene (*Nicotiana tabacum* cv. SR1, T0 generation, S1 and S2 lines). *NtCAT1* = catalase1. *NtGSTU1b* = tau class glutathione S-transferase. *NtGPX* = glutathione peroxidase. Gene expression was analyzed by RT-PCR, a tobacco actin gene (*NtAct*) served as a reference. RT0 = negative control of reverse transcription (no RNA template). PCR0 = negative control of polymerase chain reaction (no DNA template). SR1 = non-transformed tobacco

sonii (Cole et al., 2004). Inoculation of agroinfiltrated plants with TMV revealed that *ZmCat2* confers enhanced resistance to HR-type necrosis during TMV infection in both *N. edwardsonii* and *N. edwardsonii* var. Columbia. Transient overexpression of this catalase reduced the number of TMV-induced HR-type necrotic lesions and total necrotized leaf area by at least 40–50% (Fig. 3). It is worth mentioning that in TMV-inoculated *N. edwardsonii* leaves agroinfiltration of *ZmCat2* reduced the necrotized leaf area to ca. the extent found in TMV-inoculated and control-infiltrated (*A. tumefaciens*, without *ZmCat2*) var. Columbia leaves, while *ZmCat2* was able to further reduce the extent of TMV-induced HR-type necrotization in var. Columbia (Fig. 3).



N. edwardsonii

N. e. var. Columbia

Fig. 3. Transient overexpression of a maize catalase gene (*ZmCat2*, “CAT”) by agroinfiltration in *Nicotiana edwardsonii* and *N. edwardsonii* var. Columbia; effect on localized necrosis (HR) caused by Tobacco mosaic virus (TMV) in inoculated leaves. Left leaf halves: control-infiltrated (*Agrobacterium tumefaciens* LBA4404, without *ZmCat2*). Right leaf halves: infiltrated with *A. tumefaciens* LBA4404 carrying *ZmCat2*. Plants were inoculated with TMV two days after agroinfiltration. Photos in the upper and lower panels were taken six and seven days after TMV inoculation, respectively

Discussion

In an effort to create transgenic plants with high antioxidant capacity that could resist necrotic symptoms we produced two transgenic tobacco (*Nicotiana tabacum* cv. SR1) lines (S1 and S2) overexpressing a tomato chloroplast superoxide dismutase (*S1Ch-SOD*). This SOD gene has been shown to confer enhanced tolerance of transgenic potato shoots and roots to tissue necrosis caused by the superoxide-generating herbicide, paraquat (Perl-Treves et al., 1988; Perl et al., 1993). Our preliminary analysis of *Nicotiana tabacum* cv. SR1 S1 and S2 plants might indicate a similar tolerance to necrotic symptoms caused by abiotic stresses and pathogen infections. Although *in planta* overexpression of SOD genes often increase endogenous H₂O₂ levels, such plants are less sensitive to stress-related necrosis if sufficient H₂O₂-degradation is conferred by elevated expression of antioxidants like e.g. catalases and peroxidases (Kwon et al., 2002; Tang et al., 2006; Lee et al., 2007; Faize et al., 2011, 2012; Diaz-Vivancos et al., 2013). We found that S1 tobacco indeed displays elevated expression of an endogenous glutathione peroxidase (*NtGPX*) and a tau class glutathione S-transferase (*NtGSTU1b*), genes that may take part in the degradation of H₂O₂ and lipid hydroperoxides during oxidative stress. GST isoenzymes are encoded by a gene family divided into different classes based on sequence similarities and biochemical function (Edwards et al., 2000; Wagner et al., 2002). Most GSTs belong to the phi and theta classes and participate in detoxification reactions by catalyzing the conjugate formation between electrophilic compounds and the antioxidant glutathione (GSH) (Wagner et al., 2002; Dixon and Edwards, 2009a; Dixon et al., 2011). However, certain tau class GSTs possess a glutathione-peroxidase activity and are able to catalyze the breakdown of H₂O₂ and lipid hydroperoxides (Bartling et al., 1993; Dixon et al., 2009b). In fact, overexpression of GSTs with both GST and GPX activity conferred enhanced tolerance of tobacco to paraquat and thermal or salt stress (Roxas et al., 1997; Yu et al., 2003).

Although two genes that may encode H₂O₂-degrading enzymes (*NtGPX* and *NtGSTU1b*) were induced in our SOD-overexpressing S1 tobacco, this was not the case with an endogenous catalase gene. Notably, expression of a class 1 catalase (*NtCAT1*) was repressed in both healthy, SOD-transgenic tobaccos. It appears that in plants stressed by pathogenic infections or chemical treatments class 1 catalases are often repressed (Williamson and Scandalios, 1992; Willekens et al., 1994), although their original function is the removal of H₂O₂ produced during photorespiration in leaf peroxisomes (Willekens et al., 1995). For example, *NtCAT1* was shown to be down-regulated during virus-induced localized necrosis (HR) in several *Nicotiana* species (Dorey et al., 1998; Yi et al., 1999, 2003; Künstler et al., 2007). Repression of *NtCAT1* in virus-inoculated leaves that display HR is usually transient and begins within the first 24 hours after inoculation by TMV or *Tobacco necrosis virus* (TNV). It was also shown that *NtCAT1* is repressed in the vicinity of necrotic lesions (Dorey et al., 1998; Yi et al., 1999) suggesting a role for this gene primarily in the induction, rather than elimination of necrosis during localized virus infections. However, *CAT1* may play different roles in systemic virus infections: a recent report indicates that a tomato *CAT1* gene confers *Pepino mosaic virus* (PepMV) accumulation during systemic movement (Mathioudakis et al., 2013). Based on the above-mentioned

data, repression of *NtCAT1* in healthy, SOD-transgenic S1 and S2 tobaccos predicts that these plants could be sensitive to localized necrosis caused by virus infection.

In order to elucidate whether catalases other than *NtCAT1* might play a role in resistance of *Nicotiana* species to virus induced HR-type necrosis, transient *in planta* expression of a maize class 2 catalase (*ZmCat2*) was tested. Class 2 catalases are usually stimulated during abiotic and biotic stresses. For example, a potato *CAT2* gene is induced locally and systemically upon nematode and bacterial infection (Niebel et al., 1995). Furthermore, in tobacco displaying virus-induced HR *CAT2* is only slightly repressed in tissues close to necrotic lesions (Dorey et al., 1998). Also, *CAT2* mRNA levels are rapidly induced during oxidative damage by ozone, sulfur dioxide and UV-B radiation (Willekens et al., 1994). Together, these results point to a pivotal role of class 2 catalases in defense against various environmental (oxidative) stresses (Willekens et al., 1995; Vanderauwera et al., 2011). In this study, we have shown that transient overexpression of *ZmCat2* in *Nicotiana edwardsonii* and *N. edwardsonii* var. Columbia indeed confers enhanced resistance to HR-type necrosis during TMV infection. Previously we have demonstrated that *N. edwardsonii* is more susceptible to HR-type necrosis induced by e.g. *Tobacco mosaic virus* (TMV), than *N. edwardsonii* var. Columbia (Cole et al., 2004). Interestingly, in TMV-infected *N. edwardsonii* transient *ZmCat2* expression reduced the necrotized leaf area to ca. the extent displayed by var. Columbia plants. Our results suggest that certain endogenous class 2 catalases may contribute to the enhanced resistance of *N. edwardsonii* var. Columbia to virus-induced HR-type necrosis.

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