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FULL RESEARCH PAPER

Reactive oxygen intermediates and oxalic acid in the pathogenesis of the necrotrophic fungus *Sclerotinia sclerotiorum*

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Abstract An effective colonization of the host plant tissue by the necrotrophic fungus *Sclerotinia sclerotiorum* requires the secretion of the non-host specific toxin oxalic acid (OA), which is known to suppress the generation of reactive oxygen intermediates (ROI). A full-length cDNA coding for an oxalate decarboxylase (TOXDC), which converts OA into CO_2 and formate, was isolated from the basidiomycete *Trametes versicolor*. It was overexpressed in tobacco plants to study the role of ROI and OA in the interaction between tobacco and *S. sclerotiorum*. The transgenic plants contained less OA and showed a delayed colonization of *S. sclerotiorum*; furthermore a strong ROI accumulation and nearly no catalase activity compared to the wild type (WT) plants could be detected. In addition, inoculation experiments with transgenic catalase-deficient plants (CAT1AS) and *in vitro* studies showed that *S. sclerotiorum* copes with strong ROI stress. Our results indicate that OA supports the infection process caused by *S. sclerotiorum* and the fungus itself is able to tolerate high ROI concentrations.

The nucleotide sequence data is available from the NCBI Genbank nucleotide-sequence database under the number AY370675

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Introduction

One of the earliest plant resistance responses against invading microorganisms is the induction of the hypersensitive response (HR) followed by local cell death. This phenomenon is one of the most important factors in impeding the growth of biotrophic pathogens. Although the HR is efficient against these microorganisms, apparently it does not seem to protect plants against attack by necrotrophic pathogens, which can utilize dead cells as a nutrient source (Van Kan 2006). In this case, plant cell death may indeed promote fungal colonization of the host tissue.

The establishment of a HR is often accompanied by the generation of reactive oxygen intermediates (ROI; Greenberg 1997). Generally, ROI are produced both in compatible and incompatible plant–pathogen interactions, but in a compatible interaction, only a single rapid and weak H_2O_2 release is observed (Baker and Orlandi 1995; Grant and Loake 2000). This transient burst is non-specific and cannot prevent the pathogen from spreading in the plant tissue (Van Breusegem et al. 2001). In contrast, a second and enhanced burst could be observed in incompatible interactions (Levine et al. 1994).

ROI play an important role in the plant defence against biotrophic pathogens and accumulate within hours after infection (Lamb and Dixon 1997). During the so-called oxidative burst, superoxide anions $\left(O_2^-\right)$ and hydrogen peroxide (H_2O_2) are both produced and released at the site of pathogen ingress (Apostol et al. 1989; Wojtaszek 1997). These oxygen intermediates serve as signalling molecules and appear to be a part of the signalling cascade, resulting in the activation of plant defence mechanisms (H. Knight and M. T. R. Knight 2001; Mittler 2002). H_2O_2 triggers lipid peroxidation and contributes also to the reinforcement of plant cell walls (Bradley et al. 1992). Moreover, H_2O_2 takes part in the induction of the synthesis of pathogenesis-related (PR) proteins and phytoalexins (Hammond-Kosack et al. 1996). Together with salicylic acid, H_2O_2 weakens the plant tissue (Rao et al. 1997; Wojtaszek 1997; Orozco-Cardenas et al. 2001). The role of H_2O_2 in plant defence depends on its concentration in the tissue (Mittler 2002). Low levels of H_2O_2 induce protective antioxidative enzymes and activate the systemic signalling cascade (Solomon et al. 1999). Moderate to high H_2O_2 levels trigger programmed cell death (PCD; Tenhaken et al. 1995; Fleury et al. 2002). These observations were supported by experiments with transgenic plants with reduced catalase (CAT) or ascorbate peroxidase (APX) activity (Chamnongpol et al. 1996, 1998; Willekens et al. 1997). These plants accumulate high levels of H_2O_2, activate PCD at lower pathogen inoculum levels as compared to the control plants, and react hyperresponsively to pathogen infections (Mittler et al. 1999a).

Several mechanisms are described by which plant cells regulate the intracellular ROI level. Among those, the enzymes superoxide dismutase, APX and CAT are the key regulators (Halliwell and Gutteridge 1990). The balance between these enzymes is crucial for the steady-state level of the superoxide radicals and H_2O_2 (Bowler 1991). The different affinity for H_2O_2 indicates that APX is essential for the detoxification of low H_2O_2 concentrations, whereas CAT detoxifies H_2O_2 at high concentrations. Inhibition or suppression of both enzymes during a HR hinders the plant cell to detoxify H_2O_2 (Örvar and Ellis 1997; Mittler et al. 1998).

The induction of a HR and the production of ROI could also be independent from each other (Baker and Orlandi 1995; Glazener et al. 1996; Sasabe et al. 2000). Treatment of sugar beet plants with the biological control agent *Bacillus mycoides* leads to the release of H_2O_2 without the induction of a HR after infection with *Erwinia carotovora* pv. *betavasculorum* (Bargabus et al. 2003). However, it cannot be excluded that the H_2O_2 level was not high enough to induce an HR or that it seems to be necessary to activate the HR.

In contrast, the formation of extracellular ROI by the pathogen at early stages during an infection process can weaken the host tissue, which leads to a rapid pathogen colonization (Levine et al. 1994; Lamb and Dixon 1997). These findings were supported by observations in the *Phaseolus vulgaris*–*Botrytis cinerea* interaction in which at early stages of infection ROI production could be detected (Von Tiedemann 1997). Furthermore, some necrotrophic pathogens may produce ROI at early stages of the interaction to initiate a successful infection as described for *B. cinerea* or for *Rhynchosporium secalis* (Von Tiedemann 1997; Govrin and Levine 2000; Schouten et al. 2002; Able 2003). In contrast to these observations, Muckenschnabel et al. (2001) were not able to verify a direct role for ROI in the interaction between *Arabidopsis thaliana* and *B. cinerea*. In further studies, it was demonstrated that *B. cinerea* also produces H_2O_2 through the activity of a fungal glucose oxidase, which seems to be involved in the establishment of an infection in the host tissue (Van der Vlugt-Bergmans et al. 1997).

While necrotrophs may use disease resistance mechanisms evolved in a plant to defend against biotrophs to their advantage, they may also secret toxins to facilitate host cell death (Thomma et al. 2001; Van Kan 2006). *Sclerotinia sclerotiorum* is a ubiquitous necrotrophic fungal pathogen which can infect over 400 different plant species in all stages of their development (Purdy 1979). This necrotrophic ascomycete causes significant yield losses of economical important crops including sunflower, oil seed rape, tomato, tobacco and soybean (Hu et al. 2003; Bolton et al. 2006). During pathogenesis, *S. sclerotiorum* secretes large amounts of the non-host specific toxin oxalic acid (OA), which causes wilting symptoms in infected plants (Noyes and Hancock 1981; Hegedus and Rimmer 2005). This phenomenon might be influenced by the induction of stomatal opening and the inhibition of abscisic acid-induced stomatal closure (Guimaraes and Stotz 2004). In

addition, the toxin favours plant cell wall degradation by shifting the apoplastic pH close to the optimum for cell wall-degrading enzymes and removes the calcium ions that are bound to pectins (Bateman and Beer 1965). *Sclerotinia sclerotiorum* mutants lacking the ability to produce OA were found to be non-pathogenic on plants after inoculation (Godoy et al. 1990). Studies on tobacco and soybean cell cultures have shown that OA inhibits the plant oxidative burst and that the inhibitory effect is independent of the cytosolic pH value and also independent of the affinity of OA for calcium ions (Cessna et al. 2000).

Two enzymes, oxalate oxidase (OXO) and oxalate decarboxylase are able to detoxify OA (Dutton and Evans 1996). Constitutive expression of wheat OXO, which converts OA into CO_2 and H_2O_2, was successfully established in soybean, tobacco, poplar and sunflower and reveals enhanced resistance against OA-producing pathogens (Berna and Bernier 1999; Donaldson et al. 2001; Liang et al. 2001; Hu et al. 2003). Similarly, overexpression of an oxalate decarboxylase gene from the basidiomycete *Collybia velutipes*, which was successfully incorporated into the tobacco and tomato genome, led to an enhanced resistance against *S. sclerotiorum* by converting OA into CO_2 and formate (Kesarwani et al. 2000). In contrast to the OXO-enzyme activity, OA conversion into CO_2 and formate does not lead to a development of additional H_2O_2.

Research presented in this paper focuses on the role of OA and ROI in the pathogenesis of *S. sclerotiorum* on tobacco plants. For this, we isolated and expressed an oxalate decarboxylase from the basidiomycete *Trametes versicolor* (TOXDC) in tobacco plants and challenged these plants with *S. sclerotiorum*. In addition, we investigated how *S. sclerotiorum* copes with high H_2O_2 concentrations *in vivo* as well as *in vitro*.

Materials and methods

Plant material, bacterial strains, plasmids and chemicals

Nicotiana tabacum cv. Xanthi nc, transgenic CAT1AS, and the corresponding wild-type SR1 were grown in plastic pots in the greenhouse in climate-controlled chambers at 22°C and a 16 h photoperiod. *Escherichia coli* strain DH10B was grown at 37°C in Luria broth (LB; Sambrook et al. 1989). *Agrobacterium tumefaciens* strain LBA4404 was also grown in LB but at 28°C. Agar (0.8%, *w*/*v*) was added to media for plate culture. Antibiotics were used for selection at the following concentrations: Ampicillin, 100 $\mu g/ml^{-1}$ (Roth, Karlsruhe, Germany), Rifampicin, 30 $\mu g/ml^{-1}$ (Fluka, Buchs, Switzerland), Streptomycin 50 $\mu g/ml^{-1}$ (Serva, Heidelberg, Germany), and Kanamycin, 50 or 100 $\mu g/ml^{-1}$ (Duchefa Biochemie BV, Haarlem, Netherlands).

Fungal growth and plant inoculations

Sclerotinia sclerotiorum isolates Dachbrand (SSD) and HOH0 (SSHOH0) and *T. versicolor* isolate KL82070 were maintained at 20°C on BMP medium composed of malt extract (2%, *w*/*v*), peptone (0.2%, *w*/*v*) and agar (2%, *w*/*v*). For inoculation experiments, *S. sclerotiorum* was grown on 1/2 PDA as described by Donaldson et al. (2001). Mycelial agar plugs of 5 mm diam were excised and inverted on the upper surface of fully expanded detached leaves of 7–9 week-old plants. Infected leaves were kept in plastic boxes containing wet filter paper to ensure high humidity and were incubated at 20°C and a 16 h photoperiod. Disease symptoms were observed every 24 h over a period of 5 days.

Cloning of oxalate decarboxylase

Liquid cultures of *T. versicolor* were induced with OA 14 days after incubation at 20°C. Total RNA was extracted from mycelia 48 h after induction following the method of Chomzynski and Sacchi (1987). In brief, 100 mg leaf material were homogenized in liquid nitrogen and resuspended in buffer containing 4 M guanidinium thiocyanate, 0.025 M sodium citrate and 0.1 M β-mercaptoethanol. RNA was extracted with saturated phenol and precipitated twice in ice-cold isopropanol. The remaining genomic DNA in the sample was eliminated by DNAse digestion according to the manufacturer's instructions (MBI Fermentas, St. Leon-Rot, Germany). First strand cDNA was synthesized using the RevertAid First strand cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany). Second strand synthesis and amplification was performed using degenerate primers based on amino acid alignments between known oxalate decarboxylases. All primer

pairs used were obtained from MWG Biotech (Ebersberg, Germany) except for dye-labelled primers (Amersham Bioscience, Freiburg, Germany). The primer pair first used was OL218 (5′-GGH GACCTBTGGTACTTCCC-3′) and OL220 (5′-GTT CTCSAYGTAGTGDCCCA-3′). The amplified PCR product was analyzed on a 1.2% (*w*/*v*) agarose gel and cloned into a pDrive vector (Qiagen, Hilden, Germany) named as pDrive-TVF. Sequencing reactions were performed on ALF Express II (Amersham Biosciences, Freiburg, Germany) using the Cycler Reader DNA Sequencing Kit (MBI Fermentas, St. Leon-Rot, Germany) and dye-labelled M13 primers. Data analysis was performed with Lasergene (DNAstar Inc., Wisconsin, U.S.A) and ALFwin Sequence Analyser 2.10 (Amersham Biosciences, Freiburg, Germany). The complete sequence of the oxalate decarboxylase gene was generated with the SMART RACE cDNA Amplification Kit according to the manufacturer's protocol (Clontech, Heidelberg, Germany). Specific RACE-primer used for generation of the 3′-end was GSP3-1 (5′-GGAACAGCCATAGCGCAGACC TTC-3′). A combination between touchdown and nested-PCR was necessary to obtain the entire 5′-end of the transcript. Nested primers used were GSP5-1 (5′-GTC ATC CCA GTC GGC TAT GTT GTC C-3′) and GSP5-2 (5′-CGT GAG MAA AAG CGT GTC GTC GTC G-3′). The amplified PCR products were cloned into a pDrive vector and analyzed as described before. A final PCR to obtain the complete oxalate decarboxylase-cDNA was performed using the primer pair OL300 (5′-GCT CTA GAA TGG GCA AGT TCC TCG CCA CC-3′) and OL301 (5′-CGG GAT CCG AGA AGA AAC ATT CGC ACC-3′). The entire cDNA was cloned again into a native pDrive and named as pDrive-TOXDC.

Expression *in planta*

The plasmid p707 CD3-327 [smRSGFP] was double-digested by *Eco*RI and *Hin*dIII and the released fragment CaMV35S-GFP-pAnos was cloned into pUC19. The new vector was designated as pUC19-35S-GFP. In the next step, a restriction of pDrive-TOXDC and pUC19-35S-GFP was done with *Bam*HI and *Xba*I, and the TOXDC was first gel-purified and then ligated into pUC19-35S-GFP. The new plasmid and the binary vector pGpTV (Becker et al. 1992) were subsequently digested with *Eco*RI and *Hin*dIII. The 35S-TOXDC-GFP fragment was again gel-purified and ligated into the binary vector to finally obtain the vector p221-TOXDC. The construct included the neomycin phosphotransferase gene which allows selection on kanamycin containing media.

Plant transformation

Seeds from *N. tabacum* cv. Xanthi nc were surface-sterilized with hypochlorite and grown on Murashige and Skoog basal medium (Murashige and Skoog 1962). The construct p221-TOXDC was transformed into *A. tumefaciens* by electroporation (Nagel et al. 1990). Plant transformation was carried out by a leaf-disk method (Horsch et al. 1985). Transformed plants were kept in growth chambers at 18°C and a 16 h photoperiod under high humidity, tested for integration and expression of TOXDC as described below, and finally propagated and adapted to greenhouse conditions (20°C, 16 h photoperiod). The transgenic tobacco plants were selfed and T_1 seeds were used in all experiments.

Molecular and biochemical analysis of transgenic plants

Total DNA was isolated from transgenic and WT tobacco plants using the protocol described by Dellaporta et al. (1983). In brief, leaves were homogenized in liquid nitrogen, resuspended in extraction buffer and precipitated with isopropanol. After extraction with 1 volume phenol/chloroform and 1 volume chloroform/isoamylalcohol, DNA was precipitated with 1/10 volume sodium acetate and 0.7 volume isopropanol.

PCR was carried out using the primer pair OL360 (5′-ATGGGCAAGTTCCTCGCCACC-3′) and OL361 (5′-TACAGAAGAAACAT TCGCACC-3′). Total RNA was isolated as described by Chomzynski and Sacchi (1987). cDNA synthesis was done with 1 μg total DNase-treated RNA by using the RevertAid H Minus First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). PCRs were carried out as described above. The oxalate decarboxylase assay was performed according to Lang and Lang (1972). Enzyme activity was indirectly estimated by the production of formate after addition of OA as a substrate. For this purpose, plant leaf material was

homogenized using 0.2 M sodium acetate buffer (pH 5.2).

OA quantification was done according to the method described by Kesarwani et al. (2000). Four leaves from wild-type and transgenic plants were excised and homogenized in liquid nitrogen. The fine powder was extracted for 10 min in boiling 0.25 M H_2SO_4. Samples were cooled to RT and after centrifugation the supernatant was used for further analysis. The organic acid content was estimated on a Synergi Polar RP18 column (250×46 mm; 4 μm particle; Phenomenex, Aschaffenburg, Germany) by HPLC.

Detection and quantification of ROI *in planta*

Superoxide anions were detected by their ability to transform nitro-blue tetrazolium (NBT) to formazan with the method adapted from Doke (1983) and Doke and Ohashi (1988). H_2O_2 production in plants infected with *S. sclerotiorum* was assayed by the oxidation of 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Lu and Higgins 1998). In both cases, leaf pieces were either vacuum-infiltrated in a syringe with either NBT or DCFH-DA. The resulting blue spots (NBT) or fluorescence (DCF) were analyzed with a Zeiss Axioskop II microscope (excitation 485 nm; emission 525 nm).

Catalase activity staining

Total protein was extracted from leaves with an extraction buffer containing 50 mM potassium phosphate (pH 7.0), 1 mM EDTA, 1 mM phenylmethyl-sulfonyl-fluoride, 5 mM sodium ascorbate and 5% (*w*/*v*) polyvinyl pyrrolidone K25. After centrifugation at 17,000×*g* for 10 min at 4°C, protein concentration in the supernatant was determined according to the method described by Bradford (1976). A small amount of 33 μg of the crude protein extract was loaded onto a 10% (*w*/*v*) native polyacrylamide gel. In-gel detection of catalase activity was performed according to Clare et al. (1984). In brief, the gel was incubated in a solution containing 0.05 M potassium phosphate, pH 7.0, and 50 $\mu g/ml^{-1}$ peroxidase at room temperature for 45 min in the dark; 0.005 M H_2O_2 was given to the solution and the gel was incubated for 5 min. Finally, the gel was rinsed with water and placed in the 0.05 M potassium phosphate, pH 7.0, supplemented with 0.5 g/l 3,3-diaminobenzidine. As a result, CAT bands appeared on a brown background.

H_2O_2 sensitivity assay

The H_2O_2 assay was carried out as described by Schouten et al. (2002). *Sclerotinia sclerotiorum* isolates were cultivated for 3 days on water agar (1.5%, *w*/*v*). Agar plates containing H_2O_2 in concentrations between 0 and 20 mM in 2.5 mM intervals were then inoculated with 5 mm diam mycelial agar plugs.

Data analysis

Data were statistically analyzed for significant differences using one-way analysis of variance (ANOVA), followed by Tukey test (α=0.05), using SPSS for Windows.

Results

Structure of the oxalate decarboxylase gene from *T. versicolor* and its expression *in planta*

Sequence analysis of the full length TOXDC-cDNA revealed an open reading frame that encodes a 455 amino acid polypeptide with a predicted molecular mass of 48.4 kDa. Data base search of the TOXDC-amino acid sequence using BLASTP displayed a strong similarity with the oxalate decarboxylase from *C. velutipes* (Genbank accession number AF200683; identity: 61%; *E* value: 4e-161), three *Ceriporiopsis subvermispora* frames (CAD91553.1; 60%; 5e-159; CAD91552.2; 64%; 1e-156; CAG34245.1; 66%; 1e-135), and a hypothetical protein from *Coprinopsis cinerea* (EAU85209.1; 59%; 4e-147).

Tobacco leaf discs were successfully transformed with p221-TOXDC (Fig. 1), and the regenerated plants were rooted in soil following an *in vitro* culture. These plants were screened for the presence and expression of the TOXDC gene. PCR was carried out using genomic DNA and cDNA derived from transgenic and wild-type plants. A single band of 1.4 kb was obtained in transgenic tobacco plants representing the same size of the expected decarboxylase-cDNA (Fig. 2a). The tested plants also showed TOXDC transcript accumulation, which was analyzed by RNA isolation and RT-PCR (data not shown).

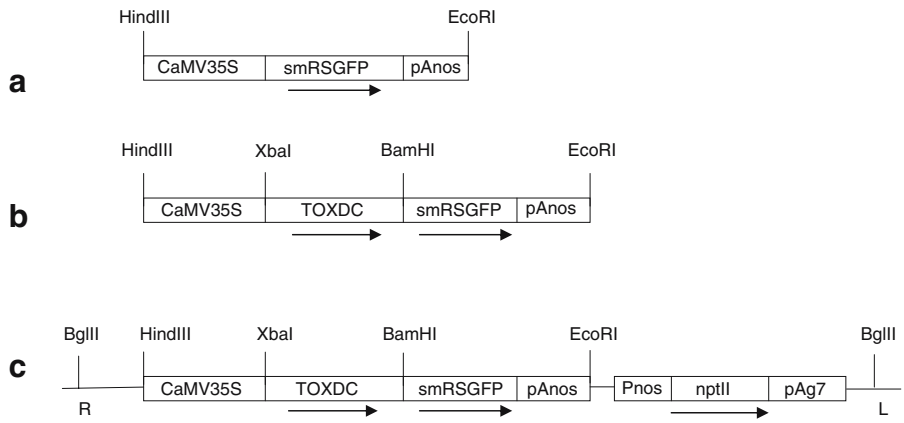


Fig. 1 Diagram of plasmids used in this study. *Arrows* indicate the orientation of the genes. **a** p707 CD3-327 (smRSGFP). **b** pUC19-35-TOXDC-GFP. **c** p221-TOXDC. All constructs were under the control of the CaMV35 promotor. The plasmid p221-TOXDC included the neomycin phosphotransferase gene (nptII). *L* Left border; *R* Right border sequence

Protein extracts of 33 regenerated transgenic plants and the corresponding wild-type (WT) were analyzed for oxalate decarboxylase activity. All transgenic plants tested produced detectable amounts of formate, the decarboxylation product of OA (data not shown). According to the results obtained by this screening, we tested several transgenic plants from the generation T1, whereas line TD16 and TD18 showed highest enzyme activities (Fig. 2b). Lines TD14, TD16 and TD18 were significantly different from the other group (WT, TD8, TD10, TD19).

Growth of *S. sclerotiorum* on transgenic TD18 tobacco leaves

Leaves of the transgenic line TD8, TD16, and TD18, exhibiting different TOXDC enzyme activities and WT plants were inoculated with *S. sclerotiorum* agar plugs and incubated for 5 days in plastic boxes under high humidity. Leaves of WT plants exhibited typical symptoms at 24 hpi. In contrast to WT plants, fungal growth and development were delayed in the transgenic TD16 and TD18 leaves (Fig. 2c). Neither mycelia nor sclerotia, which were frequently found on WT leaves, were macroscopically observed during colonization of the transgenic TD18 expressing tissue within the time period examined. In the TD16-tissue, fungal development was delayed, but still showed a visible mycelial growth. In contrast, TD8-inoculated tissue showed no delay in fungal development compared to the control. These observations were supported by the measurements of fungal spread in several tissues 3 and 5 days after inoculation (Table 1). Therefore line TD18 was significantly different from the other lines and selected for further experiments.

OA concentration in infected and non-infected tobacco leaf tissues

Total OA content was analyzed by HPLC. The transgenic TD16 leaves showed 44.49% and TD18 leaves 60.29% decrease in the level of oxalic acid content compared to the WT leaves (Table 2).

Oxygen intermediates and catalase activity in the tobacco–*S. sclerotiorum* interaction

In order to evaluate whether the fungal production of OA suppressed the oxidative burst, WT and transgenic leaves were inoculated with agar plugs containing 4 day-old mycelium of *S. sclerotiorum* and stained for superoxide anion production (Fig. 3). Spots of oxidized NBT were detected in the inoculated transgenic tissue at 24 hpi. We could also detect a few blue spots in the WT interaction, which indicated superoxide anion production in these plants. However, local H_2O_2 production was detected in transgenic plants at 24 hpi, whereas H_2O_2 production in the WT interaction occurred only within the first 24 hpi and decreased at later times (data not shown). In addition to the histochemical observations, catalase activity of the WT and TOXDC-expressing plants was

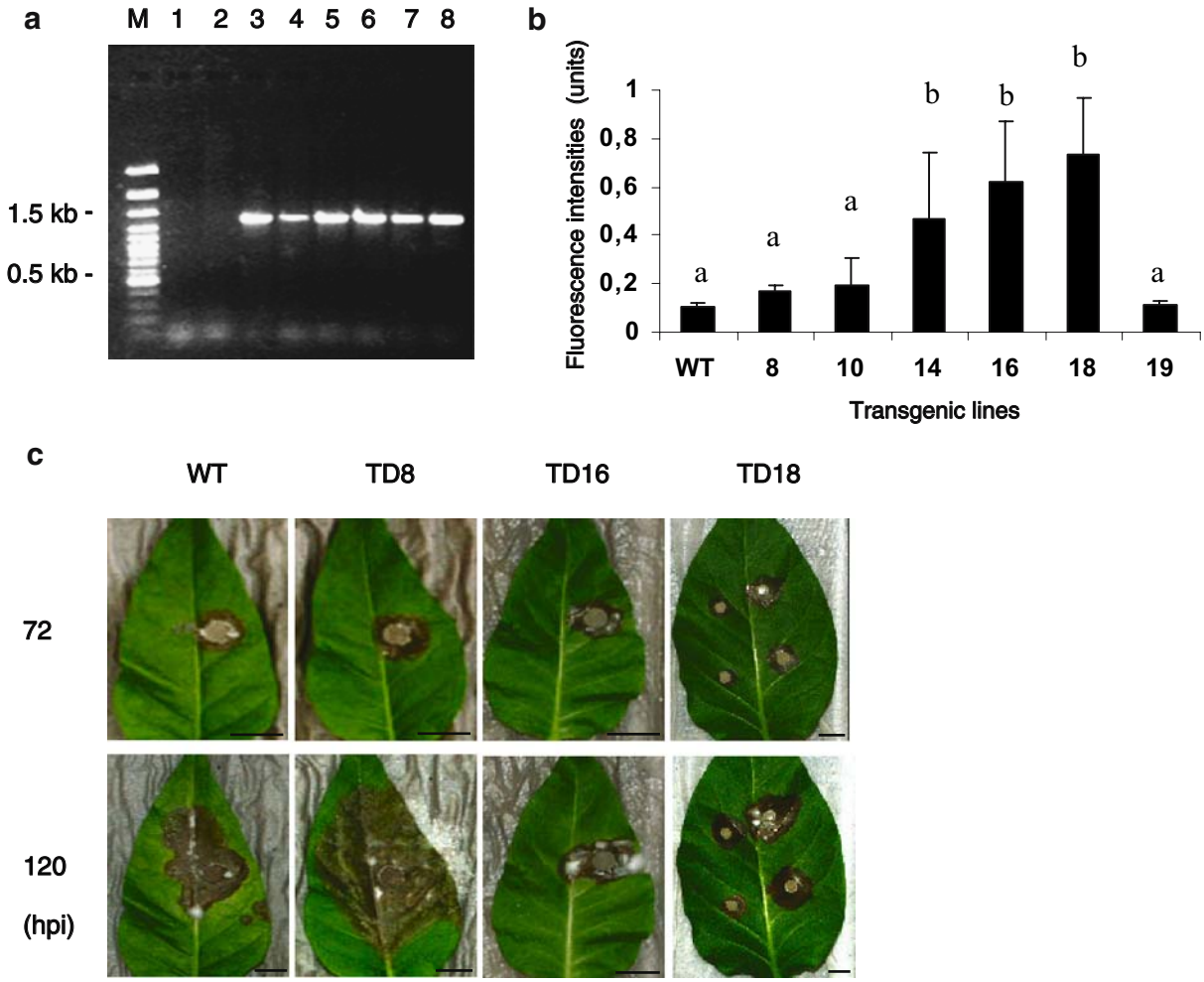


Fig. 2 Analysis of transgenic TOXDC expressing (TD) and WT tobacco plants. **a** Molecular analysis of transgenic TOXDC plants. Genomic DNA was extracted from WT plants and several TOXDC plants. After PCR amplification of the TOXDC gene, samples were electrophoresed on a 1.8% (*w*/*v*) agarose gel. *M* Molecular weight marker, *lane 1* PCR negative control; *lane 2* WT; *lane 3–8* transgenic plants TD8, 10, 14, 16, 18, 19. **b** Analysis of TOXDC activity in transgenic and WT tobacco plants. TOXDC enzyme activity was indirectly detected by the production of the enzymatic by-product formate in the plant tissue. One unit was given as increased values at 515 nm related to 100 μg crude plant protein extract. Six transgenic lines and the corresponding WT plant were investigated. The results are means with standard deviation of three independent experiments. *Bars with different letters* are significantly different. **c** Phenotype of *S. sclerotiorum*-inoculated WT- and TOXDC leaves at different time points after infection. Inoculation was done with mycelial agar plugs. *Top row* 72 hpi, *bottom* 120 hpi; *bars* represent 1 cm

monitored on native PAGE. A high enzyme activity could be observed within the first 24 hpi in wild-type plants, which decreased during the following 24 h (48 hpi). Only a weak catalase activity was found in the transgenic plants between 0 and 24 hpi, and at 48 hpi nearly no activity was detected.

We also investigated transgenic CAT1AS tobacco plants with reduced catalase activity. In these plants, H_2O_2 accumulation differed from that of WT plants (Fig. 4a–d). Infection of these plants resulted in lesion development of comparable size as in the WT plants (data not shown). Strong H_2O_2 production and nearly no catalase activity were detected in the transgenic tissue at the early stages compared to the WT tissue after inoculation with the fungus (Fig. 4e).

Table 1 Disease development in WT and the TOXDC expressing tobacco plants TD16 and TD18 after inoculation with *S. sclerotiorum* as measured by lesion size

Plant sample	Lesion size (cm) 72 hpi	Lesion size (cm) 120 hpi
WT	1.8±0.497	2.3±0.471
TD16	0.7±0.283[a]	1.8±0.792
TD18	0.8±0.185[a]	1.2±0.333[a]

Data represent means of lesion sizes from three replicates including standard deviation.

[a] Significantly different from the WT plant sample.

Sensitivity of *S. sclerotiorum* to H_2O_2 stress

To investigate fungal H_2O_2 tolerance, two *S. sclerotiorum* isolates were selected and analyzed for their sensitivity to H_2O_2 *in vitro* (Fig. 4f). The radial growth rate of both was measured daily on medium containing different concentrations of H_2O_2. Both isolates were able to grow in the presence of 2.5 mM H_2O_2 at the same rates as in the absence of H_2O_2. Higher concentrations between 2.5 and 10 mM led to a delayed growth rate. Growth rate was markedly reduced at concentrations between 12.5 and 20 mM H_2O_2 and was completely inhibited at concentrations higher than 20 mM.

Discussion

Necrotrophic fungi have evolved many mechanisms to penetrate cells and to spread within the plant tissue (Mayer et al. 2001). The production of oxygen radicals in these interactions seems to be important as they weaken the host tissue at early stages of infection and consequently, enhance the growth and development of the pathogen (Lamb and Dixon 1997; Van der Vlugt-Bergmans et al. 1997; Von Tiedemann 1997; Govrin and Levine 2000). Furthermore, some necrotrophic or facultative saprophytic pathogens produce toxins leading to a degradation of the plant tissue and inducing cell death (Markham and Hille 2001). Toxins support fungal colonization and suppress plant defence mechanisms (Wolpert et al. 2002; Walton 1996). The non-host specific toxin OA affects several mechanisms, of which the suppression of the plant oxidative burst seems to be the most important in facilitating the colonization of the host tissue (Dutton and Evans 1996; Cessna et al. 2000). The exact role of OA in the pathogenesis of several necrotrophic fungi still remains unclear. OA is often described as an important factor in the pathogenesis of *S. sclerotiorum*, where OA-mutants which produce nearly no OA are non-pathogenic (Godoy et al. 1990). However, several other reports have defined the non-host specific toxin as being unimportant in the infection progress (Callahan and Rowe 1991; Vannini et al. 1993). These authors speculate that another toxic fungal compound is involved in the early stages of the interaction, as has been described for Botrydial, produced by *B. cinerea* in addition to OA (Deighton et al. 2001; Colmenares et al. 2002). Recently, a selective phytotoxin named Sclerin, produced by *S. sclerotiorum*, was described (Pedras and Ahiahonu 2004). Sclerin seems to provoke phytotoxic effects on at least three cruciferous species. In addition, synergistic effects between OA and the activity of polygalacturonases during degradation of the plant cell wall have been described (Bateman and Beer 1965; Noyes and Hancock 1981; Marciano et al. 1983). Another effect of OA is the ability to decrease the cellular pH and bind calcium ions from the middle lamella (Bateman and Beer 1965; Maxwell and Lumsden 1970). However, recent reports suggest that this is a less important factor. According to Cessna et al. (2000), the suppression of the oxidative burst by OA plays a central role in the infection process. Other OA-producers, for example *B. cinerea*, exploit plant defence mechanisms including ROI-production for its pathogenicity (Govrin and Levine 2000). Thus, in some host–pathogen interactions, OA suppresses

Table 2 Measurement of total OA content in leaves of WT, TD16 and TD18 plants

Plant sample	OA content	Decrease (%)
WT	129.7±4.985	–
TD16	72.0±4.886[a]	44.49
TD18	51.5±9.970[a]	60.29

OA content is given as mg OA/100 g fresh leaf material. Data are means of four samples with one replication per sample including standard deviation.

[a] Significantly different from the WT sample.

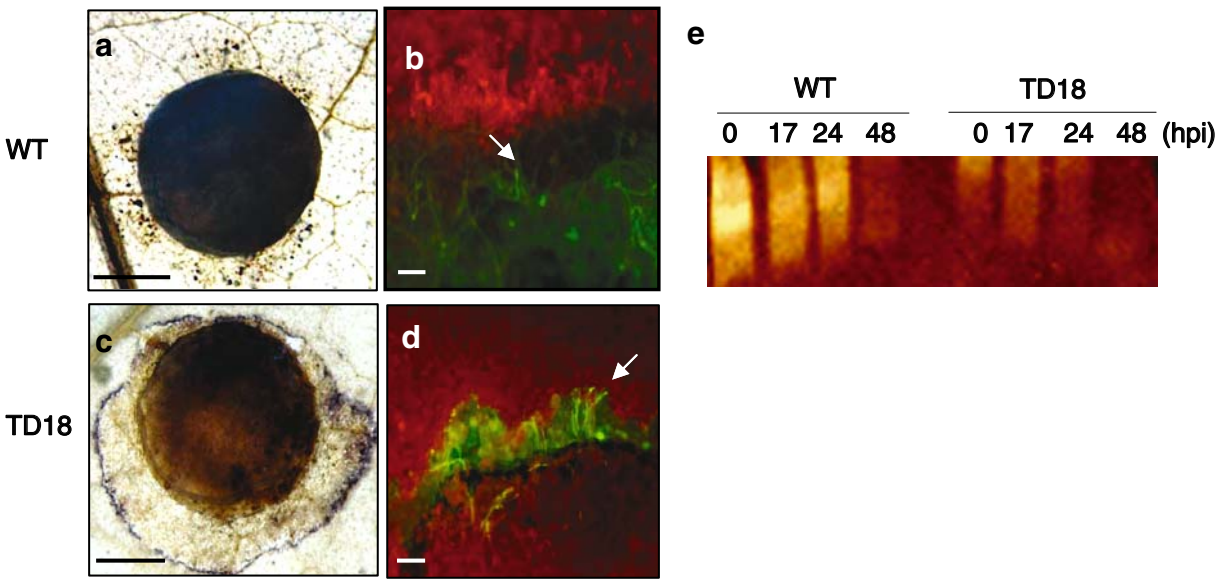


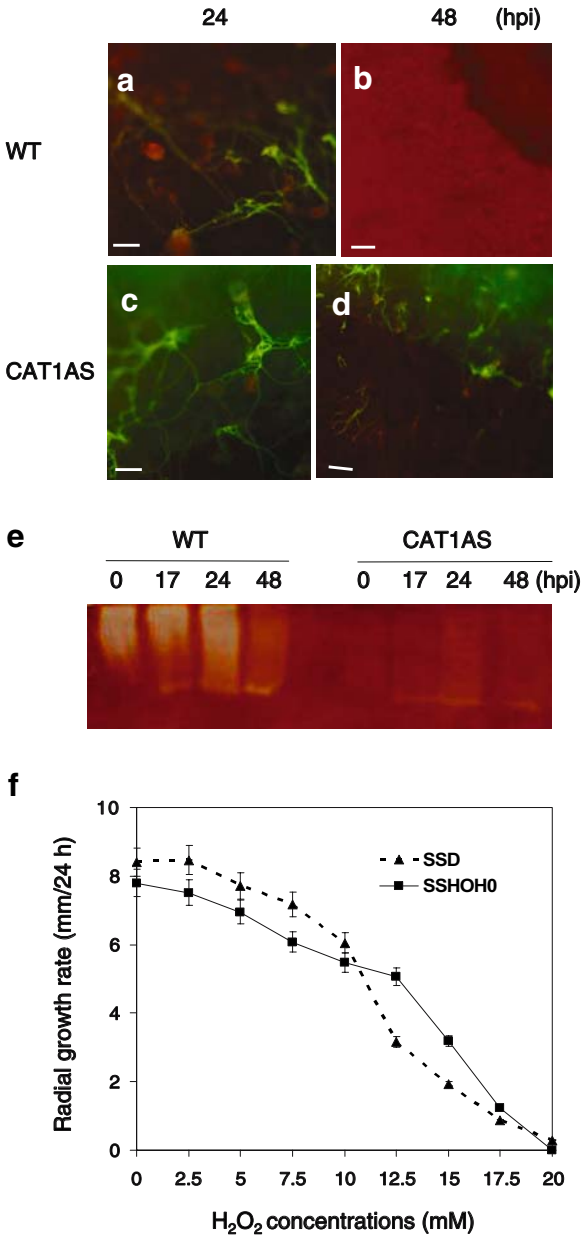
Fig. 3 ROI accumulation and detoxification in WT and TD18-plants after inoculation with *S. sclerotiorum*. **a** and **c** Inoculated tobacco leaves infiltrated with NBT 24 h after inoculation. The insoluble oxidized form of NBT (formazan) accumulates around the inoculation site and reflects the accumulation of superoxide anions. Leaves were washed in ethanol to remove the chlorophyll. **a** Diffuse formazan deposition in infected WT plant. **c** TD18 leaves stained with NBT show a localized accumulation of superoxide anions. The *scale bar* represents 3 mm. **b** and **d** Double staining of H_2O_2 by the oxidation of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and fungal hyphae. **b** Diffuse H_2O_2 production in WT plants at 24 hpi. **d** Strong and localized H_2O_2 accumulation in TD18 plant tissue at 24 hpi. The *scale bar* represents 120 μm. *Arrows* indicate fungal hyphae. **e** Native PAGE-analysis and activity staining for catalase in WT and TD18 leaf tissue at different time periods after inoculation (hpi). Identical protein concentrations were loaded in each lane (33 μg)

plant defence mechanisms, whereas in others, OA-producing fungi exploit plant defence for their own growth and ability to spread in the host tissue.

There a number of reports about transgenic plants degrading OA, e.g., soybean, tobacco, poplar and sunflower, which use oxalate oxidase (Oxo) to convert OA into CO_2 and H_2O_2 (Donaldson et al. 2001; Berna and Bernier 1997; Liang et al. 2001; Hu et al. 2003) or oxalate decarboxylase (OXDC) from *C. velutipes* (Kesarwani et al. 2000). In most cases, inoculation of these plants with OA-producing fungi such as *S. sclerotiorum* led to a delayed pathogen growth in the host tissue, and these observations are in accordance with our results.

Under normal growth conditions the production of ROI in cells is low. As demonstrated, when under attack by OA-producing pathogens, the ROI level still stays low. It has been shown that low to moderate H_2O_2 levels lead to the induction of cellular protective genes, whereas higher levels (between 6 to 8 mM H_2O_2) cause hypersensitive cell death (Tenhaken et al. 1995). Very high levels are lethal to plant cells (Apostol et al. 1989). Thus, an increased ROI level in the transgenic tissue after an infection could activate several plant defence mechanisms. ROI could serve as a secondary messenger involved in the defence signal transduction pathway, thus providing an explanation for the observed delay of fungal growth in the transgenic tissue. Oxidative stress seems to play an important role in the interaction between plants and *S. sclerotiorum*. Our findings concerning ROI production confirm other results, which had shown that OA is able to suppress ROI generation and that OA-deficient mutants of *S. sclerotiorum* could not colonize the host tissue (Cessna et al. 2000; Godoy et al. 1990). Consequently, ROI production in inoculated TOXDC-tissue or in tissue inoculated with OA-deficient mutants was not affected. Furthermore, and in contrast to other reports, we also observed a moderate ROI accumulation in inoculated WT plants within the first 24 hpi (Cessna et al. 2000).

In order to examine whether H_2O_2 production in the WT and TD18-tissue after infection coincided



◀ **Fig. 4** Accumulation and detoxification of H_2O_2 in WT and CAT1AS plants 24 and 48 h after inoculation with *S. sclerotiorum*. **a** to **d** Leaves were inoculated and assayed by oxidation of 2′,7′-dichlorofluorescein diacetate, which show double staining of ROI production and fungal hyphae. Images are taken from the border of the lesions. **a** and **b** H_2O_2 production in WT plants was only detectable at 24 hpi. **c** and **d** Strong H_2O_2 accumulation in inoculated CAT1AS plants 24 and 48 hpi; *bar* represents 100 μm. **e** Native PAGE-analysis and activity staining for catalase activity in WT and CAT1AS plants at different time points after inoculation. Identical protein concentrations were loaded in each lane (33 μg). **f** Sensitivity of two *S. sclerotiorum* isolates to H_2O_2. Mycelial agar plugs were placed on agar plates containing H_2O_2 in different concentrations (0–20 mM in 2.5 mM intervals). Radial growth was measured over 4 days in 24 h intervals. All results are means of three independent experiments

with the induction of antioxidative enzymes, the activity of the enzyme catalase was assayed. Catalase plays an important role in the detoxification of high levels of H_2O_2 in plant tissues (Solomon et al. 1999; Mittler 2002). Our results confirm that although *S. sclerotiorum* suppresses the oxidative burst by OA, low H_2O_2 levels could nevertheless be detected. Interestingly, nearly no catalase activity was found in the inoculated TD18-tissue, although H_2O_2 production was detected. Such observations have been previously described for tissues exhibiting the HR defence, in which strong ROI production and a complete suppression of ROI-detoxifying enzymes had been observed (Dorey et al. 1998; Mittler et al. 1999b). We assume that H_2O_2 stress in the transgenic TD18-tissue suppresses the catalase activity and that the fungus cannot cope with this high ROI level resulting in delayed fungal growth in the tissue.

Our observation that ROI levels increase after *S. sclerotiorum* inoculation is in contrast to other results in which no oxidative stress for the fungus *in planta* could be discovered (Cessna et al. 2000). A source of ROI could be represented by fungal-derived oxygen radicals. Fungal pathogens are able to produce ROI by several mechanisms (Von Tiedemann 1997; Deighton et al. 1999; Mayer et al. 2001). *Botrytis cinerea* seems to produce superoxide anions in hyphal tips and the production of H_2O_2 could be shown in penetrated tissues (Schouten et al. 2002; Tenberge et al. 2002; Govrin and Levine 2000). Likewise, fungal ROI production was also detected in the interaction between barley and *Pyrenophora teres* (Able 2003). Recently, a *B. cinerea* glucose oxidase gene was described, which is induced by oxidative stress. Also a fungal NADPH-oxidase gene from *Aspergillus nidulans* was isolated, which is involved in ROI generation (Lara-Ortiz et al. 2003; Rolke et al. 2004). These findings lead to the conclusion that the fungus itself induces the oxidative stress observed in the *S.*

sclerotiorum-inoculated WT tissue. However, to our knowledge, no oxidative stress-producing enzyme from *S. sclerotiorum* has so far been isolated and characterized.

Even though H_2O_2 stress in *S. sclerotiorum*-inoculated transgenic CAT1AS tobacco plants was high, fungal development was not influenced. In order to examine whether the oxidative stress coincided with a general fungal growth restriction, we assayed *S. sclerotiorum* growth on agar plates containing different concentrations of H_2O_2. In contrast to our observations, *B. cinerea* seems to tolerate higher levels of H_2O_2 *in vitro*, namely up to 100 mM (Schouten et al. 2002). It should be noted that under stress conditions, the plant cellular homeostasis can be disrupted and the production of H_2O_2 is enhanced up to 8 mM (Levine et al. 1994; Tenhaken et al. 1995). The levels tolerated *in vivo* are lower compared to the concentrations we used in the *in vitro* experiments. However, we have not been able to measure fungal tolerance towards different levels of H_2O_2 *in vivo*.

Our data strongly support the role of catalase in controlling H_2O_2 levels during *S. sclerotiorum* infection. Enhanced H_2O_2 generation during *S. sclerotiorum* infection seems not to influence the spread of the pathogen in plant tissues. However, Govrin and Levine (2000) showed that the infiltration of *Arabidopsis* leaves with glucose oxidase and glucose, leading to production of H_2O_2 prior to inoculation with *S. sclerotiorum*, lead to an enhanced spread of the pathogen in the infiltrated tissue compared to the non-infiltrated control. Our results also contradict the accelerated and enlarged lesion formation observed after infection of CAT1AS tobacco plants with *B. cinerea* (Govrin and Levine 2000). Even though OA is secreted by the fungus to suppress the oxidative burst *in planta*, *S. sclerotiorum* is able to tolerate high ROI stress *in vivo* and *in vitro*. Nevertheless, growth of *S. sclerotiorum* is reduced due to high H_2O_2 levels *in vitro*.

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