Expression of an Oxalate Decarboxylase Impairs the Necrotic Effect Induced by Nep1-like Protein (NLP) of *Moniliophthora perniciosa* in Transgenic Tobacco

Leonardo F. da Silva,¹ Cristiano V. Dias,¹ Luciana C. Cidade,¹ Juliano S. Mendes,¹ Carlos P. Pirovani,¹ Fátima C. Alvim,¹ Gonçalo A. G. Pereira,² Francisco J. L. Aragão,³ Júlio C. M. Cascardo,¹ and Marcio G. C. Costa¹

¹Centro de Biotecnologia e Genética, Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Ilhéus, BA 45662-900, Brazil; ²Instituto de Biologia, Departamento de Genética e Evolução, Universidade Estadual de Campinas, Campinas, SP 13083-970, Brazil; ³Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF 70770-916, Brazil

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Oxalic acid (OA) and Nep1-like proteins (NLP) are recognized as elicitors of programmed cell death (PCD) in plants, which is crucial for the pathogenic success of necrotrophic plant pathogens and involves reactive oxygen species (ROS). To determine the importance of oxalate as a source of ROS for OA- and NLP-induced cell death, a full-length cDNA coding for an oxalate decarboxylase (FvOXDC) from the basidiomycete *Flammulina velutipes*, which converts OA into CO₂ and formate, was overexpressed in tobacco plants. The transgenic plants contained less OA and more formic acid compared with the control plants and showed enhanced resistance to cell death induced by exogenous OA and MpNEP2, an NLP of the hemibiotrophic fungus *Moniliophthora perniciosa*. This resistance was correlated with the inhibition of ROS formation in the transgenic plants inoculated with OA, MpNEP2, or a combination of both PCD elicitors. Taken together, these results have established a pivotal function for oxalate as a source of ROS required for the PCD-inducing activity of OA and NLP. The results also indicate that FvOXDC represents a potentially novel source of resistance against OA- and NLP-producing pathogens such as *M. perniciosa*, the causal agent of witches' broom disease of cacao (*Theobroma cacao* L.).

Successful colonization of host tissues by necrotrophic plant pathogens requires the induction of plant cell death to provide the nutrients needed for the establishment of pathogenesis (Van Kan 2006). Thus, a main determinant for pathogenicity of these phytopathogens is the production of secreted metabolites and proteins which enable them, ultimately, to kill host tissues (Choquer et al. 2007; Van Kan 2006). Oxalic acid (OA) has long been known to be a pathogenicity determinant of important plant-pathogenic fungi, including several species of *Sclerotinia* and *Botrytis* (Van Kan 2006).

Pathogen-produced OA has multiple modes of action that affect the host. OA favors 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, leading to a loss of cell wall integrity (Bateman and Beer 1965). OA can also inhibit the plant oxidative burst (Cessna et al. 2000; Guo and Stotz 2007), one of the earliest and most universal plant defense responses against invading microorganisms, probably through pH changes that occur during the pathogen ingress into host tissues (Kim et al. 2008). The importance of the multifunctional OA is such that *Sclerotinia sclerotiorum* mutants lacking the ability to produce OA are essentially nonpathogenic, even though they still maintain their full arsenal of cell-wall-degrading enzymes (Godoy et al. 1990). A recent study showed that OA acts as an elicitor of programmed cell death (PCD) in plants and is responsible for the induction of apoptotic-like features in the plant during disease development, in a mechanism that involves the generation of reactive oxygen species (ROS) (Kim et al. 2008). The induction of this fungal-mediated PCD process is required for the pathogenic success of *S. sclerotiorum*.

Another group of PCD elicitors found in many plant pathogens consists of necrosis and ethylene-inducing peptides (NEP) (Pemberton and Salmond 2004). NEP1 was first discovered in *Fusarium oxysporum* culture filtrates as a 24-kDa protein causing necrosis and induced ethylene production in the coca plant; in addition, NEP1-like proteins (NLP) have been identified in diverse organisms in the past few years, including bacteria, fungi, and oomycetes (Bae et al. 2006; Bailey 1995). Transformation of *Colletotrichum coccodes* with *F. oxysporum* Nep1 has increased virulence and the spectrum of hosts that could be infected (Amsellem et al. 2002). In *Erwinia carotovora*, the expression of the *Nip* gene that encodes an NLP resulted in larger lesions and more rotting of host tissues (Mattinen et al. 2004). In *S. sclerotiorum*, a reduced or abolished SsNep2 expression correlated with a partial or total, respectively, loss of virulence (Bashi et al. 2010). NLP induce the elevation of internal Ca²⁺ levels, activation of mitogen-activated protein kinases, formation of ROS, and synthesis of ethylene and pathogenesis-related proteins (Bae et al. 2006; Fellbrich et al. 2002; Jennings et al. 2001; Keates et al. 2003; Qutob et al. 2006; Veit et al. 2001). The cellular process or pathway that is required for the necrosis-inducing activity of NLP remains unknown.

The hemibiotrophic basidiomycete *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora is the causal agent of witches' broom disease (WBD), one of the most important diseases of cacao (*Theobroma cacao* L.) in the Americas (Purdy

Corresponding author: M. G. C. Costa; Fax: +55.73.3680.5226; E-mail: marciogc.costa@gmail.com

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and Schmidt 1996). This fungus seems to have originated from the Amazon basin and is known to infect at least five families of dicotyledons, including *Malvaceae* and *Solanaceae* (Griffith et al. 2003). Recent evidence has indicated that both OA and NLP are key determinants in *M. perniciosa* pathogenicity. During the progression of the disease, *M. perniciosa* triggers a PCD pathway involving calcium oxalate crystal accumulation and its subsequent degradation into hydrogen peroxide (H_2O_2) in infected plants, resulting in plant cell death and the generation of a suitable environmental niche for fungal nutrient acquisition and conversion from biotrophic to necrotrophic phase (Ceita et al. 2007). Production of oxalate by mycelia of *M. perniciosa* was recently observed in in vitro studies (Rio et al. 2008), as well as the expression of a gene coding for oxaloacetate acetylhydrolase, which catalyzes the hydrolysis of oxaloacetate to oxalate and acetate in biotrophic-like mycelia. NLP have also been detected in *T. cacao* tissues infected by *M. perniciosa* (Garcia et al. 2007). The authors have demonstrated that the fungus contains three putative genes encoding NLP, which are apparently located at the same chromosome. MpNEP1 is expressed in the biotrophic and necrotrophic phases, whereas MpNEP2 is only expressed in the biotrophic phase. Both NLP were able to induce necrosis and ethylene emission in tobacco and cacao leaves (Garcia et al. 2007), thus indicating their active involvement in WBD.

Because OA and NLP are elicitors of PCD, which involves ROS (Bae et al. 2006; Fellbrich et al. 2002; Jennings et al. 2001; Kim et al. 2008; Qutob et al. 2006; Schouten et al. 2008), and oxalate can be a potential source of ROS (Shetty et al. 2008), we have tested the hypothesis that oxalate degradation could delay or even inhibit the plant cell death caused by both PCD elicitors. Two major enzymes, oxalate oxidase (OXO) and oxalate decarboxylase (OXDC), are able to degrade OA (Svedruzic et al. 2005). OXO converts OA into CO_2 and H_2O_2 , whereas OXDC promotes the conversion of oxalate into CO_2 and formate. To test our hypothesis, we have generated transgenic tobacco plants expressing an OXDC from *Flammulina velutipes* (FvOXDC) which, in contrast to OXO, does not generate additional H_2O_2 (Kesarwani et al. 2000; Walz et al. 2008). Our results showed that FvOXDC was able to confer resistance to both OA and MpNEP2 in all transgenic plants evaluated, which correlated with the inhibition of ROS formation. These results suggest that the induction of PCD by OA and MpNEP2 involves the generation of ROS, which is provided by the conversion of oxalate into H_2O_2 . Our results also show that FvOXDC may be considered as a potential candidate gene for resistance to OA- and NEP-producing pathogens such as *M. perniciosa*. Indeed, the successful prevention of diseases caused by OA-producing fungi has already been well demonstrated in transgenic plants expressing FvOXDC (Cunha et al. 2010; Dias et al. 2006).

RESULTS

Expression of FvOXDC in transgenic tobacco.

Tobacco leaf discs were transformed using the construct pCambOxDc, designed for high FvOXDC expression in plants (Dias et al. 2006). This construct contains an OXDC cDNA from *F. velutipes* (FvOXDC) under control of the doubled *Cauliflower mosaic virus* (CaMV) 35S promoter and a sequence enhancer of *Alfalfa mosaic virus* (AMV). Twelve regenerated tobacco plants in hygromycin-containing medium representing distinct transformation events were screened for the presence of the transgene. Polymerase chain reaction (PCR) was carried out using a pair of primers specifically designed to amplify a 502-bp fragment of the FvOXDC cDNA. A single band of the expected size was obtained in all transgenic plants evaluated (Fig. 1A). We have also carried out genomic Southern hybridization

with FvOXDC cDNA as probe, confirming that only the transgenic plants gave hybridization bands (data not shown). All the transgenic tobacco plants exhibited a normal phenotype and were fertile.

Analysis of the FvOXDC expression was carried out in up to seven transgenic plants by reverse-transcriptase (RT)-PCR. All the analyzed plants have expressed FvOXDC but differences in the transcript levels were observed among the transgenic plants (Fig. 1B). The transgenic lines L7, L6, L5, and L4 showed higher levels of FvOXDC transcripts, whereas L10 and L3 had lower transcript levels.

Transgenic tobacco plants can degrade OA and generate formic acid in vivo.

The ability of transgenic plants to degrade OA and generate formic acid by the action of FvOXDC was tested in vivo. Total OA and formic acid contents were analyzed in leaves of transgenic and control plants by high-performance liquid chromatography (HPLC). The transgenic plants showed a significant decrease in the levels of OA compared with the control plants, which varied from 18 to 75%, depending on the transgenic line (Fig. 2A). On the other hand, the formic acid content increased from 9 to 27× in the transgenic plants compared with the control plants (Fig. 2B). The results demonstrated that all transgenic plants evaluated possess an enzymatically active FvOXDC.

Transgenic tobacco expressing FvOXDC are resistant to exogenous OA application.

To test the effects of the exogenous application of OA in the transgenic plants, photosynthetically active leaves from control and transgenic plants were excised and the petioles were immediately dipped and vacuum infiltrated in OA solution. Initially, leaves of control plants were dipped into solution containing different OA concentrations that established 20 mM OA (pH 4) as the necessary concentration to induce leaf wilting and necrosis (data not shown). After 24 h, OA-treated leaves of most transgenic plants did not wilt or necrose like those of control plants. Leaves of the transgenic plants were resistant to OA even after 48 h, whereas leaf tissues of OA-treated control plants was found to be completely wilted and necrosed (Fig. 3). This result shows that the expression of FvOXDC in transgenic plants confers resistance to wilting and necrosis induced by OA.

FvOXDC-expressing tobacco plants are resistant to MpNEP2.

Similarly to NPP1 of *Phytophthora* spp. (Fellbrich et al. 2002), MpNEP are able to induce necrosis in tobacco, as previously described (Garcia et al. 2007). Based on the different results obtained in the tests carried out above (Figs. 2 and 3), three transgenic plants were selected for the assay. MpNEP2 was inoculated in leaves from control and transgenic plants as described for OA. Leaves of control plants were treated with either phosphate (PB) buffer or MpNEP2. Typical symptoms caused by MpNEP2 were observed after 48 h in leaves of the control plants. The symptoms were characterized by chlorosis followed by necrosis, starting near the margin and then proceeding to the center of the leaves (Fig. 4). Transgenic leaves showed remarkable resistance to MpNEP2 in comparison with control leaves. L5 and L7 transgenic leaves were resistant to MpNEP2 even after 96 h, when control leaves turned completely necrosed; on the other hand, L3 leaves exhibited severe symptoms of water soaking at that time. The results seem to be consistent with the levels of expression (Fig. 1B) and in vivo activity of FvOXDC (Fig. 2) in the transgenic plants.

The extent of cell death in MpNEP2-treated leaves of control and transgenic plants was assessed after 96 h by measuring the water leakage caused by membrane damage. As expected,

all the transgenic plants showed a significantly reduced (25 to 90%) water leakage when compared with the control plants (Fig. 5). L5 leaves showed more resistance to water leakage than L3 and L10, which correlated with the symptoms observed in Figure 4. Taken together, these data indicate that high FvOXDC activity is closely associated with the inhibition of cell death induced by MpNEP2.

FvOXDC confers resistance to OA and MpNEP2 by inhibiting ROS generation.

A growing body of evidence suggests that ROS can activate PCD in mammalian and plant cells (Jabs 1999). In plant tissues, some conditions leading to ROS formation and subsequent PCD include senescence, cold, osmotic stress, UV radiation, and inoculation with avirulent pathogens leading to hypersensitive response (HR) (Van Breusegem and Dat 2006). ROS also appear

to trigger PCD in OA-treated (Kim et al. 2008) and NLP-treated plant tissues (Bae et al. 2006; Fellbrich et al. 2002; Jennings et al. 2001; Qutob et al. 2006; Schouten et al. 2008). Therefore, we were interested in determining whether the resistance conferred by FvOXDC to OA- and MpNEP2-induced cell death, or PCD, involves the inhibition of ROS generation. Leaf disks of control and L6 transgenic plants were treated with OA, MpNEP2, and a combination of both elicitors for 24 h; then, the disks were stained with 3,3'-diaminobenzidine (DAB), in order to detect the production of H₂O₂ (Kim et al. 2008). L6 was selected for this assay based on the high level of expression and in vivo activity of FvOXDC. Nontreated leaf disks of control and transgenic plants showed a lightly staining mainly at the periphery, as a result of wounding (Fig. 6). OA- and MpNEP2-treated leaf disks of control plants were more heavily stained throughout and clearly distinct from the nontreated disks. The intensity of stain-

Fig. 1. Molecular analysis of representative transgenic tobacco plants. **A**, Polymerase chain reaction (PCR) amplification of transgene using genomic DNA from nontransformed (wild-type [WT]) and transgenic (L1 to L12) plants. C+, pCambiaOxDe plasmid DNA. Molecular weight marker (M) is indicated on the left. **B**, Analysis of *Flammulina velutipes* oxalate decarboxylase mRNA expression in WT and transgenic plants. Expression profiles were determined by relative quantitative reverse-transcription PCR using the QuantumRNA 18S Internal Standards kit (Ambion Inc., Austin, TX, U.S.A.). Data are presented as means $\pm$ standard deviation of three biological replicates.

ing has increased when the control leaf disks were treated simultaneously with OA and MpNEP2, suggesting a synergic effect of both elicitors on the ROS generation. Conversely, the treated leaf disks of transgenic plants were not stained under any circumstance. This observation coincides with the resistance of the transgenic plants to OA- and MpNEP2-induced cell death. Thus, there is a strict relationship between the inhibition of ROS generation and the prevention of OA- and MpNEP2-induced cell death by FvOXDC.

DISCUSSION

OA and NLP play a key role in the induction of host cell death by necrotrophic fungi, which is crucial for the establish-

ment and progression of pathogenesis (Van Kan 2006). Recent data indicate that the hemibiotrophic basidiomycete *M. perniciosa* also shares these conserved PCD elicitors with necrotrophic ascomycetes in order to assist the transition from biotrophic to the necrotrophic phase, nutrient acquisition, fungal development, and life-cycle completion (Ceita et al. 2007; Garcia et al. 2007; Rio et al. 2008). A common mechanism mediating OA- and NLP-induced PCD is the generation of ROS (Bae et al. 2006; Fellbrich et al. 2002; Guo and Stotz 2010; Jennings et al. 2001; Kim et al. 2008; Qutob et al. 2006; Schouten et al. 2008), which may derive from the degradation of oxalate by OXO (Shetty et al. 2008). In the present study, the question of whether the overexpression of an OXDC in plants can make them resistant to cell death caused by both OA

Fig. 2. Measurement of total oxalic acid (OA) and formic acid contents in leaves of wild-type (WT) and transgenic plants. **A**, OA and **B**, formic acid. Data are means $\pm$ standard deviation of three biological replicates; ** indicates significantly different from WT, according to Student's *t* test ($P \leq 0.01$).

and NLP was analyzed. Toward this end, we have introduced an *OXDC* gene from *F. velutipes* (*FvOXDC*) in transgenic tobacco plants. A number of independent transformants were obtained that exhibited a normal phenotype and were fertile. The transgenic plants were demonstrated to express high levels of functional enzyme. Our data indicate that the transgenic plants can resist wilting and necrosis caused by OA. Foliar wilting in response to OA is known to occur via opening of stomatal pores (Guimaraes and Stotz 2004), in a regulation process that involves the activation of the plasma membrane H^+ -ATPase and the concomitant inhibition of abscissic acid-induced ROS accumulation in the guard cells (Guo and Stotz 2010). Consistent with our observations, several reports have shown that transgenic plants overexpressing OXO (Berna and Bernier 1997; Donaldson et al. 2001; Dong et al. 2008; Hu et al. 2003; Liang et al. 2001; Livingstone et al. 2005; Thompson et al. 1995) and OXDC (Cunha et al. 2010; Dias et al. 2006; Kesarwani et al. 2000) are resistant to OA-producing pathogens. However, the exact mechanism involved in this resistance remains unclear. Based on our findings and in the recent observation that OA is an efficient elicitor of PCD (Kim et al. 2008), we propose the suppression of OA-induced PCD as a key mechanism inhibiting the colonization of host tissues by necrotrophic fungi in transgenic plants overexpressing OXO and OXDC.

A major breakthrough was the observation that high *FvOXDC* activity is also able to inhibit cell death induced by MpNEP2. To our knowledge, this is the first demonstration of a gene conferring resistance against NLP in plants. Our result suggested that plants with the ability to degrade oxalate may be protected from this PCD elicitor, establishing that oxalate is an important determinant of NLP-induced cell death. Oxalate

is a substrate for the germin family of plant proteins that have been isolated from many higher plants, including both dicotyledonous and monocotyledonous species (Hu et al. 2003). Yet, only those from monocots are referred to in the literature as “true germins” by possessing OXO activity (Davidson et al. 2009). Germin-like proteins (GLP) present in dicots are of unknown enzyme activity or do not possess OXO activity (Dunwell et al. 2008). On the other hand, only dicots have been shown to be susceptible to the effects of NLP, whereas monocots are insensitive (Bailey 1995; Fellbrich et al. 2002; Gijzen and Nürnberger 2006; Keates et al. 2003; Mattinen et al. 2004; Pemberton et al. 2005; Qutob et al. 2006; Schouten et al. 2008; Staats et al. 2007; Veit et al. 2001). Taken together with these observations, our result suggests that the insensitivity of monocots to NLP may be correlated with their inherent ability to efficiently degrade oxalate. We are currently testing this hypothesis.

In animals, ROS such as H_2O_2 , superoxide ion (O_2^-), and nitric oxide (NO) are well-recognized triggers of cell death (Jabs 1999). In contrast, the role of ROS in the induction, signaling, and execution of plant PCD has been revealed only recently (Van Breusegem and Dat 2006). OA and NLP also appear to activate ROS in order to cause PCD in the host (Bae et al. 2006; Fellbrich et al. 2002; Jennings et al. 2001; Kim et al. 2008; Qutob et al. 2006; Schouten et al. 2008), which is consistent with the strategy of necrotrophic plant pathogens. To understand the underlying mechanism involved in the resistance of the transgenic plants to OA- and MpNEP2-induced cell death, or PCD, DAB staining of leaf discs was used to demonstrate production of H_2O_2 as an indicative for ROS generation (Kim et al. 2008). Our data confirmed a positive DAB staining induced by OA and MpNEP2 in control plants, which

Fig. 3. Sensitivity of tobacco plants to oxalic acid (OA)-induced wilting and necrosis. Excised leaves from wild-type (WT) and transgenic plants were treated with 20 mM OA (pH 4.0) or phosphate (PB) buffer (as a control). Symptoms were recorded after 48 h. Representative results of experiments performed in triplicate are shown.

was even enhanced by the synergic effect of both PCD elicitors on the ROS formation. On the other hand, the production of ROS was completely abolished in the transgenic plants. This result suggests the inhibition of ROS as the mechanism involved in the resistance of the FvOXDC-overexpressing plants to OA- and MpNEP2-induced cell death. In addition, it

provides evidence of the cellular process or pathway required for the PCD-inducing activity of OA and NLP: ROS activation through the conversion of oxalate to H_2O_2 . As discussed, this reaction is catalyzed by OXO, whose activity has not been demonstrated for GLP of dicotyledonous species, although proteins containing OXO activity other than the germin-type

Fig. 4. MpNEP2-induced necrosis in tobacco plants. Excised leaves from wild-type (WT) and transgenic plants were treated with 1.4 μ M MpNEP2 (pH 6.0) or phosphate (PB) buffer (as a control). Symptoms were recorded after 48, 72, and 96 h. Results shown are representative of experiments performed in triplicate.

have been recently discovered in beet and spinach (Davidson et al. 2009). Thus, a possibility is that the OA- and NLP-induced reaction of oxalate oxidation could also be catalyzed by the pathogen in order to trigger ROS and the cell death

process. Equally, the pathogen may regulate the OXO activity of dicot proteins other than germins. The observation that the basidiomycete *Ceriporiopsis subvermispora* contains functional *OXO* genes (Aguilar et al. 1999; Escutia et al. 2005) is consis-

Fig. 5. Water leakage in tobacco leaves treated with MpNEP2. Excised leaves from wild-type (WT) and transgenic plants were treated with 1.4 μ M MpNEP2 (pH 6.0) and water leakage was recorded after 96 h. Data are means $\pm$ standard deviation of three biological replicates; * and ** indicate significantly different from WT at $P \leq 0.05$ or 0.01, respectively, according to Student's *t* test.

Fig. 6. Involvement of reactive oxygen species (ROS) in oxalic acid (OA)- and MpNEP2-induced cell death in tobacco leaf disks. Leaf disks from wild-type (WT) and transgenic plants were treated with 20 mM OA, 1.4 μ M MpNEP2, or 20 mM OA + 1.4 μ M MpNEP2. H₂O₂ production (revealed by reddish-brown stain) was detected by 3,3'-diaminobenzidine (DAB) staining 24 h after the treatments. Control treatments were treated with water or water + DAB. Results are representative of experiments performed in triplicate.

tent with the first premise. Analysis of the *M. perniciosa* draft genome has also revealed the presence of a putative gene encoding OXO (data not shown).

Hemibiotrophic pathogens compose a diverse group of organisms with an initial biotrophic phase when infection is established, followed by a necrotrophic phase when the pathogen completes its life cycle (Oliver and Ipcho 2004). Our study reinforces the previous findings about the contribution of oxalate and MpNEP2 for PCD induction, which is required for the establishment of a necrotrophic relationship of *M. perniciosa* with host tissues in the later stage of infection (Ceita et al. 2007; Garcia et al. 2007; Rio et al. 2008). The significance of having both OA and NLP to initiate host PCD and tissue necrosis has been well illustrated for successful infection by the necrotrophic plant pathogens *S. sclerotiorum* and *Botrytis cinerea* (Choquer et al. 2007; Van Kan 2006). We also go further than the initial findings to demonstrate that this PCD is ROS dependent, in a pathway involving the conversion of oxalate to H_2O_2 . The observation that a large amount of H_2O_2 can be observed in the infected tissues during the late stage of infection, at the same sites where calcium oxalate crystals had previously been detected (Ceita et al. 2007), is consistent with this idea. A correlation between hemibiotrophic pathogen growth at the late stages of their life cycle and large quantities of H_2O_2 has also been reported in other host-pathogen systems (Shetty et al. 2008). It has been concluded that H_2O_2 was necessary for successful infection in such host-pathogen systems. This seems to be a valid conclusion in the *M. perniciosa* compatible interaction with host plants (Ceita et al. 2007). The ability to colonize and proliferate in an environment with high concentrations of ROS shows, on the other hand, that such pathogens have efficient systems enabling them to neutralize these harmful compounds. Indeed, a complete ROS detoxification system has been found in the genome of *M. perniciosa*, including genes encoding to superoxide dismutases, catalases, peroxiredoxins, glutathiones, thioredoxins, and manganese-dependent peroxidases (Mondego et al. 2008).

In conclusion, our results suggest that ROS generation induced by OA and NLP is a key mechanism triggering PCD in tissues infected with these PCD elicitors, in a pathway that relies on the conversion of oxalate into H_2O_2 . Our data also show that FvOXDC may represent a potential source of resistance against OA- and NEP-producing pathogens such as *M. perniciosa*. We are currently testing this hypothesis through the overexpression of OXDC in tomato and cacao.

MATERIALS AND METHODS

Plant material.

Wild-type (WT) tobacco (*Nicotiana tabacum* 'Havana') was grown from seed in a growth room at $26 \pm 2^\circ\text{C}$ with 16-h light periods. Surface-sterilization was performed by immersion of the seed in 70% (vol/vol) ethanol for 1 min, followed by 20 min in a 2.5% (vol/vol) sodium hypochlorite solution containing 0.1% (vol/vol) Tween-20 and then four rinses in sterile distilled water. Seed were germinated in Magenta boxes (Sigma-Aldrich, St. Louis) with 50 ml of half-strength Murashige and Skoog ($1/2$ MS) medium containing $1/2$ MS salts (Murashige and Skoog 1962), myo-inositol at 50 mg/liter, Nitsch vitamins (Nitsch 1969), sucrose at 20 g/liter (pH 5.8), and solidified with 0.8% agar (Sigma-Aldrich). Leaf disks were obtained from 8-week-old plants using a 10-mm-diameter cork borer (Boekel Scientific, Feasterville, PA, U.S.A.).

Agrobacterium strain and plasmid.

Agrobacterium tumefaciens EHA 105, containing the vector pCambiaOxDc, was used in all tobacco transformations. This

vector contains an oxalate decarboxylase (*FvOXDC*) coding sequence of *F. velutipes*, under the control of the doubled 35S promoter from CaMV and a sequence enhancer from AMV, and the hygromycin phosphotransferase II (*hptII*) selectable marker gene, driven by the CaMV 35S promoter (Dias et al. 2006).

Plant transformation.

Leaf discs from in vitro-grown tobacco plants were transformed as previously described (Alvim et al. 2001). The shoots obtained were rooted on phytohormone-free medium containing hygromycin at 10 mg/liter. All transformed plants (T_0 plants) were micropropagated and maintained in the growth room at $26 \pm 2^\circ\text{C}$ with a 16-h photoperiod.

Molecular analysis of transgenic plants.

Total DNA was isolated from tobacco leaves using the cetyltrimethylammonium bromide method (Doyle and Doyle 1987). PCR was carried out using the primers OXDC 873 (5'-TGGGCTCGACAGAGGAGAAG-3') and OXDC 371 (5'-CTCGGCAGCAGAATGAGGTC-3'), as described (Dias et al. 2006). Total RNA was isolated from tobacco leaves using the phenol method (Jones et al. 1985). Reverse transcription was carried out using the RETROscript kit (Ambion Inc., Austin, TX, U.S.A.), following the manufacturer's instructions. Prior to RT-PCR reactions, total RNA was treated with DNase I (FPLC pure; Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) to avoid DNA contamination (DNase I at 100 U/ μg of total RNA). The relative abundance of *OXDC* mRNA in the transgenic plants was assessed employing the QuantumRNA 18S Internal Standards kit (Ambion Inc., Austin, TX, U.S.A.) and the pair of primers OXDC 873 and OXDC 371. Reactions were performed in triplicate and the PCR products were quantified on agarose gels using the program KODAK 1D v 3.5. In each sample, the signal obtained for the *OXDC* amplicon was divided by the signal obtained for the 18S amplicon.

OA and formic acid measurements.

Organic acid extraction and quantification was carried out as previously described (Keates et al. 2000), with some modifications. Approximately 6 mg of the freeze-dried and ground leaves (triplicates) of each individual plant was suspended in 1 ml of extraction buffer (H_2SO_4 at 4 mmol/liter, dithiothreitol at 5 mmol/liter, and 0.01% insoluble polyvinylpyrrolidone at 10 mg/ml). Extracts were agitated for 15 min in the vortex and then centrifuged at $15,000 \times g$ for 20 min at room temperature. The supernatant was filtered (0.45 μm) and 100 μl was applied in the HPLC for analysis and detection of OA and formic acid (column of ionic exclusion Bio-Rad Aminex HPX-87H, 300 by 7.8 mm, with flow of 0.7 ml/min, at 25°C , using the automated system of HPLC ÄKTAbasic) (GE Healthcare, Piscataway, NJ, U.S.A.). The HPLC data were integrated and analyzed using the software UNICORN v. 5.0. OA and formic acid were measured at 210 nm. Standard aliquots of OA and formic acid with known concentrations were used for the standard curve preparation.

OA and MpNEP2 sensitivity assay of leaves.

Heterologous expression and purification of MpNEP2 were previously described (Garcia et al. 2007). Photosynthetically active leaves (triplicates) from WT and transgenic plants were excised, and the petioles were immediately dipped in 20 mM OA (pH 4.0) or 1.4 μM MpNEP2 (pH 6.0) solutions and subjected to vacuum infiltration for 20 min. Negative control was imposed by dipping and vacuum infiltrating the leaves in PB buffer with pH adjusted to 4.0 (OA) or 6.0 (MpNEP2) using hydrochloric acid. Leaves were incubated in a growth room for

96 h at $26 \pm 2^\circ\text{C}$ under a 16-h photoperiod. The extent of necrosis was recorded after 24, 48, 72, and 96 h.

Measurement of cell death.

Cell death was quantified in leaves from WT and transgenic plants treated with MpNEP2 by measuring the water leakage resulting from membrane damage. The experiment was carried out as described above. Water leakage was expressed as percentage of total water release, quantified by the difference in the fresh weight of leaves between 0 and 96 h after MpNEP2 inoculation.

Detection of ROS.

In situ H_2O_2 was detected by DAB staining as described (Kim et al. 2008). Leaf disks (triplicates) from WT and transgenic plants were treated with i) 20 mM OA, ii) 1.4 μM MpNEP2, or iii) 20 mM OA + 1.4 μM MpNEP2. The samples were immersed in DAB solution (pH 7.5) at 1 mg/ml and infiltrated using vacuum for 20 min. After incubation in the dark at room temperature for 24 h, samples were boiled in a solution containing 96% ethanol for 20 min and rinsed twice with 50% ethanol. Leaf disks of control treatments were vacuum infiltrated with water alone or water plus DAB.

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