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# *Tc*CYS4, a cystatin from cocoa, reduces necrosis triggered by *Mp*NEP2 in tobacco plants

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**ABSTRACT.** In Brazil, most cocoa bean production occurs in Southern Bahia. Witches' broom disease arrived in this area in 1989 and has since caused heavy losses in production. The disease is caused by the basidiomycete fungus *Moniliophthora perniciosa*, a hemibiotrophic fungus that produces the necrosis and ethylene-inducting protein (*Mp*NEP2) during infection; this protein can activate cysteine proteases and induce programmed cell death. Cysteine proteases can be modulated by cystatin. In this study, we overexpressed *Tc*CYS4, a cocoa cystatin, in tobacco plants and evaluated the effect on *Mp*NEP2 in model plants. *Tccys4* cDNA was cloned into the pCAMBIA 1390 vector and inserted into the tobacco plants via *Agrobacterium tumefaciens*. Transgene expression was analyzed by reverse transcription-quantitative PCR and Western blot analysis. Transcript and protein levels in *Tcccys4*:tobacco lines were 8.9- and 1.5-fold higher than in wild-type plants (wt). *Tcccys4*:tobacco lines showed no change in growth compared to wt plants. $CO_2$ net assimilation (*A*) increased in *Tcccys4*:tobacco lines compared to wt plants. Only one line showed statistically significant stomatal conductance (*gs*) and transpiration rate (*E*) changes. *Mp*NEP2 was infiltered into the foliar mesophyll of *Tcccys4*:tobacco lines and wt

 

plants, and necrotic lesions were attenuated in lines highly expressing *Tccys4*. Our results suggest that cocoa cystatin *Tc*CYS4 affects *Mp*NEP2 activity related to the progression of programmed cell death in tobacco plants. This may occur through the action of cystatin to inhibit cysteine proteases activated by *Mp*NEP2 in plant tissues. Further studies are necessary to examine cystatin in the *Theobroma cacao-M. perniciosa* pathosystem.

**Key words:** Cysteine proteases; Phytocystatin; Programmed cell death; Witches' broom

## INTRODUCTION

*Theobroma cacao* L. is a perennial dicotyledonous plant typically found in tropical regions of Southern and Central America (Alverson et al., 1999; Johns, 1999). Cocoa fruit is a product of high commercial value in Brazilian agriculture because of its use in various products, including chocolate, cocoa butter, cosmetics, liquors, vinegars, jams, and juices. In Brazil, cocoa has become well adapted to the edaphoclimatic conditions in the southern Bahia region and is a major source of income for the region's established monoculture (Cuenca and Nazário, 2004). The cocoa-producing region in southern Bahia has experienced a serious economic crisis since 1980s as a result of the proliferation of the phytopathogenic *Moniliophthora* (= *Crinipellis*) *perniciosa* (Stahel) Singer (Aime and Phillips-Mora, 2005) causal agent of witches' broom disease. The infectious process is initiated by germination of basidiospores in the host tissue surface followed by the penetration of fungal hyphae into the epidermis and establishment of monokaryotic mycelium (biotrophic type). This stage remains some weeks (45-90 days) with the loss of apical dominance of the branches. Then the mycelium forms dikaryotic hyphae and becomes saprophytic. The characteristic symptom at this stage is cell death and tissue necrosis. (De Oliveira et al., 2007; Meinhardt et al., 2008; Mondego et al., 2008; Rincones et al., 2008).

cDNA libraries of the *T. cacao-M. perniciosa* interaction were used to identify genes involved in metabolic pathways during the colonization process and defensive response in the host tissues (Gesteira et al., 2007). Among these, 4 cystatin sequences were identified. These proteins are involved in the inhibition of cysteine proteases, which are triggers of the process of programmed cell death (PCD) (Solomon et al., 1999). Previous studies of rice cystatin (*OcI*) indicated that cystatins may be involved in maintaining nuclear functions and the stability of the cell membrane; when overexpressed, cystatins can promote the protection of the photosynthetic apparatus (van der Vyver et al., 2003; Wang et al., 2012).

Cystatins were found to be differentially expressed in the compatible and incompatible interactions between *T. cacao* and *M. perniciosa*, which are also diverging in terms of cystatin abundance in infected tissues, during different stages in the development of green broom and dry broom symptoms (biotrophic and saprophytic phase of the disease), and subcellular localization, confirmed by the presence and/or absence of a signal peptide in the amino acid sequences (Pirovani et al., 2010; Gesteira et al., 2007) also identified the protein necrosis and ethylene-inducing protein (*Mp*NEP2) produced by the *M. perniciosa* fungus in a compatible interaction with *T. cacao*. Previous studies have demonstrated that recombinant *Mp*NEP2

induces cell death in the leaves and tobacco suspension cell culture (Garcia et al., 2007) as well as reprograms the proteome and metabolome of *Nicotiana benthamiana* cells (Villela-Dias et al., 2014). In this study, we produced tobacco plants that overexpressed a cystatin gene sequence from cocoa (*Tccys4*). We hypothesized that overexpression of cocoa cystatin in tobacco plants would affect the response of plants inoculated with *Mp*NEP2 through the action of cystatins, which inhibit cysteine proteases activated during the PCD process triggered by *Mp*NEP2.

## MATERIAL AND METHODS

### Obtaining transgenic tobacco lines

From the library of the *T. cacao-M. perniciosa* interaction (Gesteira et al., 2007), the cDNA sequence *Tccys4* from cocoa was amplified by polymerase chain reaction (PCR) using specific primers, after which amplified fragments were subcloned into the pGEMT-EASY vector (Promega, Madison, WI, USA), and later inserted in the pCAMBIA 1390 vector (Cambia Labs, Canberra, Australia). The *Tccys4* sequence was located downstream of the CaMV35S promoter in the *Eco*RI site in the pCAMBIA 1390 vector (Figure 1). The orientation of the cloned fragment in relation to the CaMV35S promoter in the pCAMBIA 1390 vector was confirmed by PCR. Two PCRs were prepared, one with forward primer (Cys46NdeIF) and another with reverse (Cys46XhoIR), which is specific for *Tccys4*, and both reactions contained the primer Nos Poly-A terminator for the reverse reaction. Amplified clones containing the F-primer corresponded to the fragment in the sense orientation, i.e., with the 5' end of the cDNA towards the CaMV35S promoter. Amplification reactions were prepared with 10 µL 10X PCR buffer containing $(NH_4)S_2O_4$ (Fermentas, Vilnius, Lithuania), 6 µL 25 mM $MgCl_2$ (Fermentas), 6 µL 10 mM dNTPs, 8 µL forward primer (10 pM), 8 µL reverse primer (10 pM), approximately 100 ng colony DNA, 1.2 µL *Taq* DNA polymerase (Fermentas), 52.8 µL sterile Milli-Q water to a final volume of 100 µL. The PCR program used was 95°C for 4 min, followed by 40 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 1 min, and 72°C for 5 min; samples were stored at 15°C. The constructs obtained in the sense orientation were identified and used in the transformation of the *Agrobacterium tumefaciens EHA*105 strain by heat shock (Brasileiro and Carneiro, 1998). Colonies were confirmed using the PCR technique described above.

The constructs confirmed to be in the sense orientation for *Tccys4* were used to transform *Nicotiana tabacum* foliar explants according to the method described by Brasileiro and Carneiro (1998). The basic transformation protocol consisted of the co-cultivation of tobacco explants in liquid culture medium, together with the *Agrobacterium* strain and the vector containing the gene to be introduced into the plant. Explants were transferred to a regeneration solid medium [Murashige-Skoog (MS)] (Murashige and Skoog, 1962) containing 500 mg/L cefotaxime and 10 mg/L hygromycin and 5 mg/L 6-benzylaminopurine. After obtaining callus during the following weeks, the transformed cells showed differences in their shoots.

Transformed plants were isolated and transferred into micropropagation containers containing solid medium rooting MS supplemented with 30 g/L sucrose, 100 mg/L I-inositol, 500 mg/L cefotaxime, and 50 mg/L hygromycin. Every 15 days, the plants were transferred to a new micropropagation container containing the same culture medium, gradually reducing the concentration of antibiotic used, until the plants presented leaves that were large enough for DNA extraction.

## Molecular analyses

### *Genomic DNA extraction*

Leaves measuring approximately 5 cm in length were used for DNA extraction according to the method described by Brasileiro and Carneiro (1998) with some modifications. The plant material was macerated in liquid nitrogen, treated with extraction buffer containing 5% cetyltrimethylammonium bromide, followed by treatment with 5 M potassium acetate, washing with cold isopropyl alcohol, and washing with 70% alcohol. The precipitate was resuspended in 30 μL sterile Milli-Q water.

A total of 10 transformed plants (referred to as *Tccys4*:tobacco lines) and wild-type (wt) plants were used for DNA extraction. After extraction, all samples were treated with 10 mg/mL RNAse A for 40 min at 37°C.

### *Detection of transgenes*

Total DNA from the *Tccys4*:tobacco lines and wt plants was used as a template for PCR using primers designed for the cocoa cystatin sequences, including the forward Cys2e4rtF and reverse Nos poly-A (Table 1). The use of the Nos poly-A reverse primer prevented the amplification of endogenous cystatin of tobacco, thus preventing the occurrence of false-positives. Amplification reactions were performed as described above. PCR products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide.

### *Total protein extraction from leaves*

Young leaves were cut from the *Tccys4*:tobacco lines and control plants for protein extraction as described by Pirovani et al. (2008). Proteins were recovered in sodium dodecyl sulfate (SDS)-loading buffer (Laemmli, 1970) and measured using 2D Quant Kit (GE Healthcare, Chalfont, UK) based on manufacturer instructions.

### *Accumulation of transcript analysis*

Total RNA was extracted using a kit (Quick-RNA™ MicroPrep, Zymo Research, Irvine, CA, USA). Cocoa cystatin expression in the *Tccys4*:tobacco lines was analyzed by quantitative reverse transcription-PCR using Power SYBER® Green PCR Master Mix in 7500 Real-time PCR System equipment (Applied Biosystems, Foster City, CA, USA). The Cys2e4rtF- and Cys2e4rtR-specific primers were 70-140 bp amplicons corresponding to the cDNAs of cocoa cystatins. Three experiments were performed; in each, 1 of the biological replicas (independent transformants) included 5 experimental replicates. The constitutively expressed β-actin gene (ID: AB158612, Yasuda et al., 2005) was used for normalization.

### *Protein analysis*

Total protein extracts from control plant tissues and *Tccys4:*tobacco lines (30 μg protein) were resolved on a 15% SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis. SDS-PAGE was performed as described by Laemmli (1970). Proteins extracted

from the leaves were measured using the 2D-Quant Kit according to manufacturer instructions (GE Healthcare).

*Tc*CYS4 accumulation in cocoa plants was analyzed using the Western blotting technique. After electrophoresis, proteins were electro-transferred on to a nitrocellulose membrane in the mini protean II Xi Cell (BioRad, Hercules, CA, USA) system. The membranes were treated as described by Sambrook (1989). Primary antibodies were rabbit IgGs against *Tc*CYS4 produced by heterologous pathways by Pirovani et al. (2010). Anti-rabbit IgG together with alkaline phosphatase (Sigma, St. Louis, MO, USA) was used as a detection system, followed by a reaction with the nitro blue tetazolium chloride/5-bromo-4-chloro-3-indoyl phosphate substrates (Gibco BRL, Grand Island, NY, USA).

## Greenhouse experiments

The *Tccys4*:tobacco lines and wt plants were subjected to acclimatization. This procedure involves removing the plants from laboratory growth conditions (nutrient medium, temperature, humidity, and controlled lighting conditions) and exposing them to adverse conditions in a greenhouse with relative humidity exceeding 70%. For each transformation, at least 5 plants were acclimatized (individualized in 2 separate experiments), which were later transferred to the Plantmax substrate and grown in 3.6-L capacity pots.

### *Tobacco plant response expressing cystatin to MpNEP2*

The recombinant protein *Mp*NEP2 that had been expressed and purified from the clone produced by Garcia et al. (2007) at a 0.2 μM concentration was injected into the foliar mesophyll of *Tccys4*:tobacco lines and controls (wt) using an insulin syringe. The leaves used were fully expanded and mature (20 days after transfer). The progress of the *Mp*NEP2 effects were evaluated by observing the intensity of necrotic lesions on the leaves 8 h after injection, after which photographs of the leaves were obtained.

### *Vegetative growth and foliage gas exchange pattern*

For *Tccys4*:tobacco lines and wt plants, we measured plant height (measured from ground level to the shoot apex) every other day for 30 days after transferring the plants to the greenhouse to determine whether physiological changes had occurred in the transformed plants. The last measurement was acquired when the plants began flowering. Data analysis was performed by analysis of variance by evaluating the average height of the wt plants compared with the average height of the *Tccys4*:tobacco lines in an entirely randomized experimental design with $P \leq 0.05$; spot measurements of leaf gas exchange were taken using the 3rd fully expanded and mature leaf, for 3 leaves per plant from 4 copies of the *Tccys4*:tobacco lines and from 4 untransformed control plants. Measurements were carried out at 9:30 am, 30 days after transferring the plants to the greenhouse, using a portable photosynthesis meter LI-6400 (infrared gas analyzer, Li-Cor Inc., Lincoln, NE, USA) equipped with a 6400-02B red and blue artificial light source. The atmospheric concentration of $CO_2$ was 400 μM under artificial saturating light of 1500 $\mu M \cdot photons \cdot m^{-2} \cdot s^{-1}$. The rate of $CO_2$ net assimilation (*A*), stomatal conductance (*gs*), and transpiration rate (*E*) were estimated from the variation values of atmospheric $CO_2$, air humidity, and temperature within the chamber. Using the infrared gas

analyzer unit, the internal $CO_2$ concentration was automatically estimated by the device based on the values of *A*, *gs*, and *E* (von Caemmerer and Farquhar, 1981).

## RESULTS

### Obtaining transgenic plants

*Tccys4* cDNA identified in the cDNA library of the *T. cacao-M. perniciosa* interaction (Gesteira et al., 2007) was previously expressed in bacteria; the protein was characterized by Pirovani et al. (2010). In this study, the cDNA was amplified by PCR using specific primers (Table 1). The *Tc*CYS4 insert was placed under the control of the CaMV35S promoter and the Nos Poly-A terminator (Figure 1).

**Table 1.** Primers used to amplify cystatins from cDNA by PCR from and for confirmations of transgenesis with their melting temperature values.

| Primer | Sequence | Tm (°C) | Reference |
|---|---|---|---|
| Cys2e4BamHIF | GTTTTCTGCTGAATGGATCCTCTGCTC | 63.2 | This study |
| Cys2e4BamHIR | CATGATGCAAGGATCCAACCATTTTTTC | 64.8 | |
| Cys46NdeIF | CTGCTCTGAACATATGGCCACCAC | 59.9 | Pirovani et al., 2010 |
| Cys46XhoIR | GGTTCAACCTCGAGCAATATACAGC | 57.7 | |
| NosPoly-The | CCGATCTAGATACATAGATG | 49.3 | |
| Cys2e4rtF | ACGGTGGAAGCTATTGATGC | 57.6 | |
| Cys2e4rtR | GGAAACATCAGCATCACCAGC | 56.6 | |


**Figure 1.** Expression cassette inserted into the modified pCAMBIA 1390 vector.

Clones in sense orientation were used for *Agrobacterium* transformation. After that, performing co-cultivation of leaf explants with *Agrobacterium*, callus, and regenerated shoots was kept in a micropropagation container with MS medium containing antibiotics and growth regulators. These samples were successively transferred to obtain young plants with developed leaf and root systems. These plants were referred to as *Tccys4*:tobacco lines (Figure 2A).

### Molecular analysis of transformed plants

#### *Transgene confirmation*

Young leaves from the control and *Tccys4*:tobacco lines were used for DNA extraction. The presence of the transgene was confirmed by PCR using the Cys46Nde1F and the Nos Poly-A primers as shown in Table 1 to avoid the amplification of endogenous cystatin tobacco genes. The results confirmed the insertion of the transgene based on the presence of an amplified fragment of the expected size of 600 bp from *Tccys4*:tobacco plants (Figure 2B). Reactions performed using DNA from control plants (non-transformed) as a template showed no amplification (Figure 2B).

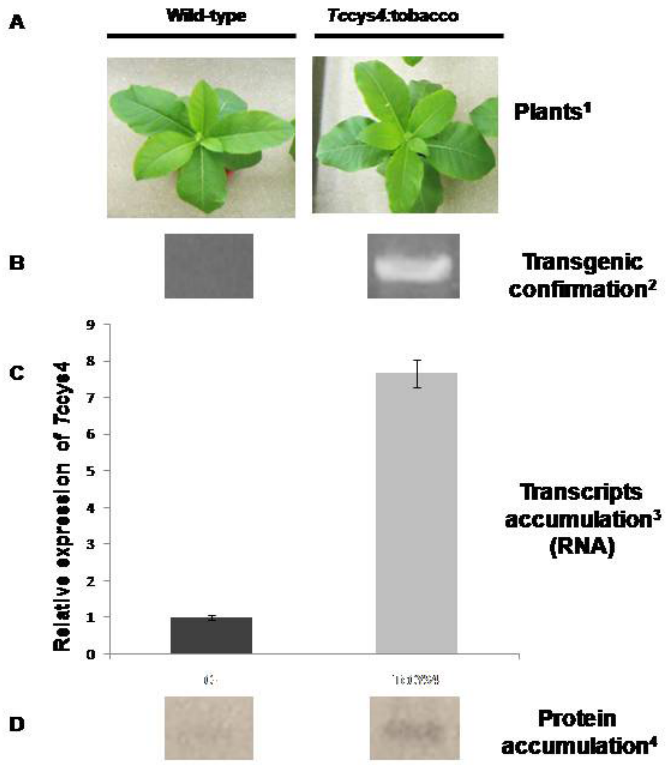


**Figure 2.** Transformation process was confirmed. **A.** Control plant specimens and *Tccys4*:tobacco acclimated in soil. **B.** Confirmation of transgenic plants by amplification of genomic DNA via PCR with primers specific to the expression cassette. **C.** Analysis of the relative accumulation of transcripts by qRT-PCR for five experimental replicates. **D.** Detection of *Tc*CYS4 protein by Western blot with specific polyclonal antibodies produced by Pirovani et al. (2010).

## *Gene expression at RNA level*

Quantification of *Tccys4*:tobacco gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001) for 3 experimental repetitions. The Dissociation Curve program 1.0 (Applied Biosystems, Foster City, CA, USA) was used to verify the melting temperature of the products amplified by PCR. This result did not allow for comparisons between the expression levels of the *Tccys4*:tobacco lines and those of non-transformed plants, as there were distinct amplification peaks for the *Tccys4*:tobacco lines and non-transformed plants. Thus, the transgenic line (L5) with the highest Ct was used as the calibrator (reference plant) for relative expression analysis between transgenic lines. In this manner, gene expression at the RNA level varied up to 7.9 times between transgenic plants (Figure 2C).

## *Protein accumulation analysis*

Protein expression analysis of transgenic lines by Western blotting showed a band with the expected mass of 25 kDa (Figure 2C). The densitometry of the bands revealed was

normalized, with the band intensity shown on SDS-PAGE, using the Image Master Platinum program (GE Healthcare). Relative cystatin accumulation was analyzed by taking the average of 2 wt plants as a reference. The level of protein accumulation was up to 1.5 times greater in *Tccys4*:tobacco compared to in non-transformed plants (Figure 2D and E).

## Greenhouse experiments

### *Vegetative growth and foliage gas exchange pattern*

Heights of the *Tccys4*:tobacco lines and wt plants acclimated in the greenhouse were for a 34-day period. The presence of exogenous genes caused no significant changes in growth compared with transgenic and wt lines until the appearance of the first flower buds. The data was plotted to compare treatments (Figure 3A). The *Tccys4*:tobacco and wt lines both were of average height when they began flowering (last day of measurement) and were 137.5 and 140.6 cm, respectively.

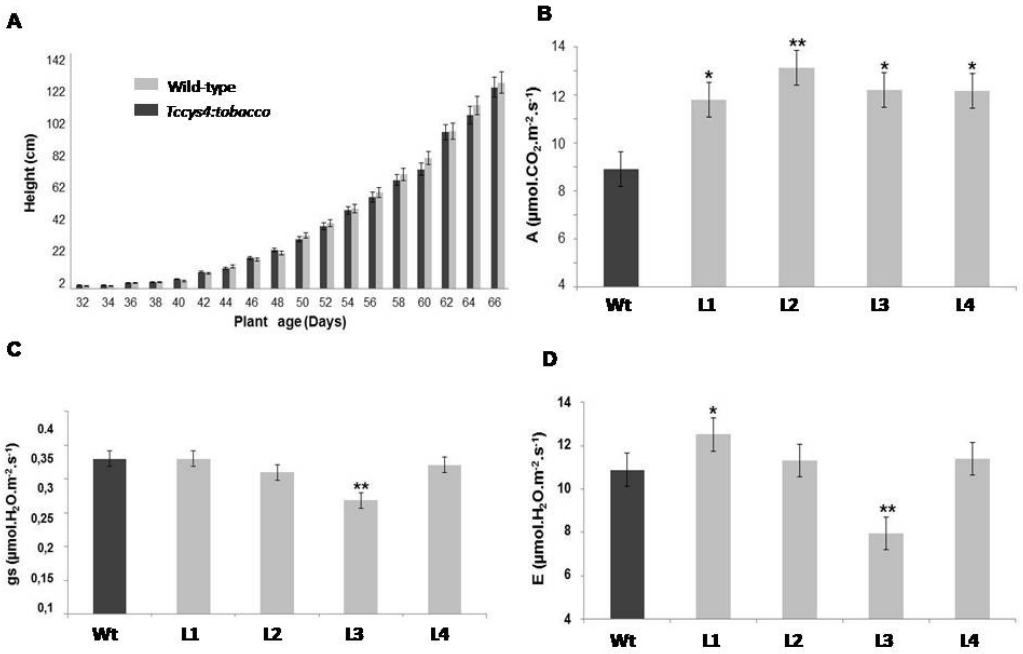


**Figure 3.** Phenological analyses and gas exchange in *Tccys4*:tobacco and control plants. **A.** Measurement of plant height from the end of the acclimation period up to the onset of flowering. **B.** Measurements of the $CO_2$ net assimilation (A). **C.** Measurements of stomatal conductance. **D.** Measurements of leaf transpiration rate. Data from B, C, and D were collected with the aid of an infrared meter (IRGA). All data were collected from five strains, with three replicates per strain. Means were compared by the Tukey test (*$P \leq 0.05$; **$P \leq 0.01$).

For $CO_2$ net *A*, the *Tccys4*:tobacco lines were more efficient than the wt, reaching maximum *A* of 12.5 $\mu M \cdot CO_2 \cdot m^{-2} \cdot s^{-1}$, while the control plants were an average of 8.9 $\mu M \cdot CO_2 \cdot m^{-2} \cdot s^{-1}$ (Figure 3B).

The wt plants showed the highest rates of *gs*, reaching a maximum *gs* rate of 0.3 $\mu M \cdot H_2O \cdot m^{-2} \cdot s^{-1}$, whereas only line 3 of the *Tccys*4:tobacco lines showed a statistical difference when compared with the control (Figure 3C).

For the *E*, the maximum value was 5.7 $\mu M \cdot H_2O \cdot m^{-2} \cdot s^{-1}$ for *Tccys4*:tobacco; statistically significant differences were observed in 2 of the 4 *Tccys4*:tobacco lines (Figure 3D).

### *Effect of cocoa cystatin in response to MpNEP2 protein in tobacco plants*

To determine whether overexpression of cocoa cystatin in tobacco plants affects *Mp*NEP2 protein activity, recombinant protein was produced from a clone prepared by Garcia et al. (2007). The *Mp*NEP2 recombinant protein, which has been purified previously, was injected into the foliar mesophyll of 5 wt tobacco plants and 5 *Tccys*4:tobacco transgenic plants according to the method described by Garcia et al. (2007).

The appearance of necrotic lesions on the leaves was observed 8 h after the *Mp*NEP2 injection. Wt plants showed intense and expanded necrotic lesions (with increased darkening), while in *Tccys4*:tobacco lines, lesions were only in the initial stage of necrosis, where L3 was the *Tccys4*:tobacco strain showing the least intense lesion when compared with the control. There was no induction of necrosis in following control injections (phosphate buffer infiltration and dialysis buffer). Injection of the *Mp*NEP2 protein induced necrosis in all treatments, differing only in the *Tccys4*:tobacco lines regarding presented necrosis intensity (Figure 4).

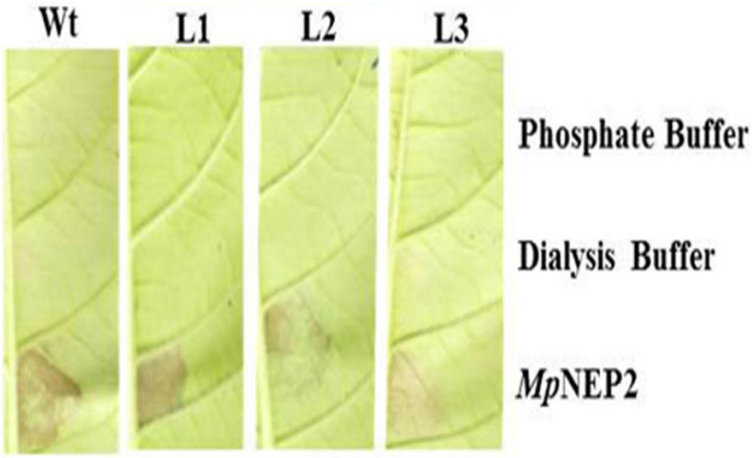


**Figure 4.** Functional studies with infiltration of *Mp*NEP2. Infiltrated leaves from each treatment are displayed 8 h after infiltration with the addition of phosphate buffer, phosphate buffer from the last dialysis exchange and 0.2 µM of *Mp*NEP2.

## DISCUSSION

Cystatins and cysteine proteases participate in plant defense processes against biotic and abiotic stresses (van der Hoorn and Jones, 2004). This occurs in 2 ways: i) direct cystatin inhibitory action against the cysteine proteases, which is dependent on the balance between

these proteins and; ii) the effect of this counterbalance on other proteases involved in cellular signaling (van der Hoorn and Jones, 2004; van der Hoorn, 2008). The cystatin *Tccys*4 fragment was cloned into a modified pCAMBIA 1390 vector (Figure 1). This gene was chosen because its respective protein features probable cytosolic localization (Pirovani et al., 2010) as well as contains an extended carboxy region with a probable inhibitory site of legumain in its structure (SNSL sequence) (Pirovani et al., 2010). To understand the role of these proteins in the *T. cacao*-*M. perniciosa* pathosystem, transgenic tobacco plants overexpressing the cystatins were produced (Figure 2A).

The young leaves of the lines obtained from the co-cultivation of explants with *A. tumefaciens*, which carried recombinant plasmids, were used for DNA extraction. The presence of the transgene was confirmed by PCR, where the reactions with *Tccys4* amplified a fragment in the expected size of 600 bp (Figure 2B) for the region bordered by specific primers. In the non-transformed plants, amplification using the same primers was not observed. Transcript analysis was carried out by quantitative qRT-PCR. Two amplification products were detected on the dissociation curve for transgenic plants, 1 of which was common with the control plants. This suggests that endogenous cystatin from tobacco may have been amplified as well as the transgene (data not shown). The level of RNA expression in the relative analysis showed variation between lines of up to 8.9 times (Figure 2C).

Accumulation of *Tc*CYS4 protein was assessed using Western blotting with specific polyclonal antibodies against recombinant *Tc*CYS4 protein by Pirovani et al. (2010) (Figure 2D). The relative expression of *Tc*CYS4 protein was 1.5-fold higher in the *Tccys*4:tobacco lines than in the wt. This level of expression is considered low, as the cystatin fragment was cloned under the control of the CaMV35S constitutive promoter; however, the vector structure used (pCAMBIA1390-modified) with 2 sequences of an adjacent constitutive promoter and an inverted orientation may have affected the initial events of transcription, contributing to the relatively low expression of the transgene (Figure 1).

The bands observed on Western blot analysis for the control plants corresponded to the likely cross-reactivity of the anti-*Tc*CYS4 polyclonal antibody against the endogenous cystatin of the tobacco (Figure 2D). According to Pirovani et al. (2010), phytocystatins of different plant species may have epitopes in common because of the overall sequence conservation or because of functional regions conserved within this class of cystatins. Thus, accumulation of only 1.5 times measured by Western blotting may be associated with the reduced homologous *Tccys4* expression present in the tobacco genome in the presence of the transgene.

The growth of transformed and control plants kept in the green house showed no significant differences between treatments (Figure 3A).

Joint analysis of the patterns obtained with the infrared gas analyzer revealed the quantum yield of photosynthetic rates and factors related to photosynthesis. These values varied with temperature and $CO_2$ concentration because of reactions between carboxylase and rubisco oxygenase (Taiz and Zeiger, 2010). Among the physiological parameters evaluated, the $CO_2$ net assimilation increased in *Tccys*4:tobacco lines compared to wt plants (Figure 3B).

Studies by van Der Vyver et al. (2003) examining rice cystatins showed that plants overexpressing cystatins (kept at low temperatures) had lower net photosynthetic rates compared to wt plants. In this study, transformed plants were grown in a greenhouse at a temperature of 28° ± 2°C, relative humidity of 70 ± 4%, and 50% global radiation. The plants showed an increased *A* value in *Tccys4*:tobacco when compared to wt plants.

For other parameters analyzed, only L3 of the *Tccys4*:tobacco showed *gs* and *E* lower than in wt plants, while the L1 showed elevated *E* (Figure 3C and D). This is the first study to evaluate the effects of genetic transformation using cystatins. These variations may be related to levels of transgene expression and to its insertion site in the genome.

Most genes that respond to treatment with necrosis and ethylene-inducting proteins (NEPs) are associated with stress, activating cellular death pathways (Bae et al., 2006; Arenas et al., 2010). During PCD, activation of cysteine proteases occurs, which have been shown to be inhibited by overexpression of cystatins in plants (Solomon et al., 1999; Belenghi et al., 2003). For the response of transgenic and control plants following exposure to *Mp*NEP2, the fungal protein that induces PCD, all *Tccys*4:tobacco lines showed the necrosis inhibition phenotype in the area in which *Mp*NEP2 protein was injected when compared to control plants (non-transformed) (Figure 4). Experimental units with higher levels of transgene expression also showed greater necrosis inhibition in a functional study with *Mp*NEP2 (Figure 4), as occurs for the L3 for *Tccys*4:tobacco. This result suggests that overexpression of *Tccys4*:tobacco reduces the activity of proteases induced by *Mp*NEP2. Notably, Gutiérrez-Campos et al. (2001) suggested a mechanism for cysteine protease regulation by cystatins at the cytoplasmic level, leading to variations in the cellular differentiation and elongation processes. A previous study showed that cystatins are induced by harm (Hwang et al., 2010); thus, during the foliar infiltration process of *Mp*NEP2, there may have been increased local expression of endogenous cystatins and reduced protein activity that induces necrosis. Previous studies showed that overexpression of *At*Cys1 inhibited PCD caused by increased hydrogen peroxide, among other factors (Belenghi et al., 2003). Our results suggest that overexpression of cystatin from cocoa in tobacco reduces PCD induced by *Mp*NEP2. The association between *Mp*NEP2 protein and the necrosis that occurs during the development of witches' broom symptoms in cacao have been demonstrated by several studies (da Silva et al., 2011; Villela-Dias et al., 2011; Zaparolli et al., 2011). In addition to solving the *Mp*NEP2 protein structure, Zaparolli et al. (2011) found that the respective gene showed elevated transcript levels during the *T. cacao*-*M. perniciosa* interaction and necrosis. This indicates interference in the pathosystem because of overexpression of cystatins, and may interfere with symptom development and disease progression, which is directly related to cellular death induced by *Mp*NEP2. Villela-Dias et al. (2014) observed responses similar to necrosis in cell cultures of *N. benthamiana* (expressing green fluorescent protein) treated with *Mp*NEP2, which is characterized by swelling of organelles and plasma membrane rupture among other processes, indicating direct damage to the plasma membrane. Studies by Mandal et al. (2006) confirmed that cystatin expression can neutralize the effect of fungal proteases and block PCD during pathogenesis of the fungus.

Further studies should be conducted to determine role of *Tc*CYS4 in the context of cellular death, as the data obtained in this study indicate the participation of *Mp*NEP2 in PCD, as well as strongly suggests that cystatins control this process.

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