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Black shank resistant tobacco by silencing of glutathione S-transferase

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ABSTRACT

A glutathione S-transferase gene was amplified from cDNA of *Nicotiana tabacum* roots infected with *Phytophthora parasitica* var. *nicotianae*. The gene was cloned in sense and anti-sense orientation to an RNAi vector for induced gene silencing, and reduced expression of the gene was detected by RT-PCR. A statistically significant increase in resistance of *N. tabacum* to infection following gene silencing was found for glutathione S-transferase-silenced plants compared with control plants. Some defense genes were up-regulated in glutathione S-transferase-silenced plants during the interaction with the pathogen. This is the first evidence of the role of glutathione S-transferase as negative regulator of defense response.

During infection by pathogens, plant cells respond by expressing a battery of disease response genes, which can result in the production of various toxic plant products, including active oxygen species [1]. One response of plants is the increased expression of glutathione S-transferase (GST) genes following infection by pathogens [2–5].

Although *Nicotiana tabacum* GSTs are known to be involved in responses to cold, salt stress, and aluminum toxicity [6,7], little is known about their role during pathogen infection. Recently, a cDNA clone, homologous to glutathione S-transferase gene showed a high induction during the interaction of *Phytophthora parasitica* var. *nicotianae* with some resistant and susceptible Nicotiana species and varieties, in all time-course evaluated [8].

Subsequently, four GST genes were amplified from cDNA of *Nicotiana benthamiana* leaves infected with *Colletotrichum destructivum* using primers based on conserved regions of *N. tabacum* GST sequences [4]. Each of the four genes was cloned into a PVX vector for virus-induced gene silencing. A statistically significant increase in susceptibility of *N. benthamiana* to infection following gene silencing was found only for NbGSTU1-silenced plants, which had 130% more lesions and 67% more colonization by *C. orbiculare* compared with control plants [4]. The results demonstrated that the different GST genes respond in different ways to fungal infection, and at least one plant GST gene could have an important role in disease development [4].

In order to determine if the *N. tabacum* GST gene is involved in the host response to fungal infection, in the present work this was silenced by transforming *N. tabacum* with an RNAi vector. The susceptibility and resistance of the plants to infection by *P. parasitica* var. *nicotianae* in GST silenced plants was determined.

Materials and methods

Inoculation of tobacco with P. parasitica var. nicotianae. The susceptible tobacco specie *N. tabacum* cv. 'Sumatra' (seed provided by the Tobacco Research Institute, Cuba), was used in the all experiments. A field-isolate of *P. parasitica* var. *nicotianae* (provided by Plant Health Institute) was used for all inoculations throughout this study. The inoculum and the protocol for inoculation under greenhouse conditions were prepared as described previously [9]. Finally, the stem disease rating was calculated according to Csinos [10].

Construction of RNAi plasmids and N. tabacum transformation. Sense and antisense GST gene fragments (200 bp) was PCR-amplified from cDNA using forward and reverse primers that added the XhoI/BamHI (sense) and KpnI/XbaI (antisense) restriction sites, respectively (Table 1). PCRs were carried out under the following conditions: an initial denaturation step for 2 min followed by denaturation for 15 s at 94 °C, annealing for 30 s at 57 °C and extension for 1 min at 72 °C for 30 cycles, followed by a final elongation step at 72 °C for 5 min. PCR products were separated on 1% agarose gels and were purified using the DNeasy kit (Qiagen, Valencia, CA). Subsequently, PCR products were cloned into the pBPΩ8 plasmid. This plasmid contained the castor bean catalase intron kindly supplied by Dr. Wang (CSIRO, Australia). The resulting CaMV 35S promoter/sense GST, intron, antisense GST/T nos fusions were inserted into the site HindIII/PstI of the binary T-DNA

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Table 1
List of primers used in real time RT-PCRs.

N. tabacum target gene	Primers	Product size (bp)
GST	5′-TGCTGTGAAGGAAAGCGAAG-3′ 5′-TCAGCACACCATGCACTCAC-3′	198
SOD	5′-CGACACTAACTTTGGCTCCCTAGA-3′ 5′-ACGTCTATTCCCAGAAGAGGAACC-3′	192
Beta-1, 3 glucanase	5′-GCCAGATTTCTCTCCCCTATTCTC-3′ 5′-ACTCTCGGACACAACAATCCCTAC-3′	161
Hsr203 J	5′-AGGAAGTATCCGGCTGGCTTAGA -3′ 5′-GAAGTAGTCATGGGGTGGGACTG -3′	113
PAL	5′-GGACAAGGGCAGCTATGCTAGTTA-3′ 5′-CATTGAGGGTCTCACCATTAGGTC-3′	237
MAPK	5′-CGAGGACGCTCCAAGGACTA-3′ 5′-CTCCGTTGGGGTGTCAGTTA-3′	151
26S rRNA	5′-CACGGACCAAGGAGTCTGACAT-3′ 5′-TCCCACCAATCAGCTTCCTTAC-3′	150

plasmid, pCambia 2300 for tobacco transformation. The tobacco transformation protocol was performed according to Ayala et al. [12]. Regenerated tobacco plants with 5 cm in height, were transferred to pots containing black turf and rice husk (4:1) and grown in growth chambers at 23 °C.

Real-time PCR analyses. Total RNA was extracted from GST-silenced tobacco and *N. tabacum* cv. 'Sumatra' plants inoculated with *P. parasitica* var. *nicotianae* at 0, 5, 7 and 14 days post inoculation (dpi) using RNeasy kit (Qiagen, Valencia, CA), including an in-column DNAse treatment (Qiagen) according to manufacturer's instruction. The cDNA were synthesized using an oligo-(dT) primer and the SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Quantitative real-time PCR was conducted using a Rotor-Gene 3000 PCR machine (Corbett, Australia) with the QuantiTect SYBR Green PCR Kit (Qiagen). All primer sequences are shown in Table 1 and designed according to Rozen and Skaletsky [11]. Real-time PCR conditions were as follows: an initial 95 °C denaturation step for 15 min followed by denaturation for 15s at 95 °C, annealing for 30s at 60 °C, and extension for 30s at 72 °C for 40 cycles and analyzed on the Rotor-Gene 3000 software (Corbett, Australia). Five replicates for each sample were used for real-time PCR analysis and the experiment was repeated two times.

Results

Based on sequence of *N. tabacum* GST gene reported by Chacon et al. [8] obtained from a cDNA library constructed during constructed the *P. parasitica* var. *nicotianae*—tobacco interaction, pairs of primers were designed to amplify GST fragment, and the hairpin plasmid constructed based on the RNAi approach for GST is shown in the Fig. 1A.

Relative RT-PCR was done for the GST gene using roots and stems samples following infection by *P. parasitica* var. *nicotianae*. At 0 dpi, prior to fungal inoculation, the GST gene in wild-type plants showed basal expression compared with *NtGST*-silenced plants, where the relative expression was reduced (Fig. 1B). Following infection by *P. parasitica* var. *nicotianae*, the relative expression of GST increased from 1.8 to 8 in WT plants during all the time-points evaluated (Fig. 1B). However, the GST-silenced plants (RNAi::GST1 and RNAi::GST3 transgenic lines) did not show an increase of GST after challenge with *P. parasitica* var. *nicotianae* and showed silencing of this gene during all the times-points (Fig. 1B). Additionally, the phenotype was not affected in the GST-silenced plants (data not shown).

On the other hand, the pathogen infected the stems, resulting in necrotic stem lesions in WT plants at 7 dpi (Fig. 2A). Meanwhile, GST-silenced plants did not show significant symptoms of the

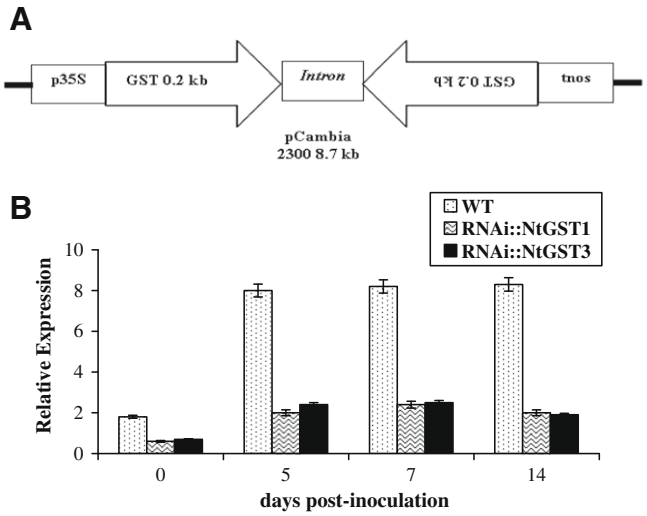

Fig. 1. Function evaluation of GST gene in the plant resistance to *P. parasitica* var. *nicoteanae*. (A) Schematic map of *A. tumefaciens* vector constructed for RNAi approach of GST gene in tobacco plants. (B) Relative expression of the GST gene in WT plants and RNAi transformants silenced for GST, as compared to the constitutively expressed 26S rRNA gene as an endogenous control. $N = 5$; ±SD.

disease in all the times evaluated (Fig. 2A). On the other hand, the GST silenced-plants had less stem disease rating compared with the WT plants (Fig. 2B).

Subsequently, the relative level of expression of several genes associated with disease resistance was evaluated in GST-silenced transgenic tobacco plants. Beta-1,3-glucanase, superoxide dismutase (SOD), mitogen-activated protein kinase (MAPK) and *Nicotiana tabacum* cell death associated protein (Hsr203J) genes were found to be significantly induced in RNAi transformed lines during the interaction with *P. parasitica* var. *nicotianae*, compared to WT plants, where the expression was either delayed or lower (Fig. 3). The expression of phenylalanine ammonia-lyase (PAL) gene was down-regulated in RNAi transformed lines; meanwhile it was induced in WT plants (Fig. 3). The results showed that the silencing of GST gene contribute to the disease resistance phenotype.

Discussion

GSTs expression in plants is up-regulated in many different stress situations [3]. A number of studies have reported an increase in the expression of GSTs after pathogen attack [5,13,14] and it has been postulated that antioxidative activity of GSTs plays a role in the reduction of damage caused by pathogens or in limiting the extent of cell death during the hypersensitive response (HR) [15].

In this study, we have analysed the biological relevance of GST gene during the compatible *P. parasitica* var. *nicotianae*—tobacco interaction by silencing in transgenic tobacco. Previously, a cDNA clone, homologous to glutathione S-transferase gene, showed highest induction during compatible *P. parasitica* var. *nicotianae*—tobacco interaction [8]. Expression of GST showed a major increase following infection by *P. parasitica* var. *nicotianae* in WT plants during the time evaluated according to previous report [8]. However, following silencing treatment of the GST gene, an examination of gene expression in the silenced plants showed that GST gene was always silenced compared with the controls in the *P. parasitica* var. *nicotianae* -infected plants (Fig. 1).

The level of resistance of GST-silenced plants compared with the WT control when were inoculated with *P. parasitica* var. *nicotianae* was surprising. GST-silenced plants inoculated with *P. parasitica* var. *nicotianae* exhibited a reduction in the stem disease rate

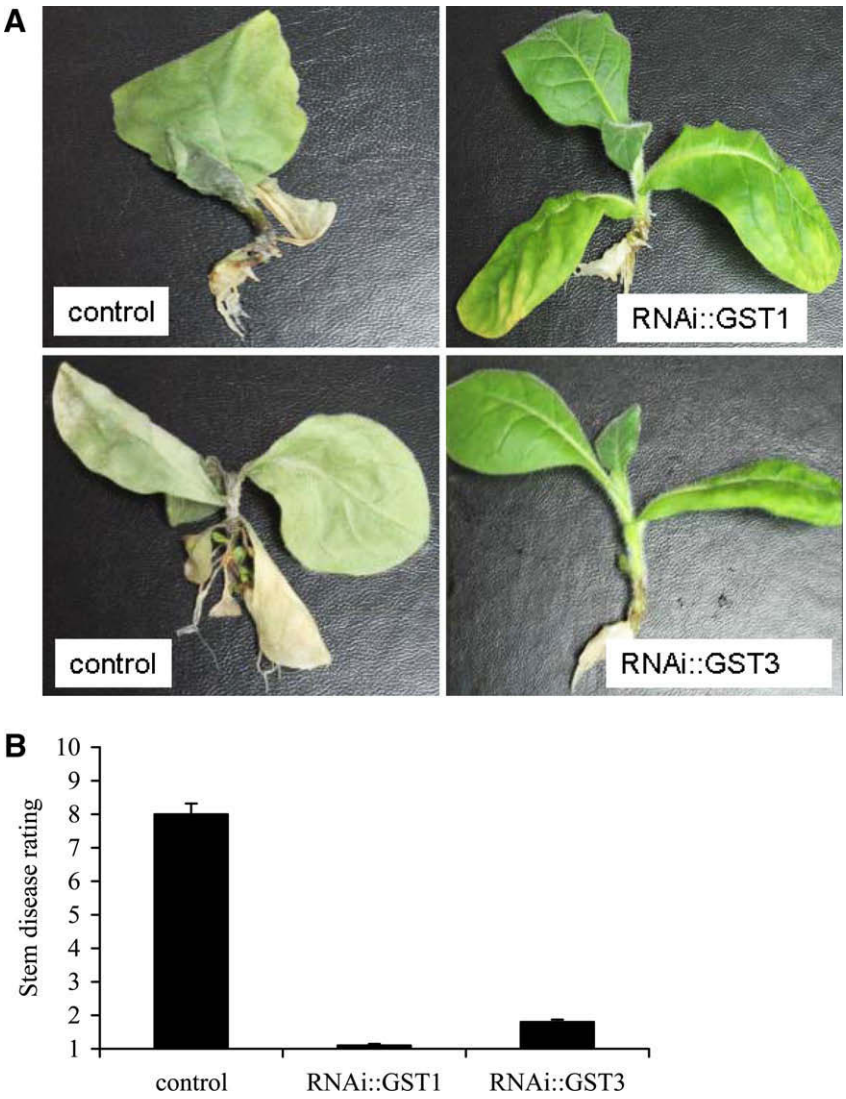

Fig. 2. Greenhouse evaluation of tobacco plants silencing the GST gene inoculated with *P. parasitica* var. *nicotianae*. (A) Phenotype (A) and stem disease rating (B) of control tobacco and transgenic tobacco plants with suppressed expression of GST gene interacting with *P. parasitica* var. *nicotianae* at 7 dpi. Stem disease rating was calculated according to Csinos [10]. $N = 50$; ±SD.

compared with the WT control plants (Fig. 2). Therefore, GST appeared to play a significant role in the plant response to the pathogen.

One possible reason for this result is that the GST expression during the compatible interaction could be limiting the HR, according to the expression of some genes involved in HR, like SOD and Hsr203 gene (Fig. 3). Studies of the hypersensitive response in a variety of experimental systems have implicated reactive oxygen intermediates, such as superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2), as important signals and/or effectors of program cell death [1]. To put it another way, $\cdot O_2^-$ can either promote the HR by providing H_2O_2 or repress the HR by scavenging nitric oxide and inducing antioxidants. In this scheme, SOD is an important mediator of these countervailing roles [16].

SOD plays an important inductive role by converting superoxide to H_2O_2, which synergizes with nitric oxide to trigger rapid cell death and other defense responses. Thus, superoxide is allowed to accumulate, and dampens HR-promoting stimuli by scavenging nitric oxide to peroxynitrite and by inducing antioxidant enzymes such as GST. Note that this regulatory balance could easily be shifted towards cell death by activation of SOD [16]. Meanwhile, the Hsr203 gene is regarded as a marker of the HR induced by a range of stimuli [8,17,18].

In addition, the silencing of GST gene reduced the expression of the PAL gene encoding a key enzyme in the pathway for phytoalexin and salicylic acid (SA) biosynthesis [19]. Besides Hsr203J, SOD and MAPK, plants silencing GST also showed an increased expression of beta-1,3-glucanase a well-described gene encoding a pathogenesis-related protein (Fig. 3). Possibly, the resistance we have observed in this study using silencing of GST is the results from a combined action of several gene products that might be under the direct or indirect control of GST.

Other pathogen-plant interactions have shown altered GST expression due to pathogen attack, and a variety of roles have been proposed for the GST genes in the host response. Gullner and Komives [20] concluded that although GSTs appear to have a variety of functions in plant metabolism, the most likely role for GSTs in pathogen-infected plants was to suppress necrosis by detoxifying lipid hydroperoxides produced by peroxidation of membranes. In potato, Prp1-1, a GST, was induced 2 h post-inoculation with *Phytophthora infestans* with maximum expression between 48 and 56 h post-inoculation [21]. They speculated that Prp1-1 was

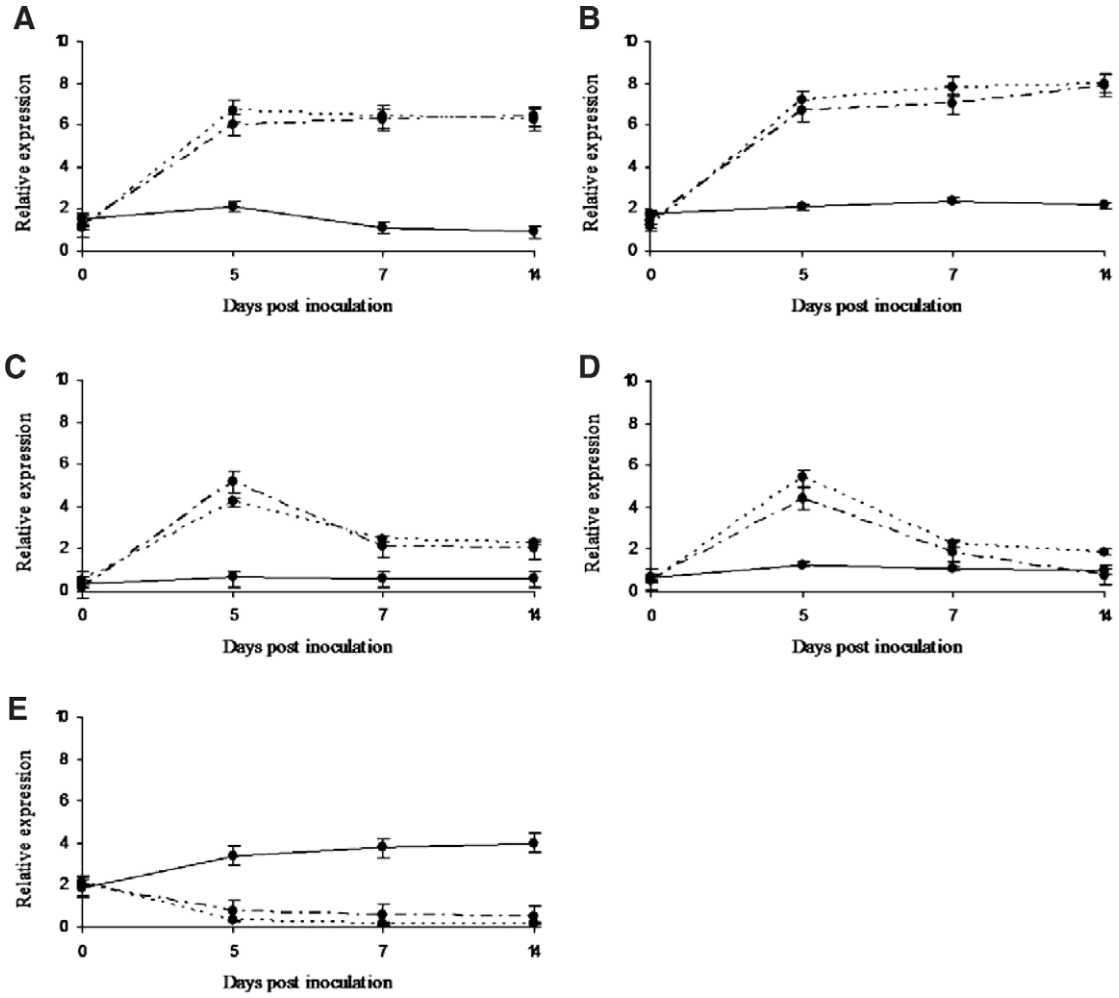

Fig. 3. Relative expression to detect *in planta* transcription of several genes involved in plant disease resistance in *N. tabacum* cv. 'Sumatra'—*P. parasitica var. nicotianae* compatible interaction (—) and tobacco RNAi transformants GST1 (···) and GST3 (–·–) silenced for GST gene. (A) Beta-1,3-glucanase, (B) SOD, (C) MAPK, (D) Hsr203J, (E) PAL. $N = 5$; ±SD.

induced during the disease as a result of auxin produced by *P. infestans*, which competitively binds PRP1-1, thereby inhibiting GST function and causing an increased need for GST. In wheat, a phi GST, Gsta1, was induced dramatically by 2 h post-inoculation with *Erysiphe graminis* f. sp. *tritici*, and the expression level remained high for at least 2 days in both compatible and incompatible interactions [2].

Moreover, after inoculation of *A. thaliana* with a compatible or incompatible strain of *Peronospora parasitica*, higher expression of phi, tau, and zeta GST genes was observed, and these may have roles in restricting cellular damage by functioning in antioxidative reactions [3]. Dean et al. [4] demonstrated that not all of the *N. benthamiana* GST genes play a role in disease susceptibility. This demonstrates that although induced gene expression may indicate involvement, it is still necessary that gene expression be altered, such as by virus-induced gene silencing during an infection process, to demonstrate the significance of a gene in the plant response to fungal infection.

Finally, the proposed function of GST could involve the detoxification of active oxygen species to prevent continuing cell death caused by free radicals produced during the hypersensitive response in the compatible interaction. The GST gene identified here appears to be involved in the defense response of tobacco to *P. parasitica* var. *nicotianae*. This gene seems to be crucial for an efficient defense against *P. parasitica* var. *nicotianae* during compatible interaction and this might now be exploited in strategies to develop durable resistance in cultivated tobacco plants through either marker-assisted breeding or biotechnological approaches.

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References

[1] C. Lamb, R.A. Dixon, The oxidative burst in plant disease resistance, Annu. Rev. Plant Physiol. Plant Mol. Biol. 48 (1997) 251–275.
[2] F. Mauch, R. Dudler, Differential induction of distinct glutathione S-transferases of wheat by xenobiotics and by pathogen attack, Plant Physiol. 102 (1993) 1193–1201.
[3] U. Wagner, R. Edwards, D.P. Dixon, F. Mauch, Probing the diversity of the Arabidopsis glutathione S-transferase family, Plant Mol. Biol. 49 (2002) 515–532.
[4] J.D. Dean, P.H. Goodwin, T. Hsiang, Induction of glutathione S-transferase genes of *Nicotiana benthamiana* following infection by *Colletotrichum destructivum* and *C. orbiculare* and involvement of one in resistance, J. Exp. Bot. 56 (2005) 1525–1533.
[5] P.G. Sappl, A.J. Carroll, R. Clifton, R. Lister, J. Whelan, A.H. Millar, K.B. Singh, The Arabidopsis glutathione transferase gene family displays complex stress regulation and co-silencing multiple genes results in altered metabolic sensitivity to oxidative stress, Plant J. 58 (2009) 53–68.

[6] V. Roxas, R. Smith, E. Allen, R. Allen, Overexpression of glutathione S-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress, Nat. Biotechnol. 15 (1997) 988–991.
[7] B. Ezaki, M. Katsuhara, M. Kawamura, H. Matsumoto, Different mechanisms, of four aluminum Al-resistant trangenes, for Al toxicity in Arabidopsis, Plant Physiol. 127 (2001) 918–927.
[8] O. Chacon, I. Hernandez, R. Portieles, Y. Lopez, M. Pujol, O. Borrás-Hidalgo, Identification of defense-related genes in tobacco responding to black shank disease, Plant Sci. 177 (2009) 175–180.
[9] J.M. Sullivan, T.A. Melton, H.D. Shew, Fitness of races 0 and 1 of *Phytophthora parasitica* var. *nicotianae*, Plant Dis. 89 (2005) 1220–1228.
[10] A.S. Csinos, Relationship of isolate origin to pathogenicity of race 0 and 1 of *Phytophthora parasitica* var. *nicotianae* on tobacco cultivars, Plant Dis. 89 (2005) 332–337.
[11] S. Rozen, H.J. Skaletsky, Primer3 on the WWW for general users and for biologist programmers, in: S. Krawetz, S. Misener (Eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology, Humana Press, Totowa, NJ, 2000, pp. 365–386.
[12] M. Ayala, J. Gavilondo, M. Rodríguez, A. Fuentes, G. Enríquez, L. Pérez, J. Cremata, M. Pujol, Production of Plantibodies in *Nicotiana* plants, in: L. Faye, V. Gomord (Eds.), Methods in Molecular Biology: Recombinant Proteins from Plants, Humana Press, Springer Science, Totowa, NJ, 2009, pp. 103–134.
[13] R. Dudler, C. Hertig, G. Rebmann, J. Bull, F. Mauch, A pathogen induced wheat gene encodes a protein homologous to glutathione S-transferases, Mol. Plant-Microbe Interact. 4 (1991) 14–18.
[14] A. Levine, R. Tenhaken, R. Dixon, C. Lamb, H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response, Cell 79 (1994) 583–593.
[15] M.E. Alvarez, R.I. Pennell, P.J. Meijer, A. Ishikawa, R.A. Dixon, C. Lamb, Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity, Cell 92 (1998) 773–784.
[16] E.P. Beers, J.M. McDowell, Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues, Curr. Opin. Plant Biol. 4 (2001) 561–567.
[17] D. Pontier, S. Gan, R.M. Amasino, D. Roby, E. Lam, Markers for hypersensitive response and senescence show distinct patterns of expression, Plant Mol. Biol. 39 (1999) 1243–1255.
[18] E. Gilroy, I. Hein, R. van der Hoorn, P. Boevink, E. Venter, H. McLellan, F. Kaffarnik, K. Hrubikova, J. Shaw, M. Holeva, E. Canales, O. Borrás-Hidalgo, L. Pritchard, G. Loake, C. Lacomme, P. Birch, Involvement of Cathepsin B in the plant disease resistance hypersensitive response, Plant J. 52 (2007) 1–13.
[19] J.P. Métraux, Recent breakthroughs in the study of salicylic acid biosynthesis, Trends Plant Sci. 7 (2002) 332–334.
[20] G. Gullner, T. Komives, The role of glutathione and glutathione-related enzymes in plant–pathogen interactions, in: D. Grill, M. Tausz, L. Kok (Eds.), Significance of Glutathione in Plant Adaptation to the Environment, Kluwer Academic Publishers, Dordrecht, NL, 2001, pp. 207–239.
[21] C. Hahn, G. Strittmatter, Pathogen-defense gene prp1-1 from potato encodes an auxin responsive glutathione S-transferase, Eur. J. Biochem. 226 (1994) 619–626.