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Over-expression of *CsGSTU* promotes tolerance to the herbicide alachlor and resistance to *Pseudomonas syringae* pv. *tabaci* in transgenic tobacco

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Abstract

Glutathione transferases (GSTs) mainly catalyze the nucleophilic addition of glutathione to a large variety of hydrophobic molecules participating to the vacuole compartmentalization of many toxic compounds. In this work, the putative tolerance of transgenic tobacco plants over-expressing *CsGSTU* genes towards the chloroacetanilide herbicide alachlor was investigated. Our results show that the treatment with 0.0075 mg cm^{-3} of alachlor strongly affects the growth of both wild type and transformed tobacco seedlings with the sole exception of the transgenic lines over-expressing *Cs*GSTU2 isoform that are barely influenced by herbicide treatment. In order to correlate the *in planta* studies with enzyme properties, recombinant *Cs*GSTs were *in vitro* expressed and tested for GST activity using alachlor as substrate. The recombinant GSTU2 enzyme was twice more active than GSTU1 in conjugating alachlor to GSH thus indicating that *Cs*GSTU2 might play a crucial role in the plant defense against the herbicide. Moreover, as a consequence of the infiltration with a bacterial suspension of the *P. syringae* pv. *tabaci*, transgenic tobacco plants but not wild type plants bestowed the capability to limit toxic metabolite diffusion through plant tissues as indicated by the absence of chlorotic halos formation. Consequently, the transgenic tobacco plants described in the present study might be utilized for phytoremediation of residual xenobiotics in the environment and might represent a model for engineering plants that resist to pathogen attack.

Additional key words: biotic stress, glutathione transferase, host-pathogen interaction, phytoremediation.

Introduction

Water deficit, high temperature, salinity, cold, heavy metals, mechanical wounding, and cytotoxic xenobiotics (*e.g*. herbicides) often impose constraints on plant growth and development under field conditions (Hu *et al*. 2011). Moreover, susceptibility to infectious diseases caused by pathogens affects most plants in their natural habitat and leads to yield losses in agriculture. Plants in order to overcome any stress have evolved sophisticated and coordinated defense responses incorporating a three-phase detoxification mechanism (Coleman *et al*. 1997). Phase I reactions introduce or reveal functional groups into the toxic molecules which can undergo to the successive phase II. Glutathione S-transferases (GSTs, EC.2.5.1.18) are a superfamily of multifunctional enzymes active in the phase II of the plant defense pathway (Labrou *et al*. 2015). They mainly catalyze the conjugation of glutathione (GSH) to a large variety of hydrophobic endobiotic and xenobiotic compounds containing electrophilic centers (Lo Piero *et al*. 2006, 2009, Öztetik 2008, Allocati *et al*. 2009). The reaction of xenobiotics with GSH facilitates their excretion from the cytoplasm to vacuoles by means of specialized tonoplast localized ATP-binding cassette (ABC) transporters acting in phase III (Coleman *et al*. 1997, Cummins *et al*. 2011). Most GSTs exist as soluble or cytosolic enzymes (cGST) being the most extensively studied subfamily. To date, seven classes of cGSTs are recognized in plants: lambda (L), phi (F), tau (U), dehydroascorbate reductases (DHAR), theta (T), zeta (Z), and tetrachlorohydroquinone dehalogenase (TCHQD) (Edwards *et al*. 2000, Dixon *et al*. 2002, Öztetik 2008, Mohsenzadeh *et al*. 2011). Plant GSTs fulfill their functions during normal development, from early embryogenesis to senescence, in every tissue type examined (Soranzo *et al*. 2004). Furthermore, there are many other specific factors that can cause induction of gene expression: hormones,

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Abbreviations: CDNB - 1-chloro-2,4-dinitrobenzene; GST - glutathione transferase; WT - wild-type.

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xenobiotics, and a range of biotic and abiotic stresses indicating that GSTs undergo to major transcriptional regulation (Marrs 1996). A recent *in silico* study revealed that specific signature profiles related mainly to protein phosphorylation are in the GST classes thus suggesting that they might be subjected to reversible activation by phosphorylation-mediated regulation (Puglisi *et al.* 2013a,b). Due to their role in plant defense mechanisms, the overexpression of heterologous *GST* genes is widely used to enhance the crop qualitative and quantitative traits (Karavangeli *et al.* 2005, Ji *et al.* 2010, Benekos *et al.* 2010, Dixit *et al.* 2011, Jha *et al.* 2011, Choi *et al.* 2013, Liu *et al.* 2013, Lo Cicero *et al.* 2015). In addition to their role in the abiotic stress relief, it has been reported the biological relevance of GSTs towards pathogen attack. Many authors (Dudler *et al.* 1991. Greenberg *et al.* 1994, Levine *et al.* 1994) emphasize that expression of specific GSTs (Mauch and Dudler 1993, Dean *et al.* 2005) is strongly induced by pathogenic attack and they postulate that the GST antioxidant activity could play an important role to limit the damages and the extent of programmed cell death during the hypersensitive response (Dudler *et al.* 1991, Alvarez *et al.* 1998). Bai *et al.* (2012) investigated the expression profile of 16 rice proteins by Western blot analysis in the rice response against *Xanthomonas oryzae* pv. *oryzae*. The comparison of their expression pattern in resistance, susceptible, and mock responses revealed that GST expression is up-regulated during the resistance process indicating that GST represents a positive regulator in resistance response. Recently, the generation and characterization of transgenic tobacco plants over-expressing tau glutathione transferases from *Citrus sinensis* (*Cs*GSTU1 and *Cs*GSTU2) have been reported (Lo Piero *et al.* 2010, 2011, Lo Cicero *et al.* 2015). In this work, transgenic tobacco plants overexpressing *CsGSTU* genes were assessed for their ability in detoxifying the herbicide alachlor. Simultaneously, the *CsGSTU* genes were *in vitro* expressed using an *E. coli* cell-free system. The catalytic activity of the GST isoforms were determined against alachlor in order to correlate the response to the herbicide observed *in planta* with enzyme properties exhibited *in vitro*. Moreover, in order to investigate the putative role of GSTs in the response to biotic stress, tobacco plants over-expressing *CsGSTU* were subjected to infection by the pathogen *Pseudomonas syringae* pv. *tabaci* (Hirano and Upper 1990, Heath 2000, Melotto *et al.* 2006, 2008).

Materials and methods

Plant materials and alachlor treatment: Tobacco (*Nicotiana tabacum* L. cv. Wisconsin) seeds were surface sterilized in 100 % ethanol solution for 30 s, then in 0.03 g cm^{-3} NaOCl aqueous solution for 10 min, rinsed in sterile distilled water and placed for germination on Murashige and Skoog (1962; MS) medium. Tobacco plants were grown in a growth chamber under a16-h photoperiod, an irradiance of 100 μmol m^{-2} s^{-1} and a temperature of 23 °C. Tobacco transformation was achieved by inserting two *GST* genes belonging to the GST tau class, namely *GSTU1* and *GSTU2*, that have been previously isolated from sweet orange leaves. The encoded proteins differ only in three amino acids, all of them included in the hydrophobic co-substrate binding site of the enzymes (R89P, E117K, I172V) (Lo Piero et al. 2009). Plasmid construction, tobacco transformation and verification of the transgenic plants were carried out as described in Lo Cicero *et al.* (2015). The transgene expression analysis was performed by real time PCR as detailed in Lo Cicero *et al.* (2015). Transgenic T_1 plants growing normally under the selective pressure of kanamycin were used in the herbicide tolerance study as well as in analysis of pathogenesis induced by *Pseudomonas syringae* pv. *tabaci*.

Alachlor tolerance experiments were performed *in vitro*. Three-week-old transgenic and wild type (WT) tobacco seedlings were transferred to *Magenta GA-7* (*Sigma Aldrich*, St. Louis, MO, USA) vessels containing MS medium supplemented with 0.0075 mg cm^{-3} alachlor (*Sigma Aldrich*). The effect of herbicide treatment was assessed after 40 d of growth by visual inspection and by measuring shoot and root elongation (Benekos *et al.* 2010).

Measurement of glutathione transferase activity: Tobacco leaf crude extract was prepared using aliquots (0.2 g) of leaves from WT and transgenic tobacco seedlings grown *in vitro*, as detailed in Lo Cicero *et al.* (2015). The GST assay using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate was performed as described in Lo Piero *et al.* (2006). The assay for determination of GST activity was based on the colour formation resulting by the reaction of $Hg(SCN)_2$ with the released halogen ions of the substrate in the presence of Fe^{3+} (Iwasaki *et al.* 1952). The reaction mixture (final volume 0.25 cm^3) containing 25 mM MES buffer, pH 6.5, 2.5 mM GSH, 0.5 mM alachlor, and 0.05 cm^3 of enzyme was incubated at 37 °C for 30 min. Following incubation, maleimide, prepared by dissolving solid maleimide in absolute ethanol, was added in each tube (10 mM final concentration) and mixed by pipetting. Colour development was accomplished by the addition of $Hg(SCN)_2$ (2 mM final concentration), prepared by dissolving solid mercury thiocyanate in absolute ethanol and $Fe(NO_3)_3$ (20 mM final concentration), prepared in 6 M nitric acid. All measurements were adjusted by subtracting the absorbance values obtained for the non-enzymatic conjugation of substrate and, when necessary, by subtracting the absorbance of samples containing the leaf extract but not alachlor. Unit activity was defined as the amount of enzyme that catalyses the reaction of 1 μmol of substrate per minute. Protein content was determined by the method of Bradford (1976) using bovine serum albumin as standard. The orange colour due to the presence of halogen ions was measured spectrophotometrically (*Lambda 11, Perkin Elmer,* Waltham, MA, USA) at 460 nm. Halogen quantitation was carried out using the linear range of a standard curve obtained from the graph of absorbance at 460 nm *versus* different concentration of NaCl (Skopelitou and Labrou 2010).

***In vitro* GSTs expression:** *In vitro* expression of functionally active GSTs was achieved by using cell-free *Escherichia coli* expression system (*Expressway*, *Invitrogen*, Carlsbad, CA, USA) as detailed in (Lo Piero *et al.* 2009). The recombinant protein, containing the 6× His-tag at the N-terminus, was purified by loading the cell-free extract onto *His-graviTrap* column prepacked with *Ni-Sepharose 6* fast flow (*GE Healthcare*, Little Chalfont, Buckinghamshire, UK) (Lo Piero *et al.* 2009).

Pathogenicity test: *Pseudomonas syringae* pv. *tabaci* strain DAPP-PG 676 was grown on King's medium B (King *et al.* 1954; KB) plates at 26 °C for 24 h. Cells were washed in sterile water and suspended at a concentration of 10^8 cfu cm^{-3} in 10 mM phosphate buffer, pH 7.0 (Sambrook *et al.* 1989). Inoculum content was determined by measuring absorbance at 600 nm, confirmed by plating serial dilutions on KB plates, and adjusted at the appropriate concentration in 10 mM phosphate buffer. Bacterial suspensions were infiltrated into fully developed WT and transgenic tobacco leaves using a 5 cm^3 plastic syringe without a needle (Klement 1963). The syringe was placed on the abaxial side of the leaf in the laminar area between two lateral veins while the area opposite the site of contact was supported with the other hand; the inoculum was then slowly injected into the leaf intercellular space, forming an infiltrated area of approximately 20 × 20 mm.

A time-course experiment was performed to assess the *P. syringae* pv. *tabaci* bacterial population in *Cs*GSTs over-expressing plants at 0, 24, 48, 72, 96, and 120 h after inoculation. Tobacco plants were maintained in the growth chamber under above mentioned conditions. Leaf disks were punched from the infiltrated area with a cork borer and sampled for each genotype at each sampling date. The six disks were divided into three sets of two and grinded in 1 cm^3 of 10 mM phosphate buffer. The bacterial populations in the leaves were determined by plating serial dilutions of the disc extract on KB plates supplemented with 0.1 mg cm^{-3} nitrofurantoin and 0.1 mg cm^{-3} of cycloeximide.

P. syringae pv. *tabaci* strain DAPPG-PG 676 was inoculated into 1 000 cm^3 batch of a chemically defined medium according to Woolley *et al.* (1952) which contains 10 g of sucrose, 5 g of KNO_3, 0.8 g of K_2HPO_3, 0.8 g of $NaH_2PO_4 . H_2O$, 0.2 g of $MgSO_4 . 7 H_2O$, 0.1 g of $CaCl_2 . 2 H_2O$, and 20 mg of $FeS0_4 . 7 H_2O$ per 1000 cm^3. The culture was grown in 100 cm^3 of medium in 250 cm^3 Erlenmeyer flask on a rotary shaker at 25 °C for 4 d. The cells were removed by centrifugation at 3 000 *g* and 4 °C for 20 min at and the culture supernatant was infiltrated into fully developed tobacco leaves of all the genotypes by using a 5 cm^3 plastic syringe without the needle.

Statistical analysis: All data are presented as means and standard deviations. Statistical analysis was performed with *SPSS*. Significance of differences between WT and transgenic lines were analyzed by Student's *t*-test.

Results and discussion

In our previous work, the generation and characterization of transgenic tobacco plants over-expressing tau glutathione transferases from *Citrus sinensis* (*Cs*GSTU1 and *Cs*GSTU2) have been reported (Lo Piero *et al.* 2010, Lo Cicero *et al.* 2015). *In planta* studies demonstrated that tobacco plants transformed by *CsGSTU2* tolerate the herbicide fluorodifen and exhibited both drought and salinity stress tolerance. In this work, both the WT and transformed T_1 tobacco lines were used to ascertain their remediation capability towards alachlor as well as the tolerance against the infection by the compatible pathogen *Pseudomonas syringae* pv. *tabaci*.

Alachlor [2-chloro-N-2,6-diethylphenyl-N-(methoxymethyl) acetamide] belongs to the chloroacetanilide herbicide family. It is a pre-emergence herbicide that is applied to young plants, absorbed through the roots and transferred to the upper parts of the plant through the apoplast. It represses the elongation of the root system and the development of the shoots (Karavangeli *et al.* 2005, Jo *et al.* 2011). To study the *in planta* tolerance towards this chloroacetanilide herbicide, we treated tobacco seedlings with 0.0075 mg cm^{-3} alachlor and 40 d after the treatment the growth inhibition induced by the herbicide was evaluated both by visual inspection (Fig. 1*A*) and by directly measuring stem and root length (Fig. 1*B*). Alachlor negatively influenced the growth of roots and stems of WT tobacco seedlings. The *Cs*GSTU1 transgenic tobacco plants were as susceptible as untransformed plants, whereas the transgenic plants overexpressing *Cs*GSTU2 were unaffected by herbicide treatment (Fig. 1*A*). These findings deriving from the visual inspection of tobacco plants have been confirmed by the direct measurement of stem and root length (Fig. 1*B*). As regards the stem growth, the WT and the transgenic lines overexpressing *GSTU1* show comparable reduced values (Fig. 1*B*). On the contrary, the stem length of the tobacco plants transformed by *Cs*GSTU2 was decreased only to 81 % of the untreated plants thus exhibiting an evident tolerance against 0.0075 mg cm^{-3} alachlor, a concentration that is 6.9 fold greater than that normally applied in the field (Benekos *et al.* 2010). Similar results have been gained in the case of root length

(Fig. 1*B*).

In order to correlate the acquired tolerance towards alachlor of *Cs*GSTU2 transformed plants with the enzymatic activity, the GST activity of the tobacco leaf extracts using alachlor as substrate was measured. The GST activity towards the universal non-specific substrate CDNB is similar between the transgenic and WT plants. The WT tobacco plants do not exhibit GST activity in the case when alachlor is supplied as substrate, suggesting that they were not able to conjugate this herbicide to GSH. On the contrary, the leaf extracts of both transformed lines show GST activity against alachlor, the specific activity of *Cs*GSTU2 transformed plants was about 2.5-fold higher than that detected in the *Cs*GSTU1 overexpressing plants (Fig. 2).

Furthermore, recombinant *Cs*GSTU1 and *Cs*GSTU2 was *in vitro* expressed as described in Materials and methods, and once the proteins have been purified, the specific activities of the enzymes were determined against both CDNB and alachlor. GSTU2 isoform exhibited a specific activity towards alachlor twice higher than that of GSTU1 suggesting that *Cs*GSTU2 might play a major role in the detoxification pathway of alachlor.

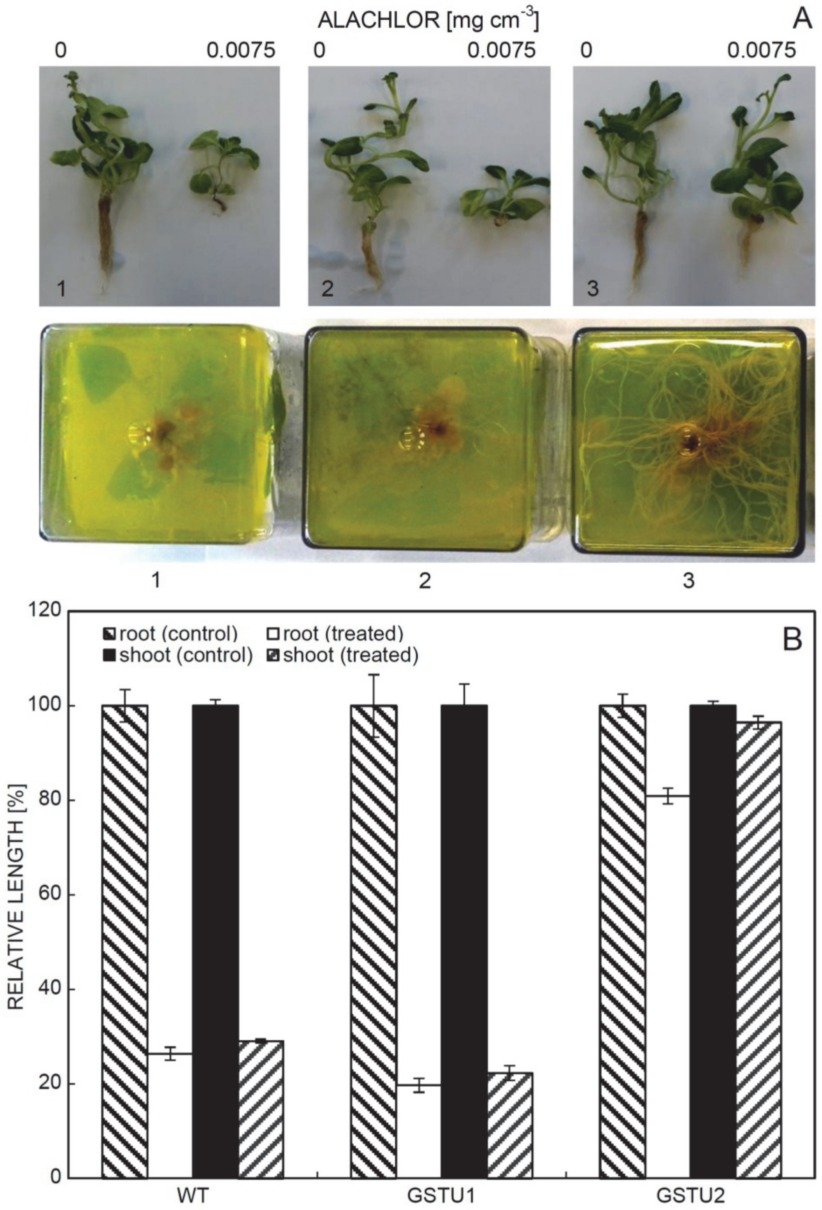

Fig. 1. *A* - Phenotype of whole seedlings and roots of wild-type (WT) and transgenic plants over-expressing *Cs*GSTU1 or *Cs*GSTU2. Plants were grown for 40 d in MS medium supplemented with 0 or 0.0075 mg cm^{-3} alachlor. *B* - Shoot and root length of WT and transgenic lines. Comparison of control and alachlor treated plants (values relative to control plants) Means ± SE, *n* = 4.

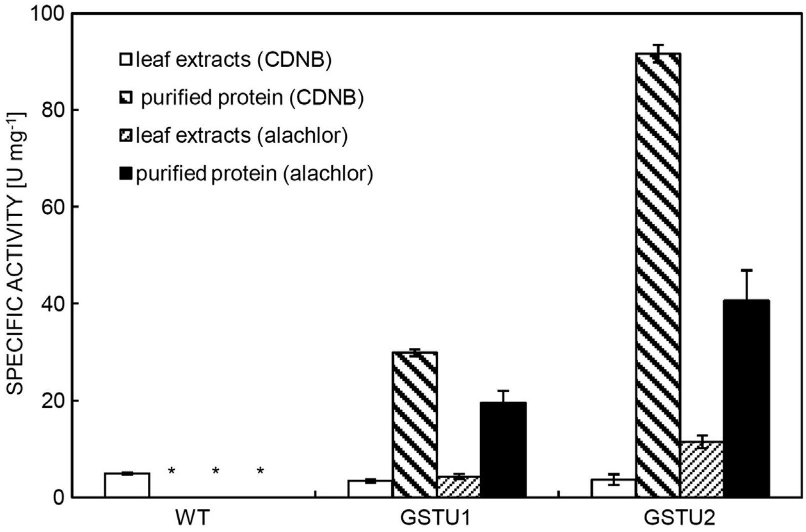

Fig. 2. GST specific activity towards CDNB and alachlor of both leaf extracts and of the *in vitro* expressed and purified recombinant *Cs*GSTUs. Means ± SD, *n* = 4, * - not detectable.

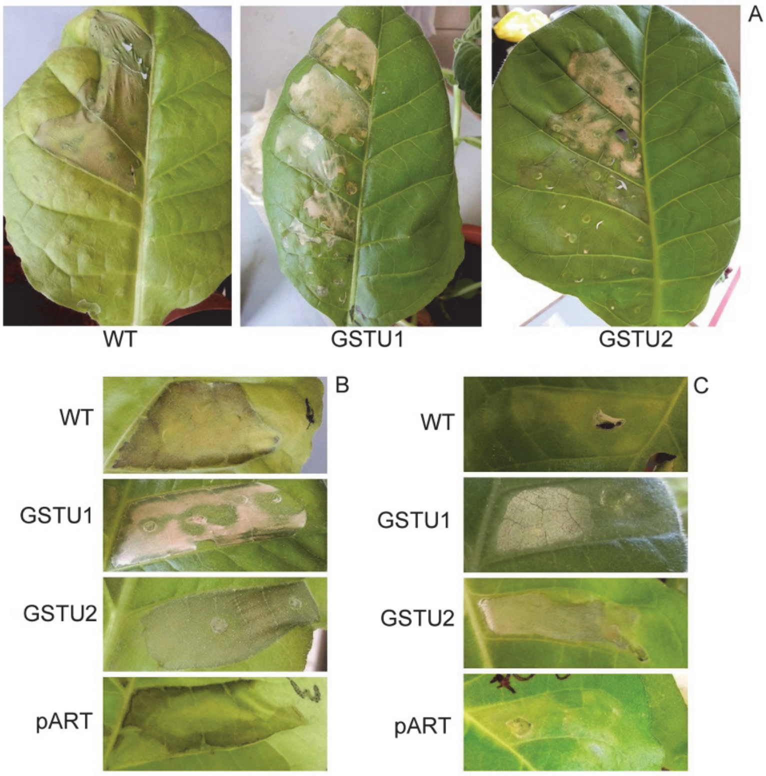

Fig. 3. Visual inspection of the host-pathogen interaction. *A* - Leaves from 8-week-old WT and transgenic tobacco plants GSTU1 and GSTU2 infiltrated with different concentrations (from 10^3 to 10^8 cfu cm^{-3}) of bacterial suspension of the *P. syringae* pv. *tabaci* DAPPG-PG 676 strain. *B* - Symptoms at three days after inoculation with bacteria, of untransformed and transformed tobacco plant overexpressing *GSTUs* genes. *C* - Inoculated leaf sections with culture filtrates from *P. syringae* pv. *tabaci* DAPPG-PG 676 (three days after inoculation). pART - plants transformed with the empty vector.

The mechanism involved in alachlor tolerance seems to require a precise enzyme-substrate interaction as it is achieved exclusively in tobacco transformed with *CsGSTU2* despite the very high similarity existing between the amino acid sequence of GSTU1 and GSTU2 (Lo Piero *et al.* 2010). These results become more relevant when the expression data of the transgenes are also considered. It has previously shown that in the *CsGSTU1* transgenic T1 lines, the *GSTU1* transcripts are about 8 times more abundant than that of *GSTU2* transcripts detected in the *GSTU2* transformed plants (Lo Cicero *et al.* 2015). This finding emphasizes that the detoxifying ability of transformed lines against alachlor is not due to a higher gene expression but to GSTU2 intrinsic kinetic properties. The different catalytic properties observed between the two *Cs*GSTU isoforms have already been highlighted in our previous work (Lo Cicero *et al.* 2015) in which the tolerance against fluorodifen of transgenic tobacco plants was assessed. Similarly, tobacco plants overexpressing *CsGSTU2* were found to be more tolerant to the herbicide than those overexpressing *CsGSTU1*, emphasizing that the mismatch of only three amino acids (R89P, E117K, I172V) might result in a diversified catalytic response (Lo Cicero *et al.* 2015). In order to understand the response of tobacco plants overexpressing the *CsGSTU* genes to *P. syringae* pv. *tabaci* inoculation, fully expanded leaves from 8-week-old plants were infiltrated with a bacterial suspension of the DAPPG-PG 676 strain and assayed by visual inspection. In a first assay directed to choose the appropriate bacterial dose-response in relationship with the potential diversity among transformants, an inoculum titration experiment was performed (Fig. 3*A*). Three days after inoculation the infiltrated tissues showed different symptoms depending on the inoculated genotype and the dose-inoculum. Symptoms included chlorosis, water-soaking and desiccation of the infiltrated area. The first concentration at which the genotypes showed interaction-symptoms is 10^6 cfu cm^{-3}. In particular, plants over-expressing *CsGSTU1* showed symptoms starting from a bacterial concentration of 10^4 cfu cm^{-3}, whereas untransformed plants and those over-expressing *CsGSTU2* showed symptoms starting from 10^6 cfu cm^{-3}, although at this concentration the leaf sections of *CsGSTU2* transformed plants were only partially damaged (Fig. 3*A*). Consequently, all the genotypes were inoculated with a *P. syringae* pv. *tabaci* DAPPG-PG 676 strain bacterial suspension at 10^6 cfu cm^{-3} concentration in multiple sites of at least three leaves distributed in different plants. The representative symptoms are summarized in Fig. 3*B*. Three days after inoculation, the untransformed tobacco plants disclosed water soaked, brown to black discoloration of the infiltrated area with a black margin as well as observable chlorotic halos (Fig. 3*B*). In tobacco plants over-expressing either *CsGSTU1* or *CsGSTU2* genes the inoculated sections showed the collapse of mesophyll which appeared dry and necrotic, whereas no chlorosis was observed. Plants transformed with the empty vector (pART samples) exhibited the same interaction phenotype of the WT tobacco plants indicating that the results arise from the GSTUs overexpression rather than being a mere consequence of transgenosis. In order to assess if the differences observed in symptomatology depend on the interaction between plant tissues and metabolites secreted out by the bacteria, culture filtrates from *P. syringae* pv. *tabaci* DAPPG-PG 676 grown in chemically defined medium were prepared (Woolley *et al.* 1952) and used to inoculate leaf sections as described in Material and methods. As shown in Fig. 3*C*, the effect of infiltration of the cultural filtrates in tobacco leaves of both transformed and control plants was perceptible at three dpi. Leaf sections from the WT plants showed diffuse chlorosis and leaves of plants harboring *CsGSTU1* and *CsGSTU2* disclose mesophyll collapse and necrosis but not chlorosis thus suggesting that bacterial culture filtrates was able to induce the same effects as the inoculated bacteria. The results reported above indicate that the overexpression of *CsGSTU1* and *CsGSTU2* in tobacco plant bestow the capability to avoid toxic compound diffusion in plant tissues as suggested by the absence of chlorotic halos formation. Several pathovars of *Pseudomonas syringae* produce a phytotoxic extracellular metabolite called coronatine (COR) that suppresses plant defense responses (Bender 1999, Cintas *et al.* 2002). Interestingly, *Pseudomonas syringae* pv. *tabaci* has been shown not to produce COR. However, the culture media extracts containing secreted metabolites still actively suppress early plant defense responses such as stomatal closure and non-host HR (hypersensitive resistance) cell death induced by the non-host bacterial pathogen (Hirano and Upper 1990, Melotto *et al.* 2006, Melotto *et al.* 2008, Heath 2000, Lee *et al.* 2013). Lee *et al.* (2013) characterized the *Pseudomonas syringae* profile of extracellular metabolites, including their retention time, the specific m/z ratio of ions and the relative intensity used to differentiate unique metabolites. However, compounds were not chemically annotated. In this regards, as we showed that tobacco plants over-expressing *CsGSTU1* and *CsGSTU2* were able to limit foliar damages, we suppose that *P. syringae* unidentified toxic metabolite are channeled towards a modification and detoxification pathway in which *Cs*GSTs (GSTU1 and GSTU2) might be involved in. Since the interaction

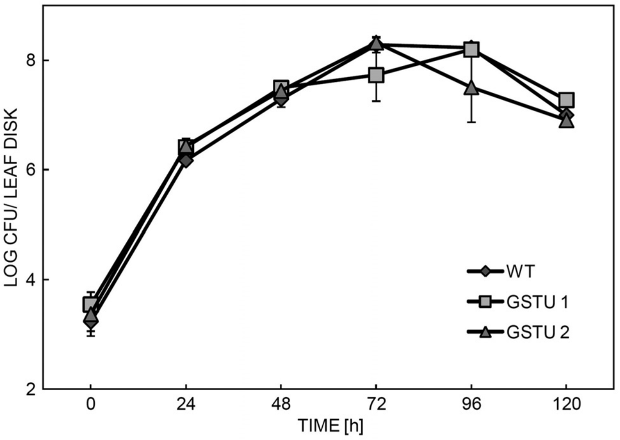

Fig. 4. Time course of growth of *P. syringae* pv. *tabaci* strain DAPP-PG 676 in leaves of tobacco WT and *CsGSTU*s transgenic lines. Fully expanded leaves of 8 week-old tobacco plants were inoculated with 10^6 cfu cm^{-3} of *P. syringae* pv. *tabaci*. Bacterial populations was determined at 0, 24, 48, 72, 96, 120 h post inoculation. Means of three samples, each consisting of two leaf discs from three different plants.

of *P. syringae* pv. *tabaci* with the tobacco plants harboring *CsGSTU1* and *CsGSTU2* was characterized by a rapid mesophyll collapse and tissue necrosis without development of observable chlorosis, the possibility that the ongoing host-pathogen interaction might involve an hypersensitive resistance (HR) response was investigated. The hypersensitive resistance response is characterized by rapid collapse and death (usually within 12 to 24 h) of the host tissue precisely confined to the inoculated area (Goodman and Novacky 1994). In a hypersensitive reaction, bacterial populations decline and the surviving bacteria are confined to the inoculation site. Thus, a time course growth experiment was performed by inoculating a bacterial suspension in the *CsGSTU1* and *CsGSTU2* transgenic tobacco lines and comparing the developing bacterial population to that of the WT plants over a 5-d experimental period. As described in Fig. 4, the growth of *P. syringae* pv. *tabaci* detected in the transgenic lines did not differ from that observed in the WT tobacco plants at any of the sampling date thus suggesting that the hypersensitive resistance response is not implicated in the *Pseudomonas syringae*-transgenic plant interaction under investigation. Increasing evidence indicates that plants, like animals, use basal resistance (BR), a component of the innate immune system, to defend themselves against foreign organisms. Contrary to the hypersensitive reaction (HR)-type cell death, recognition in the case of BR is unspecific, as intruders are recognized based on their common molecular pattern. To analyse the early events of BR in tobacco plants, a subtractive hybridisation between tobacco leaves treated with the HR-negative mutant strain *Pseudomonas syringae* pv. *syringae* and non-treated control leaves was carried out (Szatmari *et al.* 2006). Among the identified representative genes associated with BR, a member of *GST* superfamily was strongly activated after bacterial injections playing a pivotal role in the defense reactions. These results support the hypothesis that *Cs*GSTUs represent positive regulators of pathogen tolerance although their role in host-pathogen interaction still remains unclear.

Conclusions

In this work, we show that plants harboring *CsGSTU2* tolerate alachlor and the counteracting action against the herbicide is associated with the high specific conjugation activity of the *in vitro* expressed *Cs*GSTU2 protein. Therefore, the transgenic tobacco plants, which are described in the present study, can be utilized for phytoremediation of residual herbicide in the environment. An increased concern has been raised for the efficient biodegradation of alachlor in the soil (Boparai *et al.* 2006) because of its high persistence and the risk of contaminating groundwater (Yen *et al.* 2005). Due to both high growth rate and biomass of tobacco plants (Tremblay *et al.* 2010), plants over-expressing *CsGSTU2* are good candidates for designing phyto-remediation strategies in the decontamination of alachlor polluted agricultural soils. Finally, tobacco plants over-expressing *CsGSTU*s were subjected to infection with the host pathogen *Pseudomonas syringae* pv. *tabaci*, this being, as far as it is known, the first report on host-pathogen interaction upon tobacco plants over-expressing GST genes from a plant source. The lack of chlorotic halos formation during the host-pathogen interaction suggests that the over-expression of *CsGSTU1* and *CsGSTU2* avoids the active metabolite diffusion in plant tissues and this could be achieved by directing bacterial toxic molecules towards a modification pathway in which *Cs*GSTs are involved in.

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