UNIVERSITY OF CATANIA

Biological, Geological and Environmental Sciences

PhD Course in Biotechnology

XXV Cicle

Generation of CsGSTUs over-expressing tobacco plants and their role in abiotic and biotic stress tolerance

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Summary

Xenobiotics are toxic chemicals that are normally not the natural substrates for enzymes or transporters involved in plant resistance. Plants have developed a three phases detoxification system from toxic compounds. Xenobiotic are firstly activated so that certain functional groups can be exposed to the successive action of several modifying enzymes. Among them, the glutathione transferases (GSTs) catalyze the nucleophilic addition of glutathione (GSH) to the electrophilic groups of a large variety of hydrophobic toxic molecules. Previously, two gstu genes have been isolated from sweet orange leaves [(*Citrus sinensis*) L. Osbeck)] namely GSTU1 and GSTU2. The encoded proteins differ in three amino acids, all of them included in the C-terminal domain of the enzymes (R89P, E117K, I172V). In order to evaluate the contribution of the mismatched amino acids on the catalytic activity of enzymes, several cross-mutant genes were produced by site-directed mutagenesis followed by the biochemical characterization of the *in vitro* expressed enzymes. In this work, transgenic tobacco plants via *Agrobacterium tumefaciens* mediated transformation over-expressing both the wild type and mutant *Cs*GSTU genes were generated. Along with the molecular characterization of transformed plants, an *in planta* study to assess their ability in detoxifying herbicides was also performed. Therefore, transgenic plants were subjected to the action of fluorodifen, a diphenyl ether herbicide that cause photooxidative stress by inhibition of the plastid protoporphyrinogen oxidase and alachlor a chloroacetanilide

herbicide which is used to control the growth of broad-leafed weeds and grasses in many crops. The electrolytic leakage assay was carried out to test the damage caused by fluorodifen treatment upon transformed and untransformed tobacco plants. The data revealed that the transgenic lines show a sharp reduction of membrane damage compared with the wild type tobacco plants. To study the tolerance towards alachlor *in planta*, we assayed the growth inhibition of untrasformed wild type and transgenic tobacco seedlings in the presence of 7.5 mg/l of alachlor. Alachlor negatively influences the growth of roots and stems of untransformed an transformed tobacco seedlings with the exception of the transgenic plants over-expressing *Cs*GSTU2 which are clearly unaffected by herbicide treatment considering either stem or root lenght. Consequently, the herbicide-tolerant transgenic tobacco plants, which are described in the present study, can be utilized for phytoremediation of residual xenobiotics in the environment. Drought and salinity stress tolerance was also assessed. When exposed to 200 mM NaCl both the *wild type* and transgenic seedlings exhibit a reduction of root lenght, with the exception of the *Cs*GSTU2 over-expressing tobacco line whose root length is as long as untreated control roots indicating a high level of tolerance to NaCl. The effect of drought stress upon root elongation was measured by growing seedlings in the presence of 8% mannitol. In this case all treated tobacco seedlings disclose a sharp decrease of root length, although transgenic lines appear to better tolerate drought stress conditions as the mean root length is significantly higher than that of treated tobacco wild type seedlings. In order to understand the response of tobacco plants over-expressing the

*Cs*GSTU genes to biotic stress, untransformed and transformed tobacco leaves were infiltrated with a bacterial suspension of the *P. syringae* pv. *tabaci* Tox^+ DAPPG-PG 676 strain. The differences observed in symptomatology indicate that the over-expression of *Cs*GSTU1 and *Cs*GSTU2 in tobacco plant bestow the capability to avoid active toxin diffusion in plant tissues blocking chlorotic halos formation probably because tabtoxin is head towards a modification pathway in which *Cs*GSTs could be involved in. This result was confirmed when tobacco leaves was treated with culture filtrates.

Introduction

1.1 Preface

Plants grow in a dynamic environment: abiotic environmental stress conditions, including 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). Such exogenous chemicals cannot be used for nutrition or as a source of energy, but are nevertheless taken up and accumulated. Many xenobiotics are natural products that participate in the chemical interplay that takes place between species. For example, plants synthesize and release a large number of secondary compounds, which vary in structure from simple aliphatic molecules to complex polycyclic aromatic compounds. Many of these compounds defend the plant from which they have been originated, some of them counter animal predation, some others protect against microbial pathogens or suppress competition from rival plant species (Coleman et al., 1997). In turn, plants are subjected to xenobiotic challenges from non plant sources, including products of either animal or microbial origin. Furthermore, the quantity and variety of phytotoxic xenobiotics has grown as a result of the rapid increase in the number of synthetic compounds. Plants encounter these chemicals either as a result of industrial pollution or in the form of agrochemicals such as herbicides and pesticides (Coleman et al., 1997).

1.2 Plant detoxification system

Plants lack of an excretion pathway like that found in animals so they developed a detoxification system from xenobiotics (Fig. 1.1) that consists of two main processes:

- Chemical transformation.
- Compartmentation.

Generally, the chemical transformation of these molecules involves the action of enzymes that convert such foreign substances in chemical compounds usually less toxic and more hydrophilic than the original compound. This transformation decreases the ability of the compound to partition in biological membranes, thus restricting its distribution within cells and tissues. Chemical transformation process of these compounds consists in two phases (Coleman et al., 1997). During phase I, xenobiotic molecules are activated so that certain functional groups can be exposed for phase II enzymes (Lo Piero et al., 2009). The major reactions in this phase I are hydrolysis catalyzed by amidases and esterases, and oxidations conducted mainly by the cytochrome P-450 system that exist as many isoforms. This enables the plants to process a wide variety of different substrates. The phase II is characterized by the formation of a covalent linkage between the molecule activated in phase I and a bulky hydrophilic molecule, such as glucose, malonate or glutathione, to form a water-soluble conjugate. The phase III of the detoxification process involves the compartmentation of this inactive, water-soluble conjugates, from the cytosol to the vacuole by membrane-located ATP-binding cassette (ABC) transporter proteins (Coleman et al., 1997; Cummins et al., 2011). Finally, into

the vacuole further transformation processes promote substitution, degradation, and/or partial salvage of the conjugates (Bartholomew et al., 2002).

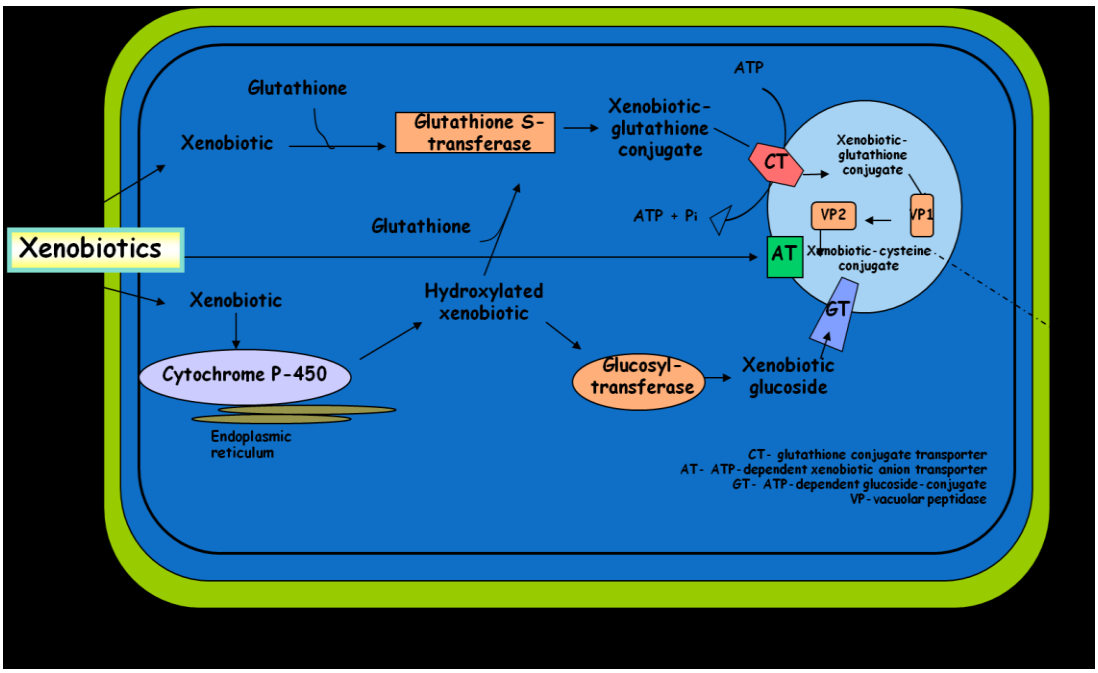


Fig. 1.1 Detoxification of xenobiotics: chemical modification and vacuolar compartmentation. Abbreviations: CT, glutathione-conjugate transporter; AT, ATP-dependent xenobiotic anion (taurocholate) transporter; GT, ATP-dependent glucoside-conjugate transporter; VP, vacuolar peptidase (Coleman et al., 1997).

1.3 Role of thiols in plant tolerance to abiotic stress

In the last decades, climate change and environmental pollution pose major problems to agriculture and increasing efforts are being made to understand plant stress response and tolerance mechanisms to develop new tools that underpin a successful agriculture. Recently, several studies, including the analysis of transcriptional regulation of stress-related genes, have been carried out, and proteomic studies have identified proteins with significance to plant stress response (Zagorchev et al., 2013). However, the

results still do not provide sufficient information on the specific mechanisms that underlie the phenomenon of stress. Many of the mechanisms of response to abiotic stress in plants were similar, if not identical, independently of the stress factor. These non-specific stress responses can help explain the cross-tolerance to various stresses of a particular species or line resistant crops. It has been established that temperature, heavy metals, salt and water deficit stress can all lead to an increased production of reactive oxygen species (ROS), with downstream alterations of oxidative signalling. Evidence is emerging that a series of non-protein and protein thiols, together with a network of sulfur-containing molecules and related compounds, also contribute substantially to the plant stress tolerance (Zagorchev et al., 2013). Low molecular weight compounds have also been found to be a key component of the stress response of plants. Glutathione (GSH) (Fig. 1.2) is the main cellular redox buffer that keeps the intracellular environment reduced.

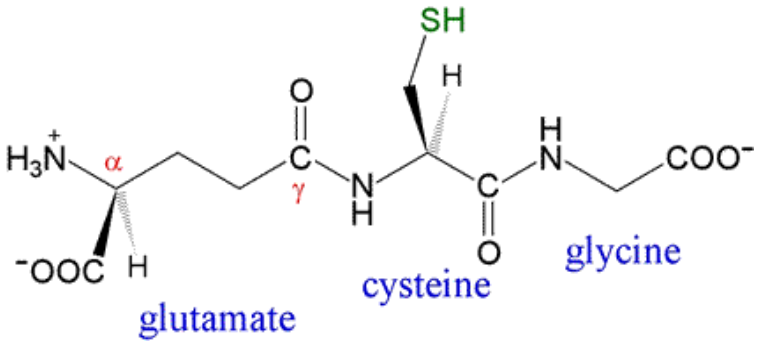


Fig 1.2 Structure of reduced glutathione (GSH)

Typically, the GSH to GSSG ratio is about 20:1 in unstressed conditions, and this ratio shifts towards GSSG upon stress, as a result of ROS scavenging under conditions that compromise the reduction of GSSG to GSH. This shift contributes to the signalling

pathways that lead to programmed cell death, an important mechanism of stress resistance. A reducing intracellular environment is needed to maintain protein structure and function. A high GSH/GSSG ratio is supported by glutathione reductase (EC 1.6.4.2), and stress-tolerant genotypes have higher ratios than stress-sensitive ones (Cummins et al., 2011; Zagorchev et al., 2013). GSH is synthesized in plants from its constituent amino acids by two ATP-dependent steps (Fig. 1.3). In the first step γ-glutamyl-cysteine is formed in a reaction catalysed by γ-glutamyl-cysteine synthetase (γ-ECS, EC 6.3.2.2), then glutathione synthetase (EC 6.3.2.3) catalyses the addition of glycine to γ-glutamyl-cysteine.

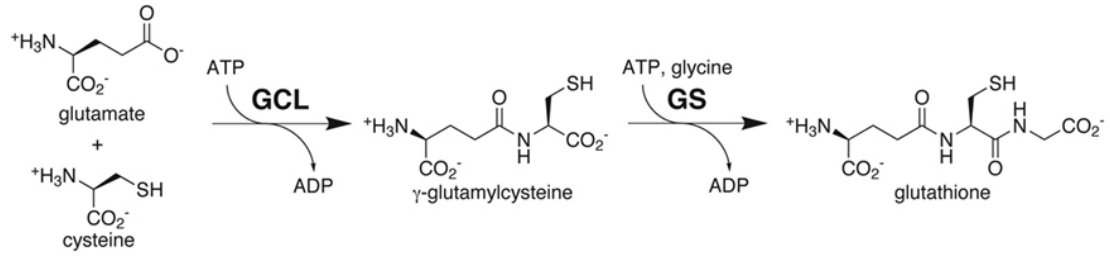


Fig 1.3 Glutathione biosynthesis.

Each of the synthetic enzymes is encoded by a single gene: *GSH1* for γ-ECS and *GSH2* for glutathione synthetase. Many factors affect the synthesis of glutathione. Several study have demonstrated that both genes are induced by heavy metals, light, salt and some stress conditions such as drought and certain pathogens. All plants contain GSH or GSH homologues, where the *C*-terminal amino acid glycine is replaced by another amino acid, such as ß-alanine (homoglutathione), serine (hydroxymethylglutathione) or glutamate (Noctor et al., 2012). These alternative thiols may be present as the dominant thiol, as is the case with homoglutathione in soybeans, with glutathione being present in only trace amounts (Cummins et

al., 2011). Alternatively, both glutathione and its variant are found coexisting, as is the case of hydroxymethylglutathione, in wheat and rice (Cummins et al., 2011).The presence of these alternate thiols allows enzyme involved in the GSH detoxification processes to potentially utilize these glutathione variants in conjugation reactions and contribute toward herbicide selectivity (see below). In soybean, the selective diphenylether herbicide fomesafen is detoxified by homoglutathione conjugation, with recombinant *Gm*GSTU1 and *Gm*GSTU21 both conjugating the xenobiotic more efficiently in the presence of homoglutathione than with glutathione (Cummins et al., 2011).

1.4 The glutatione S-transferase

Glutathione S-transferases (GSTs, EC.2.5.1.18) are a superfamily of multifunctional enzymes active in the phase II of the plant detoxification system. They mainly catalyze the conjugation of glutathione to a large variety of hydrophobic endobiotic and xenobiotic compounds containing electrophilic centers (Öztetik et al., 2008; Lo Piero et al., 2009). Plant GSTs, in addition to their glutathione transferase activities, have less well characterized roles in endogenous metabolism including functioning as GSH-dependent peroxidases counteracting oxidative stress, GSH-dependent isomerases, or have non-catalytic functions among which binding of non-substrate ligands and modulation of signaling processes (Frova et al., 2006). GSTs are ubiquitous proteins found in the cells of microbes, animals, and plants (Liu, et al., 2013). Most GSTs exist as soluble or cytosolic enzymes being the most extensively studied

subfamily. Cytosolic GST (cGST) are classified on the basis of several criteria, including aminoacid/nucleotide sequence similarity, genome organization (i.e. intron number and position) and immunoreactivity properties. To date, seven classes of cGSTs are recognized in mammals: Alpha, Mu, Pi (human specific) and the Sigma, Theta, Zeta and Omega. Plants have seven classes of cGST: Lambda, Phi, Tau, DHAR (dehydroascorbate reductases) (plant specific), Theta, Zeta and Tetrachlorohydroquinone dehalogenase (TCHQD). Five classes have been recognized in insects: Delta (insect specific), Sigma, Theta, Zeta and Omega. Bacteria certainly possess a specific class named Beta, in addition to other enzymes more related to the common Theta class and possibly other classes. Most of the soluble GSTs are active as dimers with subunit molecular weights of approximately 25 kDa, composed of identical (homodimers) or different (heterodimers) subunits, each encoded by independent genes (Fig. 1.4). Heterodimerization is restricted to subunits of the same class, as monomers of different classes are unable to dimerize because of incompatibility of the interfacial residues (Frova et al., 2006). However in addition to the aforementioned types, three other GST protein families have been identified:

- The mitochondrial family (Kappa), is also composed of soluble enzymes with some substrate specificities that are similar to the cytosolic GSTs. The Kappa class GSTs appear to be expressed in mitochondria and peroxisomes in mammals and in *Caenorhabditis elegans* (Öztetik et al., 2008; Board et al., 2012).

- The microsomal associated GST family (MAPEG) (Öztetik et al., 2008), comprises proteins involved in the synthesis of eicosanoids, leukotrienes and prostaglandins catalyzing GSH-dependent transferase or isomerase reactions (Mohsenzadeh, et al., 2011). Other member of the MAPEG family may have different functions. For instance, the first characterized microsomal GST, the human MGST1 has no part in either leukotriene or prostglandin biosynthesis, but catalyzes GSH conjugation to several halogenated arenes and also catalyzes the GSH-dependent reduction of lipid hydroperoxides. For these characteristics MGST1 is thought to be essentially a membrane bound detoxication enzyme involved in the cellular defense against toxic xenobiotics and oxidative stress (Frova et al., 2006).
- The bacterial fosfomycin-resistance proteins family is found exclusively in bacteria. Fosfomycin [1*R*,2*S*]-[1,2-epoxypropyl]-phosphonic acid) is a bactericidal broad-spectrum antibiotic effective against both Gram-negative and Gram-positive bacteria. Fosfomycin inhibits the bacterial cell-wall biosynthesis. Inactivation reaction of fosfomycin is catalysed by an enzyme that was referred to as a GST. The purified protein does not bind to the GSH–agarose matrix and does not catalyse the reaction between GSH and CDNB, indicating that it might have different properties from the canonical GSTs (Allocati et al., 2009).

Nevertheless, it is possible that the number of GST subfamilies is actually larger than so far thought. In fact, genomics and

postgenomics studies are continuously highlighting that the links with a “related families” which borders with the GSTs can be quite thin and disputable (Frova et al., 2006). Such related families include: glutaredoxins (GRX), small oxidoreductases structurally related to thioredoxins. (Rouhier et al., 2010). Among these GST related proteins we also itemize: chloride intracellular channels (CLIC) which are putative ion channel proteins sharing structural homology with members of the omega glutathione S-transferase superfamily, and showing low glutathione peroxidase activity (Singh et al., 2010), selenocysteine glutathione peroxidases (SecGPX) which are enzymes that catalyze the reduction of hydrogen peroxide, lipid hydroperoxides, and other organic hydroperoxides at the expense of oxidizing glutathione, and thus help to protect cells against oxidative damage (Fu et al., 2002), the bacterial disulfide-bond forming oxidoreductase A (DsbA) showing significant sequence identity with mitochondrial GST kappa class (Allocati et al., 2009), and finally the eukaryotic protein elongation factors (eEF1Bg) (Frova et al., 2006).

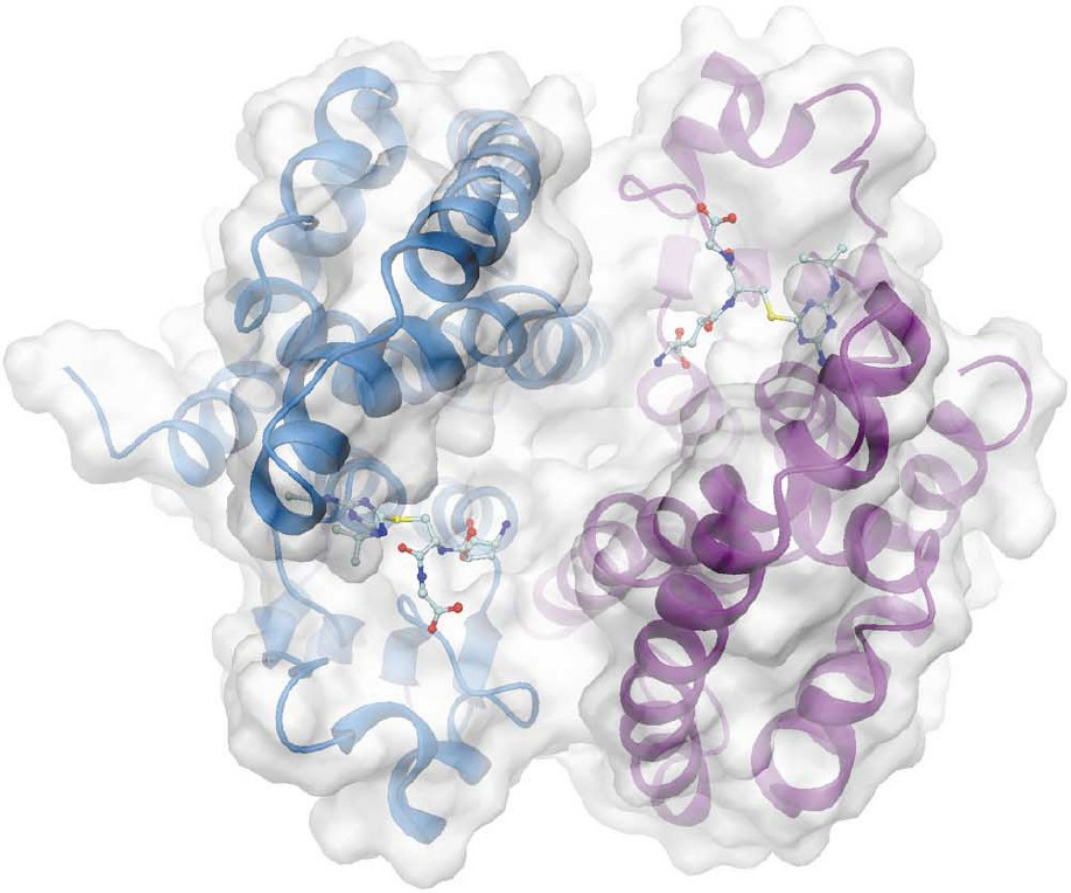


Fig. 1.4 A ribbon/surface representation of the *Zm*GSTF1 homodimer oriented with the amino-terminal domains at bottom left and top right and the subunits in blue and purple. The atrazine-glutathione conjugates are bound at the active site of each subunit the (Dixon et al., 2002a).

1.4.1 Classification, nomenclature and structure of plant GSTs

The first plant GST was discovered in maize in 1970, when, in this crop, a GST activity was shown to be responsible of the chloro-S-triazine atrazine conjugation with GSH, thereby protecting the crop from injury by this herbicide (Öztetik et al., 2008; Dixon et al., 2002b). Originally, most non mammalian GSTs were lumped into the highly heterogeneous theta class, which was viewed as closest to the original progenitor of all eukaryotic GSTs. However, due to the increasing number of studies on plant GSTs, three distinct types were recognized. Type I included GSTs with herbicide-detoxifying activity; the encoding genes have three exons and one intron. The other large group, type III, consisted mainly of auxin-induced GSTs, with the genes containing two exons. Type-II GSTs have ten exons and are much closer to the mammalian Zeta GSTs. A type IV group has been proposed for several *Arabidopsis* genes with five introns, similar to classical mammalian Theta enzymes. This scheme was revised in the following years due to the increase of plant GST sequences reported from diverse Angiosperm sequencing projects. Some plant GSTs clearly group with specific mammalian types but others were plant specific types. Since the principle of greek letter designations was widely used for non plant GSTs, it was suggested that a new nomenclature system should be adopted also for plant GST genes. Therefore, the classification of plant GSTs was be amended to include the following new classes: Phi class including plant specific GSTs previously belonging to Type I; Tau class including plant specific GSTs previously classified as Type III; Theta class enumerating GSTs previously classified as Type IV and

Zeta class comprising GSTs previously indicated as Type II. Further two GST classes were identified in *A. thaliana* which differed from all other plant GSTs by containing a cysteine in place of serine at the active site by Dixon et al (Dixon et al., 2002b): the glutathione-dependent dehydroacorbate reductases (DHARs) and the Lambda GSTs, both are plant-specific as the Phi and Tau classes are (Edwards et al., 2000; Dixon et al., 2002a; Öztetik et al., 2008; Mohsenzadeh et al., 2011). According to the recommendations of the Committee for Human Gene Nomenclature, italic letters are denoting the source organism, Consequently, GST nomenclature (Fig. 1.5) consists of the initials of the source organism (e.g. *Arabidopsis thaliana, At*), followed by GST (*At*GST), and a letter indicating the class (Z for Zeta, F for Phi, U for Tau, T for Theta, L for Lambda, so on). The subsequent numbers at the end are indicating the order of gene discovery in each class in that relevant species followed by the subunit composition at the protein level. For example, while *At* GST U1-1 is a homodimer of *At*GSTU1, *At* GSTU1-2 is a heterodimer of *A. thaliana* U1 and U2 subunits (Öztetik et al., 2008).

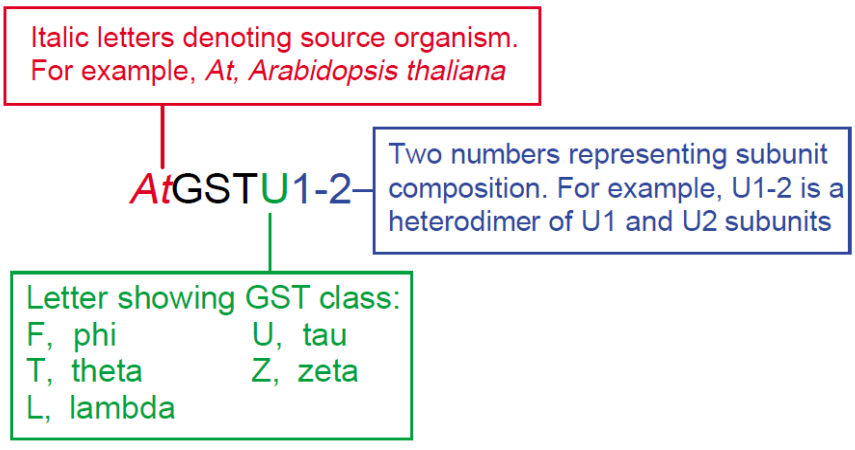


Fig. 1.5 Nomenclature for Arabidopsis and other plant GSTs (Dixon et al., 2002a).

At present, structural information about plant GSTs is available for phi GSTs from *Arabidopsis* (Reinemer et al., 1996), a tau class from wheat (Thom et al., 2002), rice GST1 (*Os*GSTU1) (Dixon et al., 2003) and maize (Neuefeind et al., 1997a, b), for a zeta-class GST from *Arabidopsis* (Thom , et al., 2001) and for the *Gm*GSTU4-4 from *Glycine max* (Axarli et al., 2009). Subunits of all known GST structures exhibit a two-domain fold, the N-terminal domain and C-terminal domain, including the highly conserved GSH-binding site (G-site) and the more divergent cosubstrate-binding domain or hydrophobic binding site (H-site), respectively (Fig. 1.6). The G-site includes both α-helices and β-strands as secondary structure elements. The topological arrangement of these elements is usually βαβαββα, similar to the thioredoxin fold of other GSH-binding or cysteine-binding proteins. The H-site is entirely helical, with a variable number of α-helices, depending on the specific enzymes. Each subunit contains an active site composed of GSH-interacting residues mainly involving amino acid residues of the N-terminus (G-site), and a hydrophobic co-substrate binding site (H-site) located in the C-terminal domain. Recently, structural biology studies have also provided evidence of additional non-catalytic ligand binding site (L-site) (Dixon et al., 2010c; Mohsenzadeh et al., 2011). The N-terminal domain contains specific residues critical for GSH binding and catalytic activity. In particular, the highly conserved Tyr7 of the mammalian Alpha/Mu/Pi classes and Ser17 of the ubiquitous Theta and Zeta, of the plant specific Phi and Tau and of insect Delta classes, have a crucial role in the catalytic activation of GSH. The Tyr/Ser hydroxyl group acts as hydrogen bond donor to the thiol group of GSH, promoting the formation and stabilization of the

highly reactive thiolate anion which is the target for nucleophilic attack of an electrophilic center. The essential role of these residues in GST catalysis has been confirmed by site-directed mutagenesis of the Tyr/Ser with Ala. In all cases the catalytic activity of the enzyme was lost. In contrast, the H-sites of GSTs exhibit a low degree of sequence identity, and hence unique structures that reflect different functions *in vivo*.

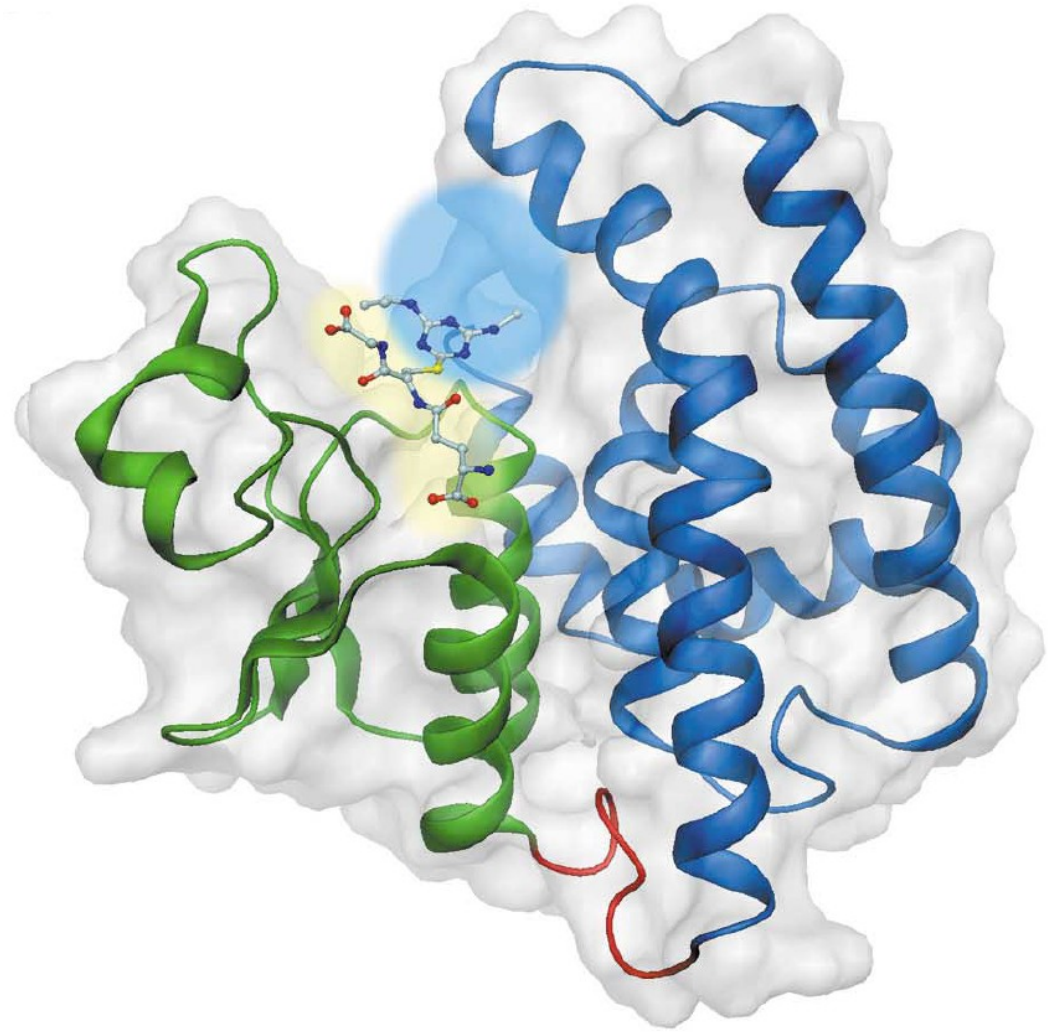


Fig. 1.6 A ribbon/surface representation of a typical GST subunit (*Z. mays* GSTF1, pdb 1BYE), with the amino-terminal domain in green, the linker region in red, the carboxy-terminal domain in blue and the protein surface in gray. A glutathione conjugate of the herbicide is shown binding at the active site; the GSH-binding site (G site) is highlighted in yellow and the hydrophobic site (H site) is highlighted in blue the (Dixon et al., 2002a).

1.4.2 Genome organization

The completion of several genome sequencing projects revealed that the genome distribution of GST genes appear to form tight clusters (Frova et al., 2006). In plants, the non-random distribution of GST genes within the genome was first noted in *Arabidopsis* (chromosome 2), which showed seven Tau GST sequences in tandem in a 14 Kb segment (Frova et al., 2003). The release of the whole genome sequence of *Arabidopsis* confirmed a general clustering tendency of GST genes. The *Arabidopsis* genome contains a total of 55 GST genes which can be divided into 8 classes. Of these genes, at least 52 are transcribed. Most classes are small and possess from 1 to 4 members: 3 Theta (T), 2 Lambda (L), 2 Zeta (Z) and 4 DHAR. However, the Phi and Tau classes have undergone to repeated gene duplication events, resulting in the generation of 13 and 28 members, respectively (Frova et al., 2006; Dixon et al., 2010a). Chromosome 1 is found as the richest with 23 sequences, followed by chromosome 2 with 13, chromosome 3 contains 5 sequences, chromosome 4 with 1 and chromosome 5 with 6 sequences (Fig. 1.7). The Tau and Phi GST classes are present in a series of clusters (enumerating between 2 and 7 members), presumably as a result of multiple duplication events provoking the induction of considerable sequence variability (Öztetik et al., 2008).

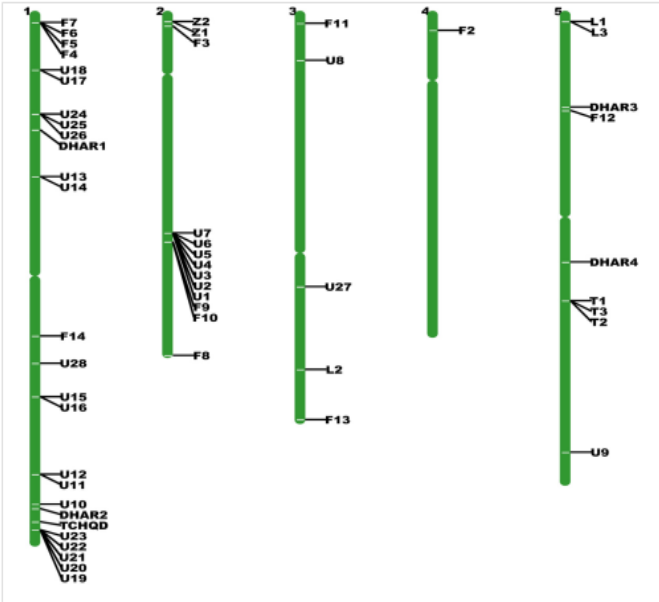


Fig. 1.7 Distribution of GST genes on the *Arabidopsis* genome, plotted using TAIR chromosome map utility (http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp) (Dixon et al., 2010a).

In 2004, Soranzo et al. (Soranzo et al., 2004), by an *in-silico* screening of the rice EST and genome divisions of the Genbank/EMBL/DDBJ database, have isolated 59 putative genes and two pseudogenes: 40 Tau, 16 Phi, 3 Zeta, 2 Theta. All the 61 genes identified *in silico* were mapped and only 15 (<25%) occupy isolated positions in the genome, The remaining 46 are grouped in clusters of various dimensions, the most prominents of which are two large clusters of 7 and 23 closely related genes on chromosomes 1 and 10, respectively (Fig.1.8) (Soranzo et al., 2004). The systematic presence of clusters of GST genes in genomes of both plant and animal species is indicative of a common organizational theme within this gene family and reflects their evolutionary history (Frova et al., 2006).

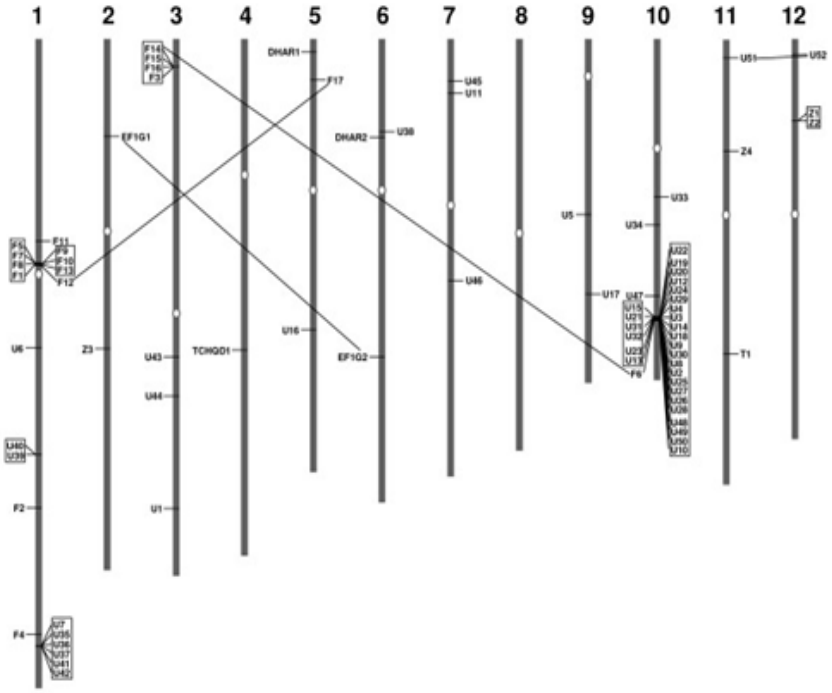


Fig. 1.8 Genome organization of rice gst genes.

1.4.3 Regulation of GST gene expression

Plant GSTs fulfill their functions during normal development, from early embryogenesis to senescence, in every tissue type examined. It has been known for some time that GSTs in major cereal crops are very highly expressed, representing up to 2% of the total protein in the foliage. Soranzo et al. (Soranzo et al., 2004) showed, in rice, that the Tau class is the most expressed in shoot and callus tissue while, in vegetative tissues, Phi genes are mainly expressed. Zeta genes display generally low but ubiquitous expression under all conditions analysed (Soranzo et al., 2004). However, this tissue-specific expression can be changed by exposing plants to chemical treatments, for example maize *Zm*GSTF2 is normally expressed only in the roots, appearing in the foliage only

after exposure to herbicide safeners or chemical treatments (Dixon et al. 1997). Furthermore, there are also some other specific factors that can cause induction of gene expression: plant hormones (such as auxins, ethylene, cytokinin), different chemicals including xenobiotics, and a range of biotic and abiotic stresses thus suggesting that GSTs undergo to major transcriptional regulation (Marrs, 1996). In some instances, the gene expression induced by stress treatments can influence the range of GST homodimerization and heterodimerization. For example, under constitutive conditions, the dominant GST in the foliage of maize is phi *Zm*GSTF1-1 homodimer. In maize, safeners induce the synthesis of the *Zm*GSTF2 subunit, which then associates with the constitutively expressed *Zm*GSTF1 subunit to form the *Zm*GSTF1-2 heterodimer (Dixon et al., 1997). By comparing to the animal GST promoters, plant GST promoters have not been found to contain Xenobiotic Regulatory Elements (XRE), Antioxidant Regulatory Elements (ARE) or Electrophilic Regulatory Elements (ERE). Therefore, the ocs (octopine synthase) elements are the only plant gene promoters found in GSTs from soybean, wheat, tobacco, *A. thaliana* and *Silene*. The ocs elements in plant GST promoters also appear to be stress-induced elements, because the ocs elements do not only respond to strong auxins and salicylic acid, but also with their weak analogues and other agents like heavy metals in *Arabidopsis*, tobacco and soybean (Öztetik et al., 2008).

In some cases the herbicides plant tolerances can be induced by treatment with safener. Herbicide safeners, are chemical agents that increase the tolerance of monocotyledonous cereal plants to

herbicides without affecting the weed control effectiveness. Various safeners were found to enhance the herbicide detoxification in the safened plants by their ability to differentially enhance the expression of members of the glutathione transferase superfamily (Jablonkai et al., 2013). The exact mechanism of the safener-mediated enhancement of GST activity is not completely understood. GSTs are induced by a diverse range of chemicals and accompanied by the production of active oxygen species. Thus the connection between safener-mediated protection of crops and oxidative stress tolerance has been suggested. Safeners may induce GST expression by mimicking oxidative insult (Jablonkai et al., 2013). The regulation of existing GSTs (post-translational regulation) by reversible covalent modification is still poorly investigated. A recent *in silico* study identified the putative correlation between structural and functional characteristics of plant GST classes belonging to different crops (Puglisi et al., 2013a). The fingerprint analysis 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., 2013b).

1.4.4 Functions

1.4.4.1 Detoxification and toxification reactions of GSTs

Much of the early work on plant GSTs focused on their important role in herbicide detoxification, and as such the

conjugation of xenobiotics (Dixon et al., 2010a). Herbicides known to conjugate with GSH include thiocarbamates, chloro-*s*-triazines, triazinone sulfoxides, chloroacetanilides, diphenylethers, some sulfonylureas, aryloxyphenoxypropionates, thiazolidines, and sulfonamides (Jablonkai et al., 2013). The role of GST in the herbicides metabolism has been demonstrated for the first time in 1971 by Shimabukuro (Shimabukuro et al., 1971). The results of this investigation indicated that the primary factor for atrazine selectivity in corn was the activity of a soluble enzyme, glutathione S-transferase (Fig. 1.9). A significant amount of unchanged atrazine accumulated only in the susceptible line, GT112, which appeared to have a low rate of glutathione conjugation because of low glutathione S-transferase activity (Shimabukuro et al., 1971).

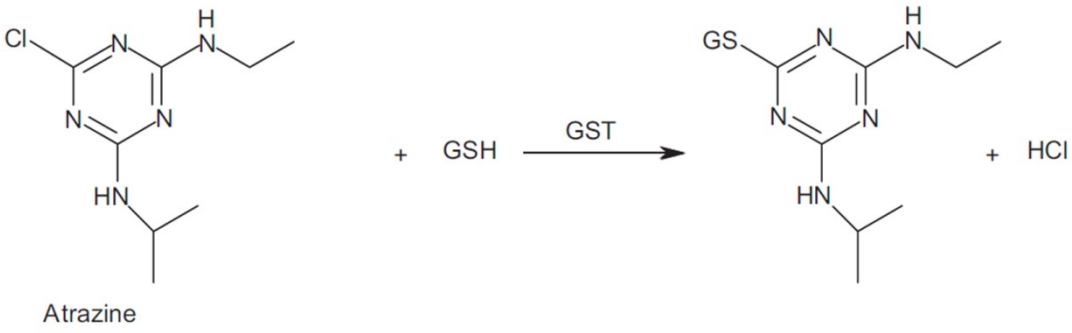


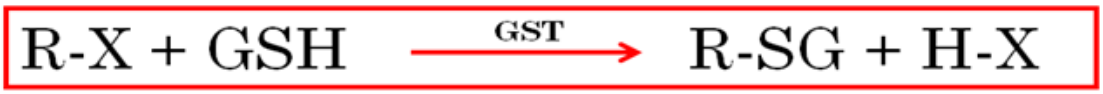
Fig. 1.9 Conjugation reaction of GSH to atrazine catalysed by GST.

Generally, detoxification reactions mediated by GST can be divided into:

- Substitution reactions on halide-bearing electrophilic centers with the displacement of the halide.
- Substitution leading to cleavage reactions.
- Addition reactions.

- Glutathione-dependent isomerizations.

The most commonly observed GSH conjugation reaction has been the nucleophilic displacement of a halogen from an electrophilic site on an aromatic ring, a heterocyclic ring, or an alkyl group in endogenous and exogenous compounds:



X = halogen ions

The glutathione conjugation of atrazine in maize showed above (Fig.1.9) is a classic example of halide substitution reaction. The GST catalyzed reactions with xenobiotics result in the formation of S-glutathionylated products which are less reactive and more hydro-soluble thus facilitating their eventual elimination. Most of the enzyme kinetic studies suggest that the GSH conjugation reactions would be prevailed by a random sequential two-substrate, two-product mechanism. However, under physiological conditions GSH addition occurs first, because the concentration of GSH in normal cells (1–10 mM) is about three orders of magnitude higher than the dissociation constant between GSH and enzyme. The main aspect of the catalytic mechanism is the lowering of the pKa of the glutathione thiol group from 9 in aqueous solution to 6-7 when bound to the protein with the effect of hydrogen-bonding activation. Here, the natures of both the G site, for binding and correct orientation of glutathione, and H site, for accepting numerous different co-substrates are responsible of carrying out a range of reactions (Öztetik et al., 2008). Often, the study of the GST-

mediated conjugation of xenobiotics has concentrated on the use of simple colorimetric assays to measure activity. The best known of these is the GST catalyzed substitution of glutathione for the chloro group of the xenobiotic 1-chloro-2,4-dinitrobenzene (CDNB) (Fig. 1.10), which was developed as a marker for similar detoxification activities observed with mammalian GSTs (Habig et al., 1974).

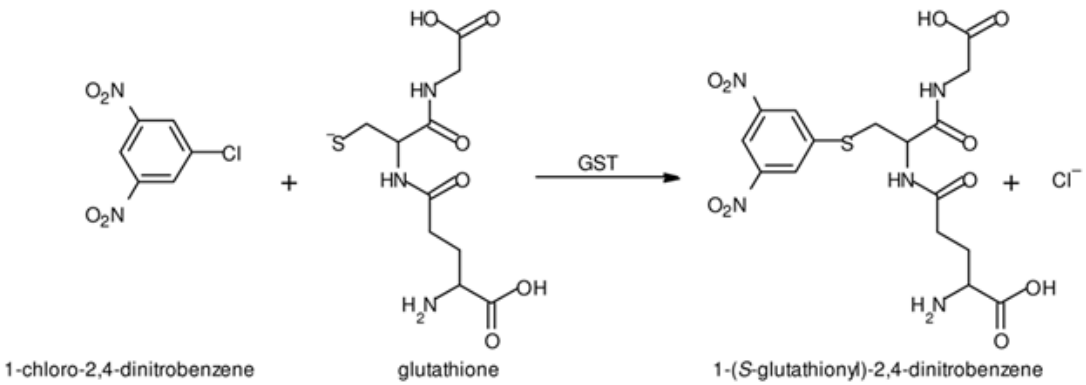


Fig. 1.10 Canonical conjugation of GSH to CDNB.

However, this xenobiotic conjugating activity may have little to do with the endogenous roles of these enzymes in plants (see Par. 1.4.4.2). Furthermore, many GST superfamily members have no, or only very low activity when assayed with CDNB, so this method cannot always be used to assess whether or not a given enzyme is catalytically active. An example of GST-mediated substitution reactions leading to herbicide cleavage occurs in wheat and in competing grass weeds. Here GST catalyze the conjugation of the aryloxyphenoxypropionate herbicide, fenoxaprop ethyl, with glutathione or hydroxymethylglutathione (hmGSH) (Fig.1.11).

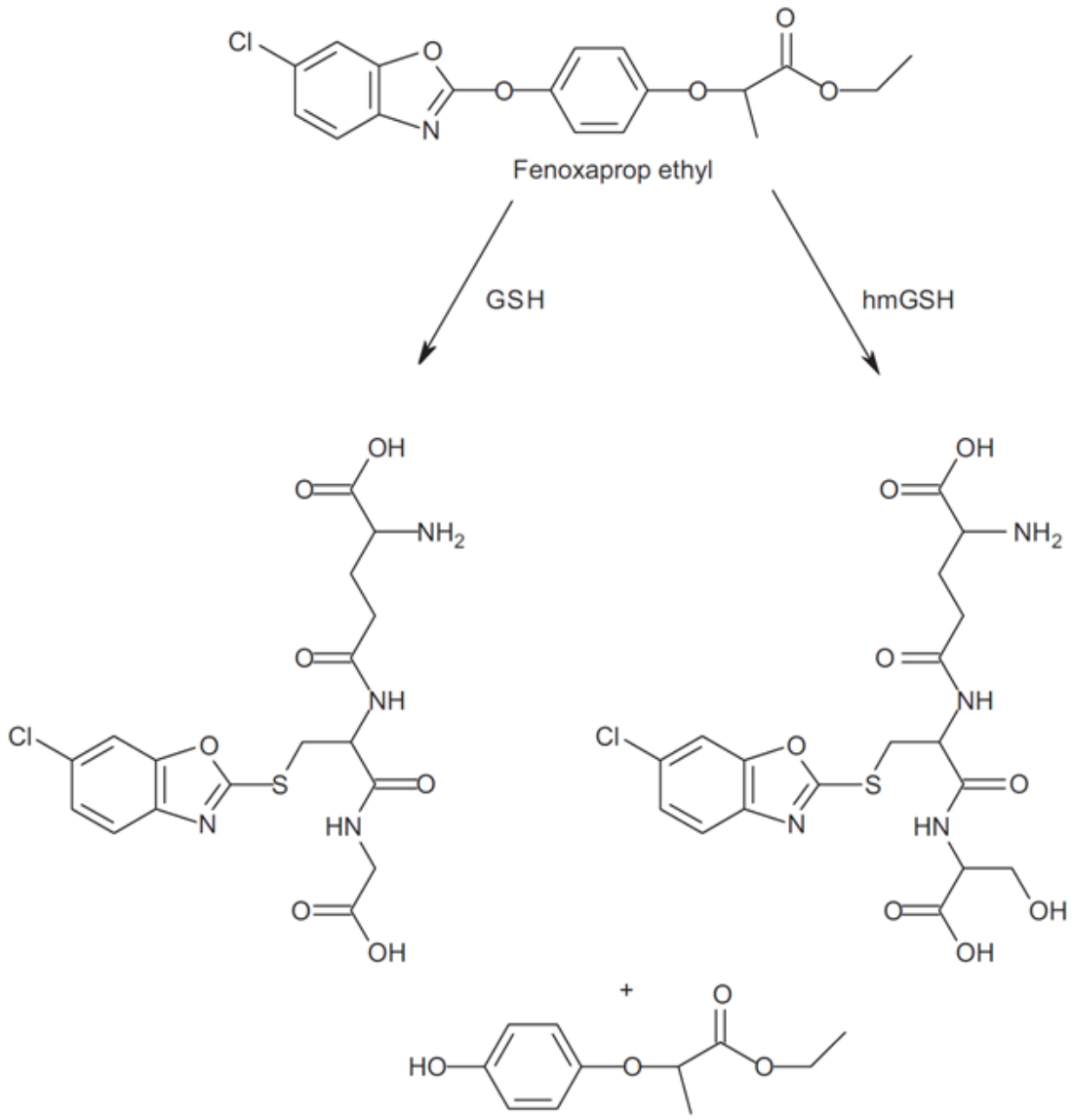


Fig. 1.11 GST-catalyzed conjugation of the aryloxyphenoxypropionate herbicide, fenoxaprop ethyl, with glutathione or hydroxymethylglutathione (hmGSH).

A typical addition reactions catalyzed by GST involves tridiphane, a synergist of herbicides (Fig.1.12) (Cummins et al., 2011). Ezra et al. (1985) showed that when used in conjunction with herbicides, such as atrazine and alachlor, tridiphane increased herbicide toxicity in weeds, such as *Setaria faberi*, whereas there was not increasing damage in *Zea mays* (Ezra et al., 1985). Subsequent work showed

that the addition of glutathione to the epoxide of tridiphane resulted in the formation of a conjugate, which was a more potent inhibitor of GSTs from *Setaria faberi* than was the case when tested against GSTFs from *Zea mays*. The increased herbicide selectivity afforded in the presence of tridiphane could, therefore, be ascribed to the selective inhibition of the weed GSTs (Lamoureux et al., 1986).

Fig. 1.12 Conjugation reaction of tridiphane to GSH catalysed by GST.

In some cases toxic products can be produced as a result of GSH conjugation. An example of toxification reaction has been described in strawberry Schuphan. A fungicide dichlofluanid is metabolized to a toxic metabolite thiophosgene derivative in a process that appears to involve two GSH conjugation steps (Schuphan et al., 1981).

1.4.4.2 Conjugation of endogenous products by GSTs in plants

Despite their well-known role in stress physiology, as well as in acute detoxification of applied chemicals, a deeper insight into matching specific GST isozymes with either their preferred substrates or their function *in vivo* in normal cellular process has

not yet been achieved. Presently, glutathione conjugates to endogenous secondary plant products, especially derivatives of the phenylpropanoid pathway such as cinnamic acids or anthocyanins, are reported (Marrs, 1996). The biosynthesis of anthocyanins occurs in the cytosol, but neither these pigments nor their intermediates have been detected there, and only the end product, the anthocyanins, but not their intermediate, are detected in the vacuoles, where stable anthocyanin pigmentation occurs (Xu et al., 2001). In maize, it has been shown that the last genetically-defined step of anthocyanin biosynthesis is encoded by bronze2 (Marrs et al. 1995). The gene product BZ2 showed high homology with glutathione S-transferases and was able to transfer glutathione to the artificial substrates 1-chloro 2,4 dinitrobenzene (CDNB) (Marrs et al. 1995). In the presence of a functional bronze2 (BZ2), anthocyanins accumulate within the vacuoles, whereas when the bronze2 gene is missing, anthocyanins were found retained in the cytosol thus conferring to the kernels a pale brown phenotype due to pigment oxidation (Marrs et al. 1995). These oxidized secondary metabolites in the cytoplasm of bz2 mutants are somewhat toxic, diminishing the overall vigor of the plant (Coe Jr et al., 1988). Therefore, it was supposed that glutathionation of anthocyanins is a prerequisite for vacuolar import because GS-conjugates are reported to be very efficient substrates of an ATP-driven ABC transporter (Goodman et al., 2004). Subsequent studies (Alfenito et al., 1998) showed that the anthocyanin9 gene (AN9) involved in the anthocyanin biosynthesis in *Petunia* also shares high homology with glutathione transferases and that BZ2 and AN9 can functionally complement each other in maize and petunia,

respectively, even though these genes are highly divergent as they belong to different subfamilies of glutathione transferases (Alfenito et al., 1998). More recently, novel *Arabidopsis* mutants, transparent testa 19 (tt19), showed a great reduction of anthocyanins in the vegetative parts as well as brown pigments in the seed coat (Kitamura et al., 2004). The TT19 gene was isolated and it was shown to be a member of the *Arabidopsis* glutathione S-transferase gene family. Heterologous expression of the putative ortholog AN9 in tt19 mutants complemented the lack of anthocyanin accumulation suggesting that the TT19 gene is required for vacuolar uptake of anthocyanins (Kitamura et al., 2004). A GST gene belonging to the GST Phi (*Cs*GSTF1) was also isolated in the blood orange flesh. The *in vitro* expression of orange GST gene leads to a GST enzyme which is active against cyanidin3-O-glucoside, the main anthocyanin of blood oranges, thus suggesting the involvement of the GSTF1 in the tagging of anthocyanins with GSH for vacuolar import (Lo Piero et al., 2006).

1.4.4.3 Ligandin Functions

In addition to their well investigated catalytic roles, GSTs also have non-catalytic roles and either may act as ligand-binding proteins or they can bind hydrophobic molecules, such as bilirubin, heme, bile salts, steroids, dyes, carcinogens, some drugs in a non-substrate manner, in both cases into a distinct site (termed L-site). Axarli et al. (Axarli et al., 2009) determined the structure of *Gm*GSTU4-4 complexed with S-(p-nitrobenzyl)-glutathione. The X-

ray structure identified an undescribed ligand-binding site (L-site) located in a surface pocket of the enzyme (Fig. 1.13).

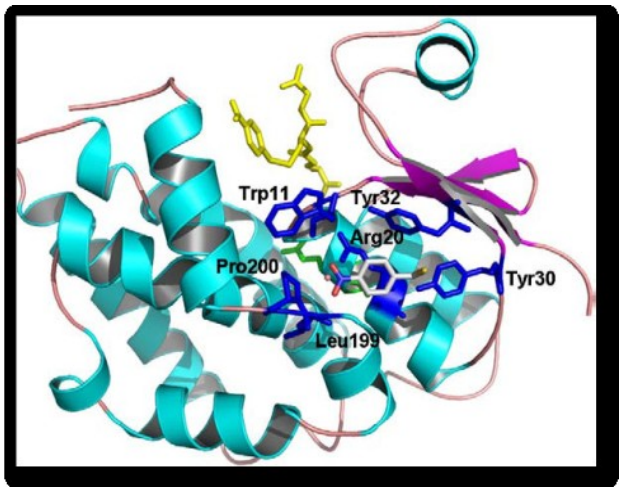
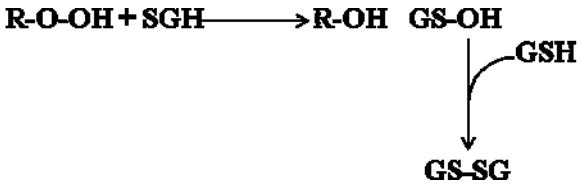


Fig. 1.13 A representation of the putative L-site of *Gm*GSTU4-4 with the ligand (4-nitrophenyl)methanethiol(Axarli et al., 2009).

This site is formed by conserved residues (W11, R20, Y30 Y32, K197, L199, P200) within the Tau GST family, indicating a biological importance for this newly discovered binding site. Although many compounds have been identified as ligands, there is little information about the exact location and nature of the L-site in GSTs of different plant sources. In the case of the *Arabidopsis* enzyme, the L-site is located next to the G-site between the side chains of helices α3"/α3"' and α5", whereas the L-site of the human Pi class GST is located in the H-site. When GSTs perform their ligandin function, their catalytic activity is usually inhibited in a non-competitive manner. The structural basis whereby catalytic function is modulated by the occupation of these sites is unclear (Axarli et al., 2009).

1.4.4.4 Peroxidase activity of plant GSTs

GSTs catalyze the nucleophilic attack of GSH on electrophilic oxygen reducing the cytotoxic hydroperoxides to less toxic monohydroxy alcohols through another non-conjugating activity. In this case the GSTs are named glutathione peroxidases (GPOXs) and protect cells from the effects of reactive oxygen species (ROS), which are produced during oxidative stress.



GST-GPOX activities have been identified in purified isoenzymes from *A. thaliana*, wheat, pea, maize and soybean (Öztetik et al., 2008). It has been suggested that GSTs might work as regulators of apoptosis. In fact, it was found that the expression of a tomato Tau GST in yeast suppresses the Bax controlled apoptosis induced by oxidative stress (Kampranis et al., 2000). Moreover, transgenic tobacco overexpressing Tau and Phi GST show a concomitant increase in GST-GPOX activities as well as an increased tolerance to chilling and salt stress, whereas enhanced herbicide resistance was observed in black grass (Öztetik et al., 2008).

1.4.4.5 Isomerase Activity of GSTs

GSTs have an additional isomerization function in plants, animals and fungi. This reaction catalyzed by GSTs involves the GSH, but not include GSH conjugate as an end product (Öztetik et al., 2008).

In plant, the recombinant *Arabidopsis* zeta GST (*At*GSTZ1) show activity as a maleylacetone isomerase (MAI). This glutathione-dependent reaction is analogous to the *cis-trans* isomerization of maleylacetoacetate to fumarylacetoacetate, which occurs in the course of tyrosine catabolism to acetoacetate and fumarate. Thus, rather than functioning as a conventional GST, *At*GSTZ1-1 appears to be involved in tyrosine degradation (Dixon et al., 2000). In contrast to the detoxification reactions described above leading to the formation of conjugates, glutathione dependent isomerization reactions performed by GSTs might result in the activation of precursor compounds to the active herbicide. The isourazole herbicide, fluthiacet methyl, act as a light-dependent peroxidizing herbicide, causing electrolyte leakage, accumulation of porphyrins, and inhibiting chlorophyll biosynthesis. Shimizu et al., showed that fluthiacet methyl was converted to the more active urazole by glutathione S-transferase (Fig. 1.14) (Shimizu et al., 1995).

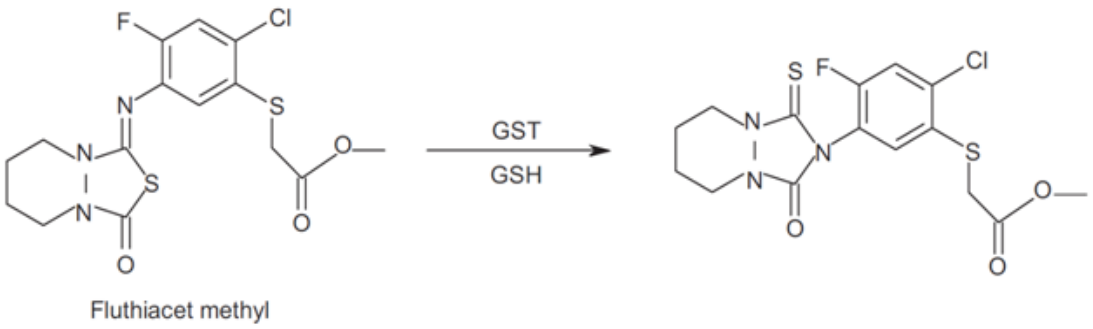


Fig. 1.14 GST-catalyzed isomerization of the proherbicide, fluthiacet methyl, to the more active urazole compound. An initial glutathione conjugate formed by the GST then spontaneously isomerizes with concomitant elimination of the thiol.

1.5 Transgenic plants

Environmental stresses, population explosion and food shortage have caused serious problems to mankind on the globe. The world population is increasing alarmingly and is projected to reach 8.5 billion by 2025. To fulfill the food demand of every individual from limited natural resources is difficult. Producing crops with improved quality and quantity is imperative for growing food demand through sustainable agriculture that could be attained using conventional selection and breeding or through genetic engineering. The biotechnology has led to opportunities and novel possibilities to enhance the qualitative and quantitative traits of organisms. Since 1990s, the major emphasis of agricultural biotechnology can be found on traits for improvement in crops related to insect and herbicide resistance, nutritional quality, virus resistance, shelf life, and biofuel production. Transgenic plants have been developed through different genetic engineering techniques but with a number of legal, political and social problems. Despite all these barriers, different countries including China, Canada, USA, Brazil and Argentina are now allowing transgenic crop production. Horsch et al. (1985) developed the first transgenic tobacco plant expressing foreign phytohormone biosynthetic gene (Horsch et al., 1985). Since then, transgenic plants of about 100 plant species have been produced which show enhanced resistance to insects and diseases, abiotic stresses etc. and the production of genetically modified plants is increasing day by day around the world. Around 25 countries are contributing to the production of biotech crops and the major portion is produced by the USA. The major biotech crops

cultivated are tomato, wheat, alfalfa, rice, soybean, maize, canola, squash, tobacco, cotton, sugarbeet, petunia, sweet pepper and carnation (Ahmad et al., 2012). Overexpression of heterologous genes is, therefore, widely used for the introduction of novel traits into transgenic crop plants or, also, in combination with down-regulation and controlled expression (e.g. induced, developmentally regulated, or tissue specific) studies as a tool for basic plant research and for functional analysis of native genes in various model plants. Two crucial components are required for the transient and/or stable expression of foreign genes in plant cells. The first is a plasmid vector capable of carrying the foreign gene-encoding sequence and the regulatory elements. The second is the biological or physical methods by which the vector can be delivered into the target plant cells. With the exception of virus mediated gene expression, biolistics, polyethyleneglycol, and *Agrobacterium* mediated gene transfer all require plasmid vectors. The *Agrobacterium* transformation is the dominant method to create transgenic plants (Fig. 1.15) (Dafny-Yelin et al., 2007). *Agrobacterium* vectors, commonly referred to as binary vectors, are composed of the borders of T-DNA, multiple cloning sites, replication functions for *Escherichia coli* and *A. tumefaciens*, selectable marker genes, reporter genes, and other accessory elements that can improve the efficiency of and/or give further capability to the system (Wang, 2006).

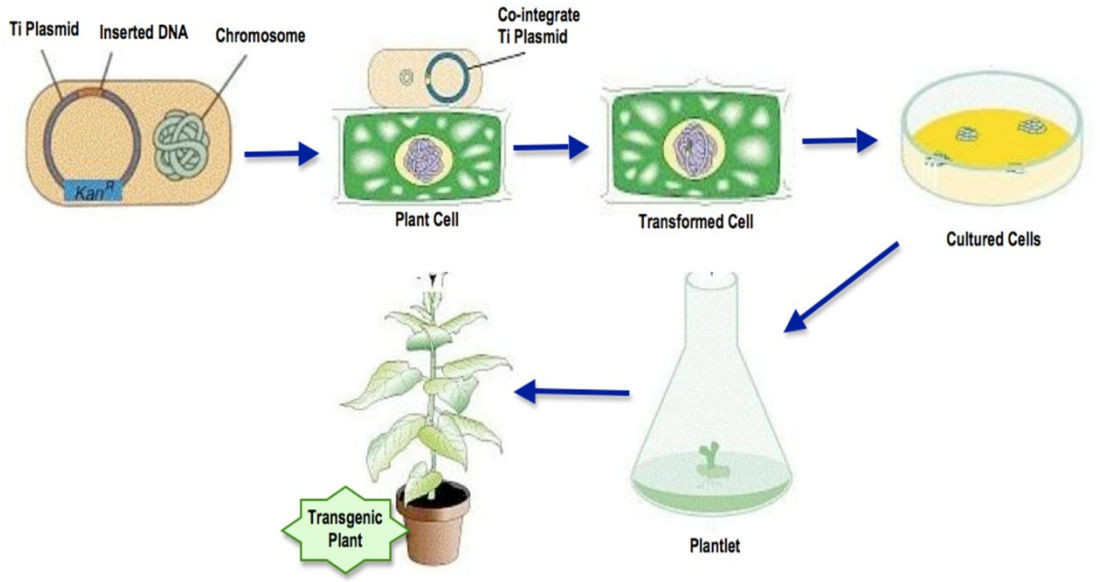


Fig. 1.15 *Agrobacterium* Transformation Overview.

1.5.1 Role of GST in tolerance of general abiotic stress

Abiotic stresses such as salt, drought, flooding, extreme temperature, oxidative stresses and herbicides often diminish plant growth and final yield. Agricultural productivity could be increased dramatically if crops were redesigned to better cope with environmental stresses (Ahmad et al., 2012). As described above, several studies paid attention to the expression regulation and function of plant GST genes. Such studies showed that the expression of GSTs can be induced by a range of abiotic and biotic stresses thus demonstrating that plant GSTs play important roles not only during normal development but also in detoxification of cytotoxic endogenous and xenobiotic compounds as well as in response to a wide variety of stress conditions. Therefore, series of GST genes were homologously or heterologously expressed, and transgenic plants showed enhanced resistance to several stress. For

instance, Li et al. (2013) generated several line of tobacco plants overexpressing a zeta GST from *Pyrus pyrifolia*. The transgenic plants showed enhanced tolerance to oxidative damage caused by drought, NaCl, and Cd stresses, compared to tobacco wild type (Liu et al., 2013). Similar results were obtained by Kumar et al. (Kumaret al., 2013). They studied the role of one member of lambda class, the *Os*GSTL2, from rice by overexpressing this gene in *Arabidopsis thaliana* as heterologous system. The insertion of *Os*GSTL2 gene provided the transgenic lines tolerance towards heavy metals and other abiotic stresses like cold, osmotic and salt stress (Kumar et al., 2013). Both the aforementioned studies and many others carried out using Tau and Phi GSTs strongly support that *gsts* can be utilized to produce novel germplasm with improved stress tolerance. (Ji et al., 2010; Dixit et al., 2011; Jha et al., 2011; Im Choi et al., 2013)

1.5.2 Role of GSTs in herbicides resistance

Herbicides are organic molecules that have chemical and physical properties that allow them to easily cross cell membranes by passive diffusion. Many of those commonly used are synthetic chemical compounds, chemically unrelated to naturally compounds present in living things. Herbicides can be classified according to the chemistry of the active ingredients, according to the target plant species or according to the mechanism of action. The herbicide's mode of action is the biochemical and physiological mechanism by which herbicides regulate plant growth at tissue and cellular level. Herbicides with the same mode of action generally exhibit the same

translocation pattern and produce similar injury symptoms. At the physiological level, they control plants by inhibiting photosynthesis, mimicking plant growth regulators, blocking amino acid synthesis, inhibiting cell elongation and cell division, etc. Several enzymes and functionally important proteins are targets sites for herbicides. Classical photosystem-II (PSII) inhibitors bind to the D1 protein, a quinone-binding protein, to prevent photosynthetic electron transfer. Inhibition of biosynthesis of aromatic amino acids relies on the enzyme 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase. Acetohydroxyacid sythase (AHAS), a target of several classes of herbicides catalyzes the first common step in the biosynthesis of valine, leucine, and isoleucine. Several different types of herbicides apparently cause accumulation of photodynamic porphyrins by inhibiting protoporphyrinogen oxidase (PPO). Formation of homogentisate via inhibition of 4-hydroxyphenylpyruvate dioxygenase (HPPD), a key enzyme in tyrosine catabolism and carotenoid synthesis, is induced by herbicides having different structure. Lipid biosynthesis is the site of action of a broad array of herbicides used in controlling monocot weeds by inhibiting acetyl-CoA carboxylase (ACC) or very-long-chain fatty acids synthesis (VLCFA). Several compounds are potent inhibitors of glutamine synthase (GS) which catalyzes the incorporation of ammonia into glutamate (Jablonkai, 2011). In agriculture, chemical control of weeds is based on herbicide selectivity and it can be defined as the differential response level among species after the application of a particular molecule. Therefore, selectivity refers to the capacity of a particular herbicide to eliminate weeds in a crop, without affecting yield or quality of the final product (Carvalho et al., 2009). The

generation of herbicide tolerant crops offers several benefits to agricultural weed control, allowing the selective chemical control of weeds in botanically related crops (Jablonkai et al., 2013). Generally, two approaches have been used to create herbicide tolerant crops: the first is to modify the degree of sensitivity of the target enzyme so that the plant sensitivity to the herbicide is inhibited, and the second is to engineer the herbicide-detoxifying pathway into the plant. An example of the first approach include glyphosate tolerance. The first adopted herbicides on field were very destructive for most plants and they created undesirable environmental impacts. New chemicals such as glyphosate have been widely recommended for use because glyphosate is environmental-friendly as soil microorganisms are able to degrade it rapidly. By introducing glyphosate tolerance genes into crops, the herbicide can now be applied over the top of crops during the growing season to control weed population more effectively (Ahmad et al., 2012). Recently, Gaines et al. (2010) developed herbicide resistant *Amaranthus palmeri* lines by overexpression of a glyphosate-insensitive form of the herbicide target gene, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that is involved in the shikimate cycle wherein it catalyzes the reversible addition of the enolpyruvyl moiety of phosphoenolpyruvate to shikimate 3-phosphate (Gaines et al., 2010). The use of transgenic plants overexpressing GST subunits active in herbicide detoxification is based on the second approach, and confirmed GST's role in crop's herbicide selectivity. Recently, Im Choi et al. (2013) generated several transgenic poplar lines harboring a chimeric p35S-PatgGSTU51 developed to understand its function in plant

defense. The transgenic lines showed increased tolerance to methyl viologen (Paraquat) as well as to inorganic mercury (Im Choi et al., 2013). Benekos et al. (Benekos et al., 2010) demonstrated the role of *Gm*GSTU4 overexpressed in tobacco plants in the detoxification against fluorodifen and alachlor (Benekos et al., 2010). Also maize GSTs are known to detoxify triazine and chloroacetanilide herbicides, and transgenic tobacco plants expressing maize GST I have been shown to remediate alachlor (Karavangeli et al., 2005). Moreover, downregulation of the expression of a rice GST (*Os*GST III) resulted in reduced tolerance of transformed rice cell lines to pretilachlor, suggesting that the *Os*GST III gene plays an important role in the detoxification of this herbicide (Deng et al., 2003). Furthermore, several plant GSTs can also function as glutathione-dependent peroxidases (GPOX) catalysing the reduction of oxidative stress products, such as organic hydroxyperoxides. This results to an indirect tolerance to herbicides by effectively scavenging toxic byproducts of herbicide activity and to the protection of the cell from lipid peroxidation as observed in transgenic tobacco plant transformed with *Gm*GSTU4-4 (Benekos et al., 2010).

1.6 Alachlor

Alachlor [2-chloro-N-2,6-diethylphenyl-N-(methoxyme-thyl) acetamide] belongs to the chloroacetanilide herbicide family. It is used to control the growth of broad-leafed weeds and grasses in corn and many other crops. It is a pre-emergence herbicide and is applied to young plants, is 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 of young plants (Karavangeli et al., 2005). The physiology of chloracetamides is poorly understood. The mode of action may include inhibition of fatty acid and lipid biosynthesis as well as protein and gibberellin synthesis. Several studies have shown that the endogenous gibberellins (GA) increase both the cell division and expansion. Alachlor appears to inhibit the biosynthesis of GAs, in particular it seems that it is acting in the first stage of the biosynthesis (Fig. 1.16), or at the level of cyclization of the first two rings of geranylgeranyl pyrophosphate, inhibiting the enzyme (CPP cyclase) (Fig. 1.16).

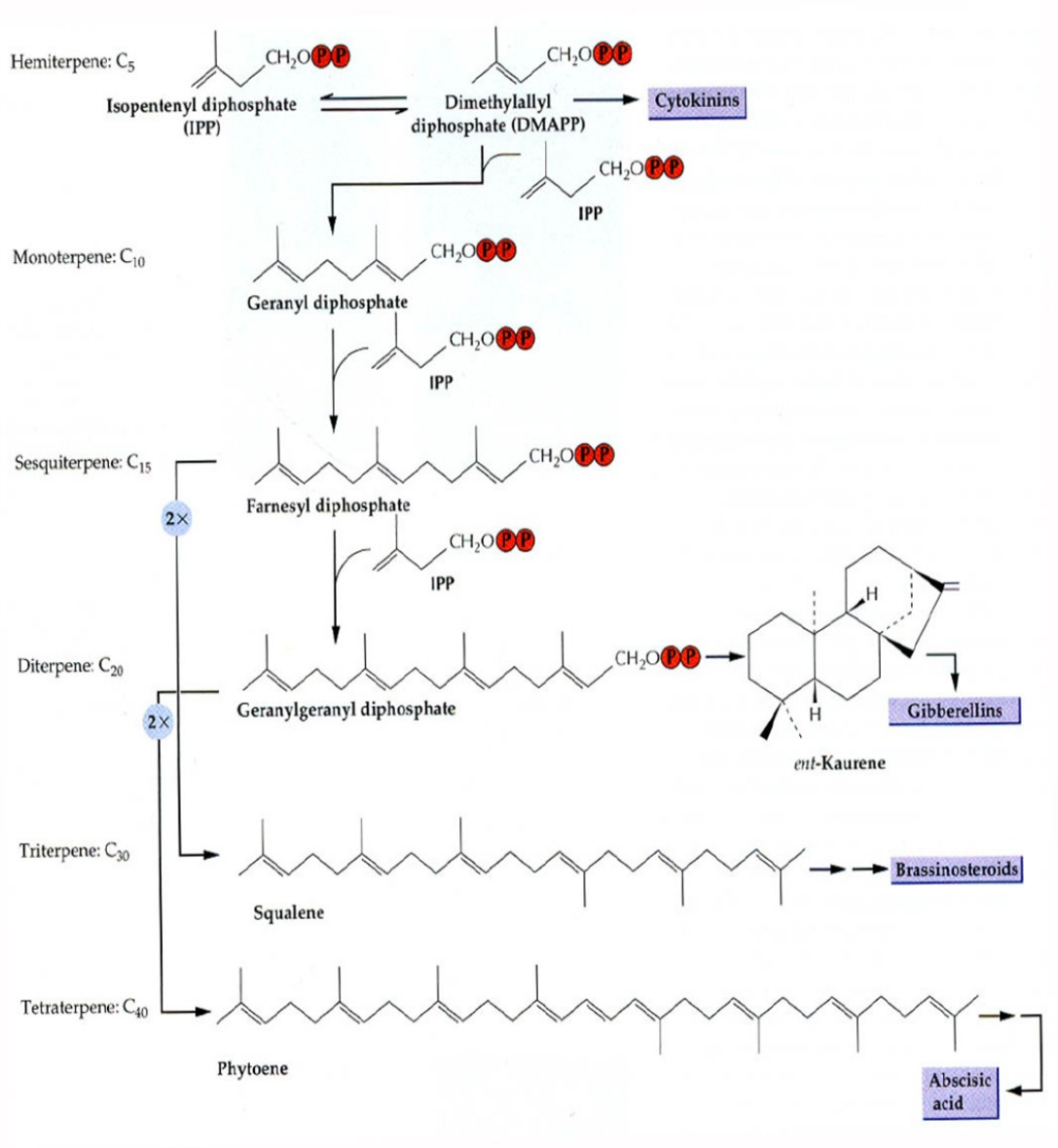


Fig. 1.16 Biosynthesis of gibberellins.

Besides the effects described above, several studies demonstrated that the alachlor is also responsible for alterations in the permeability of the membrane whereas other experiments demonstrated that it is also involved in inhibition of the absorption of different ions including Cl^- and K^+ and amino acids **(**Duk, 1985). Widely used for weed control in maize crops, alachlor detoxification

by glutathione-mediated conjugation is an example of halide substitution reaction (Fig. 1.17) (Mozer et al., 1983; O'Connell et al., 1988; Jepson et al., 1997).

Fig. 1.17 Conjugation reaction between GSH and alachlor

Although alachlor is able to conjugate readily GSH at pH values close to 7 in the absence of enzymes, some studies have shown a correlation between the herbicide tolerance and glutathione transferase activity rather than GSH content (Cummins et al., 2011).

1.7 Fluorodifen

Fluorodifen [2-nitro-1-(4-nitrophenoxy)-4-(trifluoromethyl) benzene] belongs to the diphenyl ether herbicide family. Among diphenyl ether herbicides, fluorodifen was originally developed because its selectivity is associated with its greater rate of detoxification in tolerant crops as compared with susceptible weeds, principally through GST-mediated metabolism (Dixon et al., 2003). Diphenyl ether herbicides inhibit the enzyme protoporphyrinogen oxidase (EC 1.3.3.4), a membrane-bound plastidic protein. This

enzyme converts, in a six-electron aromatic oxidation, protoporphyrinogen IX to protoporphyrin IX, a precursor molecule for both chlorophyll and heme (Fig. 1.18). When the enzyme is inhibited, the protoporphyrinogen substrate accumulates and is slowly oxidized by the high concentrations of O_2 being produced in the chloroplast, anyway producing protoporphyrin IX. Normally, the concentrations of protoporphyrin in the cell are kept very low, but in the presence of diphenyl herbicides its concentrations increase and it begin to accumulate throughout the cell. When plants are exposed to light the protoporphyrin interacts with molecular O_2 to produce singlet oxygen and other ROS, which initiate lipid peroxidation, membrane disruption and plant death (Hao et al., 2013) .

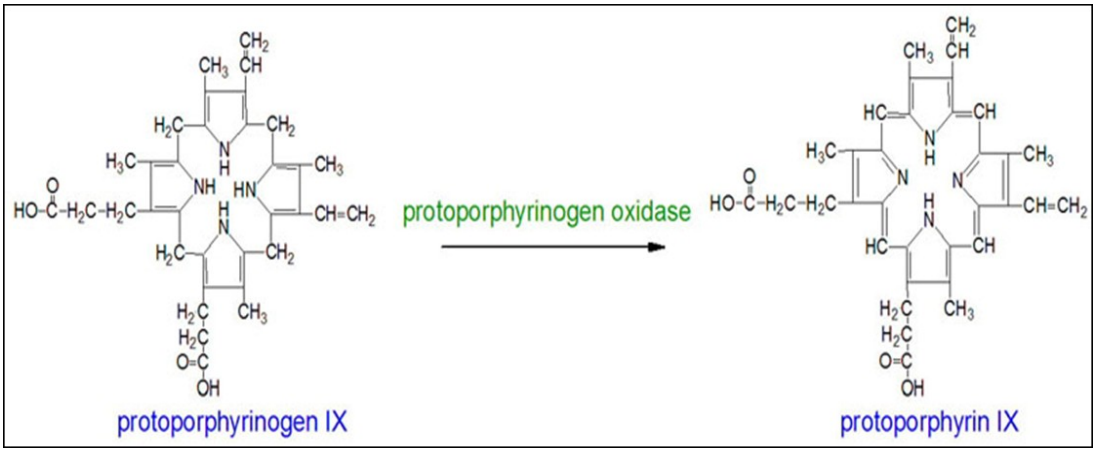


Fig.1.18 Biosynthesis of protophorphyrin IX, the common precursor for synthesis of chlorophyll and heme.

Fluorodifen is a compound formerly used for weed control in legumes. The GST-mediated substitution reaction, of this compound, leads to herbicide cleavage to form 4-nitrophenol and the

glutathione conjugate of 2-nitro-4-trifluoromethylphenol (Fig. 1.19) (Cummins et al., 2011).

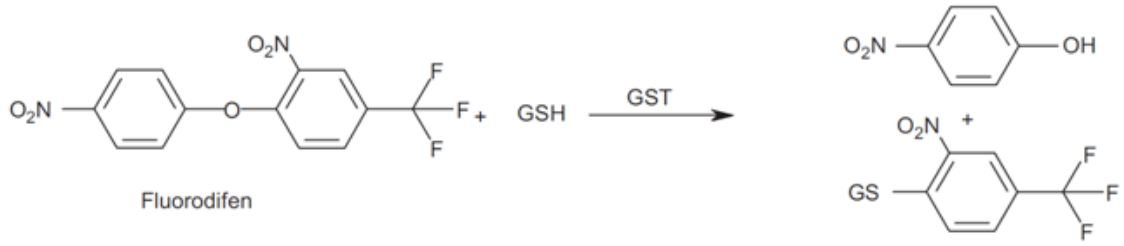


Fig. 1.19 GST-mediated substitution reactions between fluorodifen and GSH.

This reaction has been shown to be catalyzed by GSTs from a range of species (Pflugmacher et al., 2000) and is particularly associated with GSTUs (Axarli et al., 2009; Benekos et al., 2010; Jo et al., 2011).

1.8 Role of GSTs in biotic stress response

Plants exploit many different mechanism to contrast pathogenic organisms. Some of these defense systems include:

- The formation of chemical and physical barriers, that prevent pathogens from coming in host plant.
- The programmed cell death of a limited cell area (hypersensitive response) which acts to limit the infection diffusion.
- The activation of a series of metabolic pathways, that coordinately regulate the expression of many genes coding for transcription factors, enzymes involved in the "phytoalexin synthesis" as well as in other secondary metabolites and in

the synthesis of a great number of antimicrobial molecules (Schenk et al., 2000).

At least three signal molecules are associated with plant defence from pathogenic attack: salicylic acid, jasmonic acid and ethylene (Dong et al., 1998). The different responses may interact to contrast more effectively the pathogen through a combined action (Kunkel et al 2002). Genes coding for protein related to pathogenesis (PR-1) or with antimicrobial function (PDF1.2) and genes involved in the synthesis of phytoalexins (lypoxygenases) or secondary metabolites, such as glutathione tranferases are members of the so-called "pathogen-induced genes" (Campbell et al., 2003). Many authors (Dudler, et al., 1991; Greenberg et al., 1994; Levine et al., 1994) emphasize that expression of specific GSTs (Mauch at al., 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 pathogenic damages and the extent of programmed cells death during the hypersensitive response (Dudler et al., 1991; Alvarez et al., 1998). According to this hypothesis it has been demonstrated that the pathogen induced protein GSTF2 of *Arabidopsis thaliana* catalyses the glutathione dependent detoxification of the cytotoxic 13-hydroperoxy-cis-9-trans-11-octadienoic acid that is reduced to the corresponding alcohol (Wagner et al., 2002). The hydroperoxydes of fatty acids that accumulates following the inoculations of pathogen agents can be decisive for the outbreak of symptoms in the compatible as well as incompatible interactions (hypersensitive response) (Rustérucci et al., 1999). It was also demonstrated that a tomato's GST inhibits

the cell death and enhances the tolerance to oxidative stress (Kampranis et al., 2000). An interesting result is that the pathogen induction of GST expression is very fast and anticipates the induction of other genes involved in the defence systems of plants and coding for the pathogenesis-related proteins (Alvarez et al., 1998). More recently, Chronopoulou et al. (Chronopoulou et al., 2013) examined the catalytic diversification of a GST isoenzyme from *Phaseolus vulgaris* (*Pv*GSTU3-3) which is induced under biotic stress treatment (*Uromyces appendiculatus* infection). This enzyme shows high antioxidant catalytic function and acts as hydroperoxidase, thioltransferase, and dehydroascorbate reductase. In addition, its K_m for GSH is about five to ten times lower compared to other plant GSTs, suggesting that *Pv*GSTU3-3 is able to perform efficient catalysis even under unfavorable oxidative conditions. Moreover, its ability to conjugate GSH with isothiocyanates provides an additional role for this enzyme as regulator of the released isothiocyanates from glucosinolates as a response of biotic stress (Chronopoulou et al., 2013). Interestingly, as far as we know, studies on the response to biotic stress of transgenic plants overexpressing GST isoforms from whichever sources are not reported yet.

1.9 Pseudomonas syringae pv. tabaci (Pstab)

Pseudomonas syringae pv. *tabaci* is the causal agent of wildfire disease and angular spot disease in tobacco plants. As foliar bacterial phytopathogen, *Pseudomonas syringae* species survive on the plant leaf surface as epiphytes (Hirano et al., 1990). During the

initial infection process, the bacterial pathogens produce virulence factors including effector proteins and secondary metabolites, to inactivate early plant defense responses such as stomata-based immunity (Melotto et al., 2006; 2008) and hypersensitive response (HR) cell death at the site of infection (Heath et al., 2000). The failure of early pathogen recognition delays initiation of the downstream defence cascade and results in the development of disease symptoms in plants. Therefore, the suppression of early plant defence responses is one of the important steps for bacterial pathogens to successfully colonize plant tissues, leading to disease. Several pathovars of *Pseudomonas syringae* produce a phytotoxic extracellular metabolite called coronatine (COR) that has been shown to suppress plant defence responses. Interestingly, *Pstab* does not produce COR but still actively suppresses early plant defense responses. It has been known that *Pstab* produces tabtoxin that causes characteristic yellow halos around the necrotic lesions on tobacco leaves during wildfire disease development (strains Tox^{+}) (Blender et al., 1999). Angular spot is characterized by necrotic lesion delimited by veins and by the absence of chlorotic halos (strain Tox^{-}). These non toxigenic mutants of *Pstab* strains apparently are spontaneous mutants generated by the excision of tabtoxin biosynthesis gene cluster. Tabtoxin is a dipeptide which is not biologically active and needs to be hydrolyzed by peptidase for an active toxic component, tabtoxinine-ß-Lactam (TßL). TßL is released by zinc-dependent aminopeptidases in the periplasm of the bacterium or by enzymes in the plant cell, and inhibits the enzyme glutamine synthetase that results in accumulation of ammonia and plant cell death (Bender et al., 1999). Although necrosis occurring in

tissues infiltrated with *P. tabaci* is not preceded by a reduction in glutamine synthase activity or by accumulation of ammonia, the chlorosis, which is characteristic of *P. syringae* pv. *tabaci* infections, is associated with loss of glutamine synthetase activity and accumulation of ammonia, confirming the causal role of tabtoxin in this symptom (Turner et al. 1981). Self-protection of the producing bacterium has been associated with the adenylation of its glutamine synthetase, which renders the modified enzyme less susceptible to inactivation by TβL. A second potential self-resistance mechanism involves the production of β-lactamases which hydrolyse the β-lactam of TβL to liberate the nontoxic metabolite, tabtoxinine. Recently, a tabtoxin-resistance gene (ttr) has been characterized and used to enable the development of transgenic tobacco cultivars resistant to *P. syringae*. TTR is an acetyltransferase and contains the four motifs conserved in the GNAT superfamily (Hamed et al., 2013).

1. 10 Tau Glutathione transferases from Citrus sinensis

In 2009, Lo Piero et al. (Lo Piero et al., 2009) isolated from *Citrus sinensis* the genes coding for two Tau GSTs sharing a 92% homology at nucleotides levels and containing a 651 bp open reading frame which encode proteins of 216 amino acid residues each. The encoded proteins differ only for three amino acids: the triplet R89, E117 and I172 found in the isoform named GSTU1 (REI) is replaced by the triplet P89, K117 and V172 in the GSTU2 (PKV) isoform. The mismatches between the orange GST isoforms fall within the protein sequence involved in the dimer interface

(position 89) and in the hydrophobic substrate binding site (position 117), whereas the third mismatch is located in a less important region of the proteins (position 172). Sequence analysis and comparison with sequences in the data bank revealed high identity of the deduced amino acid sequences with higher plant GSTs previously isolated (Fig. 1.20) (Lo Piero et al., 2009). The conserved N-terminal domain including the GSH binding site (G-site) and a more divergent C-terminal domain as well as the topological arrangement ßαßαßßα of the G-site secondary structure elements have been detected on the orange GST isozymes (Fig. 1.21). A serine residue (Ser13), which is supposed to stabilize the thiolate anion of GSH and enhance its nucleophilicity, is also present in the putative active site of the enzymes. By comparing the genomic sequences with that of their respective cDNAs, two exons of 315 and 336 bp were identified, separated by one intron of 765 bp. Twenty mismatches were found between the sequences of GSTU1 and GSTU2, 14 of them located in the intron region (Lo Piero et al., 2009).

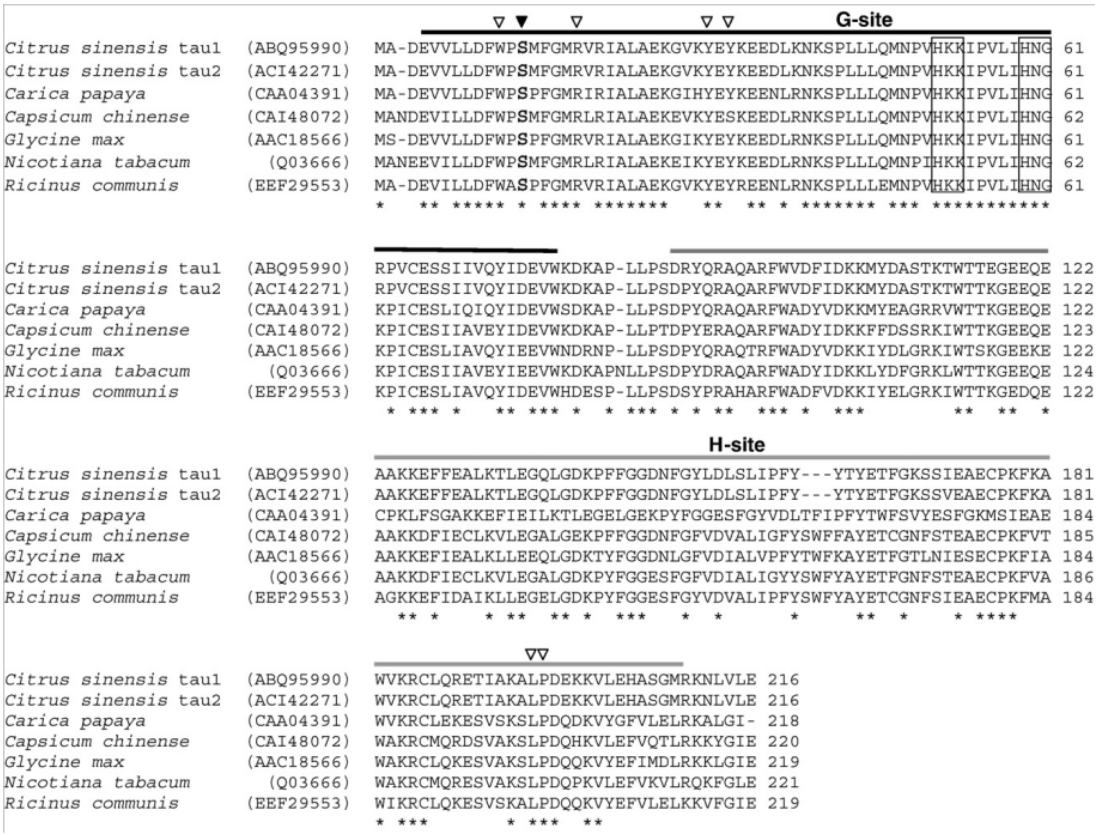


Fig. 1.20 Alignment of the deduced amino acid sequences of orange leaves GSTU1 and GSTU2. Asterisks indicate identical amino acids in all sequences. The boxed regions represent conserved amino acid residues distinctive of the tau-type GSTs. The catalytic serine is marked with a black arrow head, whereas open arrow heads indicate residues putatively involved in the L-site constitution (Axarli et al., 2009). The regions predicted to be related to substrate specificity are also highlighted (G-site and H-site) (Edwards et al., 2000) (Lo Piero et al., 2009).

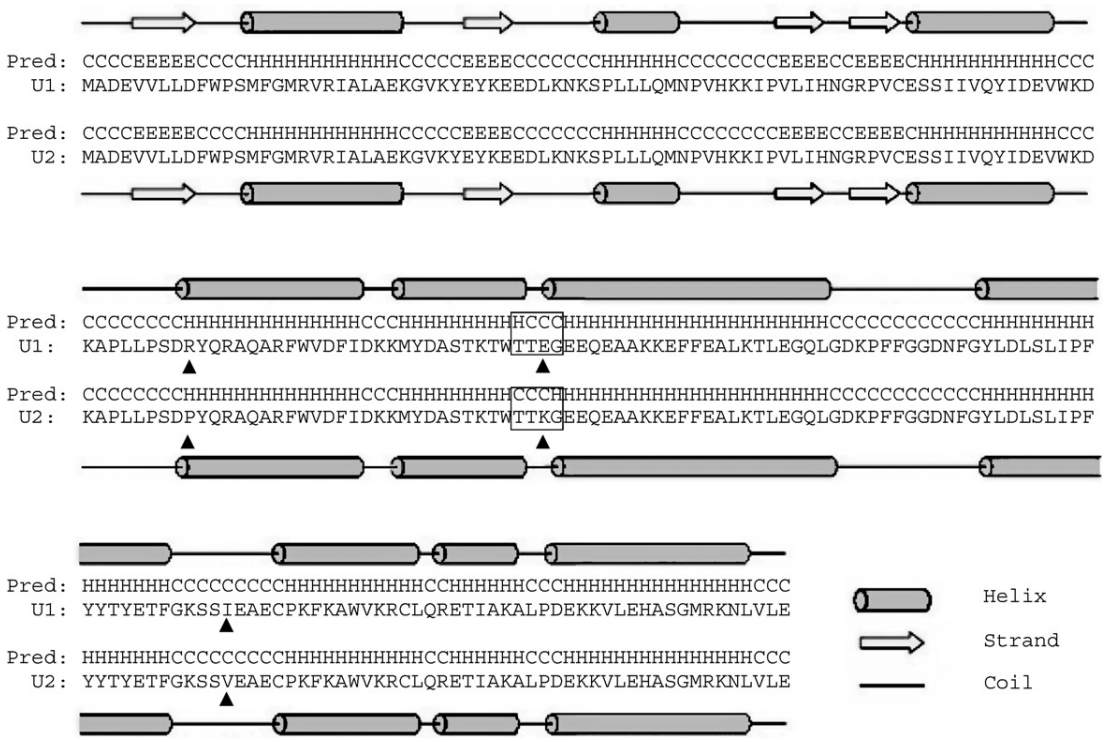


Fig. 1.21 Secondary structure prediction of the orange GST isoforms and topological arrangement of the structural elements (α-helices and β-strand). The different amino acid residues between GSTU1 and U2 are marked with black arrow heads; the boxes highlight their distinctive secondary structure regions (Lo Piero et al., 2009)

In order to determine their functional identity, the orange GST isoforms were *in vitro* expressed and purified, then the specific activities of both isoforms were determined against the artificial substrate CDNB. The results showed that two isoforms exhibit different specific activity, in fact GSTU1 showed values three fold lower than that observed for the GSTU2 enzyme. These observations suggested that one or more than one amino acidic mismatches (R89P, E117K and I172V) should be responsible for the different ability of the enzymes to conjugate GSH to CDNB (Lo Piero et al., 2009). Furthermore, evidence suggests that GSTs are implicated in plant response to environmental stresses, especially the GSTU1, the expression of which was strongly induced by

cadmium sulphate, CDNB, cyhalotrin and cold stress mainly in red orange leaves. On the contrary, the GSTU2 gene expression did not increase in response to any of the imposed stress conditions thus suggesting that the U2 isoform is constitutively expressed in sweet orange cells (Fig. 1.22) (Lo Piero et al., 2009).

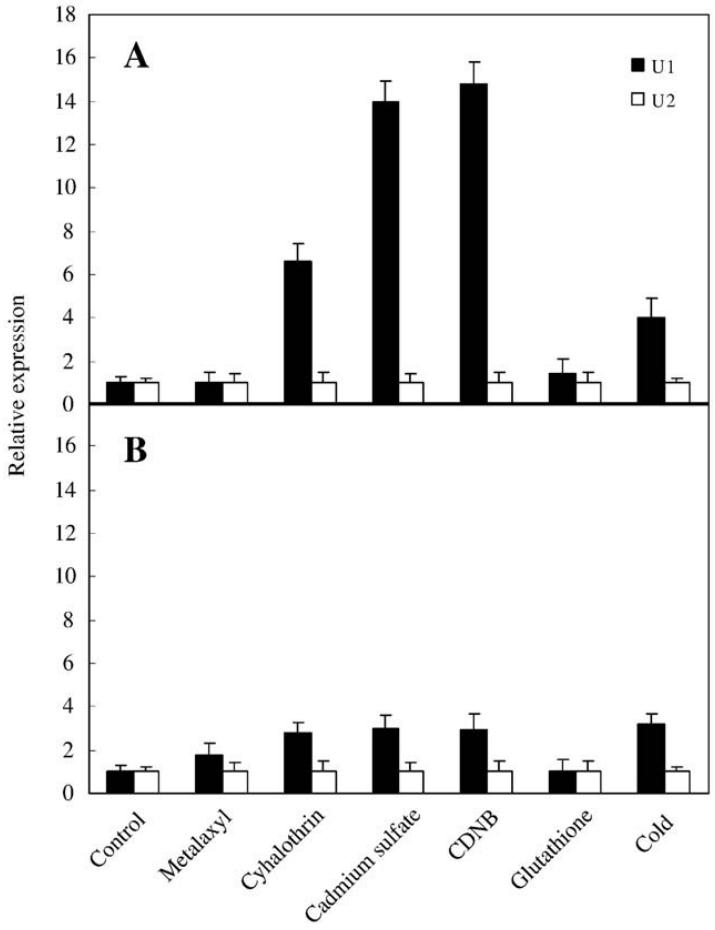


Fig. 1.22 Effect of different stressful condition upon the expression of gsts in red (A) and blond (B) orange leaf discs by RT-real time PCR. Sweet orange leaf discs (3 g) were incubated with effective concentrations of stress inducing molecules (100 µg/ml metalaxyl, 15 µg/ml λ-cyhalotrin, 0.3 mM cadmium sulphate, 1 mM CDNB, 1 mM GSH) at 16 °C for 40 h (Lo Piero et al., 2009).

The steady-state kinetic characterization of both isoforms with respect to CDNB and GSH was also performed. The results showed that GSTU1 had a lower apparent K_m for CDNB than that exhibited by GSTU2, suggesting a higher affinity of GSTU1 for this substrate.

As expected on the basis of the complete identity of the G-site sequences, the same K_m for GSH was registered for both isoforms. However, in the case of both substrates, GSTU2 showed higher catalytic efficiency (k_{cat} /K_m) (Tab. 1.1). In order to evaluate the contribution of the mismatched amino acids on the catalytic activity of enzymes (R89P, E117K and I172V, respectively), several cross-mutant genes were produced by site-directed mutagenesis followed by the biochemical characterization of the *in vitro* expressed enzymes (Lo Piero et al., 2010). Hereafter wild type and mutant GSTs are referred by their distinctive amino acid triad: GSTU1 is referred to as REI, GSTU2 is referred to as PKV. The analysis of kinetic parameters regarding the natural *in vivo* substrate GSH led to the conclusion that, the mutants containing K117 showed apparent K_m values similar to those reported for the wild-type GSTs (Tab. 1.1). However, the RK and PK enzymes (PKV, RKV and RKI), both wild type and mutants, showed a strong increase in the k_{cat} /K_m ratio with respect to the E117-containing enzymes (REI and PEI) (Tab. 1.1), indicating that the substitution E117K is crucial to the formation of enzymes with higher catalytic efficiency.

	CDNB				GSH			
GST	K_m (mM)	V_{max} ($\mu M \cdot min^{-1}$)	k_{cat} ($s^{-1} \times 10^3$)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	K_m (mM)	V_{max} ($\mu M \cdot min^{-1}$)	k_{cat} ($s^{-1} \times 10^3$)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
GSTU1 (REI)	0.75 ± 0.03	2.0 ± 0.10	23.8 ± 1	31.7 ± 1.45	0.5 ± 0.02	1.0 ± 0.1	13.8 ± 0.24	27.7 ± 1.15
GSTU2 (PKV)	1.0 ± 0.07	4.0 ± 0.06	108.1 ± 1	108.1 ± 5.4	0.5 ± 0.03	4.0 ± 0.15	76.9 ± 0.4	153.8 ± 6.94
RKI	5.0 ± 0.2	9.0 ± 0.5	257.1 ± 2	51.4 ± 2.0	0.6 ± 0.03	5.0 ± 0.20	147.0 ± 0.9	245.0 ± 4.26
RKV	4.0 ± 0.4	8.0 ± 0.25	119.4 ± 1	29.8 ± 0.6	0.35 ± 0.02	3.3 ± 0.10	129.4 ± 0.5	369.7 ± 21.12
PKI	4.0 ± 0.4	4.5 ± 0.15	160.7 ± 1.6	40.0 ± 1.5	0.7 ± 0.04	4.0 ± 0.15	64.0 ± 0.63	91.4 ± 2.16
PEI	0.85 ± 0.05	3.0 ± 0.07	28.8 ± 0.07	33.9 ± 2.4	4.0 ± 0.02	7.0 ± 0.3	70.7 ± 0.7	17.6 ± 0.24

Tab. 1.1 Steady-state kinetic constants of wild-type and mutant GSTs. (Lo Piero et al., 2010).

The substrate specificity of the sweet orange GSTs was also investigated in order to identify catalytic activities that may be related to their biological function. To this end some substrates, in addition to CDNB, were examined including the compound NBD-Cl, the alkyl halide 4-nitrophenethyl bromide (4-NPB) and ethacrynic acid (ECA), a phenylacetic derivative that contains an electrophilic group, similar to the cytotoxic α-ß-alkenals which may be formed during oxidative stress. Overall, CDNB is the preferred substrate of both naturally occurring GSTU1(REI), GSTU2 (PKV) and mutant GSTs, with RKV and PKV enzymes showing the highest specific activity (Tab. 1.2). Relatively lower activity was detected with the mammalian GST substrate NBD-Cl. Interestingly, the mutant enzyme RKV is able to conjugate 4-NPB to GSH whereas wild types as well as other mutant GSTs are not (Tig. 1.2), thus suggesting the crucial role of V172 but not of I172 in creating the active site architecture suitable to accommodate the aforesaid substrate. This finding is of special interest since alkyl halides have toxicological interest in view of their occurrence as environmental pollutants. Therefore, RKV due to the distinguishing ability to conjugate 4-NPB to GSH and to the extremely high catalytic efficiency towards GSH exhibits great potential for the development of enzymes with novel properties. None of the enzymes are active with ECA suggesting that wild type and mutant GSTs might not be directly involved in the removal of harmful oxidative stress by-products (Lo Piero et al., 2010).

	Activity [nmol (min·mg^{-1})]			
GST	CDNB	4-NPB	NBD-Cl	ECA
GSTU1 (REI)	45.7 ± 1.2	ND	26.1 ± 0.8	ND
PEI	56.3 ± 1.4	ND	23.1 ± 0.2	ND
RKI	66.8 ± 1.8	ND	53.6 ± 0.9	ND
RKV	81.1 ± 1.7	148 ± 1.5	17.7 ± 0.2	ND
PKI	75 ± 1.5	ND	38.3 ± 0.6	ND
GDSTU2 (PKV)	79.2 ± 1.6	ND	27.2 ± 0.3	ND

Tab. 1.2 Specific activity of the wild-type and mutant GSTs towards different substrates (Lo Piero et al., 2010).

1.11 Aim of work

The studies previously carried out upon wild type and mutate *Cs*GSTs laid the basis for the present work of PhD thesis. From those data emerged that the GSTU1 and GSTU2 genes from *Citrus sinensis* showed different expression levels in response to different types of abiotic stress (Lo Piero et al., 2009). The successful *in vitro* expression of the proteins and their subsequent biochemical characterization highlighted that *Cs*GSTU2 is catalitically more efficient than the *Cs*GSTU1. Among the mutants, GST-RKV showed the greatest catalytic efficiency towards glutathione and, more importantly, it is the sole enzymatic form able to conjugate glutathione to the alkyl halide 4-nitrophenethyl bromide (4-NPB), known as dangerous environmental pollutant. In this work, transgenic tobacco plants via *Agrobacterium tumefaciens* mediated tranformation overexpressing both the wild type and mutant *Cs*GSTU genes were generated. Along with the molecular characterization of transformed plants, *in planta* study to assess their ability in detoxifying herbicides (alachlor and fluorodifen) and in toleranting by salt and drought stress were also performed. Simultaneously, the *Cs*GSTUs genes, were *in vitro* expressed, using an *E. coli* cell-free system. The catalytic activity the GST isoforms were determined against herbicides in order to correlate the response to the herbicides observed *in planta* with enzymes properties exhibited *in vitro*. Moreover, in order to investigate the putative role of GSTs in the response to biotic stress, tabacco plants over-expressing *Cs*GSTUs, either wild type or mutate forms, were subjected to infection with the host pathogen *Pseudomonas syringae*

tabaci, known as the causal agent of wildfire disease in tobacco plants.

Material and methods

2.1 Plant material

Tobacco, *Nicotiana tabacum,* var. Wisconsin seeds were surface sterilized in 100% ethanol solution for 30 s, then in 3 % NaOCl aqueous solution for 10 min, rinsed in sterile distilled water and placed for germination on MS medium (Murashige et al., 1962). Tobacco plants were grown in a growth chamber under 16 h light, (100 $\mu Em^{-2}s^{-1}$)/8 h dark photoperiod at 23 °C. Transgenic T_1 plants that grew normally under the selective pressure of kanamycin were used in all the experiments of molecular characterization of transgenic plants, in enzymatic assays and herbicide tolerance experiments.

2.2 Plasmid constructs

Standard recombinant DNA methods were adopted in the construction of the plant transformation vector pART27-*Cs*GSTUs which were used for the transformation of tobacco plants. The cloning of sweet orange GSTU1 (REI), GSTU2 (PKV) and mutant (PKI, RKV) genes and as well as their transfer into the expression vector pEXP1–DEST (Invitrogen) was performed as described by Lo Piero et al. (2009). The sweet orange wild type and mutant *Csgstu* genes were amplified by PCR, using the Phusion High-Fidelity DNA polymerase (Finnzymes) and specific primers which added the restriction sites for EcoRI and XbaI on the borders of the coding sequences. The primers used were:

(forward)

5’GGCTCGAGGAATTCATGGCGGACGAAGTGGTTCT3’ (EcoRI)

(reverse)

5’GGTCTAGAGGATCCCTACTCCAATACTAAATTTT3’ (XbaI).

Thermocycling conditions were as follows: 98 °C for 30 s, followed by 30 cycles with denaturation at 98 °C for 10 s, annealing at 45 °C for 15 s and extension at 72 °C for 15 s, with a final 1 min cycle at 72 °C. The PCR products were purified, cloned into the TOPO TA pCR 2.1 (Invitrogen) vector (Fig. 2.1), and sequenced.

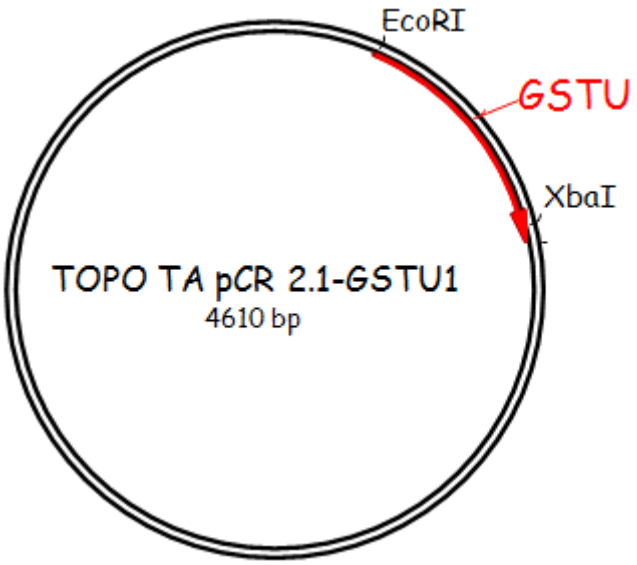


Fig. 2.1 TOPO TA pCR 2. Vector 1 (Invitrogen)

Then, *E. coli* Mach1™-T1R Chemically Competent cells (Invitrogen) were transformed and spread in LB plates containing kanamycin (50 mg/ml), X-Gal (40 mg/ml) and grown overnight at 37°C. Two white colonies per clone were randomly picked and directly inoculated in 5 ml of LB broth supplemented with kanamycin and grown overnight at 37°C with constant shaking. Plasmid

purification was undertaken using the NucleoSpin Plasmid Kit (Macherey Nagel). The sweet orange genes were excised from the plasmid TOPO TA pCR 2.1 using the restriction enzymes EcoRI and XbaI (New England Biolabs). The digestion reaction was incubated for 1 h at 37°C and analyzed on 1 % agarose gel in TBE 1x as running buffer, then stained with GEL RED (Biotium). The NucleoSpin Extract II kit (Macherey Nagel), was used to extract fragments of interest from the agarose gel. GSTUs were ligated, using T4 ligase, into the pART7 EcoRI/XbaI restriction sites thus giving rise to the primary cloning vector pART7-GSTUs, in which the *Csgstus* open reading frames were under the control of CaMV 35S promoter (Fig.2.2).

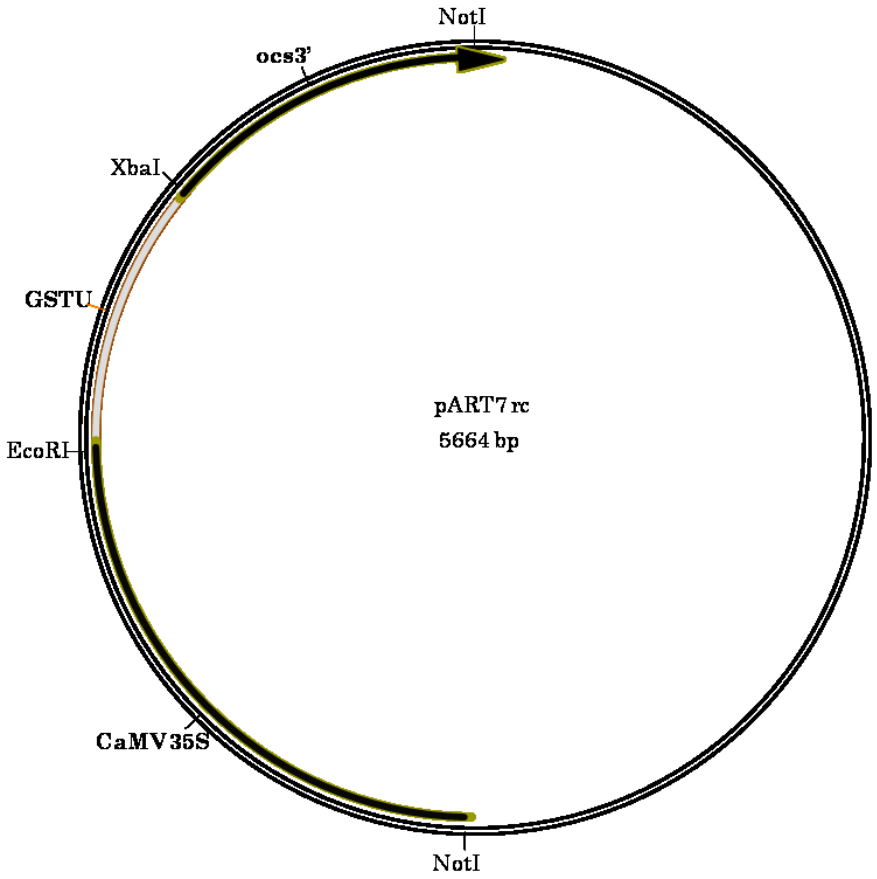


Fig. 2.2 Primary cloning vector pART7-GSTUs

The expression cassette was excised from pART7 using NotI restriction enzyme and then ligated in the T-DNA region into the NotI restriction sites of the pART27 binary vector. The ligase reaction was analyzed by PCR. The used primers were: GSTU cds for 5'ATGGCGGACGAAGTGGTTCTGT3' and Ocs rev 5'GGCGTCTCGCATATCTCATTAAAGC3' which amplified a region between the GSTUs coding sequence and the 3'-untranslated region of octopine synthase gene *(ocs)* which includes the polyadenylation signal. Thermocycling conditions were as follows: 94 °C for 2 min, followed by 35 cycles with denaturation at 94 °C for 30 s, annealing at 48 °C for 20 s and extension at 72 °C for 30 s, with a final 1 min cycle at 72 °C. All the amplicons were analyzed in 1% agarose gel in 1x TBE as running buffer, and stained with GEL RED. pART27-GSTUs (Fig. 2.3), harboring the 5'–3' oriented transgene, was then transferred into *Agrobacterium tumefaciens* strain GV3101 by electroporation according to the protocol described below.

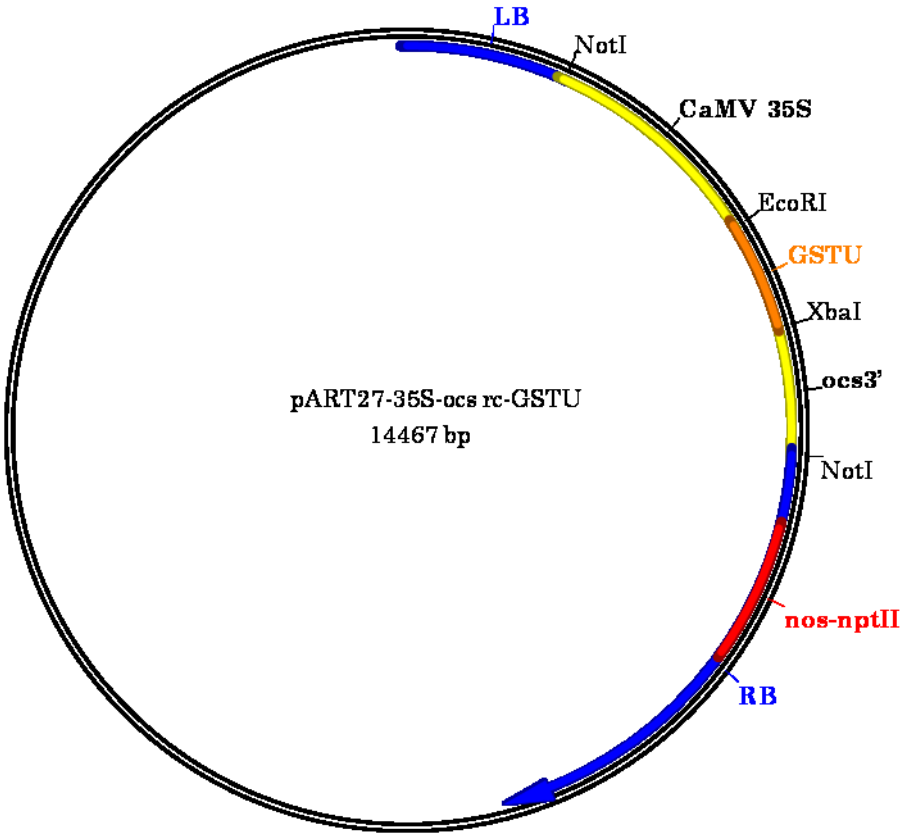


Fig. 2.3 Binary vector pART27-GSTUs

2.3 Agrobacterium transformation

Cells were thawed on ice and 200 ng of plasmid DNA were added. Successively, 40 µl of this suspension were placed in a electroporation device cuvette provided with an electrode distance of 0.2 cm (Bio-Rad). A single electrical pulse of 2.5kV initial voltage using the 25 µF capacitor (Gene Pulser, Bio-Rad) was immediately applied. Following the electrical pulse the cells were then transferred into 1 ml LB medium and incubated at 28°C for 2 hour. Aliquots were then spread on LB plates containing 100 µg/ml spectinomycin and 20 µg/ml kanamycin. After incubation at 28°C

for 48 hours, positive colonies were checked by performing PCR amplification with gene specific primers.

2.4 Tobacco transformation

For tobacco transformation the leaf disk method was adopted (Rogers et al., 1985). The protocol, basically, consists in immersing the leaf disks in a liquid culture of *Agrobacterium* carrying the chosen transformation vector. The plant tissue and *Agrobacterium* are then cocultivated on regeneration medium for a period of 1 day at the end of which the leaf disks are transferred to a regeneration medium supplemented with an antibiotic, to remove living bacteria (cefotaxime), and a selective agent against untransformed plant cells. Normally, it took about 2 months to obtain rooted plantlets that can be transferred to soil.

Day 1

PCR positive colonies of AtGV3101-pART27-GSTUs were directly inoculated in 5 ml of LB broth containing 20 μg/ml kanamycin and 100 μg/ml spectinomycin and grown overnight at 28°C with shaking.

Day 2

Four to six leaves from 1-2 month old *N. tobacum* plants growing under sterile conditions were used to generate several leaf disks. Explants were placed onto the preculture medium (MS, sucrose, pH 5.8). The day before, *Agrobacterium* cells were harvested by centrifugation (4000 rpm), suspended in 500 μl of preculture

medium and transferred to Petri dishes containing the tobacco explants. Precultured explants were inoculated overnight at 25°C.

Day 3

After the inoculation step, the explants were washed with sterile water, dried on sterile paper and placed, adaxial side up, onto solidified regeneration medium (MS, sucrose, agar, 100 µg/ml cefotaxime, pH 5.8), overnight at 25°C.

Day 4

The explants were transferred adaxial side down onto solidified regeneration medium supplemented with the selection agents and growth factors (MS, sucrose, agar, 100 µg/ml cefotaxime, 50 µg/ml kanamycin, 1 mg/l benzylaminopurine, 0,1 mg/l 1-naphthalene acetic acid, pH 5.8) and incubated at 28°C under an 18-h light regimen.

The explants were transferred onto fresh regeneration medium every 2 weeks. After 2 weeks of selection, shoots arising from callus cluster were excised and transferred onto Magenta GA-7 vassel (MS, sucrose, agar, pH 5.8). The rooting stage was carried out following the same experimental conditions used during the regeneration step.

2.5 Verification of the transgenic plants

Total genomic DNA was isolated from leaf tissue of putative transformants using the DNeasy Plant Mini Kit (Qiagen). PCR reactions were carried out to amplify a fragment containing part of

the promoter region in the expression cassette of *Csgstus*. The primers were:

(forward) 5'ATCATTCAAGATCTCTCTGC3'

(reverse) 5'AATCCACTTGCTTTGAAGAC3'.

Thermocycling conditions were as follows: 95 °C for 4 min, followed by 35 cycles with denaturation at 95 °C for 30 s, annealing at 49 °C for 20 s and extension at 72 °C for 15 s, with a final 1 min cycle at 72 °C. PCR products were visualized on 1 % agarose gel in TBE 1x, then stained with GEL RED.

2.6 Transgene expression analysis with RT-Real Time PCR

Total RNA was isolated from leaves of plants grown *in vitro* using the RNeasy plant RNA isolation kit (Qiagen), according to the manufacturer's instructions. First-strand cDNA was synthesized (1.5 μg) using SuperScript VILO cDNA Synthesis Kit (Invitrogen). The housekeeping gene elongation factor (*Nt*EF-1α), which has been reported to be constitutively expressed (Schmidt et al., 2010), was used as an endogenous reference. The elongation factor specific primers were:

(forward) 5'TTAATGAGCCCAAGAGGCCCAC3'

(reverse) 5'TTTCCACACGACCAACAGGGAC3'

The *gstu* genes were:

(forward) 5'AAGACCTTGGAAGGGCAGCTTG3'

(reverse) 5'TTTGAACTTGGGGCACTCAGC3'.

The amplification was performed for both genes in the following thermocycling conditions: 50 °C for 2 min, 95°C for 8 min, 30 s followed by 40 cycles of 15 sec at 95 °C, 20 s at 61 °C and 60 sec at 60 °C. The relative quantitation of GST expression among samples was calculated using the comparative threshold (C_T) method (Lo Piero et al., 2006). Three independent triplicates of quantitative PCR experiments were performed for each gene to generate an average C_T and to calculate standard deviation. ΔC_T was calculated by subtracting the average *Nt*EF C_T from the average C_T of the gene of interest. This value was calculated for each sample (tobacco *wild type* and transgenic tobacco plants cDNA), and then the comparative expression level of the GST genes was given by the formula $2^{-\Delta\Delta C_T}$, where $-\Delta\Delta C_T$ was calculated by subtracting the transgenic tobacco ΔC_T from the tobacco *wild type* ΔC_T, where no *Cs*GST expression was detected (C_T 40) **(**Lo Piero et al., 2006).

2.7 Herbicides treatment of tobacco plants

Fluorodifen (Sigma) was applied by uniformly spraying 8-week old transgenic and wild type tobacco plants grown in the greenhouse. For the treatment of the tobacco plants, fluorodifen was dissolved in aqueous solution of 1.2% acetone and 0.05% Tween 20 at a concentration of 200 μM. Herbicide injury was assessed 5 days after treatment, by measuring the electrolyte leakage from leaf disks of treated and untreated control tobacco plants. Twelve leaf disks were collected from the first fully expanded leaf counting from

the top of each plant, and floated to 5 mL of distilled water. After subsequent vacuum infiltration for 7 min at -70 kPa in a vacuum chamber and incubation for 16 h at room temperature in the dark, the electrical conductance of the bathing medium was measured using a conductivity meter (ORION RESEARCH). The samples were then autoclaved at 121 °C for 5 min, so that the leaf disks would release all their solutes and after cooling to room temperature the conductivity was measured again. Relative electrolyte leakage was calculated by dividing the conductivity of the bathing solution before autoclaving to the conductivity after autoclaving (Benekos et al., 2010).

Alachlor (Sigma) tolerance experiments were conducted *in vitro*. Three-weeks old transgenic and wild type tobacco seedlings were transferred to Magenta GA-7 vessels containing MS medium supplemented with 7.5 mg/L alachlor. The tobacco plant tolerance was assessed after 40 days of growth in the presence of the herbicide, by measuring growth parameters such as shoot and root elongation (Benekos et al., 2010).

2.8 Saline and drought stresses conditions

Wild-type and *Cs*GSTU transgenic tobacco seeds were surface sterilized and sown on MS medium supplemented with 50 μg/ml kanamycin. Seeds that completely penetrated the seed coat were considered as germinated. To test the effect of salt and drought stress, germinated seeds were transferred to MS medium supplemented with 150, 200 mM NaCl and 8 % (w/v) mannitol,

respectively. The seedlings were grown with a day/night cycle of 16/8 h at 25 °C. After 2 weeks, the root length of transgenic and untransformed seedlings was directly growth measured and compared with that of untreated control samples (Ji et al., 2010).

2.9 Preparation of tobacco leaf crude extract

Aliquots (200 mg) of leaves from wild type and transgenic tobacco seedlings grown *in vitro*, were homogenized, using liquid mitrogen and micropestle, then the powdered tissue was resuspended in a 400 µl aqueous solution of 50 mM potassium phosphate buffer pH 7.0. The slurry was centrifuged at 18,000×g for 15 min at 4 °C and the supernatant was assayed for GST activity against different substrates.

2.10 Measurement of glutathione transferase activity

The GST assay was performed as described in Lo Piero et al. (2006). The reaction mixture (final volume 0.5 ml) containing 50 mM potassium phosphate buffer pH 7.5, 1 mM glutathione, 1 mM CDNB, or 1 mM fluorodifen and enzyme solution (50-100 µl) was incubated at 30 °C for 20 min. GS-conjugates were detected by measuring the absorbance of samples at 340 nm for CDNB, and at 400 nm for fluorodifen. Molar extinction coefficients of 9600 $M^{-1}cm^{-1}$ (CDNB) and 17200 $M^{-1}cm^{-1}$ (fluorodifen) were used to calculate specific activity. All measurements were adjusted by subtracting the absorbance values obtained for the non enzymatic conjugation of substrates. Protein concentration was determined by the method of

Bradford using bovine serum albumin as standard (Bradford et al., 1976).

2.11 Glutathione transferase activity towards alachlor

Generally, GST activity towards alachlor is conventionally assayed by measuring the GS-conjugate by RP-HPLC (reverse-phase high-performance liquid chromatography). Alternatively, a recently developed direct and sensitive endpoint assay may be applyed. The assay is based on the color formation resulting by the reaction of $Hg(SCN)_2$ with the released halogen ions of the substrates in the presence of Fe^{3+} (Iwasaki et al., 1952). The reaction mixture (final volume 0.25 mL) containing 25 mM MES buffer pH 6.5, 2.5 mM GSH, 0.5 mM alachlor and 50 μl 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. Color 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 N nitric acid. All measurements were adjusted by subtracting the absorbance values obtained for the non enzymatic conjugation of substrates. The orange color due to the presence of halogen ions was measured spectrophotometrically 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 et al., 2010).

2.12 Measurement of glutathione peroxidase activity

The glutathione peroxidase (GPOX) assay was performed by slightly modifying the method described in Yokota et al. (Yokota et al., 1988). The reaction mixture (final volume 0.5 mL), containing 50 mM potassium phosphate buffer pH 6.5, 1.0 mM glutathione, 0.2 mM NADPH, 1.0 mM NaN_3, 1.0 mM EDTA, 1.0 mM H_2O_2, 2 units of glutathione reductase and enzyme solution was incubated at 30 °C for 12 min. The peroxidase activity was detected spectrophotometrically by measuring the decrease of absorbance at 340 nm due to NADPH consumption.

2.13 In vitro expression and purification of sweet orange wild-type and mutant GSTs

In vitro expression of functionally active GSTs was achieved by using Expresswa Cell-Free *E. coli* Expression System (Invitrogen) by incubating the plasmid GSTU-pEXP1-DEST (1 µg) (Lo Piero et al., 2009) in:

- *E. coli sly*D- Extract 20 µl
- 2.5X IVPS E. coli Reaction Buffer 20 µl
- 50 mM Amino Acids (-Met) 1.25 µl
- 75 mM Methionine 1 µl
- T7 Enzyme Mix 1 µl
- DNase/RNase-free Distilled Water To a final volume of 50 µl

for 6 h at 37 °C (300 rpm) to promote proper protein folding. Higher protein yields has been obtained by adding one half-volume of Feed

Buffer (2X IVPS Feed Buffer 25 µl, 50 mM Amino Acids (-Met) 1.25 µl, 75 mM Methionine 1 µl, DNase/RNase-free distilled water to final volume of 50 µl) at 30 minutes and one half-volume of Feed Buffer again at 2 hours after initiating the protein synthesis reaction. The recombinant protein, containing the 6xHis 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). The unspecific bound proteins were removed by washing the column with 20 mM phosphate buffer pH 8.0, 500 mM NaCl, 20 mM imidazole. The His-tagged protein was eluted with 500 mM imidazole by recovering 0.2 ml fractions. Fractions were tested for protein content using the Bradford method (Fig. 2.4), and, those included in the peak core were collected and assayed for GST activity (Lo Piero et al., 2009).

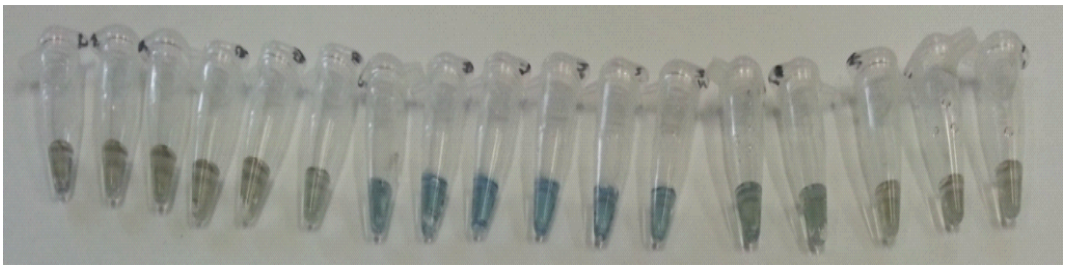


Fig. 2.4 Fractions tested by Bradford method

2.14 Pathogenicity test with P. syringae pv. tabaci

Pseudomonas syringae pv. *tabaci* strain DAPP-PG 676 was grown overnight on King's medium B plates (King et al., 1954) for 24 h at 26 °C. Cells were washed in sterile water and resuspended at a concentration of 10^8 cfu ml^{-1} in 10 mM phosphate buffer pH 7,0 (10 mM PB, pH 7,0; Sambrook et al., 1989). lnoculum concentration was determined by optical density at OD_{600}, confirmed by plating serial dilutions on KB plates and adjusted at the appropriate concentration in 10 mM PB. Bacterial suspensions were infiltrated into fully developed WT and transgenic tobacco leaves using a 5-mL 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 x 20 mm.

2.15 Assessment of bacterial populations in tobacco leaves

To investigate *P. syringae* pv. *tabaci* growth in tobacco genotypes leaf disks were punched from the infiltrated area with a cork borer. Six-disks were sampled for each genotype at each date of sampling. The six disks were divided into three sets of two and grinded in 1 ml of 10 mM PB. The bacterial populations in the leaves were determined by plating serial dilutions of the disc extract on KB plates supplemented with 100 mg/L nitrofurantoin and 100 mg/L of cycloeximide. Tobacco plants were maintained in

the growth chamber under 16 h light (100 $\mu Em^{-2}s^{-1}$)/8 h dark photoperiod at 23 °C. Two experiments were performed: 1) all genotypes were sampled 3 and 10 days after inoculation (dpi); 2) time-course assessment of bacterial population in *Cs*GSTs over-expressing plants at 0, 24, 48, 72, 96 and 120 h after inoculation.

2.16 Production of culture supernatant

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

2.17 Statistical analysis

All data is presented as mean valueand its standard deviation. Statistical analysis was performed with SPSS. Statistical differences between WT and transgenic lines were analysed by Student's t test.

Results and discussion

3.1 Generation and verification of transgenic plants

3.1.1 Construction of the pART27 binary vector

The first step in the construction of pART27 binary vector was the PCR amplification of the *Csgstu* genes, both wild type and mutants, previously cloned in pEXP1-DEST expression vector (Lo Piero et al., 2009). As described in "Material and Methods", primers were designed to add two restriction sites for EcoRI and XbaI at the *Csgstu* coding sequence ends. PCR products were subjected to electrophoretic analysis, which showed a band at approximately 679 bp (Fig. 3.1), whose identity was confirmed by sequencing, and purified.

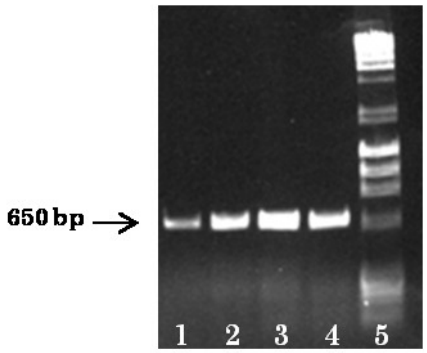


Fig. 3.1 Electrophoretic analysis of the PCR product. Line 1: GSTU1, line 2: GSTU2, line 3: PKI, line 4: RKV, line 5: DNA ladder Lambda Hind III / phiX174 Hae III.

The amplicons were inserted into the cloning vector pCR 2.1 TOPO TA and subsequently digested by EcoRI and XbaI enzymes. The reaction was subjected to electrophoretic analysis onto 1% agarose gel, then the fragments having length corresponding to that expected for the *Cs*GSTUs (Fig. 3.2) were extracted from the gel and purified.

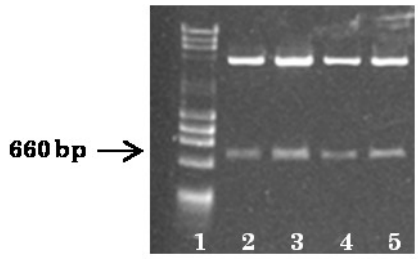


Fig. 3.2 Enzymatic digestion of the vector pCR 2.1 TOPO TA with EcoRI and XbaI. Line 1: ladder, line 2: GSTU1, line 3: GSTU2, line 4: PKI, line 5: RKV.

The purified fragments were inserted into the expression cassette of the pART7 vector. The entire expression cassette was then isolated by enzymatic digestion and inserted between the left and right borders of the pART27 vector through a ligation reaction. The ligation was subsequently verified by PCR using a couple of primers that amplifies a region comprised between the GST encoding sequence and the octopine synthase gene present in the vector DNA. The PCR products were visualized on agarose gel 1% (Fig. 3.3).

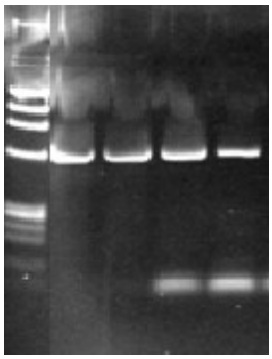


Fig. 3.3 Gel of verification of the ligation reaction

Plant transformation vector pART27-GSTUs were then used for the transformation of *Agrobacterium tumefaciens* strain *At*GV3101.

3.1.2 Agrobacterium tumefaciens transformation

The agrobacteria transformation with the binary vector pART27 was carried out by electroporation. Positive colonies were selected by growing on plates containing spectinomycin and kanamycin as selective antibiotics, and further verified by PCR as described in the Material and Methods section. The electrophoretic analysis on 1% agarose gel, confirmed the successful transformation reaction, showing the expected band of about 693 bp (Fig. 3.4).

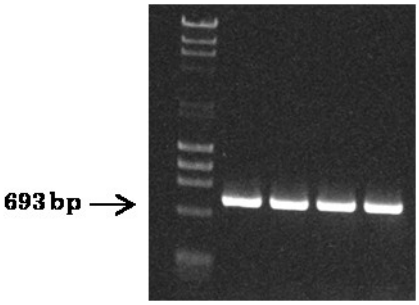


Fig. 3.4 PCR on colony

3.1.3 Transformation of Nicotiana tabacum plants

Transgenic tobacco plants carrying the *Cs*GSTU genes were generated via *A. tumefaciens* using the leaf disk method (Rogers et al., 1985). The selection of putative transgenic plants was achieved by growing explants on MS containing kanamycin. To test the selectivity of kanamycin, a negative control using leaf disks excised from tobacco *wild type* leaves was simultaneously performed. After 3/4 weeks of growth in climatic chamber, the transformed explants manifest callus formation and, successively, shoot formation is also observable (Fig. 3.5C); the control explants show no cell proliferation and appear strongly chlorotic (Fig. 3.5G). The shoots

are then excised from the callus and transferred to Magenta GA-7 vassel. After 3/4 weeks of growth on MS medium, putatively transformed plants were subjected to appropriate analysis to verify the presence of the transgenes.

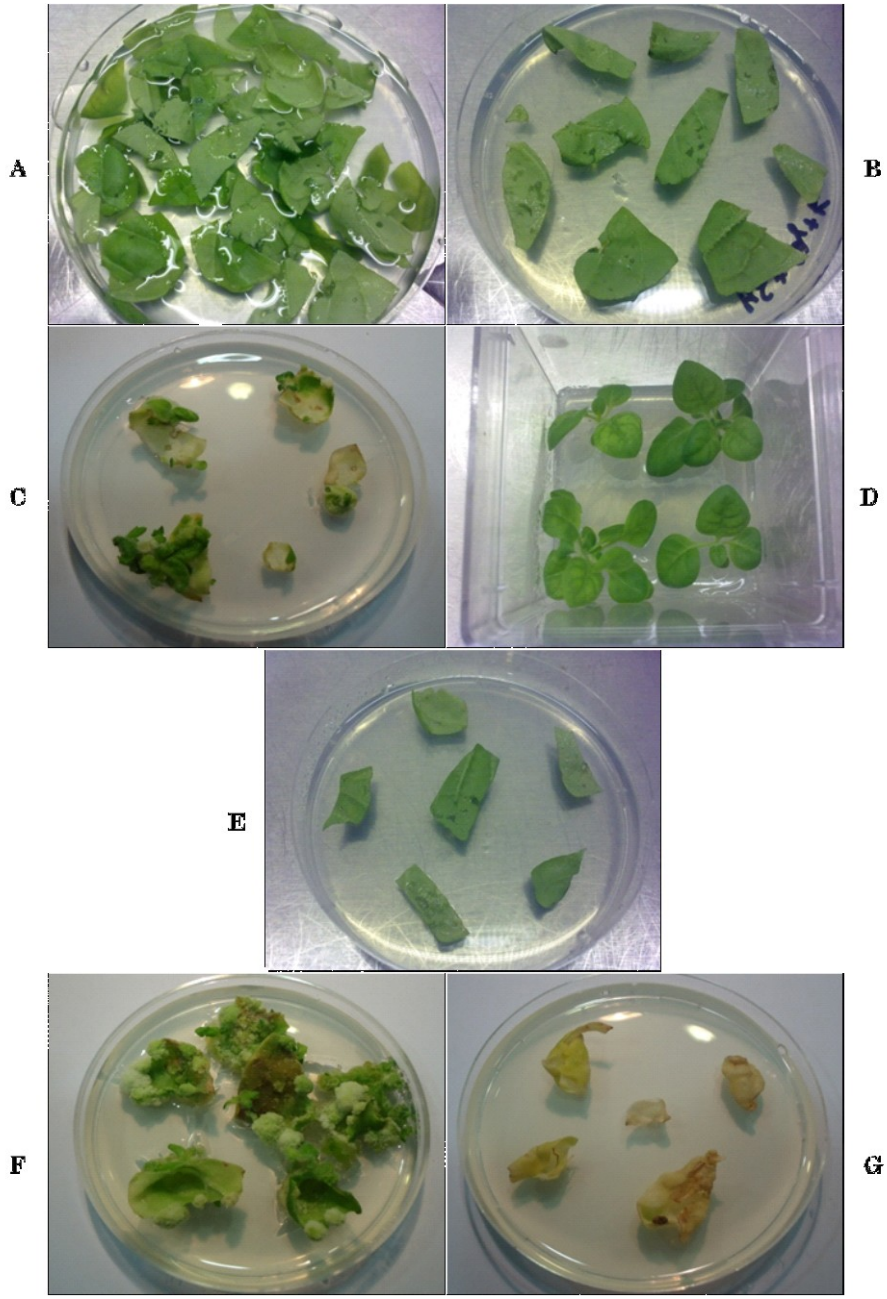
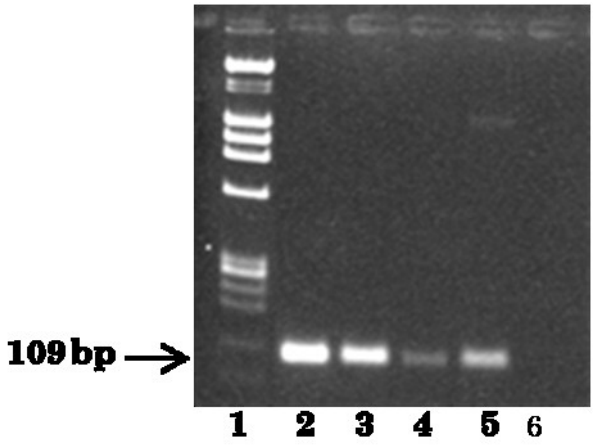


Fig. 3.5 A. Tobacco in cocoltivation media (MS), B. Transformed tobacco in regeneration media (MS + selection agents and growth factors), C. Shoot arising from a callus, D. *N. tobaccum* transformed plants. Negative control: E. Untransformed tobacco, F. MS + Cefotaxime , G. MS + Cefotaxime and Kanamycin

3.1.4 Verification of the transgenic tobacco plants

Putative transformed plants were verified for the presence of the transgene by PCR using the tobacco genomic DNA as template and primers that amplify an internal region of the CaMV 35S promoter whose expected length is 109 bp. The electrophoretic analysis performed on the PCR reaction products shows a band of about 109 bp, thus confirming the transformation of tobacco plants. No amplification product is detected in the case wild type genomic DNA serves as template (Fig. 3.6).



Figu. 3.6 PCR analysis for the verification of the transgene insertion. Lane 1: DNA ladder; Lanes 2-5: tobacco plants transformed with pART27-GSTU1/GSTU2/PKI/RKV; lane 6: untransformed tobacco plant.

3.2 Expression analysis of transgenic plants

The expression of GSTs from *Citrus sinensis* in transformed tobacco plants was evaluated using RT- real time PCR. The results reported in Table 3.1, reveal very high levels of expression for *Cs*GST genes in transgenic tobacco plants, whereas the *wild type* tobacco do not show expression of transgenes. Among the transformed plants, those harboring the GSTU1 isoform or the GST-RKV mutant show

higher expression levels than the transformed plant containing the other GST genes (Tab. 3.1). Control amplifications with the elongation factor specific primers were successful for both untransformed wild type and transgenic plant revealing equal cDNA loading. The data suggests that the insertion of the transgenes occurred in transcription active regions of the tobacco genome. Moreover, it likely that the tobacco genome does not contain correlated GST sequences that might cross react with *Citrus* specific GST primers as indicated by the absence of amplification products in the untransformed tobacco plants (Tab. 3.1).

Tobacco plants	**Normalized amount relative to *wild type* simple $2^{-\Delta\Delta CT}$**
Wild type	1
Empty pART27	1
GST U1	$6{,}77 \cdot 10^{6} \pm 3{,}42 \cdot 10^{6}$
GST U2	$8{,}56 \cdot 10^{5} \pm 4{,}09 \cdot 10^{5}$
GST PKI	$1{,}54 \cdot 10^{5} \pm 0{,}88 \cdot 10^{5}$
GST RKV	$1{,}03 \cdot 10^{6} \pm 0{,}64 \cdot 10^{6}$

Tab. 3.1 Relative expression of GSTs in both wild type and transformed tobacco plants measured by real time PCR. Data are mean ±SE (n = 3).

3.3 Tolerance of transgenic tobacco plants to fluorodifen herbicide

In order to determine whether the *in planta* overexpression of *Cs*GSTUs provokes an increased tolerance to fluorodifen, transgenic and untransformed tobacco plants were sprayed with a sublethal dose of fluorodifen (200 μM). The herbicides injury was assessed 5 days after the treatment both by visual inspection of plants and by evaluating the leaf electrolyte leakage. The results show that even at this sublethal concentration, fluorodifen causes many chlorotic and necrotic lesions on the *wild type* and empty pART27 transformed tobacco leaves, whereas transgenic lines show a less severe symptoms than untrasformed tobacco plants (Fig. 3.7-3.8).

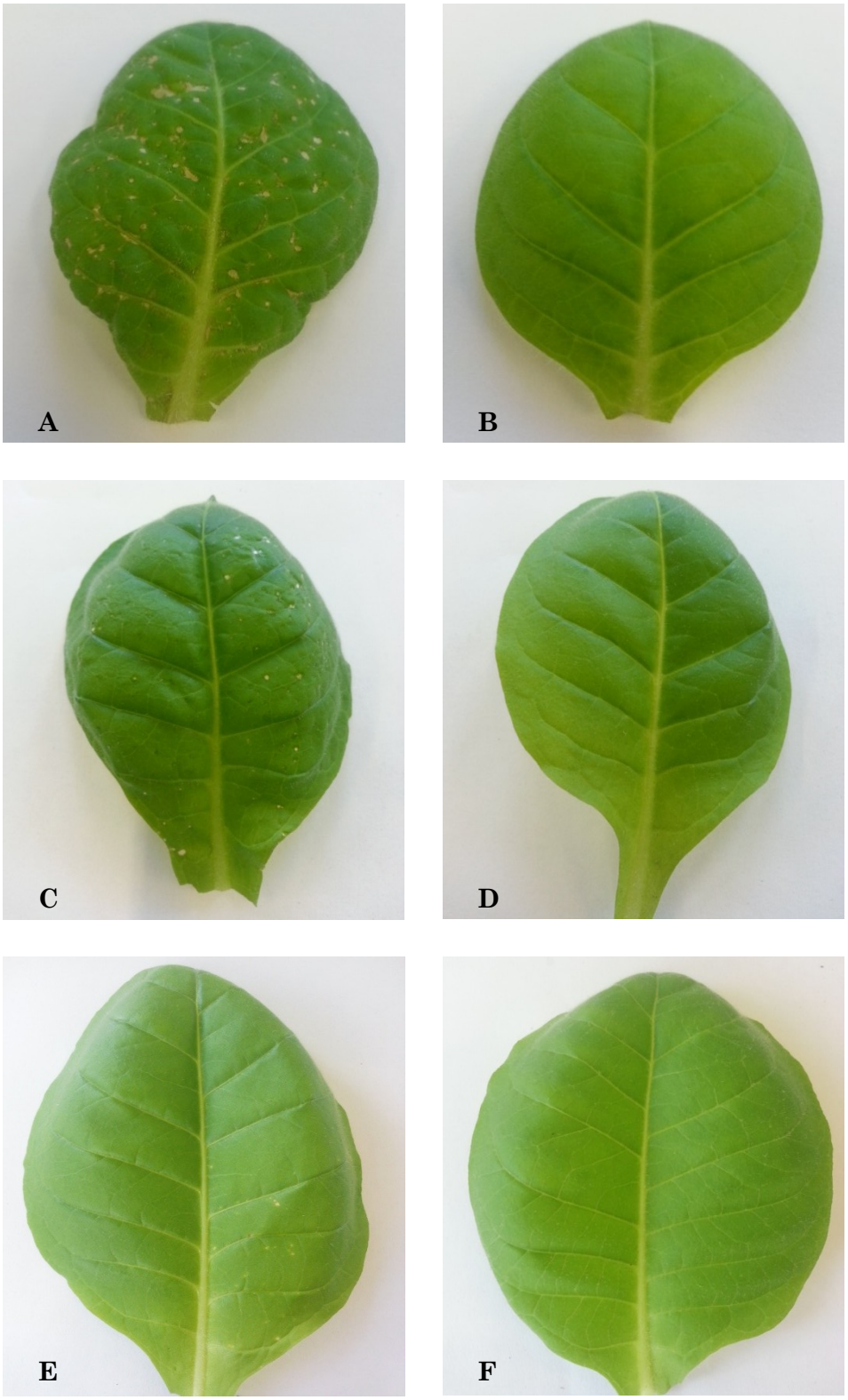


Fig. 3.7 Effect of *in planta* herbicide treatment (200 µM fluorodifen). A), C), E) *wild type,* GSTU1, GSTU2 treated. B), D), F)) *wild type,* GSTU1, GSTU2 untreated.

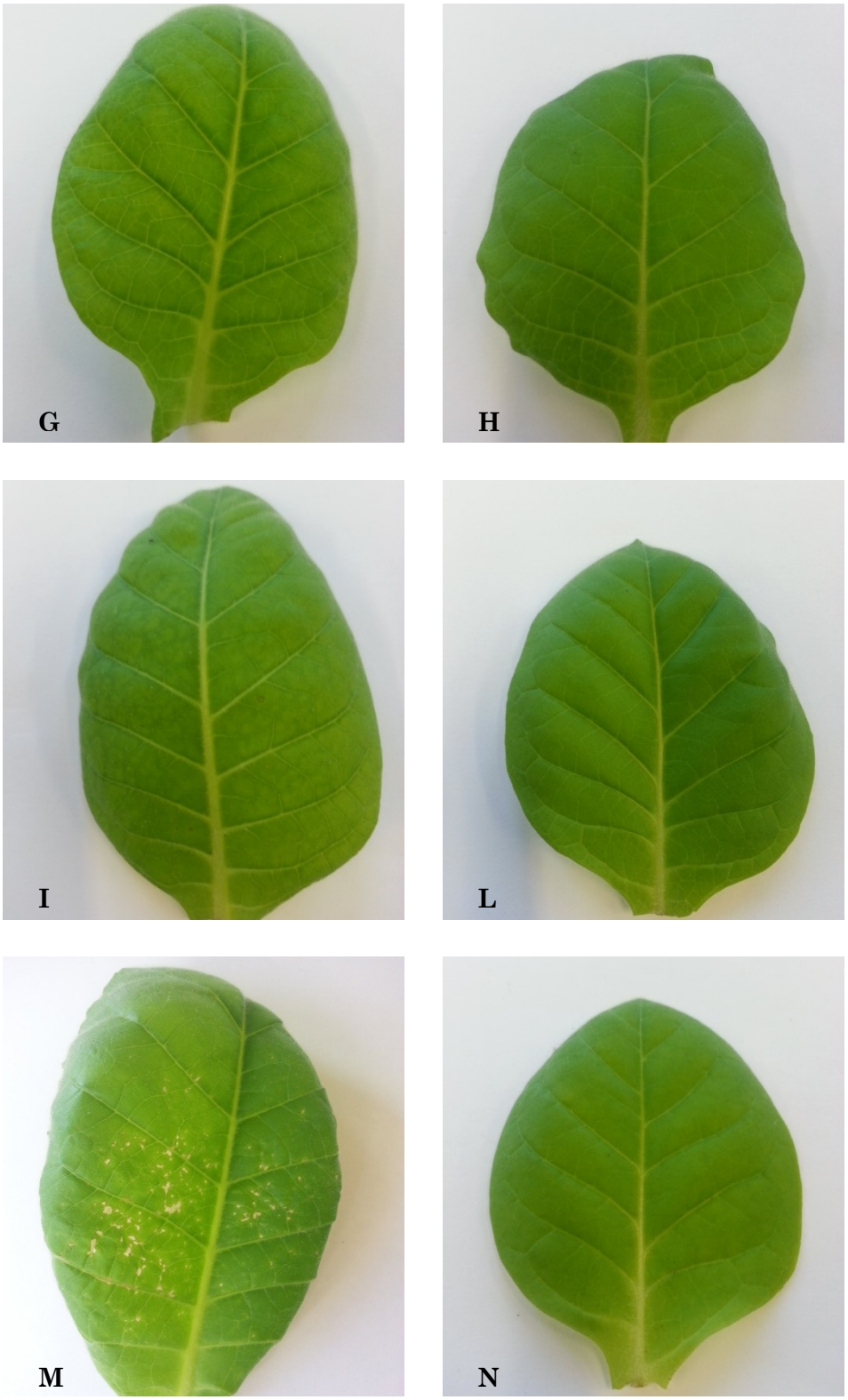


Fig. 3.8 Effect of *in planta* herbicide treatment (200 µM fluorodifen). G), I), M) PKI, RKV, empty pART27 treated. H), L), N) PKI, RKV, empty pART27 untreated.

When leaf electrolyte leakage is measured, *Cs*GSTU overexpressing lines exhibit significant reduced membrane damage compared to untrasformed plants indicating a higher degree of tolerance to the herbicide. As shown in Figure 3.9 in which the relative electrolyte leakage with respect to untreated plants is reported, tobacco overexpressing all the *Cs*GSTUs disclose a significant decrease of the relative electrolyte leakage values ranging between 3-13 % compared to 31-33 % of *wild type* and empty pART27 transgenic tobacco plants (Fig. 3.9).

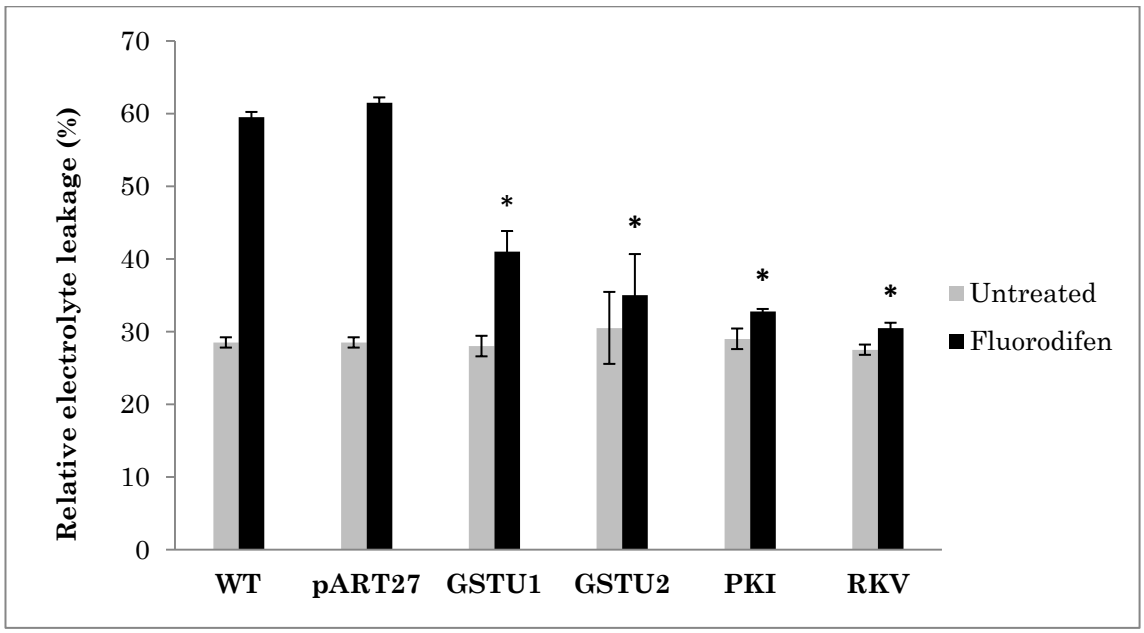


Fig. 3.9 Tolerance of wild type and *Cs*GSTUs overexpressing tobacco transgenic lines to 200 µM fluorodifen, measured by relative electrolyte leakage. Relative electrolyte leakage values of wild type and transgenic plants were converted to percent values. Data are mean±SE (n = 4). Asterisks depict significant differences at P≤0.05 of the transgenic lines compared to wild type plants.

The GST activity was measured against either CDNB or fluorodifen as substrates in tobacco leaf extracts as described in "Material and Methods" (Par. 2.10). The GST activity towards CDNB is similar between the transgenic and the untransformed plants, with the

exception of those harboring the GST-RKV mutant which show an increase of 1,9-fold compared to the tobacco *wild type*, accordingly to previous results showing that GST-RKV, exhibits the highest catalytic efficiency towards this substrate than wild type and mutate GSTs (Lo Piero et al., 2010). Conversely, GST activity towards fluorodifen of all *Cs*GSTU overexpressing transgenic plants is higher compared to the wild type plants, thus indicating that transformed plants are able to efficiently conjugate fluorodifen to GSH (Tab. 3.2). Furthermore, the sharp increase of specific activity towards fluorodifen compared to that observed with CDNB is probably the result of the higher affinity of the *Cs*GSTUs for this herbicide as substrate.

Specific Activity (nmol/min/mg)		
	CDNB	**Fluorodifen**
WT	4,98 ± 0,14	45,78 ± 5,02
GSTU1	3,34 ± 0,59 **(-)**	75,29 ± 15,53 **(+64%)***
GSTU2	3,97 ± 1,36 **(-)**	52,10 ± 5,97 **(+14%)***
PKI	4,12 ± 0,00 **(-)**	71,40 ± 4,12 **(+56%)***
RKV	9,64 ± 0,39 **(+94%) ***	96,14 ± 5,73 **(+110%)***

Tab. 3.2 GST specific activity of both wild type and tranformed tobacco plant extracts. In brackets the percentage relative increase in specific activity compared to tobacco wild type.

In order to confirm that fluorodifen is a suitable substrate for *Cs*GSTUs and mutant, the sweet orange GSTU1, GSTU2 and GST-RKV were successfully *in vitro* expressed and purified, as detailed in the "Materials and Methods" section (Par. 2.13). The specific activities of the enzymes were determined against the CDNB and fluorodifen (Tab. 3.3). The results obtained by measuring the GST activity towards the universal non-specific substrate CDNB, confirm our previous data indicating that GSTU2 and GST-RKV exhibit higher catalytic activity than the GSTU1 isoform (Par. 1.10). Using fluorodifen as substrate, the *Cs*GSTUs show catalytic activity towards this herbicide, and, in agreement with the data obtained by measuring the GST activity on transformed leaf crude extract, CsGSTU1 and GST-RKV exhibit higher levels of conjugating activity. As described in the Introduction (Par. 1.7), fluorodifen is a strong photobleaching herbicide which inhibits the plastid protoporphyrinogen oxidase and generates photooxidative stress. As shown by microarray analysis in wheat treated with cinidon-ethyl, a herbicide that also inhibits protoporphyrinogen oxidase, the primary response of wheat is the upregulation of genes coding for antioxidant enzymes, thus indicating the importance of counteracting oxidative stress caused by these peroxidizing herbicides (Pasquer et al., 2006). The dual GST/GPOX function of many GSTs might contribute to peroxidizing herbicides tolerance in different crops alleviating abiotic stresses mediated oxidative stress. As a consequence, the GSH-peroxidase activity of the *in vitro* expressed CsGSTUs was also measured using hydrogen peroxide as substrate, as described in Materials and Methods (Par. 2.12). The experiment revealed that none of the assayed CsGSTUs show GSH

peroxidase activity (data not shown) indicating that the protective effect against fluordifen is likely due to the conjugating acivity of the herbicides to GSH and not to a putative scavenging activity of the oxidative stress byproducts, as instead shown in the case of tobacco plants over-expressing *Gm*GST4-4, which has been identified as a GST/GPOX enzyme (Benekos et al., 2010).

Specific Activity (nmol/min/mg)		
	CDNB	**Fluorodifen**
GSTU1	45,65 ± 0,07	71,75 ± 1,34
GSTU2	76,9 ± 3,25	38,9 ± 16,40
RKV	82,1 ± 1,41	54,85 ± 9,40

Tab. 3.3 GST activity towards CDNB and fluorodifen of *in vitro* expressed and purified CsGSTU1, CsGSTU2 and RKV mutant. Data are mean±SE (n = 4).

3.4 Tolerance of transgenic tobacco plants to alachlor herbicide

As detailed in the Introduction of this thesis (Par. 1.6), alachlor is a pre-emergence herbicide applied to young plants to repress both the elongation of the root system and the development of the shoots. To study the tolerance towards chloroacetanilide herbicide *in planta*, we assayed the growth inhibition of untrasformed *wild type* and transgenic tobacco seedlings in the presence of 7.5 mg/l of alachlor. Forty days after the treatment the growth inhibition induced by the herbicide was evaluated both by

visual inspection (Fig. 3.9-3.10) and by directly measuring stem and root length (Fig. 3.11-3.12). As shown in the aforementioned figures, alachlor negatively influence the growth of roots and stems of wild type tobacco seedlings which appear sharply reduced. The transgenic tobacco plants are as susceptible as untrasformed plant, except the transgenic plants overexpressing *Cs*GSTU2 which are clearly unaffected by herbicides treatment considering either stem or root lenght (Fig. 3.9-3.10).

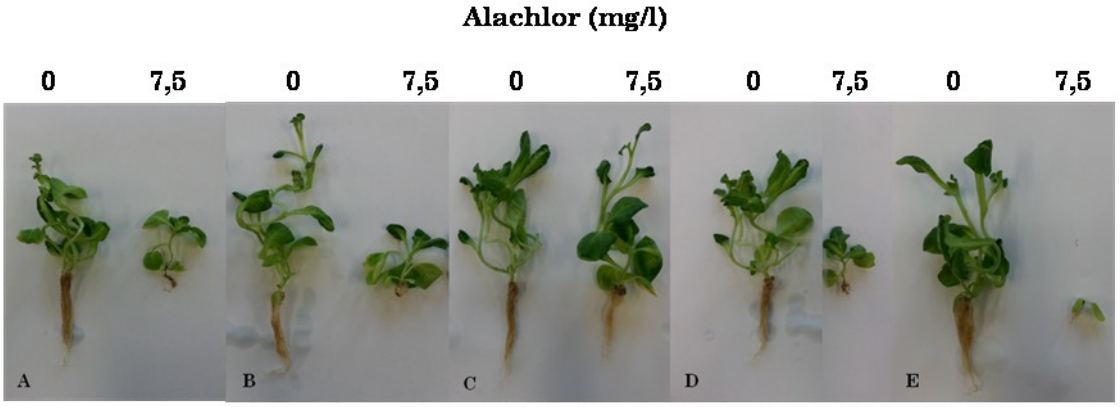


Fig. 3.9 Phenotype of wild type plants and transgenic line grown for 40 days in MS medium supplemented with 7.5 mg/L alachlor. A) WT, B) GSTU1, C) GSTU2, D)PKI, E) RKV.

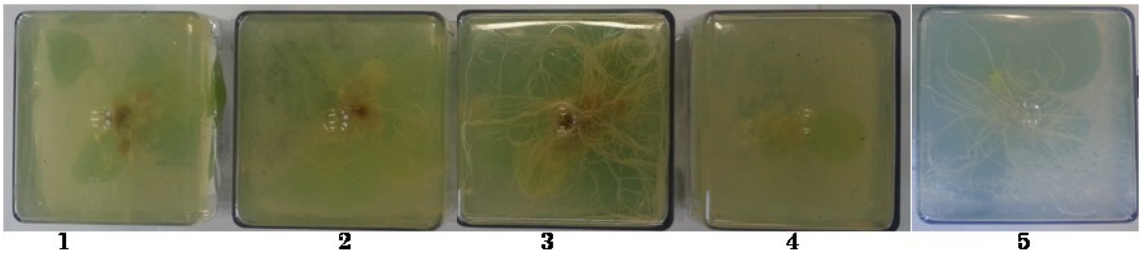


Fig. 3.10 Root phenotype of wild type plants and transgenic line grown for 40 days in MS medium supplemented with 7.5 mg/L alachlor. 1) WT, 2) GSTU1, 3) GSTU2, 4)PKI, 5) RKV.

The findings derived from visual inspection of tobacco plants have been confirmed by the direct measurement of stem and root lenght.

As regards the development of the stem, the *wild type* tobacco plants and the transgenic lines overexpressing GSTU1, GST-PKI showed comparable values of shoot length, whereas stems of plants transformed with GST-RKV are even shorter than wild type tobacco seedlings (Fig. 3.11-3.12). On the contrary, the stem length of the transgenic lines overexpressing *Cs*GSTU2 is the 81% of the untreated CsGSTU2 transformed plants exhibiting an evident tolerance against 7.5 mg/ml 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 that root lenght was measured, since the values of transgenic plants overexpressing *Cs*GSTU2 are around the 80% of untreated transformed plants (Fig. 3.11).

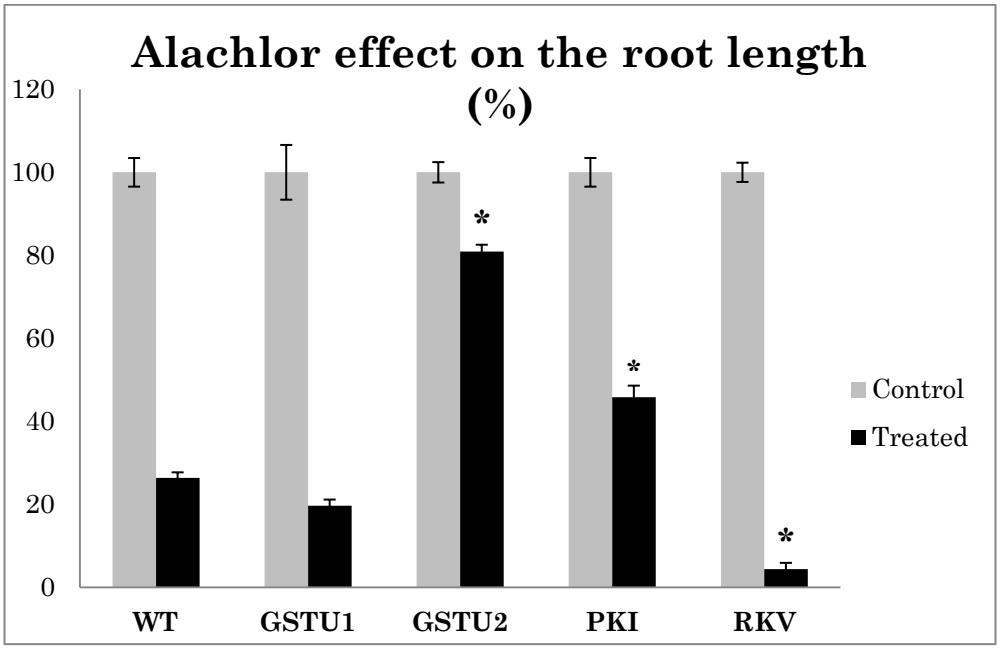


Fig. 3.11 Comparison of average root length of wild type and *Cs*GSTU overexpressing tobacco transgenic lines grown for 40 days in MS medium supplemented with 7.5 mg/l alachlor. Relative average root length values of wild type and transgenic plants were converted to percent values, compared to average root length of untreated plants. Data are mean±SE (n = 4). Asterisks depict significant differences at P≤0.05 of the transgenic lines compared to wild type plants.

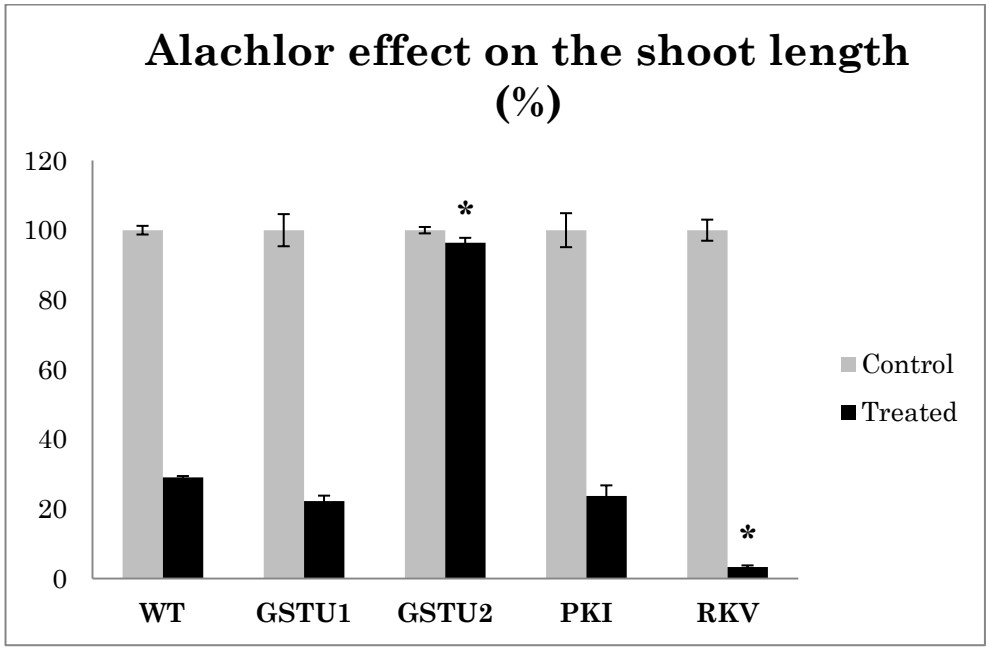


Fig. 3.12 Comparison of average shoot length of wild type and *Cs*GSTU overexpressing tobacco transgenic lines grown for 40 days in MS medium supplemented with 7.5 mg/l alachlor. Relative average root length values of wild type and transgenic plants were converted to percent values, compared to average root length of untreated plants. Data are mean±SE (n = 4). Asterisks depict significant differences at P≤0.05 of the transgenic lines compared to wild type plants.

In order to correlate the acquired tolerance towards alachlor of *Cs*GSTU2 transformed plants with the enzymatic activity, *Cs*GSTU1 and *Cs*GSTU2 were *in vitro* expressed and purified on affinity column as described in Material and Methods. Once the proteins have been purified, the GST activity towards alachlor was measured using a novel method which measures the amount of released alongen atom as a consequence of the conjugation reaction. As detailed in Tab. 3.4, GSTU2 isoform exhibits a specific activity towards alachlor twice higher than that of GSTU1 thus confirming the crucial role played by *Cs*GSTU2 in the tolerance to this chloroacetanilide herbicide. These results become more relevant when the expression data of the transgenes are also considered. It has previously shown that GSTU1 transcripts are about 8 times higher than that observed for the GSTU2 isoform, thus emphasizing that the detoxifying ability against alachlor of transformed line is not due to a higher level of gene expression but to GSTU2 intrinsic kinetic properties. These results support the correlation of specific GST catalytic activity towards xenobiotics, rather than increased overall GST activity, with herbicide tolerance (Deng et al., 2002). Furthermore, the characteristic lack of GPOX activity in the *Cs*GSTU isoforms suggests that this kind of activity has no significant contribution to alachlor tolerance, at least under the conditions that were used, as also shown in tobacco plants overexpressing the *Gm*GST4-4 (Benekos et al., 2010). This is possibly due to the fact that chloacetanilide herbicides act primarily by inhibiting metabolic processes such as very long chain fatty acid synthesis which does not mainly involve generation of oxidative stress (Böger et al. 2000).

Specific Activity (mmol/min/mg)	
	Alachlor
GSTU1	19,50 ± 2,54
GSTU2	40,70 ± 6,24

Tab. 3.4 Values of specific activity obtained by colorimetric assay. Data are mean±SE (n = 4).

3.5 Drought and salinity stress tolerance

To understand the function of *Cs*GSTs under drought and salt stress conditions, root elongation of *Cs*GSTU transgenic and *wild type* seedlings was evaluated during growth on NaCl and mannitol containing substrates, as described in Material and Methods. When exposed to 150 mM NaCl *wild type* seedlings show evident root growth retardation, whereas all transgenic seedlings grow normally (Fig. 3.13). However, at 200 mM NaCl both the *wild type* and transgenic seedlings exhibit a reduction of root lenght, with the exception of the *Cs*GSTU2 overexpressing tobacco line whose root length is as long as untreated control roots indicating a high level of tolerance to NaCl (Fig. 3.13). The effect of drought stress upon root elongation was measured by growing seedlings in the presence of 8% mannitol. As shown in Fig. 3.13, treated wild type seedlings disclose a sharp decrease of root length, this representing the more severe effect registered among the imposed stress conditions. Although transgenic lines undergo to a reduction of root elongation

compared to untreated samples, they appear to better tolerate drought stress conditions as the mean root length is significantly higher (+25%) than that of treated tobacco wild type seedlings (Fig. 3.13). The aforementioned results indicate that transgenic tobacco lines overall respond better than the wild type to these abiotic stressful conditions. Similar results have been found with *Pj*GSTU1 from *P. juliflora* which confer drought tolerance on the transgenic tobacco (George et al., 2010). Moreover, it has been shown that overexpression of *Arabidopsis At*GSTF10 and *S. brachiata Sb*GSTU, respectively, confer higher tolerance to salt in the transgenic plants (Ryu et al., 2009; Jha et al., 2010), and overexpression of *Pp*GST in tobacco enhanced the tolerance of transgenic lines to damage caused by drought, NaCl, and Cd stresses (Liu et al., 2013). Better performance of transgenic tobacco lines under abiotic stress is attributed to the accumulation of the antioxidant GST enzyme which efficiently protects the transgenic tobacco from the oxidative stress. But the mechanisms through which GSTs protect plants against oxidative stress are far from to be clear. The abiotic stresses including drought, salt, Cd and so on inhibit plant growth, interrupt morphological, physiological, biochemical processes, and generate ROS and lead to oxidative damage. GSTs are involved in abiotic stress tolerance, and the improved tolerance of the GSTs overexpressing plants may be due to enhanced levels of ROS scavenging enzymes which eliminate excessive H_2O_2, superoxide anion and other ROS. Some GSTs possess GSH dependent peroxidase (GPOX) activities through which the organic hydroperoxides of fatty acids and nucleic acids are reduced to the corresponding monohydroxic alcohols with GSH

as the electron donor. However, it has been demonstrated that *Cs*GSTUs, both wild type and mutants, do not function as glutathione peroxidase *in vitro* [this thesis]. Therefore, their role in counteracting oxidative injuries caused by stress remains unclear.

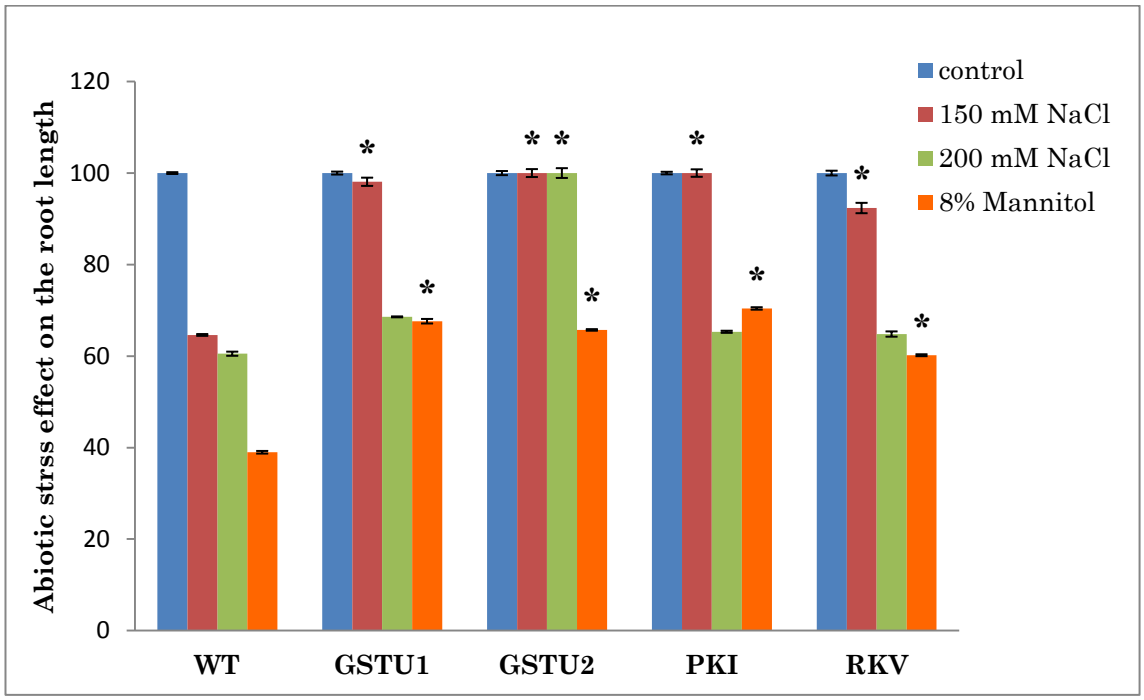


Fig. 3.13 Comparison of average root length of wild type and *Cs*GSTU overexpressing tobacco transgenic lines grown for 2 weeks in MS medium supplemented with 150 mM NaCl, 200 mM NaCl and 8% mannitol. Relative average root length values of wild type and transgenic plants were converted to percent values, compared to average root length of untreated plants. Data are mean±SE (n = 4). Asterisks depict significant differences at P≤0.05 of the transgenic lines compared to wild type plants.

3.6 Response of transformed tobacco plants to P. syringae pv. tabaci (Pstab) inoculation

It has been known that *Pstab* produces tabtoxin that induces the development of characteristic yellow halos around the necrotic lesions on tobacco leaves during wildfire disease development (strains Tox^+). In order to understand the response of tobacco plants overexpressing the *Cs*GSTU genes to *P. syringae* pv. *tabaci*

inoculation, fully expanded leaves from 8-week-old plants were infiltrated with a bacterial suspension of the Tox$^+$ 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. 3.14). As shown in Fig. 3.14, three days after inoculation the infiltrated tissues show different symptoms depending on the inoculated genotype and the dose-inoculum. Symptoms include chlorosis, water-soaking and desiccation of the infiltrated area. The first concentration at which all the genotypes show interaction-symptoms is 10^6 cfu ml^{-1}. In particular, plants over-expressing *Cs*GSTU1 (REI) and *Cs*GST-PKI show symptoms starting from a bacterial concentration of 10^4 cfu ml^{-1}, whereas untransformed plants and those over-expressing GST-RKV as well as GSTU2 (PKV) show symptoms starting from 10^6 cfu ml^{-1}, although at this concentration the leaf panels of *Cs*GSTU2 transformed plants are only partially involved (Fig. 3.14).

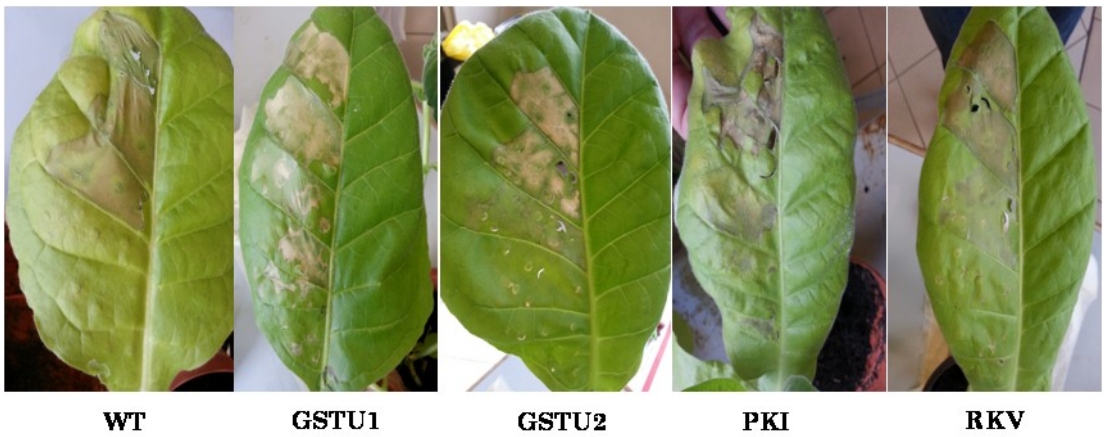


Fig. 3.14 Leaves from 8-week-old wild type and transgenic tobacco plants infiltrated with different concentrations (from 10^3 to 10^8 cfu/ml) of bacterial suspension of the *P. syringae* pv. *tabaci* Tox$^+$ DAPPG-PG 676 strain.

Consequently, all the genotypes were inoculated with a *P. syringae* pv. *tabaci* DAPPG-PG 676 strain bacterial suspension at 10^6 cfu/ml concentration in multiple sites of at least three leaves distributed in different plants. The representative symptoms are summarized in Fig. 3.15. Three days after inoculation, the untransformed tobacco plants disclose brown to black water soaking of the infiltrated area with a black margin as well as observable chlorotic halos (Fig. 3.15). Plants transformed with the empty vector exhibit the same interaction phenotype of the untransformed tobacco plants (data not shown). Leaves of transgenic tobacco plant harboring either GST-RKV or GST-PKI genes show both black water soaked lesions and chlorosis and black margins of the infiltrated areas (Fig. 3.15). In tobacco plants over-expressing either *Cs*GSTU1 or *Cs*GSTU2 genes the inoculated panels show the collapse of mesophyll and appear as dry and necrotic, whereas no chlorosis is observed. Ten day after inoculation all the infiltrated panels become necrotic and only the wild type plants and the transformed plants overexpressing GST-RKV show broad chlorotic halos (data not shown). In order to assess if the differences observed in symptomatology depend on the interaction between plant tissue and metabolites secreted out by the bacteria, culture filtrates from *P. syringae* pv. *tabaci* DAPPG-PG 676 grown in chemically defined medium already exploited to isolate tabtoxin were prepared (Woolley et al., 1952) and used to inoculate leaf panels as described in Material and Methods. As shown in Fig. 3.16, the effect of infiltration of the cultural filtrates in tobacco leaves of both transformed and control plants is perceptible at three dpi. Leaf panels of the wild type tobacco leaves as well as those belonging to plants over-expressing GST-PKI and

GST-RKV show diffuse chlorosis with a more evident effect in wild type tobacco plants. Leaves of plant harboring *Cs*GSTU1 and *Cs*GST2 disclose mesophyll collapse and necrosis but not chlorosis thus suggesting that bacterial culture filtrates are able to induce the same effects of the inoculated bacteria. The results reported above indicate that the overexpression of *Cs*GSTU1 and *Cs*GSTU2 in tobacco plant bestow the capability to avoid active toxin diffusion in plant tissues blocking chlorotic halos formation. Interestingly, this defensive role is not achieved in tobacco plants transformed with the GST mutate genes (RKV, PKI) suggesting that a specific mechanism of substrate-catalytic site identification is required to gain toxin inactivation. In this regards, we propose that probably tabtoxin is head towards a modification pathway in which wild type *Cs*GSTs could be involved in.

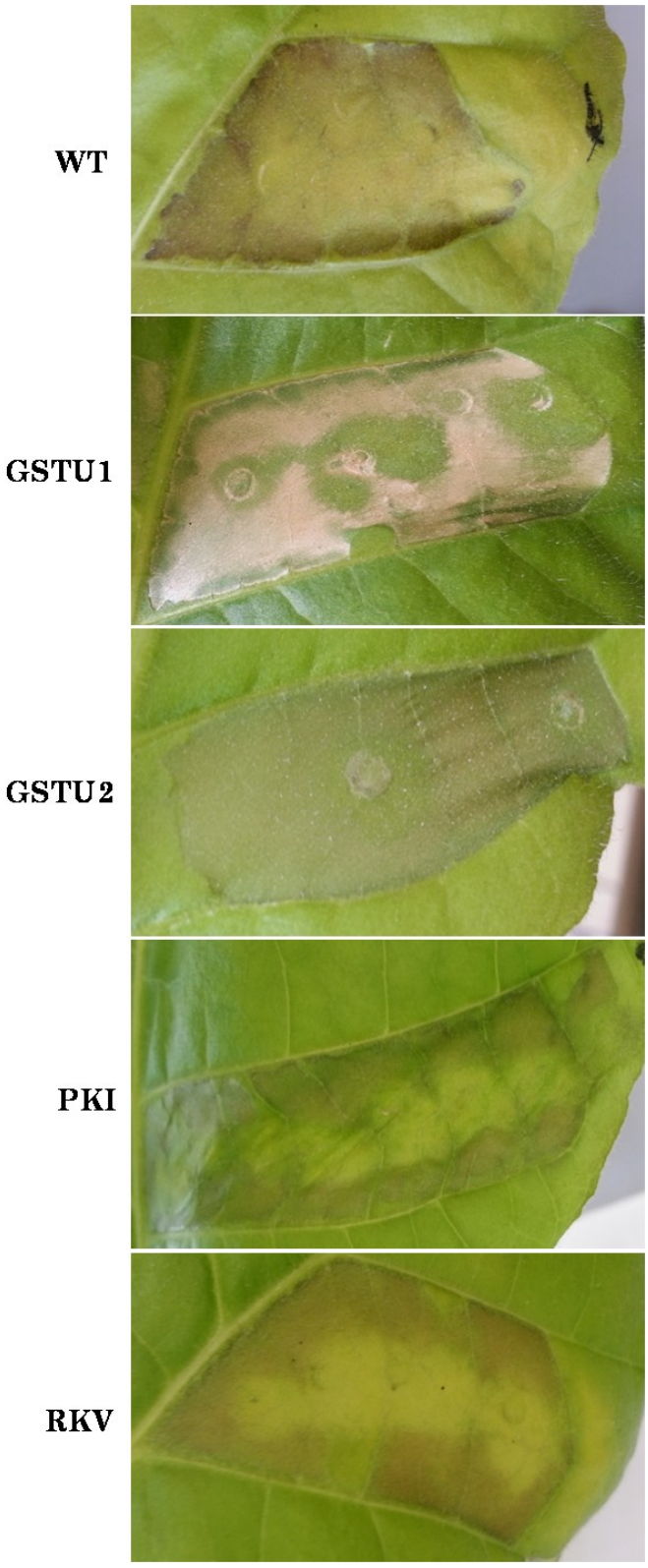


Fig. 3.15 Symptoms at three days after inoculation, of untransformed and transformed tobacco plant overexpressing GSTUs genes.

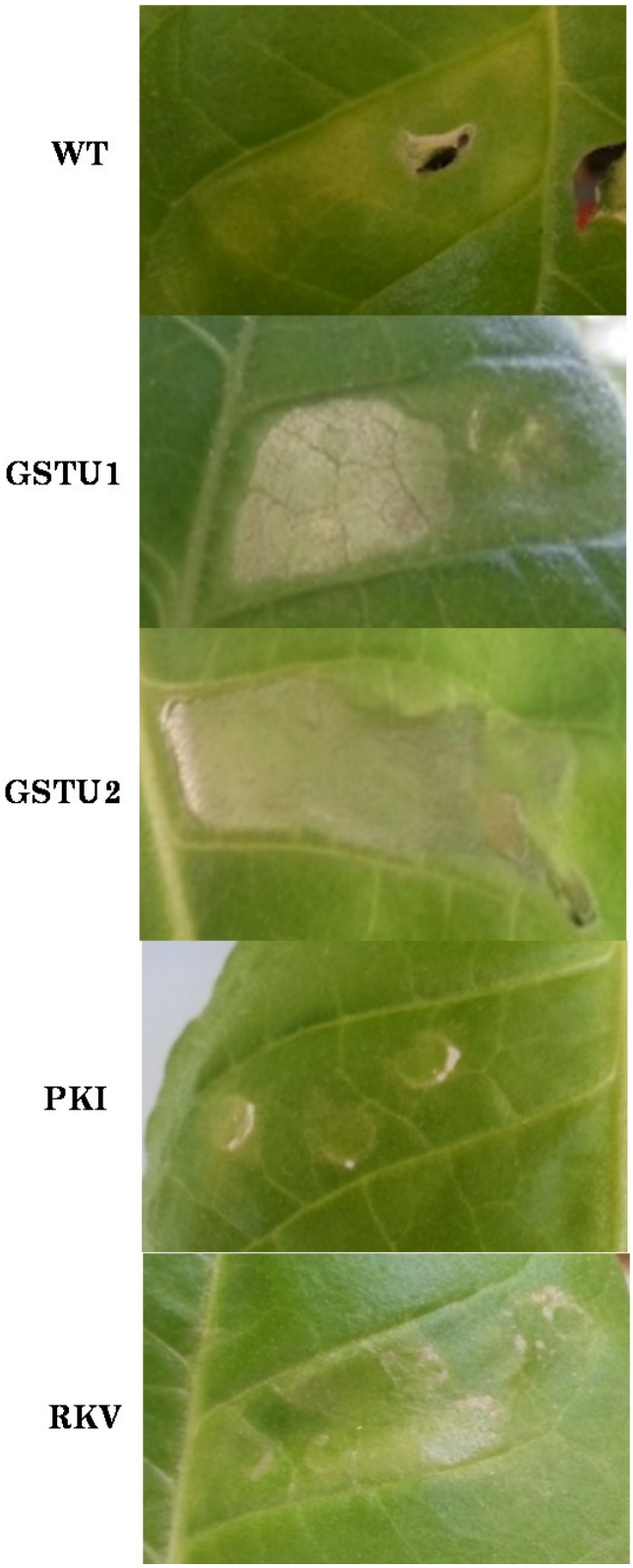


Fig. 3.16 Inoculated leaf panels with culture filtrates from *P. syringae* pv. *tabaci* DAPPG-PG 676.

3.7 Bacterial population tracking in tobacco leaves following Pstab inoculation

To determine whether the different interaction-symptoms observed among the different genotypes reflect the degree of bacterial growth, *Pstab* population was monitored in leaves of all genotypes under investigation at 3 and 10 days post inoculation (dpi).

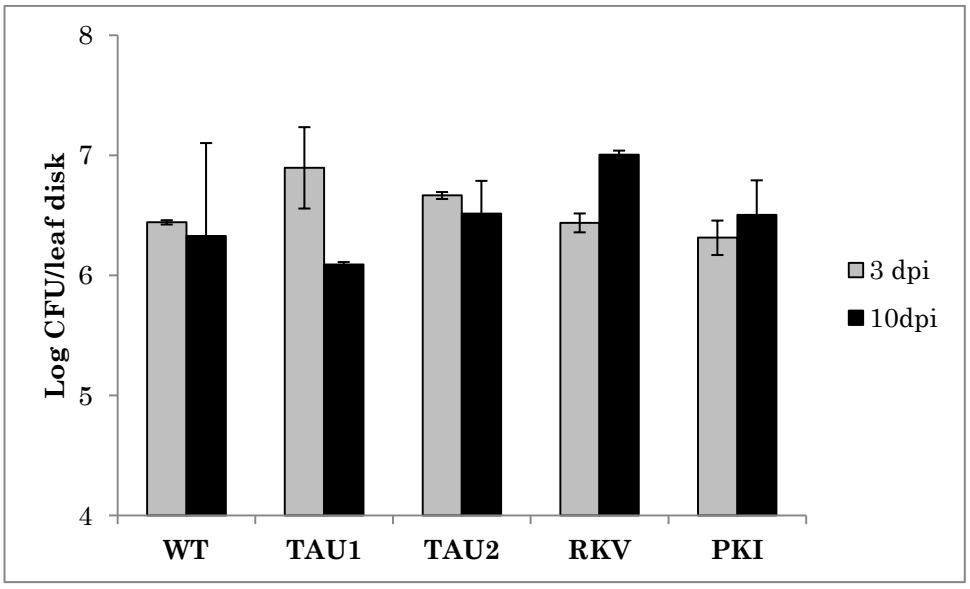


Fig. 3.17 Growth of *P. syringae* pv. *tabaci* NCPPB 676 in leaves of GSTU1, GSTU2, RKV and PKI transgencic tobacco line and wild-type tobacco. Fully expanded leaves of 8 week-old tobacco plants were inoculated with 10^6 cfu/mL of *P. syringae* pv *tabaci* DAPP-PG 676. Bacterial populations was determined three and ten days post inoculation (dpi). Values are the means of three samples, each consisting of two leaf discs from three different plants.

A significant reduction of approximately one log in bacterial population was observed exclusively in tobacco plants overexpressing CsGSTU1 at 10 dpi (Fig. 3.17) thus highlighting that in transformed tobacco plants mechanisms limiting the bacterial grow are not triggered, at least in the early phase of the infection (3 dpi).

3.8 Characterization of host-pathogen interaction

Since the interaction of *P. syringae* pv. *tabaci* with the tobacco plants harboring *Cs*GSTU1 and *Cs*GST2 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 hr) of the host tissue precisely confined to the inoculated area (Goodman et al., 1994). Hypersensitivity indicates either gene-for gene resistance or non-host resistance. In a hypersensitive reaction, bacterial populations decline and surviving bacteria are confined to the inoculation site. Thus, a time course growth experiment was performed by inoculating a bacterial suspension in the *Cs*GSTU1 and *Cs*GSTU2 transgenic tobacco lines and comparing the developing bacterial population to that of the untransformed plants over a 5-day experimental period (Fig. 3.18). As described in Fig. 3.18, the growth of *P. syringae* pv. *tabaci* detected in the transgenic lines do not differ from that observed in the wild type tobacco plant at any of the sampling date thus suggesting that the hypersensitive resistance response is not implicated in the *Pstab*-transgenic plants 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 recognised based on their common molecular patterns. 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 recently carried out (Szatmari et al., 2006). Among the identified representative genes associated with BR, a member of GST superfamily play a role in defence reactions as it was strongly activated after bacterial injections. Therefore, although the roles of GSTs in host-pathogen interaction remains still unclear, it represents a fruitful field to be studied.

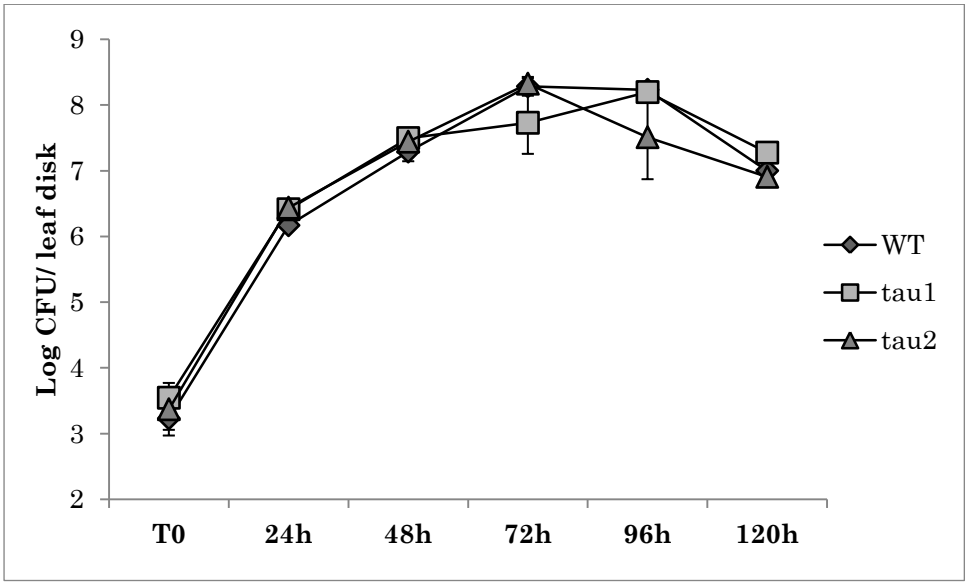


Fig. 3.18 Growth of *P. syringae* pv. *tabaci* DAPP-PG 676 in leaves of GSTU1, GSTU2 transgencic tobacco line and wild-type tobacco. Fully expanded leaves of 8 week-old tobacco plants were inoculated with 10^6 cfu/mL of *P. syringae* pv *tabaci* NCPPB 676. Bacterial populations was determined 0, 24, 48,72, 96, 120 hour post inoculation (hpi). Values are the means of three samples, each consisting of two leaf discs from three different plants.

Conclusions

In this PhD thesis it is reported the generation and characterization of transgenic tobacco (*Nicotiana tabacum* L.) plants over-expressing two different tau glutathione S- transferases from *Citrus sinensis* (*Cs*GSTUs) and several mutate forms of these genes derived from site-directed mutagenesis experiments. The transformed plants express the transgenes at high levels indicating that they have been inserted in fully transcriptional active regions of the tobacco DNA. *In planta* studies to assess the ability of transformed plants in detoxifying diphenyl ether and chloroacetanilide herbicides (alachlor and fluorodifen) were also performed. Simultaneously, the *Cs*GSTUs genes, were *in vitro* expressed and the catalytic activity the GST isoforms were determined against herbicides in order to correlate the response to the herbicides observed *in planta* with enzyme properties exhibited *in vitro*. As regards the fluorodifen tolerance analysis, the data reported here suggests that all transformed tobacco plants gain fluorodifen tolerance measured as decrease of electrolyte leakage. Among the transformed plants those harboring *Cs*GSTU2 better tolerate alachlor injury and this strong herbicides counteracting activity is positively correlated with the high specific conjugation activity of the *in vitro* expressed protein. Consequently, the herbicide-tolerant transgenic tobacco plants, which are described in the present study, can be utilized for phytoremediation of residual xenobiotics 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) and increased herbicide degradation

capacity, through *Cs*GSTU2 catalytic conjugation of alachlor with glutathione, these plants are good candidates for designing phytoremediation strategies for alachlor polluted agricultural soils. Transgenic lines over-expressing *Cs*GSTs are also able to counteract drought stress, whereas those transformed with *Cs*GSTU2 gene appear to better tolerate both salt and drought stress according to a mechanism far to clear. Generally, better performance of GST over-expressing lines under salt and drought stresses is attributed to the enhancement of antioxidant enzymes. In this study, we found that none of the *Cs*GSTs, wild type and mutants, exhibit GPOX activity. However, it has been shown that overexpression of a specific GST/GPOX gene might influence the expression of the other antioxidant genes at times. Compared to the non transgenic seedlings, the GST transgenic tobacco seedlings simultaneously show higher GST, ascorbate peroxidase (APX), and monodehydroascorbate reductase (MDHAR) activities. (Roxas et al., 2000). Consequently, we cannot exclude that the salt and drought tolerance of transgenic tobacco plants can be related with transgene induced activation of tobacco genes involved in the relief from oxidative injury. In this case, the fluorodifen tolerance could be partially due to this induction process. Finally, tabacco plants over-expressing *Cs*GSTU, either wild type or mutate forms, were subjected to infection with the host pathogen *Pseudomonas syringae tabaci*, this being, as far as it is known, the first report on host-pathogen interaction upon GST transgenic plants. The over-expression of *Cs*GSTU1 and *Cs*GSTU2 in tobacco plants avoids the active toxin diffusion in plant tissues blocking chlorotic halos formation probably because tabtoxin is head towards a modification

pathway in which *Cs*GSTs could be involved in. As, this defensive role is not achieved in tobacco plants transformed with the GST mutate genes (RKV, PKI) a specific mechanism of substrate-catalytic site identification is required to gain toxin inactivation. Thus, an important conclusion is the increased significance of detailed evaluation of the specific enzymatic activity of GST isoenzymes to be utilized in the generation of abiotic and biotic stresses resistant transgenic crops.

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