

THE USE OF HIGHLY SPECIFIC GSTS TOWARDS THE DEVELOPMENT OF STRESS TOLERANT TRANSGENIC PLANTS

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ABSTRACT

Glutathione transferases (GSTs) belong to a super-family of multifunctional proteins. GSTs play a key role in cellular detoxification from xenobiotic substances like herbicides, secondary metabolites and toxic degradation products resulting from oxidative stress and cellular metabolism. Furthermore, environmental conditions generate oxidative stress, the products of which have to be detoxified by plants.

It is anticipated that environmental stresses will worsen over the following years due to climate change. Hence, plants must adapt rapidly to the new environmental conditions in order to both survive and satisfy the constantly increasing human demand for agricultural products. Genetic engineering has been successfully used to develop plants resistant to stresses and, having taken all the necessary precautions, could offer a solution as it can help to develop plants with desirable traits in a short period of time.

We present here the use of GST isoenzymes in the development of transgenic plants. Although transgenic plants over-expressing various GSTs have been used for “*in planta*” evaluation of the enzymes used in response to different stresses, the results show that GSTs could be of great value for generating stress tolerant plants. However, the literature is limited and more studies should be performed in order to exploit their full potential.

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Keywords: Glutathione transferase, GST, transgenic plants, herbicide detoxification, salinity, drought and temperature stresses, heavy metal tolerance

1. INTRODUCTION

Biotic stresses (caused by other living organisms, such as bacteria, viruses, fungi, parasites etc.) and abiotic stresses (caused by non-living factors, like temperature, salt, water deficit, heavy metals, UV radiation etc.) affect all forms of life, but since plants are immobile they are particularly sensitive to environmental conditions and thus are forced to develop sophisticated systems to overcome them. These stresses, such as drought, salinity, high and low temperatures, fungal infections, bacteria and weeds are the most harmful factors affecting the growth and productivity of crops worldwide. It has been claimed that abiotic stresses cause the highest crop loss compared to any other factor and can result in major crops being reduced in yield by as much as 50%. It has also been speculated that this yield reduction will only increase in the future due to the dramatic climate changes expected. For example, production of cold weather crops, such as rye, oats, wheat and apples, is expected to decline by about 15% in the next fifty years and that of strawberries will drop as much as 32%, simply because of the projected rise in temperature by a few degrees [1]. Plants are extremely sensitive to such changes and do not generally adapt quickly.

Agriculture is obliged to provide increased plant material for food, feed, fuel, pharmaceuticals, fumes (as from perfumes), and products for entertainment (fun) like coffee, chocolate, tea and spices. All these have to be produced by plants cultivated in a reduced soil area, using decreasing amounts of water and chemical inputs, while the world population will continue to increase as well as the demand for agricultural products. Moreover, environmental constraints are expected to intensify as global warming and climate changes are taking place faster than anticipated. Climate change will result in increased temperatures and water shortages, hence it is expected to cause serious salinization of more than 50% of all arable lands by 2050 [2, 3]. In addition, large soil losses are expected due to the increase of the sea level and desertification [2, 4], thus a new “green revolution” will be necessary in order to feed the world population.

Biotic and abiotic stresses, like drought and temperature, cause oxidative stress in plants which results in cellular damage, yield loss and even death of plants. Plants carry an arsenal of defence mechanisms against these stresses. Some of these are enzymes, like glutathione transferases (GSTs), superoxide dismutase (SOD), catalase (CAT) and ascorbic acid oxidase (AOX), which are responsible for scavenging ROS and removing xenobiotic molecules [5-8] (Figure 1).

Glutathione transferase (GST, EC 2.5.1.18.) isoenzymes have been identified in animals, plants and fungi [9]. The plant GST family of proteins includes six distinct classes (phi, tau, zeta, theta, dehydroascorbate reductases and lamda) [7, 10]. The phi and tau class GSTs are only found in plants. In contrast, the zeta and theta class GSTs are conserved in animals and plants [6, 11].

GSTs are abundant plant proteins which belong to a superfamily of proteins, with 54 members reported in *Arabidopsis*, over 25 in soybean and 42 in maize [12, 13]. GSTs have multiple functions in stress tolerance, such as the detoxification of xenobiotic compounds and

the prevention of oxidative damage [14, 15]. GSTs are thought to play a vital role in plant tolerance to biotic and abiotic stresses.

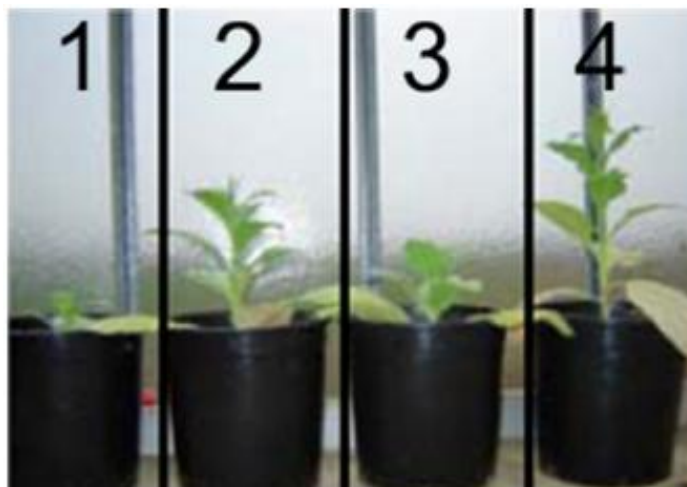


Figure 1. Tobacco over-expressing a soybean GmGSTU4 show increased tolerance against alachlor compared to wt plants 1) wild-type untransformed plants, 2 GST transgenic plants, 3) GST transgenic plants (sprayed with double dosage of alachlor compared to number 2), 4) wild-type untransformed plant (not sprayed) [5].

The mechanism of GST detoxification in the plant involves the conjugation of electrophilic substrates to the tripeptide glutathione (γ -glutamyl-cysteinyl-glycine, GSH). Plant GSTs can also reduce the levels of toxic organic hydroperoxides. Furthermore, several plant GSTs can also function as glutathione-dependent peroxidases (GPOX), catalyzing the reduction of oxidative stress products such as organic hydroxyperoxides. Moreover, GST plants are known to function in hormone homeostasis, vacuolar sequestration of anthocyanin, tyrosine metabolism, hydroxyperoxide detoxification and regulation of apoptosis.

Over the past decade, our knowledge of plant adaptation to environmental stress has grown considerably. The problem with stress tolerance is that it is multigene controlled, making it difficult to apply to conventional breeding. It is thus very important and urgent to deploy biotechnological and molecular breeding methods in order to develop stress tolerant plants and continue to supply humans with food [4].

Genetic engineering and plant transformation technologies have played a central role in studying a plethora of biological processes and, in particular, plant stress tolerance. As plant breeding and development of novel characteristics in plants, like tolerance to drought, high and low temperature, would need to be performed in a very short period of time, stress tolerant transgenic plant technology could be the only alternative method [16].

A rapid development of plants tolerant to abiotic stresses might be crucial due to the quick pace of climate changes taking place. Overall, antioxidant enzymes like GSTs are very important enzymes that could have a huge impact on plant tolerance to biotic and abiotic stresses, although there are still missing links in the field of the stress tolerance mechanism. The elucidation of them is of great interest in order to develop stress resistant plants, including herbicide tolerant plants. Developing transgenic plants and plants adaptive to stresses quickly might be of extreme importance towards this goal. There are five major developing countries cultivating biotech crops: China and India in Asia, Brazil and Argentina

in South America and South Africa in Africa, with a combined population of 2.7 billion (40% of global population). In addition, the potential of transgenic technology is emphasized by the decision of the Chinese government to invest around \$3.5 billion over 13 years on genetically modified plants (allocated in research, development and public education) [17].

2. THE USE OF HIGHLY SPECIFIC GSTs TOWARDS THE DEVELOPMENT OF STRESS TOLERANT TRANSGENIC PLANTS

2.1. Transgenic Plants Conferring Tolerance to Herbicides

The most studied group of plant enzymes involved in herbicide metabolism is by far the GSTs, which catalyze the conjugation of the major cellular thiol, GSH, to an electrophilic site of lipophilic substrates. The catalytic mechanism involves the addition of the GSH thiolate anion to the substrate or release of a leaving group (e.g. a halogen or phenolic moiety) from the substrate. GSTs are involved in the detoxification of herbicides, a highly significant function with potential use in agriculture and industry. The first report of GST isoenzymes being involved in detoxification of herbicides was published more than 40 years ago [18], but still the corresponding literature is limited. Edwards and Owen (1986) have described the tolerance conferred by GSTs against the herbicides s-triazine, chloroacetanilide and thiocarbamide in plants [19]. Nevertheless, the role of GSTs in the protection from xenobiotics was proved much later, when expression of the maize GSTIV in tobacco provided protection from metolachlor [20].

Maize GST-27, which belongs to the theta class [21], was introduced into wheat. The transgenic plants became resistant to herbicides belonging to the chloroacetanilide group (alachlor and dimethenamid) and the thiocarbamate herbicide EPTC. In contrast, GST-27 did not provide any tolerance against atrazine or oxyfluorodifen [22]. Although the research of Milligan et al aimed at the development of selectable markers, it showed the potential of GSTs for developing herbicide resistant plants.

Using an antisense approach, Deng et al [23] have transformed Lemont rice (*indica*×*japonica*) with the OsGST III subunit from rice in an antisense orientation. Transgenic lines were sensitive to pretilachlor. GST activity utilizing cinnamic acid, 1-chloro-2,4-dinitrobenzene (CDNB), and pretilachlor as substrates was reduced in transformed rice, suggesting that the OsGST III gene plays an important role in the detoxification of the herbicide pretilachlor and the metabolism of phenolic compounds.

Tobacco transgenic plants over-expressing a cotton GST gene, which had been shown to have both GST and GPX (Glutathione Peroxidase) activities, developed enhanced tolerance to methyl viologen (0.03 mmol/L) [24]. Unfortunately, no other herbicide has been tested, while, at the same time, the authors did not mention the number of transgenic copies in their lines, thus it is not possible to determine if the tolerance is the result of a “gene dosage” effect.

The maize GST I isoenzyme (belonging to the theta class), shows significant catalytic activity for the chloroacetanilide herbicide alachlor and appears to be involved in its detoxification. The gene encoding GST I was introduced into tobacco plants. The transgenic plants showed substantially higher tolerance to alachlor compared to non-transgenic plants in

terms of root, leaf and vigorous development when grown on MS medium supplemented with alachlor [8]. Thus, if GSTs are involved in the detoxification of herbicides, they could be of great use in the development of a phytoremediation system for the degradation of herbicide pollutants in agricultural fields. Tobacco plants which are susceptible to fomesafen were transformed with a soya GST gene from the U class and with a dual construct (hGSH and GST). Plants carrying the dual construct were significantly more tolerant to fomesafen compared to wild-type tobacco plants, while plants carrying only hGSH did not exhibit higher protection compared to the wild-type, suggesting that protection requires not only the GST gene but also hGSH in order to become resistant. In contrast, wild-type tobacco was highly susceptible to the herbicide fomesafen [25].

OsGSTL1 is a glutathione transferase lambda class gene from rice (*Oryza sativa* L.). Over-expression of OsGSTL1 in rice enhanced tolerance to chlorsulfuron and glyphosate. [26].

Benekos et al [5] have over-expressed a soybean GmGSTU4 in tobacco and found that the transgenic plants had increased tolerance to the diphenyl ether herbicides fluorodifen and oxyfluorfen (200 μ M) and the chloroacetanilide alachlor (7.5 mg/L). The tolerance was expressed as reduced electrolyte leakage when compared to wild -type plants.

As described above, GSTs are enzymes able to enhance plant tolerance to herbicides. The first transgenic herbicide crops were introduced in the United States in 1996. These were soybean plants resistant to glyphosate. Later, cotton and maize transgenic herbicide resistant plants were developed. Currently, almost 74 million hectares in 13 countries are being cultivated with five different genetically engineered herbicide tolerant crop species [27]. In order to develop herbicide tolerant plants, safe for both the consumer and the environment, the genes introduced into the plants must lead to a complete detoxification of the herbicide. However, the formation of herbicide conjugates with GSH fundamentally alters their physicochemical properties, rendering the compounds more polar and unable to freely diffuse across cellular compartments or translocate throughout the symplast. Furthermore, GSH-herbicide conjugates are usually the target of further metabolism and can be accepted by energized carriers for their transport out of the cytosol. In order to develop and commercialise herbicide resistant plant species, toxicity studies must be performed [28].

2.2. Transgenic Plants Conferring Tolerance to Abiotic Stresses

Environmental stresses, such as extreme temperatures, are among the major factors resulting in crop losses. GSTs have been shown to be involved in many abiotic interactions of plants with the environment and in stress tolerance mechanisms. Specific GSTs have been engineered in order to investigate their *in planta* function and the possibility of their enhancing plant stress tolerance.

Qi et al [29] have introduced a GST gene from *Suaeda salsa* into Arabidopsis. The specific gene, which was shown to have both GST and GPX activity [30], provided the transgenic plants with enhanced tolerance to salinity up to 200mM NaCl. They also showed almost normal root growth when compared to the wild-type plants despite their 1.5 fold lower photosynthetic rates. The plants also exhibited reduced lipid peroxidation and a high metabolic rate [29].

Another GST, which has dual glutathione transferase and glutathione peroxidase activity, was over-expressed in tobacco. The transgenic seedlings showed enhanced seedling growth when they were exposed to low temperatures or high salinity [31].

Later, Roxas et al [32] also over-expressed a tobacco GST in tobacco and found that the transgenic seedlings grew faster than wild-type, even under different stress conditions such as chilling, heating or salt stress and also showed reduced lipid peroxidation. These results suggest that plants over-expressing GSTs will have adaptive capability in a fast changing environment.

Takesawa et al [33] have developed transgenic rice over-expressing a rice zeta (ζ) class GST gene under the control of the ubiquitin promoter. Interestingly, the transgenic lines show increased germination and growth at low temperatures and, most importantly, even under submergence, which enables the direct sowing of rice in cooler regions, thus reducing the production cost.

Dianthus superbus transgenic plants over-expressing a *Nicotiana tabacum* GST showed enhanced acclimatization compared to the wild-type plants. The transgenic plants showed enhanced tolerance to high light intensity and increased photosynthetic rates under high light and in drought conditions. These characteristics can be extremely useful in the impending climate change conditions. In addition, the transgenic plants showed increased copper accumulation [34].

Tau class GST (GSTU) genes are plant specific, induced by different abiotic stresses, and important for protecting plants against oxidative damage. A GSTU (SbGST) gene, isolated from an extreme halophyte *Salicornia brachiata*, was over-expressed in transgenic tobacco plants, leading to enhanced seed germination and growth under salt stress compared to wild-type [35].

The GST from *Suaeda salsa* has been co-expressed, along with CAT, in rice. The co-expression resulted in a reduced generation of H_2O_2 . The plants showed significantly less oxidative damage under salt and paraquat stress conditions. It is interesting to note that GST activity increased only under the herbicide stress. The enhanced tolerance might be the result of the synergistic effect of the two enzymes and the increased SOD activity as well [36]. This suggests that a complex mechanism must be operating in plants which, depending on the diverse stresses, regulates the differential expression of GSTs. [37]. It is thus important to acquire the missing knowledge on GST regulation under different stresses in order to understand the underlying mechanisms and breed/improve plants to withstand extreme climates.

Zhao et al [38] found later that the transgenic rice plants proved to be tolerant to cold, heat and their combinations. The authors also state that the increased tolerance of transgenic plants might be attributed not only to the high levels of expression of the transgenes but also to the coordinated action of the ascorbate-glutathione cycle in the transgenic plants, probably due to differences in H_2O_2 , but this has yet to be proven.

Thus far there has been only one report on transgenic plants over-expressing a GST gene that failed to show enhanced tolerance [39]. Light et al [39] have reported that transgenic cotton seedlings over-expressing the tobacco GST (Nt107) failed to provide enhanced tolerance to salt, cold, or herbicides (atrazine and imazethapyr), even though they exhibited five to ten fold higher GST activity compared to wild-type plants. Yet, we should take into consideration that the authors have used 200 mM NaCl, while 100 to 150 mM NaCl is the usual dosage and also that they do not refer to any preliminary results on the NaCl dosage. A

phi class GST from *Arabidopsis* (AtGST10) proved to confer tolerance to salt and disturbed redox status when over-expressed in *Arabidopsis* transgenic plants. Moreover, it should be mentioned that down-regulation of AtGSTF10 via RNA interference caused reduced tolerance to abiotic stress [40].

Prosopis juliflora is a drought-tolerant tree species of Fabaceae, which is used as a model plant system in order to identify genes involved in abiotic stress tolerance, like the PjGSTU1 gene. Transgenic tobacco lines carrying a PjGSTU1 gene survived under conditions of 15% PEG stress, which acts as a drought stress, compared to wild-type plants [41].

In another study, a novel GST gene from *Limonium bicolor* (LbGST1) has been cloned and over-expressed in tobacco. Transgenic LbGST1 tobacco lines exhibited both GST and glutathione peroxidase activities. In addition, superoxide dismutase, peroxidase (POD), and catalase activities in transgenic plants were significantly higher compared to wild-type plants, particularly when grown under salt stress. LbGST1 was found to be localised in the nucleus, suggesting a possible role in mediating certain physiological pathways or protecting the DNA from oxidative damage [42].

Tobacco plants over-expressing the GsGST gene from *Glycin soja* (a soybean species which can survive in high salt and drought conditions) showed enhanced dehydration tolerance and T₂ seedlings showed higher tolerance to salt and mannitol than wild-type plants, during the seedling stage, as measured by longer root length and less growth retardation [43].

Arabidopsis thaliana has been sequenced [44] and thus far 54 GST genes have been identified [45]. Gong et al [46] investigated the “*in vivo*” function of the mustard gene BjGSTF2. The transgenic plants were tolerant to HgCl₂ and paraquat and flowered two days earlier compared to wild-type. Moreover, transgenic plants over-expressing the GST gene were highly regenerative, suggesting that GST plays a role in plant growth and development *in vivo* and shoot regeneration *in vitro*.

Finally, transgenic tobacco and tomato plants over-expressing a specific soybean GmGSTU4 isoenzyme have been created in our laboratory and show enhanced tolerance to abiotic stresses such as drought and salt (unpublished results) (Figures 2 and 3).



Figure 2. Transgenic tomato plants over-expressing a specific soybean GmGSTU4 isoenzyme show enhanced tolerance to drought stress (unpublished results).



Figure 3. Transgenic tomato plants over-expressing a specific soybean GmGSTU4 isoenzyme show enhanced tolerance to salt stress (200mM NaCl, unpublished results).

2.3. Transgenic Plants Conferring Tolerance to Heavy Metals

Transgenic tobacco plants have been engineered to express a GST gene from the fungus *Trichoderma virens*. Transgenic plants with fungal GST showed enhanced tolerance to anthracene (a recalcitrant polyaromatic hydrocarbon) compared to control plants. Remediation of ^{14}C uniformly labeled anthracene from solutions and soil by transgenic tobacco plants was higher compared to wild-type plants. Transgenic plants (T_0 and T_1) degraded anthracene to naphthalene derivatives, while no such degradation was observed in wild-type plants. The present work has shown that *in planta* expression of a fungal GST in tobacco imparted enhanced tolerance, as well as higher remediation potential of anthracene, compared to wild-type plants. [49]

The same group has performed another study using the glutathione transferase gene from this fungus. When the GST gene was introduced into tobacco plants, the transgenic plants became tolerant to different concentrations of Cadmium (Cd) compared to wild-type plants, with transgenic plants showing lower levels of lipid peroxidation. Interestingly, the levels of different antioxidant enzymes, such as glutathione transferase, superoxide dismutase, ascorbate peroxidase and catalase, showed enhanced levels in transgenic plants compared to control plants. It is also very important to note that Cd accumulation in the plant biomass in transgenic plants was similar to, or even lower than, wild-type plants [50].

Two Aluminium (Al) induced genes from Arabidopsis, AtGST1 and AtGST11, were fused to GUS and over-expressed back in Arabidopsis. Transgenic lines showed GST induction by Al treatment as well as by cold stress, heat stress, metal toxicity and oxidative damage, suggesting a common induction mechanism in response to the tested stresses but

unfortunately the authors did not investigate the levels of plant tolerance against the stresses mentioned above [51].

2.4. Engineering GSTs Directly into the Chloroplast for Enhanced Plant Stress Tolerance

In an interesting approach, Martret et al [48] expressed GST and DHAR (dehydroascorbate reductase) in tobacco chloroplasts, either alone or in combination with DHAR and glutathione reductase. Although a GST gene has been expressed directly in the chloroplast before [47], the authors here have studied the effect of GST and the combinations used, on plant stress tolerance. Furthermore, methyl viologen treatment had the same effect in wild-type and transgenic plants (transplastomic in the case of genes introduced in the chloroplast) with the single gene, but the lines expressing the double constructs were significantly tolerant as measured by chlorophyll and malondialdehyde levels, membrane damage and electrolyte leakage. Moreover, all transplastomic plants were cold tolerant as their seedlings germinated even at 4⁰C, in contrast to wild-type. Transplastomic plants were also tolerant to high salt concentrations (200mM NaCl). It is very interesting that transplastomic plants are resistant to stresses. The chloroplast is the organelle where photosynthesis takes place and thus a place where reactive oxygen species are generated. In order to achieve high rates of photosynthesis, an efficient detoxifying mechanism needs to operate. Hence, plants which over-express ROS scavenging enzymes should perform better under stress conditions.

CONCLUSION

Plant adaptation and tolerance to stresses is of high importance for farmers, the agricultural industry and the economy, as they are the main constraints for high yield. Some of the most important factors that influence plant yield and quality are the abiotic and biotic stresses and weeds.

Reduction in plant productivity due to abiotic and biotic stresses, and even weeds, is expected to worsen in the following years due to climate changes and global warming. It is of paramount importance to understand the mechanism behind stress tolerance and develop stress resistant plants. In particular, because of the rate of changes taking place, new plant varieties will have to be produced in a very short period of time. Genetic engineering technology offers a quick alternative to classical plant breeding, although we should not exclude the latter from such an effort. The area cultivated with genetically engineered plants has increased from 1.7 million hectares in 1996 to 148 million hectares in 2010, showing the potential of genetically engineered plants and making biotech crops the fastest adopted crop technology in the history of modern agriculture. The GST family of proteins is part of the xenobiotic detoxification mechanism and is involved in biotic and abiotic stress tolerance in plants. It is noteworthy that, although there is vast literature on GSTs spanning several years, the exploitation of GST isoenzymes aimed at developing stress tolerant plants is limited, as reviewed here.

Hence, studies using genetic engineering to over-express GST isoenzymes in different plants show the potential of GSTs for the development of stress tolerant plants. Moreover, GSTs could offer increased tolerance to herbicides, cold, heat, drought and salt stresses, thereby facilitating agricultural practice and preparing for the forthcoming climate change.

ACKNOWLEDGMENTS

We would like to thank General Secretariat for Research and Technology Greece and the grant THALIS for financing our research. The action THALIS-"Glutathione transferases: multifunctional molecular tools in red and green biotechnology" falls under the Operational Programme "Education and Lifelong Learning (EdLL)" and is co-financed by the European Social Fund (ESF) and National Resources.

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