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ScienceDirect
Phytochemistry 68 (2007) 2614–2624
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Glutathione *S*-transferases in *Festuca arundinacea*: Identification, characterization and inducibility by safener benoxacor

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Received 21 March 2007; received in revised form 25 May 2007
Available online 20 July 2007

Abstract

Over recent years it has emerged how certain no crop-species can be employed in phytoremediating contaminated soils or preventing herbicide pollution; in this contest *Festuca arundinacea* was investigated. Shoots of *Festuca* were submitted to fast protein liquid chromatography in order to identify their glutathione *S*-transferases (GST; EC 2.5.1.18), by a combination of anionic, affinity and RP-HPLC chromatography. The chromatographic procedure revealed satisfactory yield and four GSTs were identified: they were named FaGST I, FaGST II, FaGST III and FaGST IV. Among these, significant differences were observed in the chromatographic behaviours, structure, activity toward a ''model'' substrate, 1-chloro-2,4-dinitrobenzene, and responsiveness to the herbicide safener benoxacor. FaGST I showed the highest activity toward the above substrate, and this activity was up-regulated by the herbicide safener. Therefore, FaGST I was purified till homogeneity and was determined to be an heterodimer consisting of two subunits of 28.0 and 27.2 kDa. Each subunit of FaGST I was further characterized by means of LC–ESI–MS/MS and immunoblotting analysis, which revealed that both the subunits belong to the tau subclass.

Keywords: *Festuca arundinacea*; Glutathione *S*-transferases; Isoforms; Benoxacor; Induction

1. Introduction

Glutathione *S*-transferases are a family of enzymes present in all eukaryotes; in the plant they can constitute more than 2% of the soluble proteins (Frova, 2003). These enzymes are involved in many cellular processes and some are relevant for their involvement in metabolism of electrophilic toxic compounds, both endogenous and exogenous. The mechanism of inactivation of the toxic substrate consists in its conjugation with the natural tripeptide glutathione (GSH). The *S*-glutathionylated product can be thereafter transferred into the vacuole, by means of ATP-binding cassette transporters (De Ridder et al., 2002). On the basis of analogies in the amino acid sequence, gene sequences, and immunochemical reactivity, GSTs have been grouped into six classes: phi, tau, zeta, theta, lambda and DHAR (Dixon et al., 2002; Frova, 2003). They have been found to be active both as monomeric and dimeric proteins. The dimers can be composed by the same subunit (homodimer) or by different subunits (heterodimer). In each case, the subunits have a molecular mass in the range of 25–30 kDa. In the case of heterodimers each subunit is encoded by a different gene (Edwards et al., 2000). The GST family is very divergent and very few amino acids are conserved among GSTs of different plants. GSTs belong to phi and tau classes have a very interesting role because they are implicated in the metabolism of electrophilic herbicides, drugs and other pollutants (Dixon et al., 1998, 1999). For crop species it was demonstrated that GSTs catalyse the conjugation of glutathione with many herbicides, such as chloroacetanilides, chloro-*s*-triazines, thiocarbamates, triazinone sulfoxides, solfonylureas, aryloxyphenoxypropionates, diphenylethers, thiodiazoli-

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doi:10.1016/j.phytochem.2007.05.041

dines and sulfonamides (Edwards and Cole, 1996; Hatton et al., 1999; Scarponi et al., 2003; Hatzios and Burgos, 2004; Scarponi and Del Buono, 2005). This wide spectrum of conjugative activity of GSTs toward different chemicals is due to the possibility to form many dimeric proteins by combination of different subunits. Each subunit shows an independent active site consisting of two functional components: a GSH binding site (G-site) and a second site which is able to bind the xenobiotic hydrophobic substrate (H-site) (Reinemer et al., 1996; Van Eerd et al., 2003). GST enzymes have another important function: protection against oxidative damages. Oxidative stress can cause membrane lipid peroxidation and oxidative injury to DNA, therefore, the role of GSTs is to contrast this oxidative injury by acting as peroxidase in reducing the organic hydroperoxides of fatty acids and nucleic acids to the respective alcohols (Edwards et al., 2000).

Under normal conditions some plant GSTs are constitutionally expressed in certain tissues while in stress situations, such as those caused by herbicide treatments, a significant enhancement of GST activity has been observed (Frova, 2003). In addition, some chemicals, called herbicide safeners, can regulate the expression of GSTs by activating them in order to protect the crops from herbicide damage. Safeners specifically induce enzymes involved in the herbicide metabolism of crops in relation to competing weeds (Davies and Caseley, 1999). GST expression is differentially regulated by safeners to induce specific enzymes with greater affinity for herbicidal substrates. Consequently, the tolerance toward some herbicides is increased because of the rise in their detoxification rate (Dean et al., 1991; Farago et al., 1994; Abu-Qare and Duncan, 2002; Hatzios and Burgos, 2004). Among the more diffuse safeners, benoxacor [(±)-2,2-dichloro-1-(3,4-dihydro-3-methyl-2H-1,4-benzoxazin-4-yl)ethanone] was found to be very effective in inducing herbicide detoxification in maize (Fuerst et al., 1993, 1995; Irzyk and Fuerst, 1993; Miller et al., 1994; Del Buono et al., 2006).

The GSTs of cultivated species have been extensively investigated, while the knowledge of GSTs for weed and no-crop species remains limited to very few plants, even though the number of these species that are developing herbicide resistance is increasing. Resistance to an individual class of herbicide can be attributed to one or more modifications in its specific target site in the plant, while resistance to multiple classes of herbicides can be a consequence of an enhanced herbicide metabolism. Consequently, the role played by GSTs in herbicide detoxification in weeds and no-crop species deserves attention. Generally, weed species show a lower level of expressed GSTs, and their herbicide metabolism is slower than crop species (Andrews et al., 2005). A recent study shows that the perennial grass, *Festuca arundinacea*, possesses a consistent GST detoxifying activity toward some synthetic compounds (Scarponi and Del Buono, 2005). These behaviours potentially make *Festuca* an interesting plant to vegetate buffer strips, which are zones left along the boundaries of crops to reduce the contamination of surface runoff by various pollutants, herbicides included (Delgado et al., 1995; Patty et al., 1997; Vianello et al., 2004).

As the *Festuca* GSTs have not been characterized for their activity in relation to their isoforms composition neither for their safener inducibility, the aims of this research were: (i) to develop a procedure, by a combination of different media for protein chromatography, to extract, identify and purify GSTs from *Festuca arundinacea*; (ii) to ascertain the effect on expression of identified GSTs of the safener benoxacor; (iii) to characterize the GST having the greatest activity, and the most benoxacor-inducible, by means of immunochemical analysis and LC–ESI–MS/MS determinations.

2. Results

2.1. Purification of GSTs extracted from shoots of Festuca

GSTs were purified from shoots of *Festuca* 12 days old by combination of anion-exchange chromatography and affinity chromatography using GSH as ligand. A summary of all chromatographic steps and the yields are given in Fig. 1. When the crude protein extracts were applied to Q Sepharose FF column, the GST(CDNB) was recovered in two fractions (pool 1 and pool 2) (Fig. 2). Pool 1 was eluted from the column at low ionic strength and was responsible for the minor amount of the total recovered GST activity (1.1%). Pool 2 was eluted from the column between fractions 18 and 30 at high ionic strength and was responsible for the greatest amount of the total recovered GST activity (84.5%) (Fig. 1). Fig. 2 also reports the electrophoretic profiles of crude extract, of desalted pro-

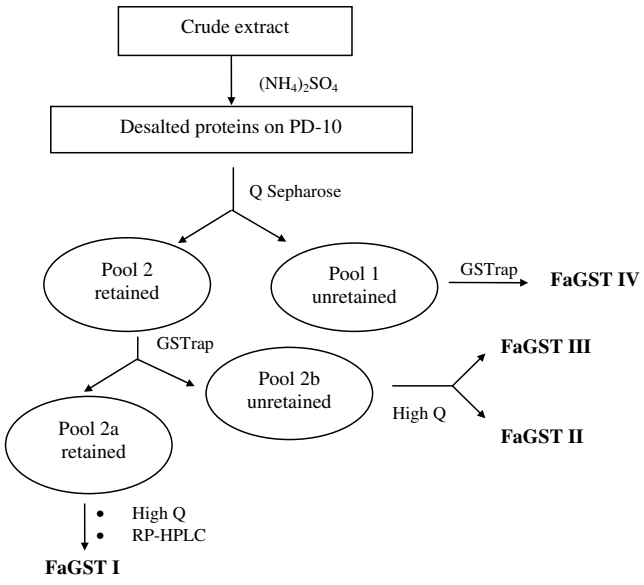

Fig. 1. Summary of the chromatographic steps performed to identify the different GST isoforms of *Festuca arundinacea*. At the bottom of the figure are reported the yields of the chromatographic steps.

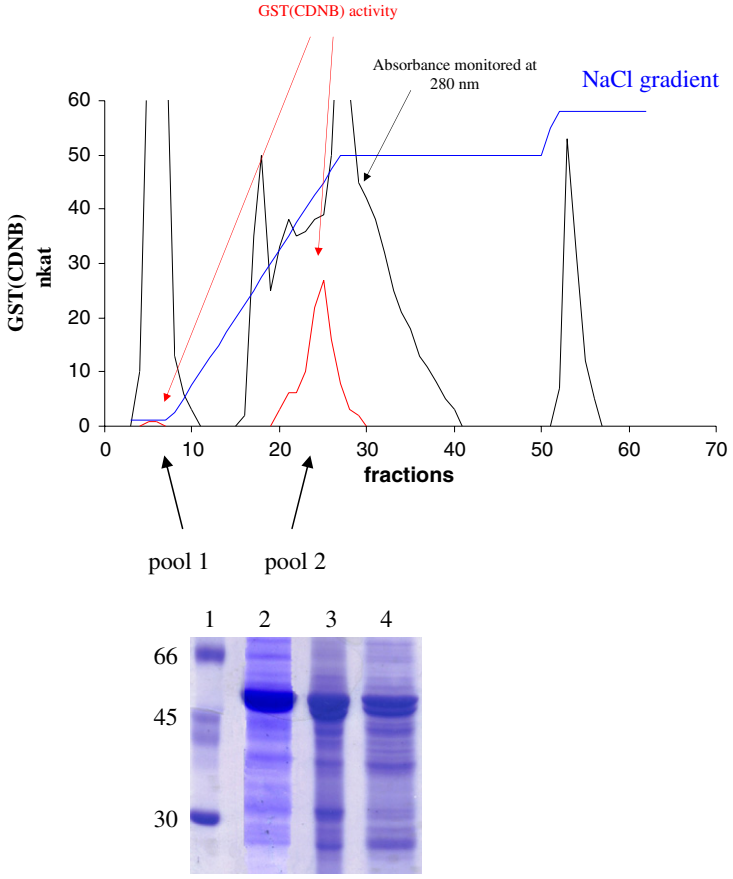

Fig. 2. The chromatographic profile, obtained using a Q sepharose FF column in gradient of NaCl, is reported at the top of the figure. Fractions were assayed for activity toward CDNB and the eluent was monitored at 280 nm. At the bottom of the figure are reported the electrophoretic analysis of the gel stained with Coomassie Brilliant Blue. Lane 1, molecular mass markers; lane 2, proteins of crude extract; lane 3, desalted proteins; lane 4, proteins fractioned by chromatography with Q Sepharose column showing activity toward CDNB.

teins and of proteins retained by the column (pool 2). From the profiles it is evident the presence of polypeptides with molecular masses in the 25–30 kDa range, which is typical for GSTs.

Pool 1 was concentrated and recharged on an Q Sepharose FF Column, in order to verify that this protein pool was not a consequence of column overloading. The same elution profile was observed, and pool 1 was charged on a GSTrap affinity column, and the fractions showing GST activity toward CDNB were eluted with 10 mM GSH and analysed by mono-dimensional SDS–PAGE. The subsequent electrophoresis analyses showed two polypeptides with molecular masses of 28.0 and 29.0 kDa, which were associated to the protein named FaGST IV (data not shown).

The proteins of pool 2 were charged on a GSH affinity column and two major activity peaks were observed (Fig. 3). The first peak of activity was loosely retained by the GSH affinity column; the corresponding fractions were named pool 2b and they were responsible for 22.5% of the total recovered GST activity. Whereas, the second activity peak was retained by the column and then eluted at 10 mM of GSH; the corresponding fractions were named pool 2a and they were responsible for 21.6% of the total GST activity. Protein pool 2a was charged on a High Q Column; all the GST activities eluted between the fractions 50 and 60, at high salt concentration. The mono-dimensional SDS–PAGE analyses showed that each fraction was composed only by two polypeptides characterized by molecular masses of 27.2 and 28.0 kDa, respectively (Fig. 4). In order to confirm that the identified protein was composed by the two individuated polypeptides, the protein was further processed by means of reversed-phase HPLC. This test confirmed that the protein of pool 2a was composed of two polypeptides, eluting between 25 and 30 min (Fig. 5). This protein, named FaGST I, showed a specific activity toward the substrate CDNB of 576.0 nkat mg protein^{-1}, with a purification fold of 162 with respect to crude extract (Fig. 1).

Pool 2b was submitted to further chromatographic analyses in order to determine the polypeptides constituting the identified GST activity. When pool 2b was applied onto a High Q column, no GST(CDNB) activity was detected in the first fractions eluted at low salt concentrations, while all the GST activities were found in the fractions eluted at high salt concentration, in particular between the fractions 40 and 63 (Fig. 6). The proteins were resolved into two different peaks of GST activity toward CDNB: the first

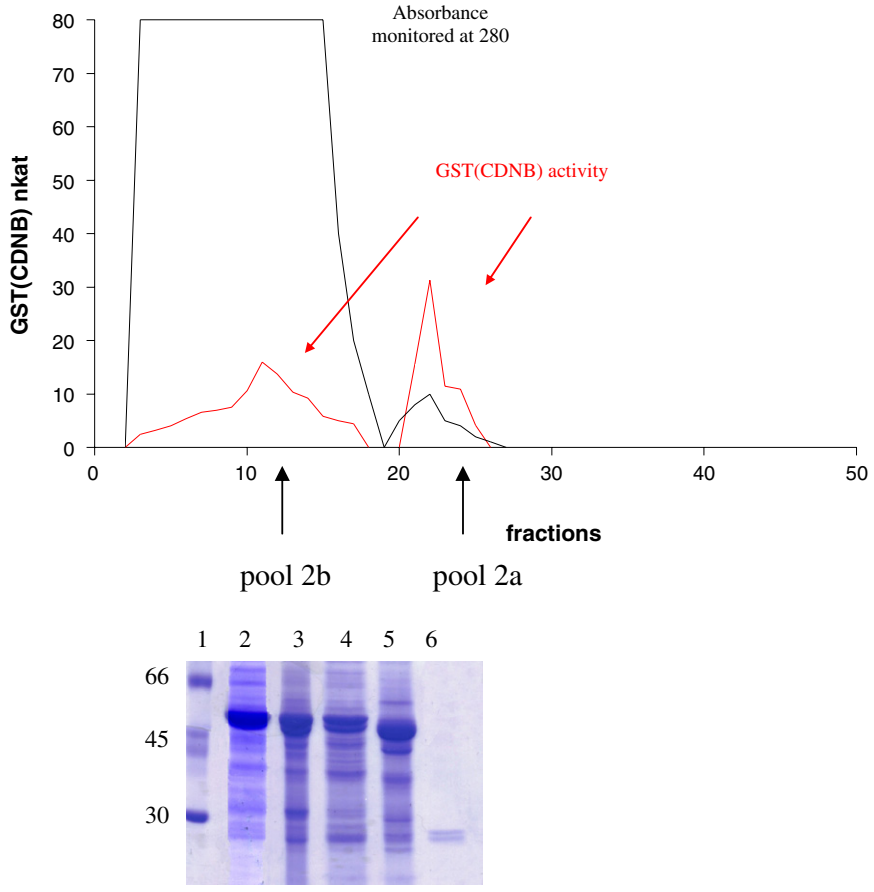

Fig. 3. The chromatographic profile obtained using GSTrap affinity column is reported at the top of the figure. Fractions were assayed for activity toward CDNB and the eluent was monitored at 280 nm. At the bottom of the figure are reported the electrophoretic analysis of the gel stained with Coomassie Brilliant Blue. Lane 1, molecular mass markers; lane 2, proteins of crude extract; lane 3, desalted proteins; lane 4, proteins fractioned by chromatography with Q Sepharose column showing activity toward CDNB; lane 5, proteins unretained by GSTrap column; lane 6, protein eluted with 10 mM GSH showing the highest activity toward CDNB.

and the second peak were responsible for 11.1% and of 0.7% of the total GST(CDNB) activity, respectively. Fig. 6 also reports the electrophoretic analysis of the more representative fractions of pool 2b: the first peak of GST activity was associated with two polypeptides of 26.0 and 27.5 kDa, eluted between the fractions 40 and 52; the second peak of GST activity was associated with two polypeptides of 27.5 and 30.0 kDa, eluted between fractions 58 and 65. The first activity peak, named FaGST II, and the second, named FaGST III, showed specific activity toward the substrate CDNB of 8.47 and 2.75 nkat mg protein^{-1}, respectively (Fig. 1).

2.2. Effect of benoxacor on the expression of GSTs

In order to determine the effect of the safener benoxacor in inducing GST(CDNB) activity for *Festuca*, the same procedure developed to identify, purify and characterise GSTs extracted from untreated *Festuca* shoots was used for the benoxacor safened *Festuca* shoots. Therefore, used samples were collected 48 h after benoxacor treatment, which is the time of maximum induction of *Festuca* GSTs (Scarponi and Del Buono, 2005). The proteins were extracted from the shoots and the GSTs were purified as described in Section 5. The activity of each GST extracted from the safened shoots was compared with the GST activity extracted from the unsafened samples. Significant increases of 200% and 30% in the content of FaGST I and of FaGST II, respectively, were ascertained in the safened shoots, while no effect in response to the benoxacor treatment was determined in the content of FaGST III and FaGST IV (Table 1).

2.3. Western blotting analyses

The availability of class specific antisera raised to tau and phi maize GSTs, *Zm*GSTU5-6 and *Zm*GSTF1-2, respectively, permitted to determine that FaGST I and FaGST II belonged to the tau and phi class, respectively (Table 1). FaGST III and FaGST IV did not react with any of the above antibodies.

2.4. Mass spectrometry

In order to characterize the more active and inducible FaGST I, which was purify till homogeneity, the subunits

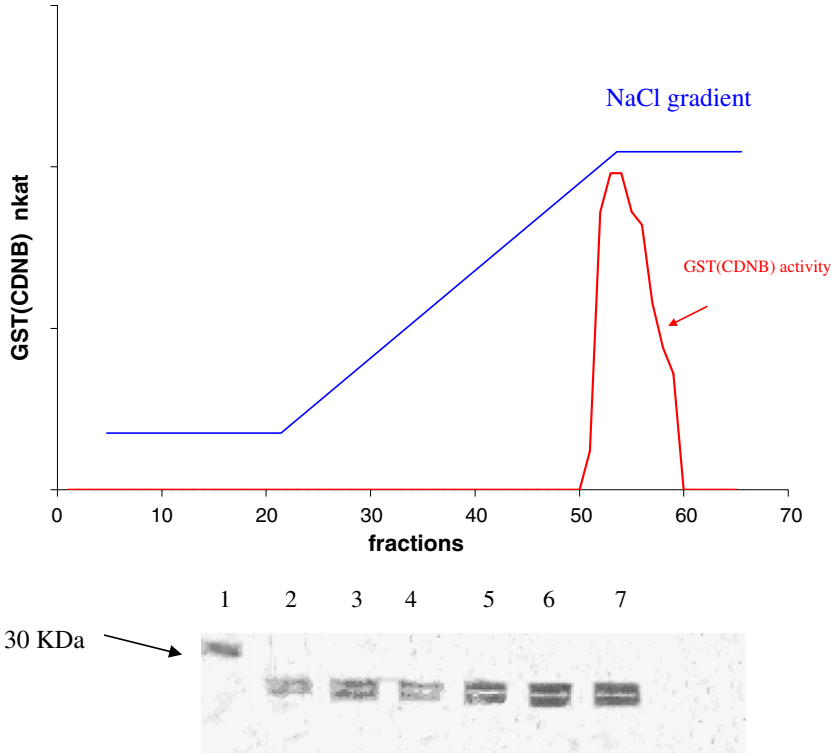

Fig. 4. The figure shows the chromatographic profile of protein pool 2a resolved by using a High Q column. The fractions were assayed for activity toward CDNB and the eluent was monitored at 280 nm. At the bottom of the figure are reported the electrophoretic analysis of the gel stained with Silver Staining. Lane 1, molecular mass marker; lane 2, proteins of fraction 50; lane 3, proteins of fraction 51; lane 4, proteins of fraction 52: lane 5, proteins of fraction 53; lane 6: proteins of fraction 54; lane 7, proteins of fraction 55.

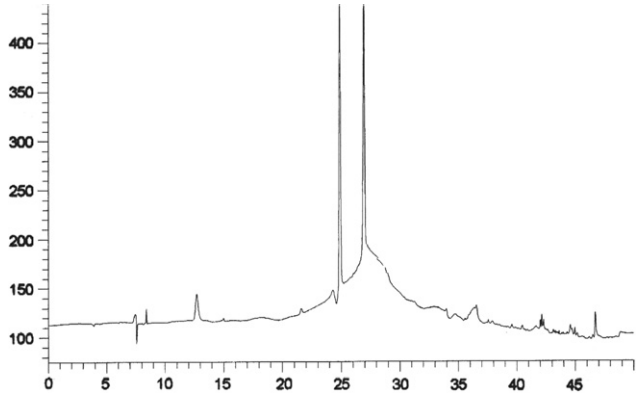

Fig. 5. The figure reports the chromatogram of HPLC analyses of sample containing FaGST I; the eluent was monitored at 280 nm. The trace shows that the protein was composed only by two polypeptides.

resolved by the mono-dimensional SDS–PAGE were excised, digested with trypsin and then subjected to LC–ESI–MS/MS analysis (Table 2). MS/MS data were searched against in the NCBI nonreduntant protein database using SEQUEST software (Bioworks 3.1, ThermoElectron). In spite of the scanty knowledge on *Festuca arundinacea* genome, it was possible to identify both the polypeptides of FaGST I as GST subunits belonging to the *tau* class, as evidenced also by Western blotting analyses.

3. Discussion

By using the Q Sepharose FF column to achieve the identification of *Festuca* GSTs, it was possible to retain the major percentage of GST activity of *Festuca* shoots in one peak of activity (pool 2), while a minor percentage of GST activity was recovered in the unretained fractions (pool 1) (Figs. 1 and 2).

The proteins of pool 1 were resolved by using a GSTrap affinity column, which possess glutathione as the ligand; the proteins of the active fractions were eluted from the column by adding glutathione to the elution buffer. The pool 1 was not further resolvable, and this GST activity was indicated as FaGST IV. In accordance with the more diffuse acidic nature of GSTs, the proteins of pool 2 were eluted from the column at high salt concentration, then pool 2 was chromatographed by using a GSTrap affinity column. This chromatography permitted to retain 21.6% of *Festuca* shoot GST activity (pool 2a), while 22.5% was unretained (pool 2b) (Figs. 1 and 3). Therefore, the loosely bounded pool 2b showed lower affinity for the GSH ligand of the column than the bounded pool 2a. The grouping of GSTs into two different groups, on the basis of their affinity to the GSH ligand, indicates that the identified enzymes could belong to different classes of GST isoenzymes. This same GST characteristic was already observed in *Triticum aestivum* (Cummins et al., 1997; Pascal and Scalla, 1999).

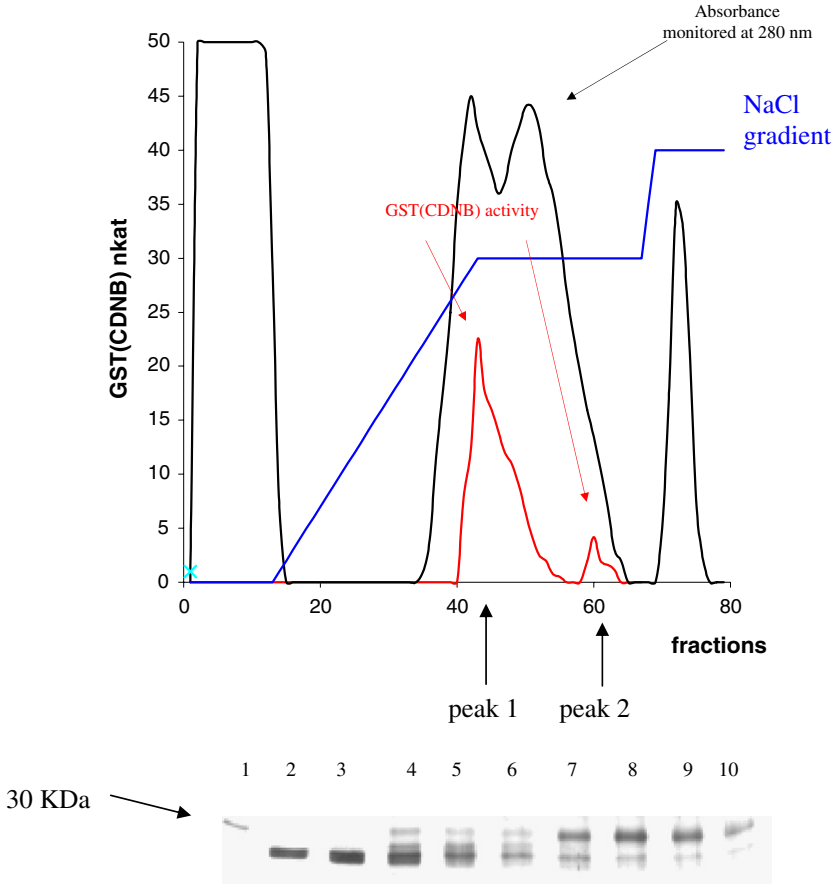

Fig. 6. The chromatographic profile of protein pool 2b resolved by using a High Q column is reported at the top of the figure. Fractions were assayed for activity toward CDNB and the eluent was monitored at 280 nm. At the bottom of the figure are illustrated the electrophoretic analysis of the gel stained with Silver Staining. The figure reports the polypeptides with masses of about 30 kDa which is the typical range of GSTs subunits. Lane 1, molecular mass marker; lane 2, proteins of fraction 42; lane 3, proteins of fraction 43; lane 4, proteins of fraction 50; lane 5, proteins of fraction 55; lane 6: proteins of fraction 58; lane 7, proteins of fraction 60; lane 8, proteins of fraction 62; lane 9, proteins of fraction 63; lane 10, proteins of fraction 64.

Table 1
Immunochemical reactivity and characteristics of FaGST I and FaGST II

Protein	Molecular masses of the subunits (kDa)	Safener inducibility	Western blotting
FaGST I	27.2–28.0	+200%	*Zm*GSTU5-6
FaGST II	26.0–27.5	+30%	*Zm*GSTF1-2

The table summarizes molecular masses, safener inducibility and Western blotting analysis obtained for FaGST I and FaGST II. In particular, for the Western blotting two antibodies raised against two maize GSTs were used: *Zm*GSTF1-2 and *Zm*GSTU5-6.
Each determination was run in triplicate.

The successive chromatographic purifications on High Q and the subsequent mono-dimensional SDS–PAGE analysis of the collected fractions of pool 2a showed the presence of a etherodimeric protein, FaGST I, endowed by the highest specific activity and purification fold. FaGST I constituted about 0.1% of the total soluble protein of the *Festuca* shoots (Fig. 1), and this result agrees with that found for other plants such as sugarcane, rice, wheat, sorghum and maize (Deng and Hatzios, 2002).

Pool 2b exhibited significant amount of the total GST(CDNB) activity (Fig. 1), therefore it was further purified by chromatography, in order to ascertain if it was resolvable in more than one GST activities. The High Q column demonstrated that the activity was attributable to two GSTs named FaGST II and FaGST III, respectively. The mono-dimensional SDS–PAGE evidenced a different composition in terms of polypeptides for the above GSTs.

3.1. Enhancement of GST isoenzymes by safener benoxacor

While it is well known that benoxacor is a safener able to enhance GST activity in maize, novel GST isoforms were not expressed in *Festuca* shoots in response to benoxacor treatments. Whereas an increase in the amount of two constitutively expressed proteins, FaGST I and FaGST II, was determined. These proteins were responsible for the greatest amounts of the conjugative activity toward CDNB (Fig. 1).

In particular, the safener treatment determined a strong induction of FaGST I coupled with lower induction of

Table 2
Characterization of FaGST I by LC–ESI–MS/MS

	Homologous protein	Species (NBCI no.)	Peptide sequences	Cov.[a] (%)	M_W[b]	M_W[c]/pI[d]
A	Glutathione *S*-transferase	*Zea mays* (gi|2288969)	FWADYVDKK	4.0	27,200	25,119/6.21
B	Glutathione *S*-transferase	*Hordeum vulgare* (gi|6683765)	GLPYEYVEEDLIAGK FWADYVDKK IPVLIHGGK	13.5	28,000	25,040/6.35

FaGST I was purified using a GSTrap, a High Q column, and then resolved by mono-dimensional SDS–PAGE. The two polypeptides composing this protein were excised form the gel, spotted, digested and finally analysed by LC–ESI–MS/MS.
[a] % Cov.: relative amino acid coverage.
[b] M_W: experimental M_W.
[c] M_W: theoretical M_W.
[d] pI: theoretical pI.

FaGST II (Table 1). The lack of significant responses to the safener treatment of FaGST III and FaGST IV could be reasonably imputed to the involvement of these proteins in other, different cellular processes. In fact, it is well known that GSTs are also implicated as antioxidant agents in response to oxidative stress, heavy metals and pathogen attacks (Mauch and Dudler, 1993; Frova, 2003).

However, safener induction of GST in *Festuca* happened in a specific manner, similar to that observed for *Arabidopsis thaliana*, wheat and maize (Edwards et al., 2005; Scarponi et al., 2006).

3.2. Mass spectrometry and Western blotting analyses

FaGST I was found to be the most active GST toward the xenobiotic substrate CDNB and the most inducible by the safener benoxacor. Therefore, this protein was submitted to further characterizations by mean of LC–ESI–MS/MS analysis in consideration of its importance as a potential factor in xenobiotic detoxification and herbicide resistance. The analysis of peptide fragments showed a significant amino acid coverage with the predicted sequences for tau subunits of glutathione *S*-transferases of *Zea mays* and *Hordeum vulgare*. In fact, the subunit with a molecular mass of 27.2 kDa showed a relative amino acid coverage of 4.0% with the predicted sequence of a GST subunits of maize (gi|2288969); while the subunit with a molecular mass of 28.0 kDa showed a relative amino acid coverage of 13.5% with the predicted sequence of a tau GST of barley (gi|6683765). The data of Western blotting analysis are in agreement with these findings, in fact, the *Zm*GSTU5-6 antibody obtained from a tau GST protein of maize, recognised both the subunits of the FaGST I protein (Table 2).

As already reported in Section 2, FaGST II was recognised by *Zm*GSTF1-2, while FaGST III and FaGST IV did not react with any of the tested antibodies.

4. Conclusions

The chromatographic procedure developed by combination of anionic exchanger columns and a GSH-affinity column, determined the recovery of a consistent fraction of the total GST(CDNB) of the crude extract. In addition, on the basis of the chromatographic behaviours, it was possible to identify four different GSTs: FaGST I, FaGST II, FaGST III and FaGST IV. The first anionic chromatography determined the resolution of GSTs into two groups: the literature reports that GSTs can be grouped into polar, with acidic behaviours, and hydrophobic proteins (Fuerst et al., 1993; Irzyk and Fuerst, 1993; Edwards, 1996; Cummins et al., 1997; Deng et al., 1997; Pascal and Scalla, 1999; Deng and Hatzios, 2002). Pool 1, containing the protein identified as FaGST IV, appeared to have a hydrophobic nature, similar to that ascertained for wheat GSTs (Cummins et al., 1997; Pascal and Scalla, 1999). Pool 2 reflected the more diffuse acidic nature of GSTs, in fact it was retained from the ammonium quaternary salt of the column. The GSH affinity column resolved pool 2 into the new subgroups differentiated on the basis of a different affinity for the GSH-ligand.

Therefore, this research evidenced that *Festuca* shoots possess at least four different GST isoenzymes showing different chromatographic behaviours and different activity toward CDNB, that reflects their involvement in many different cellular processes.

It was ascertained that the expression of GSTs can vary during plant development and in relation to the exposure to pathogens, to the changes in the environmental conditions and in response to chemical treatments (Mauch and Dudler, 1993; Marrs, 1996; Pascal and Scalla, 1999; Frova, 2003). For these reasons GST expression is considered an important marker for plant responses to many stressing agents and conditions. Generally, a high GST response to xenobiotic stresses determines a good capacity to tolerate them, as observed in the case of herbicide treatments (Rossini et al., 1996; Scarponi et al., 2003). *Festuca* tolerance to terbuthylazine herbicide has been observed and this was imputed to the conjugative GST activity, safener-inducible, toward the above herbicide (Scarponi and Del Buono, 2005). Therefore, the chromatographic procedure developed to identify and purify the *Festuca* GSTs, also permitted evaluation of the effect of the safener benoxacor on GST expression. FaGST I and FaGST II were found to be particularly inducible. The other two proteins, FaGST III and FaGST IV, were not found to be safener

responsive. Due to the highest activity toward CDNB, FaGST I was further purified till homogeneity and its subunits were investigated by LC–ESI–MS/MS and immunochemical reactivity. These polypeptides showed analogies of sequence with predicted structure of the subunits of tau GST of *Zea mays* and *Hordeum vulgare*. Among the six classes of known GSTs, tau and phi are involved in herbicide detoxification, therefore, the finding that FaGST I is a tau protein, composed by two tau polypeptides, is in accordance with its high CDNB detoxificative activity and it suggests its involvement in the detoxification of other xenobiotics. Although *Festuca arundinacea* is not a cultivated species, its GST detoxifying activity, particularly inducible by benoxacor treatment, may assume relevance in relation to practices of soil–water protection.

5. Experimental

5.1. Plant material

Festuca seeds (*Festuca arundinacea*, hybrid *Villageoise*) (50 g) were germinated in plastic flats (0.08 m^2) containing sand quartz pre-washed with a solution of hydrochloric acid (10%, v/v) and sterilised with a solution of NaClO (5%, w/v). Seedlings were grown in the dark at 23 °C (relative humidity 80%). After two days the seedlings were submitted to day–night conditions (12 h of light at 26 °C, light intensity 300 μmol m^{-2} s^{-1}, and 12 h of darkness at 21 °C) and watered daily. When the seedlings were 12 days old, the plants were divided into two groups: one group was left as the control and the second one was treated with benoxacor at the recommended field application rates (4 mg/flat). Shoots were collected 48 h after treatment, rinsed with water to remove non-adsorbed chemical benoxacor, dried by blotting with paper and underwent procedures for GST purification, characterization and activity determinations.

5.2. GST purification

All purification steps were carried out between 0 and 4 °C to avoid protein degradation.

Shoots of *Festuca* (30.0 g) were ground to a fine powder in liquid nitrogen using a mortar and a pestle. The powder was suspended in an extraction buffer (1/5, w/v), composed of 100 mM Tris–HCl (pH 7.5), containing 2 mM EDTA, 1 mM dithiothreitol and 1.5% (w/v) polyvinylpolypyrrolidone. After filtration through two layers of muslin, the homogenate was centrifuged at 15,000 rpm for 20 min and the supernatant was adjusted to 80% saturation with respect to $(NH_4)_2SO_4$ to precipitate the proteins (4 °C for 3 h). The resulting suspension was centrifuged at 15,000 rpm for 10 min and the protein pellet collected and stored at −20 °C. The pellet was dissolved in buffer A (20 mM Tris–HCl, pH 7.5, containing 1 mM dithiothreitol) and applied onto a Sephadex G-25 for desalting. The desalted proteins were applied, at a flow rate of 1 ml min^{-1}, to a Q Sepharose FF column (Amersham Biosciences – bed volume 8 ml), pre-equilibrated with buffer A (40 ml). Initially, the column was washed, at a flow rate of 4 ml min^{-1}, with buffer A (20 ml) until the absorbance, monitored at 280 nm, had returned to baseline. The column was then eluted with buffer A containing a concentration of NaCl increasing from 0 to 0.5 M (total volume of 80 ml), followed by washing with buffer A containing 1 M NaCl (80 ml). Fractions of 4 ml were collected and assayed for GST(CDNB) activity as described below.

The fractions showing GST activity were grouped in two pools: the unbounded proteins which flowed through the column without NaCl (pool 1) and the bounded proteins which were retained by a Q Sepharose FF column and eluted with NaCl (pool 2). Each protein pool was concentrated and desalted by ultrafiltration using Vivaspin Concentrator (20 ml, 10.000 MWCO PES).

Concentrated pool 1 and pool 2 were submitted to further chromatographic purifications. Both pool 1 and pool 2 were separately charged, at a flow rate of 1 ml min^{-1}, on a GSH affinity column (bed volume 5 ml), the GSTrap FF (Amersham Biosciences), pre-equilibrated with buffer A (25 ml). The column was washed at a flow rate of 1 ml min^{-1} with 20 ml of buffer A until the absorbance, monitored at 280 nm, had returned to baseline, then the retained proteins were eluted with buffer A containing 10 mM of GSH (10 ml). Fractions of 1 ml were collected and assayed for GST(CDNB) activity. The fractions of pool 1, which showed enzyme activity, were grouped, concentrated and submitted to electrophoretic analyses. Pool 2 was resolved into two groups of active fractions: the unbounded GSTs to the affinity column, showing low affinity for the GSH ligand and recovered during washing (pool 2b) and the bounded GSTs, showing high affinity to GSH ligand, and eluted with 10 mM GSH (pool 2a). Each protein pool was concentrated and desalted by ultrafiltration using Vivaspin Concentrator (20 ml, 10.000 MWCO PES).

In order to ascertain the possible presence of GST isoforms, the concentrated pools 2a and 2b, were submitted to further chromatographic purifications using a High Q column (bed volume 5 ml). Concentrated pool 2a was charged on an anion exchanger column (bed volume 5 ml), High Q Cartridge (Bio Rad), at a flow rate of 0.5 ml min^{-1}, pre-equilibrated with buffer A (25 ml). The column was then washed with buffer A until the absorbance, monitored at 280 nm, had returned to baseline (5 ml). The column was then eluted with buffer A containing a concentration of NaCl increasing from 0 to 0.5 M (total volume 15 ml), maintained with buffer A containing 0.5 M of NaCl (15 ml), and then washed with buffer A containing 1 M NaCl (15 ml). Fractions of 0.5 ml were collected and assayed for GST(CDNB) activity and the polypeptide composition of each fraction was determined by mono-dimensional SDS–PAGE, as described below.

Concentrated pool 2b was purified using the same anion exchanger column (High Q), at a flow rate of 1.0 ml min^{-1} and pre-equilibrated with buffer A (25 ml). The column was then washed with buffer A until the absorbance, monitored at 280 nm, had returned to baseline (10 ml). Thereafter the column was eluted with buffer A containing a concentration of NaCl increasing from 0 to 0.5 M (total volume 30 ml), then maintained with buffer A containing 0.5 M of NaCl (30 ml) and, finally, washed with buffer A containing 1 M NaCl (30 ml). Fractions of 1 ml were collected and assayed for GST(CDNB) activity. The polypeptide composition of each fraction showing GST activity was determined by mono-dimensional SDS–PAGE, as described below.

5.3. Assays of GST(CDNB) activity

The spectrophotometric procedure described by Edwards and Owen (1986) was used to determine GST activity of fractions obtained from chromatographic separations. The GST activity was determined by adding 25 μl of 40 mM 1-chloro-2,4-dinitrobenzene (CDNB), as a model substrate, to a solution containing 900 μl of 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 6.5), 25 μl of collected fractions and 50 μl of 0.1 M GSH (pH 7.0). The amount of conjugate formed by reaction between GSH and CDNB was evaluated spectrophotometrically at 340 nm and 35 °C. From this result the amount of conjugate formed in a reaction mixture with the enzymatic extract substituted by the buffer (non-enzymatic reaction) was then subtracted. The GST activity was expressed in nkat mg of $protein^{-1}$ employed for assay, corresponding to nmols of GSH-CDNB formed s^{-1} mg $protein^{-1}$.

The protein content of each fraction was determined according to Bradford (1976).

5.4. HPLC analyses of resolved polypeptides

The GST of pool 2a, which was resolved by the High Q column, was further processed by RP-HPLC. A Perkin Elmer Series 410 LC Pump equipped with a C 18 HPLC column (25 cm × 2.1 mm i.d., 5 μm, 300 A) was employed. The chromatographic separation was performed using trifluoroacetic acid (0.1%, v/v) (mobile phase A) and acetonitrile (mobile phase B), at a flow rate of 0.5 ml min^{-1}, and the eluent was monitored at 280 nm. The gradient profile was as follows: 1 min 95% A:5% B, followed by a linear increase in 40 min to 80% B, then the column was raised in 1 min to 100% of B and maintained for 15 min, before re-equilibrating the column with 95 A:5% B.

5.5. Mono-dimensional SDS–PAGE analyses

The mono-dimensional SDS–PAGE analyses were performed according to the Laemmli (1970). The proteins were suspended in the Laemmli buffer and denatured at 95 °C for 5 min. Each sample was submitted to mono-dimensional SDS–PAGE using a resolving gel containing 12.5% of acrylamide and a stacking gel containing 4% of acrylamide. The polypeptide were resolved under a constant current of 15 mA in an Amersham Hoefer miniVE Vertical Electrophoresis System. The gels were stained with Coomassie Brilliant Blue R-250 and Silver Staining. The electrophoretic images were processed using Gel Dool 2000 (Bio-Rad).

5.6. Western blotting

For the Western blotting analyses two antibodies raised against maize GSTs were used: *Zm*GSTF1-2 and *Zm*GSTU5-6. Proteins extracted from shoots of *Festuca arundinacea* and purified as described above were resolved by mono-dimensional SDS–PAGE, and electro-transferred onto a nitrocellulose membrane applying the following conditions: 25 V for 1.5 h at 400 mA. Blot was blocked overnight in PBS buffer containing non-fat dry milk (5%, w/v). The blots were then incubated for 1 h with the first antibody (1/5000) in PBS buffer containing Tween 20 (0.1%, v/v). After washing with three aliquots of PBS buffer containing Tween 20 (0.1%, v/v), blots were incubated for 1 h with an anti-rabbit alkaline phosphatase (1/10,000) in 100 mM Tris (pH 9.5). After washing the blots were developed by the addition of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as described by Blake et al. (1984).

5.7. Protein in-gel digestion

Protein spots were excised from Coomassie Brilliant Blue R 250 stained mono-dimensional gels, transferred to sterilized Eppendorf tubes (0.5 ml) and stored in 50% ethanol at 4 °C until digestion. After removing ethanol solution, gel pieces were incubated in 100 μl distillate H_2O (HPLC-grade) for 15 min at room temperature and then in 40 μl 50% acetonitrile for the same time. This step was repeated three times afterwards the supernatants were removed and excised gel fragments were incubated for 5 min in 40 μl 100%, for 5 min in 40 μl 100 mM NH_4HCO_3 and finally in 80 μl 50 mM NH_4HCO_3/50% acetonitrile for 15 min. After removing the supernatants, gel pieces were dried under vacuum on a centrifugal evaporator. To each tube were added 40 μl of 10 mM dithiotreitol in 100 mM NH_4HCO_3 then incubated in a water bath at 56 °C for 45 min. The solution was discarded and then 40 μl of 55 mM iodoacetamide/100 mM NH_4HCO_3 was added. The sample was washed with 100 μl of 100 mM NH_4HCO_3 for 5 min, incubated in 100 μl of 50% acetonitrile for 15 min and finally, after discarded the supernatant, it was incubated in 100% acetonitrile, until gel piece was white and stick. After removing acetonitrile, the sample was incubated in μl 100 mM NH_4HCO_3 for 5 min and, after the addition of 40 μl acetonitrile, it was incubated for further 15 min. After removing the supernatants, gel pieces were dried under vacuum on a centrifugal evaporator. For the

protein digestion, 20 μl trypsin solution [Sequencing Grade Modified Trypsin V5111, Promega, Madison; 12.5 ng/μl in digestion buffer (25 mM NH_4HCO_3 containing 2.5 mM $CaCl_2$, pH 7.8)] was added to each sample. After an incubation of 45 min at 4 °C, the supernatants were replaced with 20 μl of digestion buffer and samples were incubated for 16 h at 37 °C. In order to extract the tryptic fragments, the gel pieces were sonicated for 5 min in a cold water bath. After collecting supernatants, gel pieces were incubated for 15 min in 20 μl of 25 mM NH_4HCO_3 (occasionally the sample were sonicated for 2–3 min) and then, after the addition of the same amount of acetonitrile, incubated for further 15 min at room temperature. These steps were repeated twice. The supernatants were collected and the samples were incubated for 15 min in 20 μl of 5% formic acid and then, after the addition of the same amount of acetonitrile, incubated for further 15 min. After the repetition of these two last steps, to the pooled supernatants (about 140 μl) was added 100 mM dithiotreitol to give a final concentration of 1 mM dithiotreitol. Finally, the samples were dry under vacuum on a centrifugal evaporator and the resulting tryptic fragments were redissolved in 10 μl of 0.1% formic acid and stored at −80 °C.

5.8. Mass spectrometry analysis and protein identification

The extracted tryptic fragments were resuspended and analysed by LC–ESI–MS/MS. For all experiments a Finningan LCQ DECA XP MAX spectrometer equipped with a Finningan Surveyor MS HPLC system (Thermo Electron Corporation, CA, USA) was used. Chromatography separations were conducted on a BioBasic C18 column (150 μm ID × 150 mm length and 5 μm particle size; Thermo Electron Corporation, USA), using a linear gradient from 5% to 80% acetonitrile containing 0.05% formic acid with a flow of 2.5 μl/min. Including the re-equilibrating step, one run lasted 90 min. Acquisitions were performed in data-dependent MS/MS scanning mode (full MS scan range of 400–2000 *m/z* followed by full MS/MS scan for the most intense ion from the MS scan) and enabling a dynamic exclusion window of 3 min. Proteins identifications were conducted by correlation of uninterpreted tandem mass spectra to the entries of a non-redundant protein database and a EST database downloaded from the National Center for Biotechnology Information (NCBI; April 12, 2006) using TurboSEQUEST Bioworks™ 3.2 software (Thermo Electron Corporation, CA, USA). The software was set to allow two missed cleavages per peptide and considering cysteine carbamidomethylation and methionine oxidation. The precursor ion tolerance was set to 1.4 amu. In order to identify proteins, only peptides with X-correlation >1.5 (+1 charge), 2.0 > (+2 charge), >2.5 (+ 3 charge) and with peptide probability $<1 \times 10^{-3}$ were considered.

Each determination was run in triplicate and the paper reports the data regarding to the more representative experiment.

Acknowledgements

This research was carried out with the financial support of the Italian MIUR-PRIN 2006 program ''Study of processes and phenomena to mitigate the pesticide water bodies contamination''. Subproject: ''Use of no crop species in prevention or mitigation of water pollution from herbicides''.

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