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Detection of *Glutathione S-Transferase* gene (*GST2, GST3*) during induction of somatic embryogenesis in grape (*Vitis vinifera* L.)

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Glutathione S-transferases (GSTs) are an important group of multifunctional enzymes that belong to diverse multigene families. In plants these enzymes are involved in the detoxification of xenobiotic compounds, herbicide detoxification, and primary and secondary metaboplism and play an important role in plant growth and development. There are several reports that show that the expression of GST is linked to the developmental phases of somatic embryogenesis. This study highlights the detection of transcript abundances of glutathione S-transferase genes *GST2* and *GST3* in the process of somatic embryogenesis of *Vitis vinifera*, and the lack thereof in non-embryogenic tissue of leaf cultures of grape (control). These results indicate that the expression of *GST2* and *GST3* could be used as a molecular signal for the identification of embryogenic cultures during the early development of somatic embryos.

Key words: Dharwad, Gene expression, Karnataka, Somatic embryogenesis, Sonaka, Tas-e-Ganesh, Thompson, transcription factor, *Vitis vinifera.*

Glutathione S-transferases (GSTs) are classified as specialized multifunctional dimeric enzymes involved in enzymatic detoxification of endo and xenobiotics (Moons, 2005: Jiang *et al.* 2010). GSTs are found in nearly all forms of life, including bacteria, fungi, yeast, insects, mammals and higher plants. Plant GSTs are divided into six

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classes, namely, phi (F), tau (U), zeta (Z), theta (T), lamba (L), and dehydroascorbate reductases (Dixon *et al*. 2002; Moons, 2005; Jiang *et al.* 2010). Among them, in plant, *tau* (U) and *phi* (F), classes are the most represented whereas *theta* and *zeta* ones are found in animals (Jiang *et al*. 2010; Galland *et al*. 2007). The functions of GSTs in plants are diverse and might be due to the ability to conjugate glutathione (GSH) to various targets involved in biotic and abiotic stress (Jiang *et al.* 2010). In addition to this, plant GSTs are also involved in plant growth and development (Gong *et al*. 2005; Moons, 2005; Jiang *et al*. 2010). GSTs have been found to bind hormones such as auxin and cytokinin (Zettl *et al.* 1994; Gonneau *et al.* 1998), and can be induced by a wide variety of phytohormones, including ethylene, auxin, methyl jasmonate, salicylic acid, and abscisic acid (Wagner *et al*. 2002; Moons, 2003; Smith *et al.* 2003). All these hormones regulate many aspects of plant development which implies that plant GSTs may play vital roles in plant growth and development as well (Jiang *et al*. 2010). GST genes can be induced by various exogenous factors such as pathogen attack, heavy metals, heat shock, wounding or auxin (Singla *et al*. 2007; Jiang *et al.* 2010).

Somatic embryogenesis (SE) is the developmental phenomenon where somatic cells under *in vitro* conditions under the influence of external growth regulators are programmed towards the embryogenic pathway and reflects cellular totipotency in higher plants (Konar and Nataraja, 1965; Nataraja and Konar, 1970; Malabadi and van Staden, 2003; Malabadi *et al.* 2004, 2005, 2010; Malabadi and Nataraja, 2006a, 2007a, 2007c; Malabadi, 2006; Malabadi and van Staden, 2005a, 2005b, 2005c, 2006; Malabadi *et al*. 2009abc; Malabadi and Teixeira da Silva, 2011; Feher *et al*. 2003; Namasivayam, 2007; Aronen *et al.* 2007, 2008; Malabadi *et al.* 2011a-2011g; Malabadi *et al.* 2012ab; Mulgund *et al*. 2012). Acquisition of embryogenic competence largely relies on dedifferentiation because the existing developmental information must be erased or altered to make the cells responsive for new signals (Dudits *et al.* 1991, 1995; Pasternak *et al.* 2002; Feher *et al*. 2003; Namasivayam, 2007; Chugh and Khurana, 2002; Singla *et al*. 2007; Malabadi and van Staden, 2003; Malabadi *et al.* 2004, 2005, 2010; Malabadi and Nataraja, 2006a, 2007a; Malabadi, 2006; Malabadi and van Staden, 2005a, 2005b, 2005c, 2006; Feher *et al*. 2003; Namasivayam, 2007; Aronen *et al.* 2007, 2008; Malabadi *et al.* 2011a-2011g; Malabadi *et al.* 2012ab; Mulgund *et al*. 2012). The developmental switch from a differentiated and resting cell state to a dedifferentiated, dividing, embryogenic state may involves the general reorganization of chromatin structure, overall reprogramming of gene expression, as well as cellular metabolism (Dudits *et al.* 1991, 1995; Pasternak *et al.* 2002; Feher *et al.* 2003; Namasivayam, 2007; Konar and Nataraja, 1965; Nataraja and Konar, 1970; Chugh and Khurana, 2002; Singla *et al*. 2007). Such developmental programmes involve activation of various signal cascades and differential gene expression, which confers the ability to manifest the embryogenic potential on somatic cells (Chugh and Khurana, 2002; Singla *et al*. 2007; Malabadi and Nataraja, 2007b; Malabadi *et al.* 2011a-2011g). There are many reports on profiling of expressed genes during the somatic embryogenesis pathway (Feher *et al*. 2003; Malabadi *et al*. 2011a; Malabadi and Nataraja, 2007b). Arrays of genes that are activated or differentially expressed during somatic embryogenesis process have been identified in many plant species (Zimmerman, 1993; Chugh and Khurana, 2002; Feher *et al.* 2003; Ikeda *et al.* 2006; Raghavan, 2006; Quiroz-Figueroa *et al.* 2006; Malabadi *et al.* 2011a; Malabadi *et al.* 2009b; Malabadi and Nataraja, 2007b; Park *et al.* 2009).

The transcripts of GST were detected in abundance during auxin induction and in somatic embryos (Galland *et al.* 2007; Singla *et*

al. 2007). A GST gene (*CHI-GST1*) was specifically expressed in leaf tissues of the chicory embryogenic responsive genotype "474" from the third day of the induction phase, when the first reactivated cells were observed but not in a non-embryogenic responsive genotype (Galland *et al.* 2007). GST transcripts have been shown to accumulate in *Chicorium* (Galland *et al*. 2001), *Medicago sativa* (Thibaud-Nissen *et al.* 2003), *Triticum aestivum* (Singla *et al*. 2007), *Cyclamen persicum* (Rensing *et al*. 2005; Winkelmann *et al*. 2006; Hoenemann *et al.* 2012), cotton (Zeng *et al*. 2006), and *Crocus sativus* (Sharifi *et al*. 2012) somatic embryos and GSTs appears to be a major regulator of the interacting genes sequenced in the present case in response to auxin (Galland *et al.* 2007). Some GSTs are induced by auxin (Nagata *et al*. 1994), H_2O_2 (Levine *et al.* 1994), and might target transcription factors like *WRKY* (Du and Chen, 2000; Singla *et al*. 2007), and transport certain gene products produced during oxidative stress to the vacuole (Marrs 1996; Edwards *et al.* 2000; Singla *et al*. 2007). Reactive oxygen species (ROS) have been shown to act as second messenger during auxin and stress-induced embryogenesis (Nagata *et al*. 1994; Maraschin *et al.* 2005; Singla *et al*. 2007). These facts suggest that the GST genes could be active to attain the embryogenic competence in different plant system (Ikeda *et al.* 2006; Giroux and Pauls, 1997; Chugh and Khurana, 2002; Singla *et al*. 2007). This study aims to detect the transcript abundances of *Vitis vinifera* glutathione S-transferase genes *GST2, GST3* in the process of somatic embryogenesis of grape during the crucial step of transfer of embryogenic cultures on to maturation medium which triggers the realization of embryo development.

Materials and methods

Induction of embryogenic tissue

Leaf explants were harvested from 6-7- month- old mother plants of three *V. vinifera* L. cvs: Thompson, Sonaka and Tas-e-Ganesh. These were carefully washed in double distilled water (DDW) (Malabadi *et al.* 2010). They were surface decontaminated sequentially with 0.1% streptomycin (1 min), 70% (v/v) ethanol (5 min) and 0.1% (w/v) $HgCl_2$ (2 min) (Sigma-Aldrich, St. Louis, USA), and thoroughly rinsed with sterilized double distilled water. Leaf sections were cultured on Nitsch and Nitsch (1969) NN basal medium with 3.0% sucrose, 0.7% agar, 0.5 gl^{-1} *myo*-inositol, 1.0 gl^{-1} casein hydrosylate, 0.5 gl^{-1} L-glutamine, 250 mgl^{-1} peptone, 0.2 gl^{-1} *p*-aminobenzoic acid, and 0.1 gl^{-1} biotin, all purchased from Sigma (Malabadi *et al.* 2010). The medium was supplemented with 4.54 µM thidiazuron (TDZ) and 2, 4-dichlorophenoxy acetic acid (2, 4-D) at a concentration of 4.52 µM singly without any other growth hormones (Malabadi *et al.* 2010). The cultures were raised in 25 mm × 145 mm glass culture tubes (Borosil, Mumbai, India) containing 15 ml of the above basal medium under cool white fluorescent light (Mysore lamps, India) at 100 µmol m^{-2} s^{-1} and 25 ± 3°C with a relative humidity of 55-60% (Malabadi *et al.* 2010). The pH of the media was adjusted to 5.8 with 1 N NaOH or HCl before agar was added. Media without TDZ and 2, 4-D served as the control. The media were then sterilized by autoclaving at 121°C at 1.04 Kg cm^{-2} for 15 min. L-glutamine, biotin, and *p*-aminobenzoic acid were filter sterilized (Whatman filter paper, pore size = 0.45 µm; diameter of paper = 25 mm) and added to the media after autoclaving when the medium had cooled to below 50°C (Malabadi *et al.* 2010).

The cultures were examined for the presence of different developmental stages of somatic embryos by morphological and cytological observations of callus (Malabadi *et al.* 2010). The cultures showing different cell divisions were identified and subcultured on the initiation medium for further 6 weeks for the better development of early stages of somatic embryogenesis (Malabadi *et al.* 2010).

The full strength inorganic salts NN (Nitsch and Nitsch, 1969) basal medium supplemented with 4.52 µM 2, 4-D and 4.54 µM TDZ (induction medium) was used as an effective induction medium for producing the embryogenic tissue (Malabadi *et al.* 2010). Embryogenic tissue showing different cell divisions such as 2 to 8 celled stages was identified using microscopic observation (Malabadi *et al.* 2010). On the other hand the callus without pro-embryonic cell divisions was considered as non-embryogenic. Non-embryogenic tissue was separated immediately from the rest of the tissue to avoid the overgrowth of the tissue (Malabadi *et al.* 2010). The efficiency of plant growth regulators and their concentrations were analyzed on the basis of visual observation (callusing percentage, percentage of explants forming embryogenic tissue, callus growth and callus necrosis). The ineffective treatments were discontinued as previously reported (Malabadi *et al.* 2010).

Maintenance of embryogenic tissue

The embryogenic tissue of three of *V. vinifera* L. cvs: Thompson, sonaka and Tas-e-Ganesh showing various developmental stages of somatic embryos was maintained on full strength inorganic salts NN (Nitsch and Nitsch, 1969) basal medium supplemented with 4.52 µM 2, 4-D and 4.54 µM TDZ for the proliferation of callus (maintenance medium) (Malabadi *et al.* 2010). The embryogenic tissue was subcultured for every 4 weeks. All the cultures were maintained under a cool white fluorescent light (100µmol m^{-2} s^{-1}) at 25±3°C with a relative humidity of 55-60%. The percentage of cultures showing somatic embryogenesis was recorded (Malabadi *et al.* 2010).

RNA preparation and cDNA synthesis

Frozen samples of embryogenic tissue derived from leaf explants of three of *V. vinifera* L. cvs: Thompson, Sonaka and Tas-e-Ganesh and control (non-embryogenic tissue) were ground in a mortar and pestle with liquid nitrogen. Total RNA was isolated according to the modified method of Chang *et al*. (1993). To remove residual genomic DNA, 25 µg of RNA was treated with TURBO-DNase™ (Ambion, Austin, TX, USA) (Malabadi *et al*. 2011; Park *et al.* 2009). cDNA was generated from 1 µg of DNase-treated RNA using the Superscript II RT system (Invitrogen, CA, USA) according to the manufacturer's protocol. Each reaction was run in duplicate, generating two independent cDNA samples for each RNA sample isolated from three *V. vinifera* L. cvs: Thompson, sonaka and Tas-e-Ganesh (Malabadi *et al*. 2011; Malabadi and Nataraja, 2007g).

Cloning of grape (*Vitis vinifera*) glutathione S- transferase gene (*GST2, GST3*)

For cloning of grape (*Vitis vinifera*) glutathione S-transferase genes *GST2, GST3*, gene specific primers were designed using Primer 3.0 software based on homologous sequences publically available on NCBI databases (www.ncbi.com). The nucleotide sequences of *Vitis vinifera* glutathione S-transferase gene *GST2* (Gene Acc No-AY156049), *Vitis vinifera* glutathione S-transferase gene *GST3* (Gene Acc no-AY156050) were selected from the NCBI databases respectively. The gene specific primers (***GST2***: Forward: 5′ AGC TCT TTG ACT 3′; Reverse: 3′ TTA ACT CTT CTG CAT 5′ and ***GST3***; Forward: 5′GAT TGA GGA GAG GA 3′; Reverse: 5′ TCA CTC CAA GAG GGG CCA T3′) were custom synthesized (Bangalore Genei, Bangalore, Karnataka, India) and used for the PCR amplification of the *GST2* and *GST3* genes using cDNA from embryogenic tissue and control (non-embryogenic tissue) as the template. Amplified fragments from *grape (Vitis vinifera)* cDNA samples were excised from the agarose gel and purified with QIA quick gel extraction kit (Qiagen, USA). The eluted PCR product was cloned using the TOPO-TA cloning kit (Invitrogen, USA) (Malabadi *et al.*

2011; Park *et al.* 2009). Ligated products were then transformed into chemically competent TOPO 10 *Escherichia coli* cells using heat shock method and the transformants were selected on LB agar plates containing kanamycin (100 µg ml^{-1}) (Malabadi *et al.* 2011; Park *et al.* 2009). The expression of *Vitis vinifera* glutathione S-transferase *GST2* and *GST3* genes during induction of somatic embryogenesis were confirmed by the DNA sequence analysis**.**

Somatic embryo maturation

The embryogenic tissue of three *V. vinifera* L. cvs: Thompson, Sonaka and Tas-e-Ganesh showing different developmental stages of somatic embryos, was transferred to maturation medium to induce cotyledonary embryo development (Malabadi *et al.* 2010). The full strength (inorganic salts) NN (Nitsch and Nitsch, 1969) basal medium supplemented with 3.0% sucrose, 5 µM ABA and 0.8% agar (maturation medium) was tested for this purpose (Malabadi *et al.* 2010). All the cultures were again maintained in the dark for 4 weeks. Microscopic observation was conducted to ensure the development of somatic embryos. The total number of somatic embryos produced after 8 weeks on maturation medium per one gram fresh weight of embryogenic tissue was recorded (Malabadi *et al.* 2010).

Germination and recovery of plantlets

After maturation, the cotyledonary somatic embryos were taken from the cultures for germination (Malabadi *et al.* 2010). The germination medium used was half strength (inorganic salts) NN (Nitsch and Nitsch, 1969) basal medium with 0.7% agar without any growth regulators (germination medium). Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlet was based on the presence of an epicotyl (Malabadi *et al.* 2010). After 4 weeks on germination medium, the plantlets were directly transferred to vermiculite. Plantlets were placed in a growth room under a 16 hr photoperiod (50µ mol m^{-2} s^{-1}) for hardening. Somatic embryo proliferation in terms of root, shoot development, plant conversion was recorded (Malabadi *et al.* 2010).

Results and discussion

In the present study, leaf explants induced embryogenic tissue after 2-4 weeks of culture on the full strength inorganic salts NN (Nitsch and Nitsch, 1969) basal medium supplemented with 4.52 µM 2, 4-D and 4.54 µM TDZ (induction medium) in all the three varieties of *V. vinifera* (Malabadi *et al.* 2010). Embryogenic areas were clearly visible from the rest of the callus by their globular and glazy appearance and emerged as distinct white glossy structures. The embryogenic tissue was separated from non-embryogenic tissue for the gene expression studies. In a control study, the leaf explants did not promote callus formation (Malabadi *et al.* 2010). Leaf explants remained green for two weeks and, eventually turned brown and necrosed. Therefore, 4.52 µM 2, 4-D and 4.54 µM TDZ are the optimum concentrations for the induction of embryogenic tissue in all the 3 tested varieties of *V. vinifera* (Malabadi *et al.* 2010). Total RNA was isolated from both control (non-embryogenic tissue) and embryogenic tissue. Furthermore, cDNA preparation was done and used as the template for the identification of *Vitis vinifera* glutathione S-transferase genes *GST2, GST3* expressed during somatic embryogenesis pathway. In our present study, we used the embryogenic cultures for the gene expression studies from the induction phase which showed the early sign of somatic embryogenesis. Microscopic observation of the callus revealed the abundance of two, four and eight celled stages often mixed with the globular and heart shaped embryos. Our results clearly demonstrated the GST transcript abundances in the embryogenic cultures showing clear evidence of GST role in inducing somatic embryogenesis in grapes.

Furthermore, we can not pinpoint whether the GSTs gene expressions are stage specific or might be up-regulated or down regulated. A detailed study of suppression subtractive hybridization (SSH) library method should be used to study the role of different genes during different stages of somatic embryo development in grape, which could give a clear picture of the role of GST and other genes in down or up regulation.

A number of studies have been published, in which gene expression profiling includes GST has been used to analyse the process of somatic embryogenesis by [(Hoenemann *et al*. 2010 in . in *Glycine max;* (Thibaud-Nissen *et al.* 2003 in , *Pinus roxburghii;* (Malabadi *et al.* 2011a; Malabadi and Nataraja, 2007 in, *Picea abies* (Stasolla *et al.* 2004 in, *Oryza sativa;* (Su *et al*. 2006 in *Zea mays* (Che *et al.* 2006 in *Gossypium hirsutum;* (Zeng *et al*. 2006; Wu *et al*. 2009 in *Cichorium intybus* (Legrand *et al.* 2007 in *Triticum aestivum*; (Singla *et al*. 2007 in *Elaeis guineensis* (Lin *et al.* 2009). The possible involvement of GSTs in morphogenesis has been well documented on microspore and somatic embryogenesis (SE) (Gong *et al.* 2005). In barley, a GST homolog, ECGST, was not detected in freshly isolated microspores but the transcript accumulated in microspores undergoing early stages of embryogenesis (Vrinten *et al.* 1999; Gong *et al.* 2005). A GST gene CHIGST1 isolated from chicory has been shown to express in cultured leaves of embryogenic cultivar forming somatic embryos but not in the cultured tissues of non-embryogenic cultivar (Galland *et al.* 2001; Gong *et al.* 2005). Furthermore, a GST homolog Dcarg-1 has been isolated in carrot during SE and its expression has been associated with somatic embryo formation (Kitamiya *et al.* 2000; Gong *et al.* 2005). Although these findings indicate the possible role of GSTs in SE, it remains to be substantiated (Gong *et al.* 2005). Results of this study provids direct evidence showing that GST is involved in shoot morphogenesis *in vitro* (Gong *et al.* 2005). This has been demonstrated in culture responses of transgenic plants, where down regulation of GST expression by RNAi in GST-DS1 markedly decreases the shoot regeneration capacity of the cultured tissues (Gong *et al.* 2005). In contrast, over expression of GST promotes regeneration of GST-S6 (Gong *et al.* 2005). Furthermore, regulatory role of GSTs in shoot regeneration and SE may be related to ethylene production (Gong *et al.* 2005). The regulatory role of GST in shoot regeneration may be attributed, in part, to stress because GST expression can be up regulated in response to stress as demonstrated in this and previous studies (Gong and Pua, 2004; Gong *et al.* 2005). GST genes are not only responsive to auxin but are induced by other hormones, e.g. ABA, mJA, and under various biotic and abiotic stresses, and may have a possible role in detoxifying excessive amounts of auxin, thus regulating the intracellular concentration or its inactive analogs (Singla *et al.* 2007). In our present study, the expression of GST in abundance during auxin induction and in somatic embryogenesis suggests that the GST genes could be active to attain the embryogenic competence in the grape. Thus, the presence of GST trascripts representing potent markers of somatic embryogenesis and involved in cross-talk between auxin and SE, clearly suggest their involvement in the initiation phase of somatic embryogenesis in the grape leaf base system. In one of the study reported by Galland *et al.* (2007), a GST gene (CHI-GST1) expression was linked to the early stages of somatic embryogenesis of *Cichorium.* Therefore, a GST gene (*CHI-GST1*) was up-regulated specifically in leaf tissues of the embryogenic responsive *Cichorium* '474' from the third day of culture (Galland *et al.* 2007). They have also mentioned that the localization of GST transcripts and proteins in *Chicory* leaf sections showed that during cell reactivation, i. e. from G_0 to the end of G_2 phases, GSTs were strictly associated with

preparation for embryogenic mitosis (Galland *et al*. 2007). Therefore, GSH-dependent developmental pathway was essential for initiation and maintenance of cell division. Further GST transcripts were not observed in a similar manner in developing somatic embryos of *Chicory* (Galland *et al.* 2007). The presence of GST transcripts and proteins in reactivated cell and multicelled embryos indicated that anti-oxidation mechanisms were active during precocious phases of SE in *Chicory*. Therefore, GST transcripts could be taken as one of the molecular marker during the induction of somatic embryogenesis in plants. This study provides the direct evidence that GSTs are involved in regulation of vegetative growth and somatic embryogenesis in plants.

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