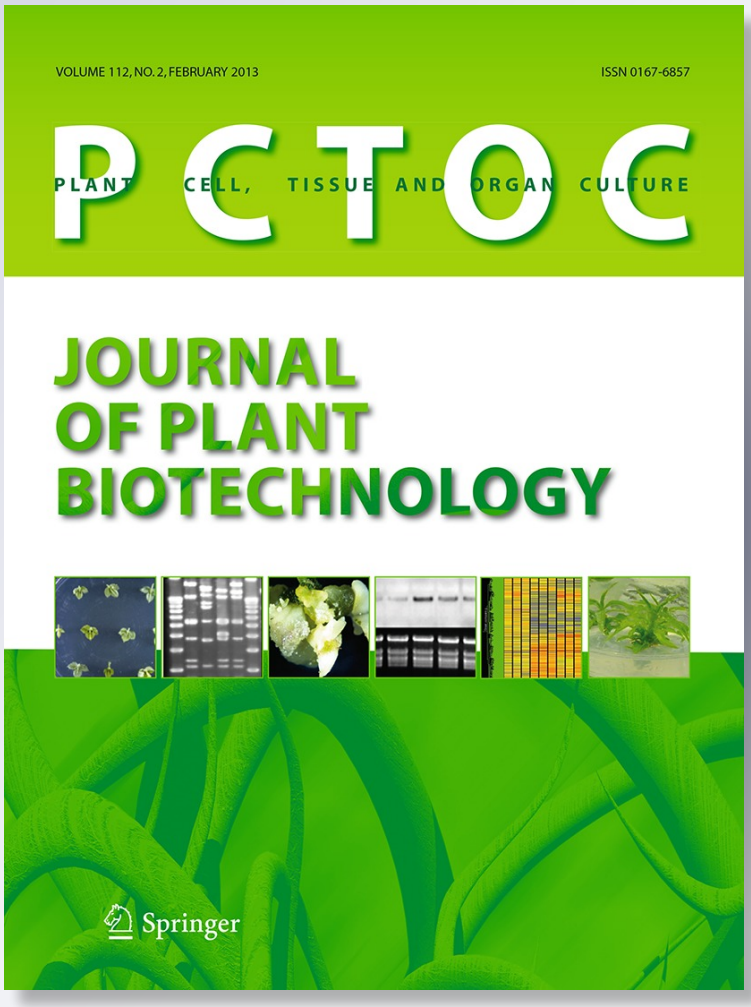
Silencing of the glutathione biosynthetic pathway inhibits somatic embryogenesis in wheat

E. Bossio, A. Díaz Paleo, M. del Vas, I. Baroli, A. Acevedo & R. D. Ríos

Plant Cell, Tissue and Organ Culture (PCTOC)
Journal of Plant Biotechnology

ISSN 0167-6857
Volume 112
Number 2

Plant Cell Tiss Organ Cult (2013)
112:239-248
DOI 10.1007/s11240-012-0228-4



Springer

Plant Cell Tiss Organ Cult (2013) 112:239–248
DOI 10.1007/s11240-012-0228-4

ORIGINAL PAPER

Silencing of the glutathione biosynthetic pathway inhibits somatic embryogenesis in wheat

E. Bossio · A. Díaz Paleo · M. del Vas · I. Baroli · A. Acevedo · R. D. Ríos

Received: 30 January 2012 / Accepted: 20 August 2012 / Published online: 1 September 2012

Abstract Somatic embryogenesis in scutella of wheat (*Triticum aestivum* L.) is a well documented phenomenon and it has been shown through transcriptome analysis that genes involved in antioxidant responses, particularly in glutathione (GSH) biosynthesis, participate in the process. Thus, we investigated the influence of post-transcriptional silencing (PTGS) of the glutathione biosynthesis genes *GSH1* and *GSH2* on somatic embryogenesis in wheat. We found that PTGS of either of the target genes drastically inhibits callus regeneration and overall efficiency of transformation, in a similar manner as the GSH biosynthetic inhibitor buthionine sulfoximine. Supplementing the medium with glutathione did not overcome the observed low efficiency of wheat transformation. Furthermore, of the small number of obtained transformants, none exhibited altered *GSH1* and *GSH2* levels of transcription. Thus, it is concluded that GSH is essential for somatic embryogenesis and, as a consequence, it is difficult to regenerate wheat plants with silenced *GSH1* and *GSH2* genes.

Keywords Antioxidant response · Glutathione biosynthetic pathway · Post transcriptional gene silencing · Somatic embryogenesis · ROS mediators of somatic embryogenesis · Wheat

Abbreviations

PTGS	post-transcriptional gene silencing
GSH	Glutathione
BSO	Buthionine sulfoximine
GST	Glutathione S-transferase
MS	Murashige and Skoog
2, 4-D	2,4-Dichlorophenoxyacetic acid
PPT	DL-Phosphinothricin
PCR	Polymerase Chain Reaction

E. Bossio (✉) · A. Díaz Paleo · I. Baroli · R. D. Ríos
Instituto de Genética "Ewald A. Favret", Centro de Investigación en Ciencias Veterinarias y Agronómicas, INTA, CC 25 (B1712WAA), Buenos Aires, Argentina
e-mail: ebossio@cnia.inta.gov.ar

A. Díaz Paleo · R. D. Ríos
Departamento de Ciencias Básicas, Universidad Nacional de Luján, CC 212, Luján, Buenos Aires, Argentina

M. del Vas
Instituto de Biotecnología, Centro de Investigación en Ciencias Veterinarias y Agronómicas, INTA, CC 25 (B1712WAA), Buenos Aires, Argentina

I. Baroli
Centro Nacional de Investigaciones Científicas y Técnicas, CONICET, Av. Rivadavia 1917 C1033AAJ, Buenos Aires, Argentina

A. Acevedo
Centro de Investigación de Recursos Naturales, INTA, CC 25 (B1712WAA), Buenos Aires, Argentina

Introduction

Somatic embryogenesis is a form of asexual reproduction whereby somatic cells under favorable in vitro conditions are induced to form an embryo. The process consists of two phases, an initial induction phase, during which differentiated somatic cells acquire embryogenic competence and proliferate as embryogenic cells, and an expression phase, when the embryogenic cells differentiate to form somatic embryos (Namasivayam 2007). The process of acquisition of embryogenic competence by somatic cells involves reprogramming of gene expression patterns as well as changes in cell morphology, physiology, and metabolism

(Sun et al. 2012). In wheat (*Triticum aestivum* L.) plant regeneration from immature embryos has been described in several reports (Ahloowalia 1982; Ozias-akins and Vasil 1982; Nehra et al. 1994; Pellegrineschi et al. 2002). Even though the molecular basis of somatic embryogenesis, particularly the transition of somatic cells into embryonic cells, is poorly understood, under specific in vitro culture conditions, the formation of new wheat plants occurs only in the upper epidermal layers of the scutella (He et al. 1990; Nehra et al. 1994). Also, the unicellular origin of somatic embryos has been widely accepted under these same specific conditions (He et al. 1990; Nehra et al. 1994). These attributes, together with the reproducible and high frequency regeneration of plants, have made the somatic embryogenesis of wheat scutella the in vitro morphogenesis procedure of preference for genetic transformation mediated by *Agrobacterium* or biolistics.

The factors that influence somatic embryogenesis of scutella and other wheat tissues have been extensively studied. The morphological description of the changes that take place during the new formation of somatic embryos (He et al. 1990) was followed by gene expression analysis. Several genes whose expression vary during wheat somatic embryogenesis encode proteins expected to participate in the process, while others encode products whose biological functions could not be so obviously related to the known morphological events that outline embryogenesis. This second group of genes includes enzymes and non-enzymatic components that are involved in antioxidant mechanisms. In particular, the tripeptide thiol glutathione (GSH) was suggested as a key player in the establishment of cell totipotency during wheat somatic embryogenesis through the action of glutathione-S-transferase (GST) (Singla et al. 2007).

As an antioxidant, GSH participates in the regeneration of reduced ascorbate in the Halliwell-Asada cycle, maintaining cell redox homeostasis, and is essential for the turnover of the cell cycle and root and nodule meristem activity. Glutathione can interact in multiple ways with proteins through thiol-disulphide exchange and related processes (Rouhier et al. 2008¸ Foyer and Noctor 2011, Noctor et al. 2012).

GSH biosynthesis is catalysed in plants by two ATP dependent enzymes: γ-Glutamylcysteine Synthase (γ-ECS, NCBI: EC 6.3.2.2) and Glutathione Synthase (GS, NCBI: EC 6.3.2.3), encoded by the *GSH1* and *GSH2* genes, respectively (Tyburski and Tretyn 2010). These are both single copy genes which have been described in Arabidopsis (NCBI: Y09944.1 and AJ243813.1), rice (NCBI: AJ508916.2 and AY453405.1) and maize (NCBI: NM_001111672.1 and AJ302784.1), among other species. In allohexaploid wheat the cDNA sequences of the *GSH2* genes present in the A, B and D genomes and a version of the *GSH1* gene have been reported (NCBI: AJ579380.1, NCBI: AJ579381.1, NCBI: AJ579382.1 and NCBI: AY864064.1, respectively). In Arabidopsis, all the mutations that lower the levels of glutathione map to the *GSH1* gene. The *cad2* mutant is almost indistinguishable from the wild type except for its sensitivity to cadmium (Howden et al. 1995). A second mutant, root meristemless 1 (*rml1*), also very sensitive to cadmium, fails to initiate cell division during germination and is therefore unable to organize an active postembryonic root meristem, but the shoot apex is not affected (Vernoux et al. 2000). Both mutants differ in their intracellular GSH content: *cad2* has 15–30 % of the GSH present in the wild type (Cobbett et al. 1998) whereas *rml1* only has 2.7 % (Vernoux et al. 2000). According to Vernoux et al. (2000), there is a critical threshold GSH concentration below which developmental effects are observed in the roots of the *rml1* genotype. A third mutant in the *GSH1* gene, *rax1*, was isolated from Arabidopsis as a regulator that constitutively expresses photooxidative stress-inducible Ascorbate Peroxidase 2 (Ball et al. 2004). Despite the apparent simplicity of the GSH biosynthetic pathway there are few reports on the engineering of GSH levels through the reduction of enzyme levels, particularly in non-model plants. Transgenic Arabidopsis plants expressing γ-Glutamylcysteine Synthetase (*GSH1*) in both sense and antisense orientations and containing glutathione levels ranging between 3 and 200 % of those of the wild type were used to describe the role of GSH during stress protection and its contribution to normal metabolic activities. Interestingly, plants that carried the *GSH1* gene in antisense orientation and had low GSH levels were smaller but developed at the same rate as wild type plants. Although other plants contained GSH levels similar to the *rml1* mutant, no developmental aberrations were described in roots or shoots besides the already mentioned difference in plant height (Xiang et al. 2001).

The aim of the present work was to evaluate the effect of the interruption of glutathione biosynthesis on wheat somatic embryogenesis by triggering PTGS to target the *GSH1* and *GSH2* genes.

Materials and methods

Plant material

Wheat (*Triticum aestivum* L.) cv. SH9856 and barley (*Hordeum vulgare* L.) cv. Golden Promise were used in this investigation.

Buthionine sulfoximine and glutathione treatments

Immature scutella of 1 mm size were dissected and cultured in Murashige and Skoog (MS) induction medium (Murashige and Skoog 1962) containing 2 mg/L 2,4-Dichlorophenoxyacetic acid (2, 4-D) and buthionine

sulfoximine (BSO) at a final concentration of 0.5 and 1 mM. The scutella were transferred every 2 weeks to new Petri dishes with fresh medium. Glutathione in filtered water solution at a 125, 250, 500 and 1,000 μM final concentration was added to the scutellum culture medium before dispensing into the Petri dishes.

Plasmid vectors for biolistic transformation

When these experiments were performed, no *GSH1* gene sequence was available from wheat. With the rationale that the unique sequences of each the *GSH1* and *GHS2* genes of (diploid) barley would have enough similarity to the three variants of the *GSH1* and *GSH2* target genes in allohexaploid wheat as to be functional in silencing, barley *cv*. Golden Promise was used to isolate the cDNA sequences of the *GSH1* and *GSH2* genes. cDNA of the barley *GSH1* transcript was obtained using primers designed on conserved sequences between the homologous rice (NCBI: AK103315.1) and maize (NCBI: AY105308.1) genes. Sequences of the three *GSH2* homologous genes present in wheat were published before starting the *GSH2* silencing construction. This sequence was not by then available in barley. So a cDNA of the barley *GSH2* was obtained (NCBI: DQ291128) using primers designed on conserved sequences on the *GSH2* genes of wheat.

The fragments of the barley genes *GSH1* or *GSH2* were subcloned in inverted repeat orientation in two plasmids to transcribe double stranded RNA in a hairpin structure (Wesley et al. 2001). The *GSH1* gene fragment used, that included 341 bp corresponding to the *GSH1* wheat sequence (NCBI: AY864064.1) between positions 625 and 965, was amplified with the gsh1-forw (5′-ggtagatggttctcccagtcatt-3′) and gsh1-rev (5′-aggaagttccgtactggtctagc-3′) primers. Similarly, a 469 bp fragment from position 866 to 1,334 of the *GSH2* barley gene (NCBI: DQ291128) and amplified with the gsh2-forw (5′-gaatggagtgcaaggcttttgat-3′) and gsh2-rev (5′-ccttatctttgttccgcaggtagg-3′) primers was used for the silencing construct. Each of these sequence fragments with the corresponding inverted repeats separated by the second intron of the *PYRUVATE ORTOPHOSPHATE DIKINASE* gene of *Flaveria trinervia* (Rosche et al. 1998) were subcloned under the control of the rice *ACTIN1* gene promoter (McEllroy et al. 1990) and the *OCS* terminator sequence (Barker et al. 1983) giving rise to the *SiECS* or *SiGS* silencing cassettes (Fig. 1). Each silencing cassette was

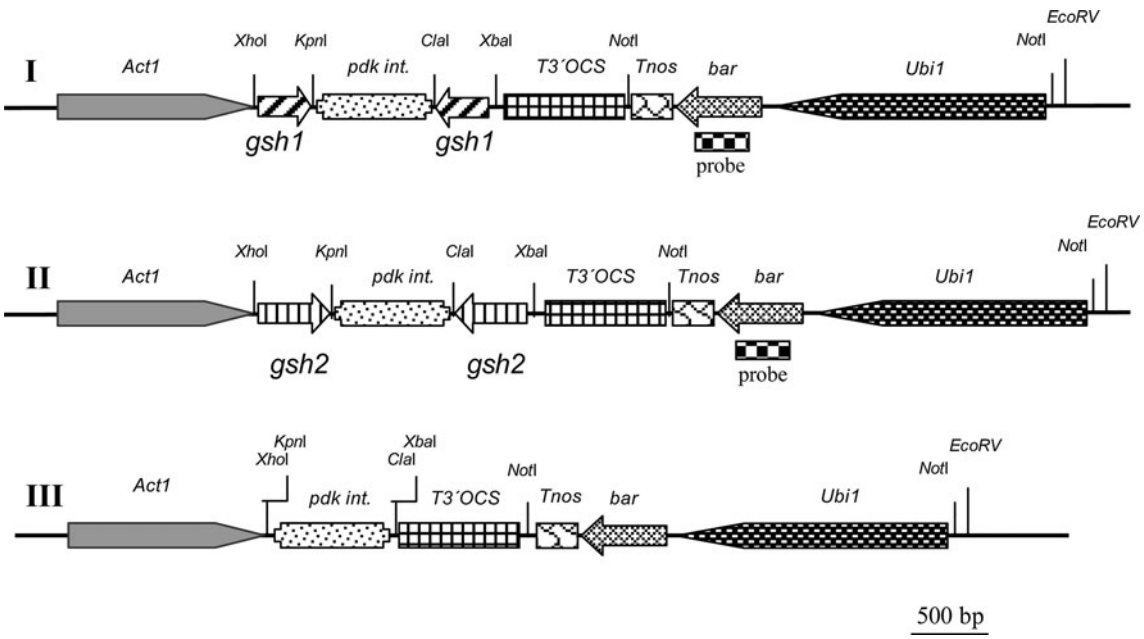


Fig. 1 Vectors used in wheat transformation experiments. Schematic representations of the plasmids, I: pKECS-UBN, II: pKGS-UBN, III: pK8. *SiECS* and *SiGS* regions, containing silencing cassettes present in pKECS-UBN plasmid (**I**) and pKGS-UBN plasmid (**II**) respectively. The *BAR* selection cassette is present in **I**, **II** and **III** (see "Buthionine sulfoximine and glutathione treatments" section). Vectors were constructed on the pBLUEScrit KS+ plasmid backbone. The 346 nucleotide probe used in Southern blot analysis is indicated as a *box* below the *BAR* sequence. The indicated *Eco*RV restriction site was used to estimate number of insertion sites of *SiECS* and *SiGS* in the transgenic wheat plants. Act1: rice *ACTIN1* gene promoter, *pdk* int: second intron of the *PYRUVATE ORTOPHOSPHATE DIKINASE* gene of *Flaveria trinervia*, OCS: *OCS* terminator sequence of *Agrobacterium tumefaciens*, Tnos: *NOS* terminator sequence of *Agrobacterium tumefaciens*, *GSH2*: *GSH2* gene fragment, *GSH1*: *GSH1* gene fragment, *bar*: complete *BAR* gene sequence, Ubi1: maize *UBIQUITIN1* gene promoter

placed adjacent to a selection cassette named UBN which consisted of the coding sequences of the *BAR* gene conferring phosphinothricin resistance (Thompson et al. 1987), under the control of the maize *UBIQUITIN* promoter (Christensen et al. 1992) and the *Agrobacterium tumefaciens NOS* terminator sequence (Depicker et al. 1982). The plasmids that included either SiECS or SiGS along with UBN were respectively named pKECS-UBN and pKGS-UBN (Fig. 1). Another vector, named pK8, containing the *bar* selection cassette (UBN) and an "empty" cassette with the same regulatory elements as in the silencing constructions but without any additional DNA sequences was used as a transformation control vector (Fig. 1).

Wheat genetic transformation

Wheat plants of the genotype SH9856 (Pellegrineschi et al. 2002) were grown in a growth chamber at 18/15 °C thermoperiod and 16/8 h photoperiod to be used as scutellum donors. Scutella of approximately 1 mm in size, separated from dissected immature embryos, were used as targets for gene transfer following the biolistic procedure described by Pellegrineschi et al. (1999) using the Particle Inflow Gun, *PIG*, as a microprojectile accelerator (Vain et al. 1993). In vitro selection was applied to cultures 20 days after gene transfer by adding 5 mg/L DL-Phosphinothricin (PPT, Duchefa, The Netherlands). Surviving rooted plantlets were transferred to pots with a soil mixture and placed in a growth chamber under the conditions already described.

Molecular characterization of transgenic plants

Plant genomic DNA was extracted from leaf tissue as described by Dellaporta et al. (1983). All the plants regenerated from in vitro culture were analyzed by polymerase chain reaction (PCR) for the presence or absence of *BAR* and *SiECS* or *SiGS* silencing cassettes. The specific primers used for DNA amplification were bar-for (5′-tgcaccatcgtcaaccacta-3′), bar-rev (5′-acagcgaccacgctcttgaa-3′), IntH-forW (5′-cgaacatgaataaacaaggtaac-3′) and tOCS-rev (5′-agaatgaaccgaaaccggcg-3′). IntH-forw and tOCS-rev annealed to specific regions of the intron and *ocs* terminator sequences of the silencing cassettes, respectively. The PCR reaction was carried out in a final volume of 25 µl with 20–50 ng wheat genomic DNA as a template.

RT-PCR and RT-qPCR

For quantitative RT-PCR, total RNA was extracted from leaf tissues with Trizol (Invitrogen, USA) following the manufacturer's instructions and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). Total RNAs were treated with RQ1 RNase-free DNase (Promega, USA) to remove contaminating DNA. The absence of DNA in RNA samples was confirmed by PCR analysis. First strand cDNA was synthesized with oligo $(dT)_{18}$ as primer and SuperScript III Reverse Transcriptase (Invitrogen, USA) following the manufacturer's recommendations. An Icycler IQ Real-Time Detection System (BioRad, USA) was used. The wheat *TaCCF* gene, which encodes a putative chromosomal condensation factor, was used as an internal reference gene (Stephenson et al. 2007). The endogenous gene transcripts *GSH1* and *GSH2* were amplified using IQ SuperMix PCR kit (BioRad, USA) with the primers: qgsh1-for (5′-tgcggaggtcaattcacatc-3′), qgsh1-rev (5′-tgcggaacatcatatcaaggc-3′), qgsh2-for (5′-gctcaacaccatctcaacatc-3′), qgsh2-rev (5′-cgcttccattattatactcaacc-3′). The PCR cycling conditions comprised one cycle at 95 °C for 5 min, followed by 45 cycles at 95 °C for 20 s and 60 °C for 40 s. A melting curve was generated to confirm the specificity of the amplification reaction. For each sample the reactions were carried out in three replicates. Statistical analyses of the results were performed with the Relative Expression Software Tool REST© (Pfaffl et al. 2002).

RT-PCR was performed to detect hairpin transcripts encoded by the *SiECS* or *SiGS* cassettes in transgenic plants. For the *GSH2* gene the target sequence was located downstream of the intron position, whereas for the *GSH1* gene it was located upstream of the intron (Fig. 2).

Southern blot analysis

For Southern blot analyses 20 µg of DNA extracted from leaf samples of each transgenic plant (Saghai-Maroof et al. 1984) were digested overnight with *Eco*RV and separated by agarose (1 %) gel electrophoresis. DNA was transferred to a positively charged nylon membrane and hybridized to digoxigenin (DIG)-dUTP labeled probes, following manufacturer's instructions (F. Hoffmann & La Roche, Switzerland). DIG-labeled probes were generated by PCR amplification of the coding region of the *BAR* gene with the previously mentioned bar-for and bar-rev primers.

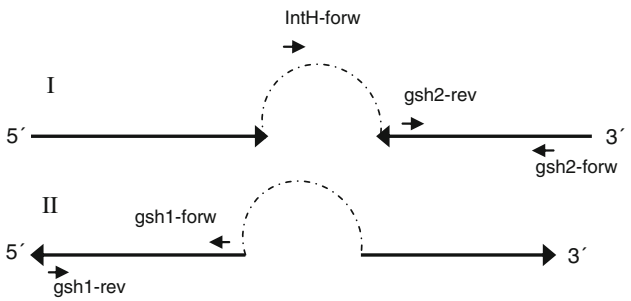


Fig. 2 Schematic representation of primer annealing sites on silencing hairpin RNA from the *SiGS* (**I**) and *SiECS* (**II**) cassettes

Table 1 Effect of BSO on wheat somatic embryogenesis

Assay	Induction medium additives	Total scutella tested	Embryogenic scutella (%)
A	1 mM BSO	385	0
A	Without BSO	362	99
B	0.5 mM BSO	350	0
B	Without BSO	350	96
C	1 mM BSO + 500 μM GSH	300	0
C	Without BSO and GSH	250	97

In all treatments the culture medium consisted of MS salts and vitamins (Murashige and Skoog 1962), 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/L sucrose, and 8 g/L agar. Assays A, B, C were conducted along a period of 6 months. The 1 mm immature scutella were dissected according to the availability of wheat spikes collected from plants grown in chambers. Scutella were screened 30 days after dissection for the presence of somatic embryos

Results

Effect of a competitive inhibitor of γ-ECS on wheat somatic embryogenesis

In order to assess if γ-ECS plays a significant role on wheat somatic embryogenesis, we evaluated the regeneration of wheat plants from immature scutella by adding BSO, a competitive inhibitor of γ-ECS (Hiratake et al. 2002), to the culture medium of developing somatic embryos. BSO has been accepted as a way of depleting intracellular GSH in many plant experimental systems (Xiang and Oliver 1998; Yanagida et al. 2004) including cell cultures (Sanità di Toppi et al. 1998), although its use has never been reported in morphogenic calli. After 30 days of treatment, there was no in vitro somatic embryogenesis on the calli derived from scutella cultured in induction media supplied with either 0.5 or 1 mM BSO (Table 1; Fig. 3). Conversely, development of somatic embryos was normal on the control induction medium without BSO. The addition of 500 μM GSH could not restore embryogenesis on scutella cultured on BSO (Table 1), therefore, the experimental approach could not demonstrate that the absence of embryogenesis was due to GSH depletion. It is important to note that the addition of GSH to the culture media in the absence of BSO did not affect normal embryo regeneration (Table 2).

Effect of *GSH1* and *GSH2* silencing on in vitro wheat plant regeneration

We then tested whether GSH depletion could be achieved by direct operation on its biosynthetic pathway by the silencing of *GSH1* or *GSH2*. To silence these endogenous wheat genes, embryogenic cells were genetically transformed with the pKECS-UBN or pGS-UBN vectors that were able to express hairpin RNA with fragments of the barley *GSH1* and *GSH2* genes, respectively. The 341 and 469 bp fragments derived from the *GSH1* and *GSH2* barley genes showed 94.7 and 100 % sequence identity as compared to the already published *GSH1* and *GSH2* (genome B) wheat genes, respectively.

The pKECS-UBN plasmid vector was bombarded to 6,750 scutella in two independent assays. Although 85 plantlets were regenerated, only four of them were confirmed as independent *SiECS* transgenic T_0 events by PCR (Table 3). Similarly, while 30 regenerated plantlets were obtained from 1,821 scutella bombarded with pKGS-UBN vector, only two were confirmed as transgenic independent T_0 *SiGS* plants. Taken together, four *SiECS* and two *SiGS* primary transgenic plants were obtained out of 8,571 scutella. However, it is worth noting that while the overall transformation efficiency with pKECS-UBN and pKGS-

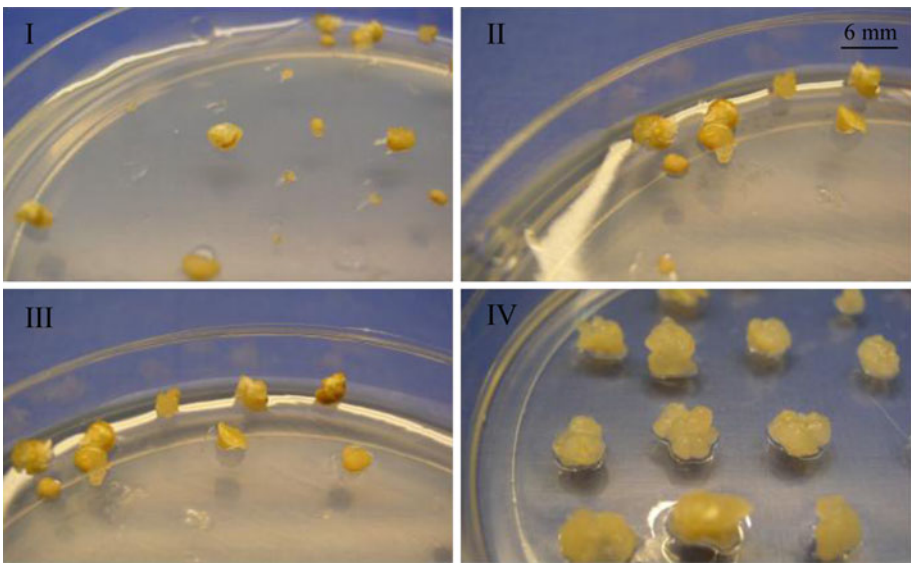


Fig. 3 Scutella development on culture media with BSO. Scutella were photographed 4 weeks after dissection in media with 0.5 mM BSO (**I**), 1 mM BSO (**II**), 1 mM BSO + 500 μM glutathione (**III**) or no BSO as a negative control (**IV**)

Table 2 Effect of GSH on wheat somatic embryogenesis

Assay	GSH (μM)	Total scutella tested	Embryogenic scutella (%)
A	125	210	99
A	0	200	98
B	250	220	99
B	0	215	99
C	500	220	98
C	0	200	97
D	1,000	210	99
D	0	200	98

In all treatments the culture medium consisted of MS salts and vitamins (Murashige and Skoog 1962), 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/L sucrose, and 8 g/L agar. Assays A, B, C, D were conducted along a period of 6 weeks. The 1 mm immature scutella were dissected according to the availability of wheat spikes collected from plants grown in chambers. Scutella were screened 35 days after dissection for the presence of somatic embryos

UBN vectors was 0.07 %, the efficiency of plant transformation with the control plasmid pK8 was 1.3 % (approximately 18 times higher). No transgenic plants could be regenerated from scutella bombarded with the pKECS-UBN and pKGS-UBN vectors and cultured on medium supplied with glutathione, in accordance with the results shown in the BSO experiments (Table 1). Plant escapes are usually obtained at a frequency that could reach 49 % of the total plants regenerated under the selection protocol routinely applied. In this investigation 109 plant escapes were detected, representing 1.27 % of the total scutella bombarded.

Molecular analyses of transgenic plants

Like the two *SiGS* primary transformants, only one out of the four *SiECS* primary transformants expressed a normal phenotype when compared to wild type plants. The three other plants remained dwarf, had severe developmental problems and died approximately 45 days after being transferred to pots.

When analyzed by PCR, all the T_0 plants gave rise to the expected 345 bp amplification fragment of the *BAR* gene and to the 476 and 604 bp amplification fragments of *SiECS* and *SiGS* hairpins, respectively.

The entire progenies of the three transgenic T_0 plants that set seeds were also analyzed by PCR. Surprisingly, all the 180 T_1 plants carried the *BAR* selection and silencing cassette sequences and no segregant non-transgenic T_1 plant could be detected, indicating none of the three T_1 progenies segregated normally, even though they derived from primary transformants.

Southern blot analysis revealed two insertion sites in all the *SiECS* T_1 plants derived from the surviving T_0 transgenic plant. The two T_1 progenies derived from the *SiGS* independent events had 2 and 1 insertion sites each (Fig. 4). These results further confirm the lack of segregation in the 3 T_1 progenies analyzed.

RT-qPCR assay showed that the transcription levels of the *GSH1* or *GSH2* target genes in the T_1 transgenic progenies were not significantly different from those in the control non-transgenic plants, indicating that *GSH1* or *GSH2* gene expression was not silenced in the transgenic plants.

To investigate whether the inability of the transgenic plants to trigger PTGS on the target genes was due to lack of transcriptional activity of the silencing cassettes, RT-PCR was performed on the transgenic *SiECS* and *SiGS* plants (Fig. 5). The fact that all the T_1 plants analyzed expressed the hairpin RNA structure demonstrated that the absence of silencing on the target genes was not due to the lack of transcription either of the transgenic cassettes (Fig. 5).

Discussion

In this investigation, we attempted to post-transcriptionally silence two genes coding for enzymes of the GSH

Table 3 Transformation experiments by biolistic bombardment

Assay	Vector	GSH (μM)	Total number of bombarded scutella	Total number of regenerated plants[a]	Total number of transgenic regenerated plants	Transformation efficiency[b]
D	pKECS-UBN	0	4,228	51	4	0.09
D	pKGS-UBN	0	1,821	30	2	0.1
D	pk8 (control)	0	1,077	38	14	1.3
E	pKECS-UBN	0	1,560	20	0	–
E	pKECS-UBN	500	962	14	0	

Transformation experiments were carried out using the PIG device following the protocol of Pellegrineschi et al. (1999)

[a] Taken together no statistical differences were found in the regenerated plants ($\chi^2 4df = 8.15$) of assays D and E

[b] Differences among the frequency of transgenic plants (Total transgenic regenerated plants/Total bombarded scutella * 100) obtained with the pKECS-UBN, pKGS-UBN and pk8 (control) plasmids were highly significant ($\chi^2 2df = 46.4$, $P < 0.01$)

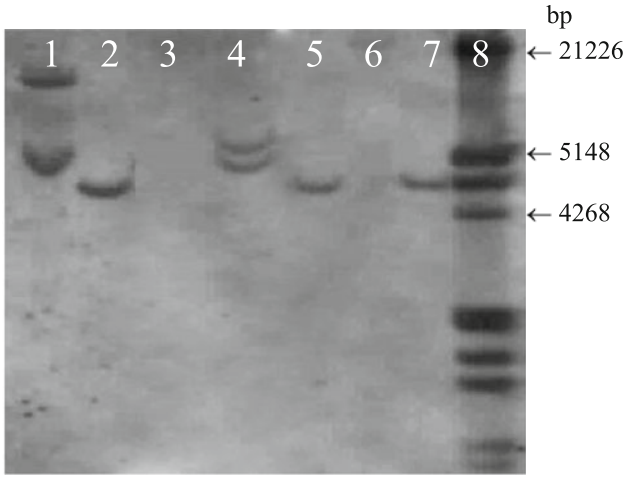


Fig. 4 Southern blot analysis of *SiECS* and *SiGS* T_1 transgenic plants. Chemiluminescent detection of digoxigenin-labeled DNA probes prepared by PCR amplification of a 346 nucleotide *BAR* gene fragment (see also Fig. 1). The probes were hybridized to genomic DNA digested with *Eco*RV. *Lane 1* a T1 transgenic *SiECS* plant. *Lane 2, 5* and *7*: three of the T1 transgenic plants corresponding to the progeny of the first of two *SiGS* events obtained. *Lane 4*: a T1 transgenic plant corresponding to the second *SiGS* event. *Lane 3* and *6*: wild type wheat. *Lane 8*: MWM (DNA Molecular-Weight Marker III, Dig-labeled, Roche Diagnostics)

biosynthetic pathway, in order to determine the contribution of GSH to wheat somatic embryo development. Wheat scutella were bombarded with vectors that transcribed hairpin RNA, so that PTGS could be induced on either of the *GSH1* and *GSH2* genes. Traditionally, transformation efficiency in our laboratory averages 1.3 %, which is in agreement with the transformation efficiency determined with the control plasmid pK8 in this work. Theoretically, if this transformation efficiency is calculated over the 8,571 scutella used in this investigation, 111 primary transgenic plants would have been expected instead of the six that we obtained. The difference in transformation efficiency obtained by using the silencing and control vectors suggests that the lack of GSH had a strong negative effect on the development of somatic embryos from scutella. As a comparison, no development of somatic embryos was observed on callus induction medium supplemented with BSO, a competitive inhibitor of γ-ECS enzyme, in which no new embryogenic structures, usually distinguished as green islands by the naked eye (Table 1; Fig. 3). The fact that the silencing of either *GSH1* or *GSH2* induces a similar inhibitory effect on wheat somatic embryogenesis as the addition of BSO suggests that the lack of intracellular GSH itself, instead of the absence of active transcripts or of the γ-ECS and GS enzymes themselves, could be the cause of the observed inhibition of somatic embryogenesis.

The three morphologically normal *SiECS* and *SiGS* transgenic T_0 plants set seeds as did the transformed control plants carrying pK8 and the non transgenic wild type plants. Unexpectedly, the self progenies of the three plants

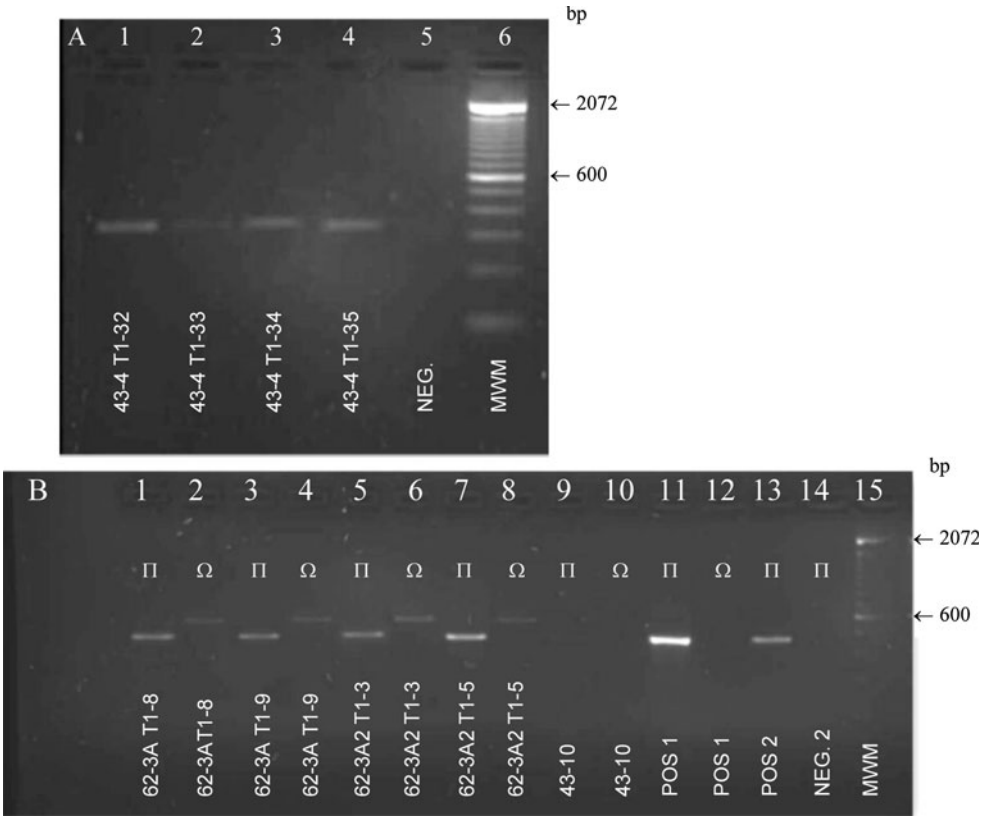


Fig. 5 Agarose gel electrophoresis of the amplification products of the RT-PCR using as templates the hairpins dsRNA corresponding to: **a** the *SiECS* transgene, **b** the *SiGS* transgene. **a** *Lane 1–4*: T_1 transgenic *SiECS* plants. *Lane 5*: wild type wheat. *Lane 6*: MWM (100 bp ladder, Invitrogen). The cDNA was synthesized with the gsh1-forw primer. PCR amplification was performed with gsh1-forw and gsh1-rev primers. **b** *Lanes 1–4* and *5–8* correspond to two T_1 plants derived from the two SiGS T_0 plants. *Lane 9–13*: wild type control plant. *Lane 11–12*: cDNA was synthesized using $[dT]_{18}$ as primer. *Lane 13*: genomic DNA. *Lane 14*: Control mix without template DNA. *Lane 15*: MWM (100 bp ladder, Invitrogen). Π: PCR amplification with gsh2-forw and gsh2-rev primers (see "Wheat genetic transformation"). Ω: PCR amplification with gsh2-forw and IntH-forw

exhibited an anomalous segregation pattern, because non-transgenic T_1 plants could not be recovered, in spite of the fact that only one or two transgene insertion sites were confirmed. Positive RT-PCRs on the hairpin transcripts demonstrated the functionality of silencing cassettes. RT-qPCR on the transcripts of the endogenous target *GSH1* and *GSH2* wheat genes showed neither total nor partial PTGS. The T_1 progenies of *SiECS* and *SiGS* transgenic plants were morphologically similar to wild type plants and they equally failed in establishing PTGS of the *GSH1* or *GSH2* genes, respectively.

It has been reported that not every hairpin RNA transcription leads to RNA silencing. Parameters such as the integration locus of the transgene, the inherent characteristics of the transcript, its intermediate processing and product interaction with various proteins of competing machineries may be critical in determining the fate of hairpin transcripts and efficient triggering of RNA silencing (Dalakouras et al. 2011). Our study demonstrates that regenerated transgenic plants harbored silencing cassettes that were transcriptionally active (Fig. 5), ruling out the occurrence of positional effects. Interestingly, even though the level of expression of these cassettes was not determined, the selection cassette, located close to the silencing one, exhibited an expression level high enough to allow for in vitro callus cells development under the pressure of the selective agent.

Transcription of stable hairpin RNA structures in the three fertile *SiECS* and *SiGS* plants stand as new examples that demonstrate that the presence of dsRNA is not always enough to induce gene silencing (Dalakouras et al. 2011) and further research is needed to explain the reason why PTGS was not established in these plants although they were expressing the hairpin constructs. The obstacle in the formation of transgenic wheat somatic embryos derived from bombarded scutella supports the idea that PTGS mechanisms are active very early in wheat somatic embryo development.

The three transgenic *SiECS* T_0 plants that died were dwarf and very poorly developed. However, PCR and RT-PCR applied on genomic and cDNA samples of these three genotypes indicated that *SiECS* was present and that endogenous *GSH1* gene was transcribed. No further experiments could be performed with the leaf material available, so the molecular results are not conclusive. We find likely that the surviving *SiECS* T_0 plant was not silenced considering the strong band detected on gel of the RT-PCR products of the endogenous *GSH1* transcript (Fig. 5).

Glutathione is at the hub of the complex antioxidant networks of plant and animal cells, participating in cellular redox signalling networks that influence growth, development and defence (Diaz Vivancos et al. 2010a, b). Knockout Arabidopsis mutants for γ-ECS are embryo lethal (Cairns et al. 2006) and morphological abnormalities have been observed in Arabidopsis genotypes with very low levels of GSH (Vernoux et al. 2000; Reichheld et al. 2007). More relevant for the interpretation of the present results is the *rml1*mutant, in which the low levels of glutathione are associated with an inhibition of cell division in root meristems after embryo formation (Reichheld et al. 2007). As demonstrated by Vernoux et al. (2000), the mutant phenotype is largely due to the low intracellular GSH concentration but not through the lack of antioxidant capacity, because shoot development is basically not affected. In the authors' interpretation, the GSH reduction in the *rml1* mutant primarily affected root meristem through developmental pathways that would need minimum GSH concentrations, below which these pathways would not be activated. Following this rationale, we can speculate that in the in vitro embryogenic scutellum cells of wheat transcribing *SiECS* and *SiGS* cassettes, GSH would not reach a threshold concentration beyond which embryogenesis is possible.

Results from several gene expression studies suggest that the induction of somatic embryogenesis is the result of oxidative stress (Dron et al. 1988; Kitamiya et al. 2000; Davletova et al. 2001; Galland et al. 2001). At the same time, there is increasing evidence in favor of an interaction of GSH and auxin in embryo development and maintenance of meristem function (Pasternak et al. 2005; Noctor et al. 2012). In Arabidopsis, it has been suggested that thiol reduction pathways interfere with developmental processes through modulation of auxin signaling at the meristem level (Bashandy et al. 2010). Therefore, considering that auxin is the major hormonal inducer of somatic embryogenesis (Namasivayam 2007), the observed effect of PTGS of the *GSH1* and *GSH2* genes reported here could be due to a disruption of the interaction between the protein thiol/disulfide status of the cell and auxin signaling. In line with our results, treatment of Arabidopsis root tips with BSO led to an abnormal auxin response and altered expression of quiescent center genes (Koprivova et al. 2010). Clearly, more research is needed to elucidate the role of the interplay between GSH and auxin in the control of somatic embryogenesis.

Genotypes with loss-of-function phenotypes have traditionally been used to settle the role of key compounds in biology. So far, this has not been the case for glutathione, since neither null glutathione mutants nor the complete silencing of its biosynthetic pathway has ever been reported. To better understand the molecular and metabolic events downstream of glutathione which determine somatic embryogenesis in wheat, a null mutant is indispensable. Alternatives to genetically engineer such null genotype in wheat are currently in progress. Briefly, they consist of using inducible promoters to drive the expression of the silencing

constructions when required or coexpressing in a wheat plant silencing constructions, similar to those reported here, and a function restorer construction containing the corresponding low identity homeologous sequence.

Acknowledgments The authors wish to thank Dr. C. Foyer for the critical reading of this manuscript. This work was funded by project AEGR 3425 from Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina.

References

Ahloowalia BS (1982) Plant regeneration from callus culture in wheat. Crop Sci 22:405–410. doi:10.2135/cropsci1982.0011183X002200020047x

Ball L, Accotto GP, Bechtold U, Creissen G, Funck D, Jimenez A, Kular A, Leyland N, Mejia-Carranza J, Reynolds H (2004) Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in Arabidopsis. Plant Cell 16:2448–2462. doi:10.1105/tpc.104.022608

Barker RF, Idler KB, Thompson DV, Kemp JD (1983) Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pti15955. Plant Mol Biol 2:335–350. doi:10.1007/BF01578595

Bashandy T, Guilleminot J, Vernoux T, Caparros-Ruiz D, Ljung K, Meyer Y, Reichheld JP (2010) Interplay between the NADP-linked thioredoxin and glutathione systems in Arabidopsis auxin signaling. Plant Cell 22:376–391. doi:10.1105/tpc.109.071225

Cairns NG, Pasternak M, Wachter A, Cobbett CS, Meyer AJ (2006) Maturation of Arabidopsis seeds is dependent on glutathione biosynthesis within the embryo. Plant Physiol 141:446–455. doi:10.1104/pp.106.077982

Christensen A, Sharrock R, Quail P (1992) Maize Poly-Ubiquitin Genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18:675–689

Cobbett CS, May MJ, Howden R, Rolls B (1998) The glutathione-deficient, cadmium-sensitive mutant, cad2-1, of *Arabidopsis thaliana* is deficient in γ-glutamylcysteine synthetase. Plant J 16:73–78. doi:10.1046/j.1365-313x.1998.00262.x

Dalakouras A, Tzanopoulou M, Tsagris M, Wassenegger M, Kalantidis K (2011) Hairpin transcription does not necessarily lead to efficient triggering of the RNAi pathway. Transgenic Res 20:293–304

Davletova S, Mészáros T, Miskolczi P, Oberschall A, Török K, Magyar Z, Dudits D, Deák M (2001) Auxin and heat shock activation of a novel member of the calmodulin like domain protein kinase gene family in cultured alfalfa cells. J Exp Bot 52:215–221. doi:10.1093/jexbot/52.355.215

Dellaporta S, Wood J, Hicks J (1983) A plant DNA minipreparation: version 2. Plant Mol Biol Rep 1:19–22. doi:10.1007/BF02712670

Depicker A, Stachel S, Dhaese P, Zambryski P, Goodman H (1982) Nopaline synthase: transcript mapping and DNA sequence. J Mol Appl Genet 1:561–573

Diaz Vivancos P, Dong Y, Ziegler K, Markovic J, Pallardó F, Pellny T, Verrier P, Foyer C (2010a) Recruitment of glutathione into the nucleus during cell proliferation adjusts whole-cell redox homeostasis in *Arabidopsis thaliana* and lowers the oxidative defence shield. Plant J 64:825–838. doi:10.1111/j.1365-313X.2010.04371.x

Diaz Vivancos P, Wolff T, Markovic J, Pallardó F, Foyer C (2010b) A nuclear glutathione cycle within the cell cycle. Biochem J 431:169–178. doi:10.1042/BJ20100409

Dron M, Clouse S, Dixon R, Lawton M, Lamb C (1988) Glutathione and fungal elicitor regulation of a plant defense gene promoter in electroporated protoplasts. Proc. Natl. Acad. Sci. USA 85: 6738–6742

Foyer CH, Noctor G (2011) Ascorbate and glutathione: the heart of the redox hub. Plant Physiol 155:2–18. doi:10.1104/pp.110.167569

Galland R, Randoux B, Vasseur J, Hilbert J (2001) A glutathione s-transferase cDNA identified by Mrna diferential display is upregulated during somatic embryogenesis in Cichorium. Biochim Biophys Acta 1522:212–216. doi:10.1016/S0167-4781(01)00314-1

He DG, Yang YM, Bertram J, Scott KJ (1990) The histological development of the regenerative tissue derived from cultured immature embryos of wheat (*Triticum aestivum* L.). Plant Sci 68:103–111. doi:10.1016/0168-9452(90)90158-K

Hiratake J, Irie T, Tokutake N, Oda J (2002) Recognition of a cysteine sustrate by *E. coli* γ-glutamylcysteine synthetase probed by sulfoximine-based transition-state analogue inhibitors. Biosci Biotechnol Biochem 66:1500–1514. doi:10.1271/bbb.66.1500

Howden R, Andersen C, Goldsbrough P, Cobbett C (1995) A Cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. Plant Physiol 107:1067–1073. doi:10.1104/pp.107.4.1067

Kitamiya E, Suzuki S, Sano T, Nagata T (2000) Isolation of two genes that were induced upon the initiation of somatic embryogenesis on carrot hypocotyls by high concentrations of 2,4-D. Plant Cell Rep 19:551–557. doi:10.1007/s002990050772

Koprivova A, Mugford ST, Kopriva S (2010) Arabidopsis root growth dependence on glutathione is linked to auxin transport. Plant Cell Rep 29:1157–1167. doi:10.1007/s00299-010-0902-0

McElroy D, Rothenberg M, Reece K, Wu R (1990) Characterization of the rice (*Oryza sativa*) *Actin* gene family. Plant Mol Biol 15:257–268. doi:10.1007/BF00036912

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Namasivayam P (2007) Acquisition of embryogenic competence during somatic embryogenesis. Plant Cell Tiss Org Cult 90:1–8. doi:10.1007/s11240-007-9249-9

Nehra NS, Chibbar RN, Leung N, Caswell K, Mallard C, Steinhauer L, Baga M, Kartha KK (1994) Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. Plant J 5:285–297. doi:10.1046/j.1365-313X.1994.05020285.x

Noctor G, Mhamdi A, Chaouch S, Han YI, Neukermans J, Marquez-Garcia B, Queval G, Foyer C (2012) Glutathione in plants: an integrated overview. Plant Cell Environ 35:454–484. doi:10.1111/j.1365-3040.2011.02400.x

Ozias-akins P, Vasil I (1982) Plant regeneration from cultured immature embryos and inflorescences *Triticum aestivum* L. (wheat): evidence for somatic embryogenesis. Protoplasma 110:95–105. doi:10.1007/BF01281535

Pasternak T, Potters G, Caubergs R, Jansen M (2005) Complementary interactions between oxidative stress and auxins control plant growth responses at plant, organ, and cellular level. J Exp Bot 56:1991–2001. doi:10.1093/jxb/eri196

Pellegrineschi A, Fennell S, McLean S, Brito RM, Velázquez L, Salgado M, Olivares JJ, Hernandez R, Hoisinngton D (1999) Wheat transformation in CIMMYT: a description of a service laboratory. In Vitro Cell Dev Biol 35:43–49. doi:10.1007/s11626-999-0042-4

Pellegrineschi A, Noguera LM, Skovmand B, Brito RM, Velazquez L, Salgado MM, Hernandez R, Warburton M, Hoisington D (2002) Identification of highly transformable wheat genotypes

for mass production of fertile transgenic plants. Genome 45:421–430. doi:10.1139/g01-154

Pfaffl M, Horgan G, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36. doi:10.1093/nar/30.9.e36

Reichheld JP, Khafif M, Riondet C, Droux M, Bonnard G, Meyer Y (2007) Inactivation of thioredoxin reductases reveals a complex interplay between thioredoxin and glutathione pathways in Arabidopsis development. Plant Cell 19:1851–1865. doi:10.1105/tpc.107.050849

Rosche E, Chitty J, Westhoff P, Taylor WC (1998) Analysis of promoter activity for the gene encoding pyruvate orthophosphate dikinase in stably transformed C4 *Flaveria* species. Plant Physiol 117:821–829. doi:10.1104/pp.117.3.821

Rouhier N, Lemaire SD, Jacquot JP (2008) The role of glutathione in photosynthetic organisms: emerging functions for glutaredoxins and glutathionylation. Ann Rev Plant Biol 59:143–166

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Sanità di Toppi L, Lambardi M, Pazzagli L, Cappugi G, Durante M, Gabbrielli R (1998) Response to cadmium in carrot in vitro plants and cell suspension cultures. Plant Sci 137:119–129. doi:10.1016/S0168-9452(98)00099-5

Singla B, Tyagi AK, Khurana JP, Khurana P (2007) Analysis of expression profile of selected genes expressed during auxin-induced somatic embryogenesis in leaf base system of wheat. Plant Mol Biol 65:677–692. doi:10.1007/s11103-007-9234-z

Stephenson T, McIntyre C, Collet C, Xue G (2007) Genome-wide identification and expression analysis of the NF-Y family of transcription factors in *Triticum aestivum*. Plant Mol Biol 65:77–92. doi:10.1007/s11103-007-9200-9

Sun L, Wu Y, Su S, Liu H, Yang G, Li S, Shan X, Yuan Y (2012) Differential gene expression during somatic embryogenesis in the maize (*Zea mays* L.) inbred line H99. Plant Cell Tiss Org Cult 109:271–286. doi:10.1007/s11240-011-0093-6

Thompson C, Movva N, Tizard R, Crameri R, Davies J, Lauwereys M, Botterman J (1987) Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J 6:2519–2523

Tyburski J, Tretyn A (2010) Glutathione and glutathione disulfide affect adventitious root formation and growth in tomato seedling cuttings. Acta Physiol Plant 32:411–417. doi:10.1007/s11738-009-0418-9

Vain P, Keen N, Murillo J, Rathus C, Nemes C, Finer J (1993) Development of the particle inflow gun. Plant Cell Tiss Org Cult 33:237–246. doi:10.1007/BF02319007

Vernoux T, Wilson R, Seeley K, Reichheld J, Muroy S, Brown S, Maughan S, Cobbett C, Van Montagu M, Inzé D (2000) The root meristemless/cadmium sensitive gene defines a glutathione: dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. Plant Cell 12:97–110. doi:10.1105/tpc.12.1.97

Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27:581–590. doi:10.1046/j.1365-313X.2001.01105.x

Xiang C, Oliver D (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in Arabidopsis. Plant Cell 10:1539–1550. doi:10.1105/tpc.10.9.1539

Xiang C, Werner BL, Christensen EM, Oliver DJ (2001) The biological functions of glutathione revisited in Arabidopsis transgenic plants with altered glutathione levels. Plant Physiol 126:564–574. doi:10.1104/pp.126.2.564

Yanagida M, Mino M, Iwabuchi M, Ogawa K (2004) Reduced glutathione is a novel regulator of vernalization-induced bolting in the rosette plant *Eustoma grandiflorum*. Plant Cell Physiol 45:129–137. doi:10.1093/pcp/pch030