Arch. Biol. Sci., Belgrade, 62 (3), 539-546, 2010 DOI:10.2298/ABS1003539G

MATURE EMBRYO-DERIVED WHEAT TRANSFORMATION WITH MAJOR STRESS-MODULATED ANTIOXIDANT TARGET GENE

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Abstract – The mature embryos of fourteen elite winter wheat cultivars have been transformed by a biolistic approach. The gene coding for **γ**-glutamylcysteine synthetase (EC 6.3.2.2) was used as a transgene in order to obtain stable transformants resistant to drought stress. A binary vector, pBinarUTRECS, was used. The gene was under the control of the CaMV35S promoter region. GUS::GFP gene fusion was used as a reporter system and *nptII* served as a selectable marker gene. A high regeneration capacity of callus tissue under the selective pressure and successful GUS assay of transformed tissue were an indication of successful insertion of a transgene into mature embryo derived wheat tissue.

Keywords: Biolistic transformation, drought stress, glutathione, γ - glutamylcysteine synthetase, mature embryo, wheat

UDC 577.2

INTRODUCTION

Immature embryos have been found to be a good source for the successful induction of callus tissue and have often been used for wheat transformations. However, it is difficult to obtain immature embryos all year round, and their isolation is a demanding procedure. Explant tissue maturity stage is one of the prevalent factors for embryogenic callus formation (Varshney et al., 1996) and therefore scientists have tried to develop a system of callus induction that led to mature embryos. Although using mature embryos creates a problem of low callus induction frequency, Ozgen et al. (1996) successfully overcame this problem by using an endosperm supported callus induction method from a mature embryo culture. However, Turhan and Baser (2004) proved that a free embryo, without the support of endosperm, could be cultured. This method proved to be easy to handle, required less space in the culture vessel and it was simpler to identify embryo derived embryogenic callus.

Several transgenic wheat cultivars differing in the introduced genes have been developed in the past decade. The first transgenic wheat plant was developed by Vasil et al. (1992), who introduced a gene for herbicide resistance. A transgenic approach by De Block et al. (1997) was used to achieve male sterility in wheat. Two years later, Alpeter et al. and Stoger et al. (1999) made contributed the introduction of transgenes that improved tolerance to insects and viruses. Gopalakrishnan et al. (2000) and Miroshnichenko et al. (2004) registered two new transgenic wheat cultivars containing the *bar*I PPT gene for resistance to the herbicide Basta. Zhou et al. (2003) succeeded in doubling the tolerance of transgenic wheat to the herbicide Roundup Ready.

The other inserted gene was HMW – GS, which improved the rheological properties of dough for bread and pasta preparation (Barro et al., 1997; Blechl et al., 1998; He et al., 1999, Rooke et al., 1999).

In the same time Baga et al. (1999) registered new transgenic wheat cultivars in which a gene for the starch-branching enzyme had been introduced in order to obtain new starch characteristics. Also,

Pellegrineschi et al. (2000) applied a transgenic approach to solve resistance to fungi and Dahleen et al. (2001) tried a transgenic approach for Fusarium head blight control in wheat and barley. Notable success was achieved by CIMMYT in the development of wheat tolerance to abiotic stress. Using the *DREB1A* gene from *Arabidopsis thaliana*, a team from CIMMYT succeeded in developing a transgenic wheat plant tolerant to drought (Pellegrineschi et al., 2004).

According to Wachter et al. (2005), the tripeptide gluthatione (GSH), the major non-protein thiol compound in the eukaryotic cell, participates in the regulation of the cellular redox status. In plants, this is especially important during exposure to oxidative stress after exposure to high light intensity, salinity, drought, low temperature or pathogenic attack. The requirement for GSH function in the detoxification of reactive oxygen species (ROS) could start early in plant development (Rausch et al., 2007).

So far, the tripeptide glutathione has not been extensively studied as a potential transgene for wheat transformation. Several cases have been reported of successful wheat transformation with a glutathione S – transferase coding gene with a strong constitutive *Ubi – 1* promoter, in order to increase tolerance to the herbicide Alachlor (Milligan et al., 2001). The same year, a German-Hungarian research team succeeded in increasing the tolerance to chloroacetanilide in transgenic poplar (Gullner et al., 2001) plants by initiating an excessive expression of γ - glutamylcysteine synthetase (γ - ECS).

As shown above, no significant progress has been made so far in plant transformation in the sphere of tolerance to abiotic stress by means of γ – ECS, and this field remains open for further research. Increasing GSH content in plants could have multiple beneficial effects because GSH is directly or indirectly involved in many stress responses (pathogen defense, drought, cold, salinity) in plants (Noctor et al., 2002).

The aim of this investigation was to apply a mature embryo-derived biolistic transformation method in order to improve wheat tolerance to drought stress by multiplication of the GSH1 (γ-glutamylcysteine synthetase) gene with its stable integration into the wheat genome.

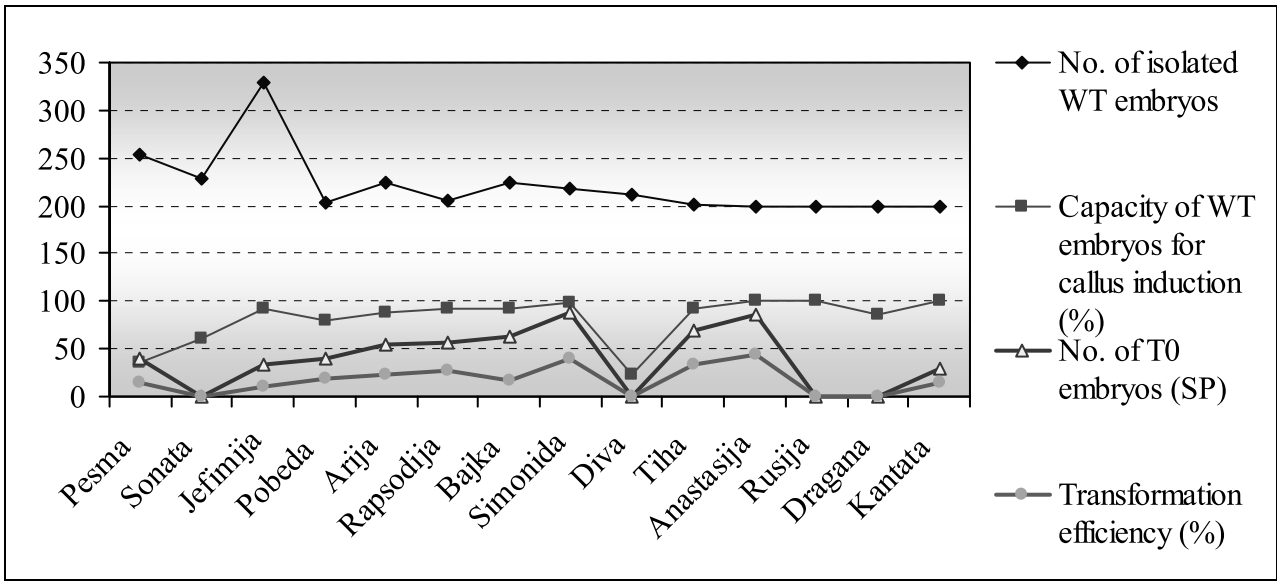
MATERIALS AND METHODS

Two hundred mature embryos were isolated from each of the 14 elite wheat cultivars from a collection of the Small Grains Department, Institute of Field and Vegetable Crops, Novi Sad, Serbia, and placed on induction medium.

The seeds were kept in 70% (v/v) ethanol for 1 min for surface sterilization, following 4.5% NaOCl for 30 min at room temperature, and rinsed with sterile water three times. The sterile seeds were imbibed in sterile water overnight at room temperature. Embryos were isolated together with scutellar tissue and placed on an induction medium, scutellar side up. Culturing was conducted in the dark, at 25° C, for 5–7 days.

The medium for callus tissue induction was prepared according to Sears and Deckard (1982) and enriched with 2 mg L^{-1} 2,4–D, 20 g L^{-1} maltose, 40 mg thiamine–HCl, and 150 mg L^{-1} L-asparagine, pH 5.8. All media were prepared with 0.25% (w/v) Phytagel. Reagents for *in vitro* culture were prepared using products manufactured by Serva Electrophoresis, Heidelberg.

Wheat transformation was applied to 7-day old callus tissue. The tissue was induced, after isolation of mature embryos, on a highly osmotic nutritive medium that contained induction medium reagents enriched with 0.4 M sorbitol. A highly osmotic nutritive medium was used as an intermediate step in the preparation of callus tissue for transformation. Each sample was bombarded three times with 5 μ L tungsten particles coated with the DNA of interest. Non-bombarded embryogenic calli were used as the control.



Graph 1. Analysis of transformation experiment with mature embryogenic calli of 10 winter wheat cultivars. WT – Wild-type; T0 – 1st generation of transformants

After bombardment, the calli were incubated under identical conditions for 16-24 h and then transferred to the induction medium enriched with 0.5 μg L^{-1} of kanamycin sulphate. The culture was maintained for three weeks in the dark, at 25° C. Selective pressure, 0.5 μg L^{-1} of kanamycin, was maintained through the period of shoot regeneration (MS salts and vitamins without 2,4-D, enriched with 1 mg L^{-1} of kinetin and BAP and 0.5 mg L^{-1} of IAA) under the conditions of a 16 h photoperiod at 25°C.

The differentiated callus tissue was transferred to a nutritive medium that contained a half of the quantities of the reagents used in the induction medium and a constant concentration of the selective agent.

Already after 48 h several transformed calli were taken for histochemical GUS analysis. Four weeks later, 10 calli of each genotype underwent GUS assay. The remaining bombarded calli were cultured until regeneration.

As a transgene, a binary vector pBinARUTRECS for heterologous plant transformation has been used. The gene was under control of the CaMV35S promoter region as well as the expression of the *nptII* gene which carries the resistance to kanamycin, as selectable agent, and GUS::GFP gene fusion that represents the reporter unit. The GUS::GFP gene fusion was used to create the possibility for a double detection of GSH1 transgenes in wheat callus tissue.

The bombardment procedure was performed in petri dishes containing 5-7 days old embryogenic calli. It consisted of shooting three times from a distance of 5 cm from a macrocarrier ring.

β-glucuronidase activity (GUS analysis) was detected histochemically as described by McCabe et al. (1988). A laser scanning confocal microscope (Zeiss LSM 510) was used for the detection of *gfp* expression in the transformed wheat calli. We used the following detection parameters: wavelength for the green fluorescent protein - 488 nm; scan detection of 505 – 530 nm and laser emission of 510 nm.

For molecular characterization of gene transfer, total genomic DNA was isolated from individually transformed and wild-type calli (T0, WT) by the procedure of Dellaporta et al. (1983). The PCR method was applied (initial denaturation at 94° C

Fig. 1. Proliferation of roots (T0) on a selective medium and shoots (WT) on a high osmotic medium two weeks after keeping the culture with and without selective pressure

for 5 min followed by 45 cycles involving: 1) 95° C, 5 min; 2) 95° C, 30 s; 3) 52° C, 30 s, and 4) 72° C, 45 s, 10 min extension, 72°C) to amplify the coding region of the transgene. PCR products were subjected to electrophoresis for 1 h at 80 V, and visualized by UV light on 1% (w/v) agarose gel.

RESULTS

The obtained results show that the cultivars differed significantly in the number of induced calli. The cultivar Diva had the lowest percentage (24%) of induced calli. Cultivars Pobeda and Arija had significantly higher percentages, 79% and 89%, respectively. The highest induction-shared cultivars were Anastazija, Rusija and Kantata with 100%, respectively (Graph 1).

The highest transformation efficiency was detected in the cultivar Anastazija (43%) followed by the cultivars Simonida (39.9%), Tiha and Rapsodija (34.2% and 27.7%, respectively). The cultivar Jefimija had the lowest transformation efficiency, 10.1%. The cultivars Pesma and Kantata had similar efficiencies, i.e. 14.2% and 14.5%, respectively. Callus tissues of the cultivars Sonata, Diva, Rusija and Dragana, showed no signs of transformation. Transformation efficiency was not significantly correlated with the induction capacity ($r=0.436$, $p=0.119$).

The first signs of callus tissue regeneration in both transformed (1st generation of transformants - T0) and non-transformed calli (wild-type - WT) were observed in the cultivar Bajka. However, these transformants exhibited degenerative morphological changes as well as the albino phenotype (Fig. 1).

All genotypes subjected to the GUS analysis produced positive results. When observing the treated callus tissues after staining, it was possible to differentiate several types of GUS expression. Representative results of GUS expression are presented in Figure 2.

Presence of GFP was determined in wheat callus tissue in the samples of forty-eight hours and four weeks after bombardment.

Figure 3 shows *gfp* expression in the layers of transverse sections of callus tissues observed through a confocal laser microscope at 488 nm wavelength. All of the observed tissues obviously possessed a very weak signal for the green fluorescent protein. It required setting the microscope to a lower stringency rate of laser emission (510 nm) which in turn resulted in lower detection accuracy.

DISCUSSION

Transformation efficiency was not significantly linked to the induction capacity. It was observed to depend significantly on the genotype. The cultivar Jefimija, which had one of the highest induction capacities (93%), exhibited the lowest transformation efficiency, only 10.1%. It deserves to be mentioned that the cultivars Pesma and Kantata, with 36% and 100%, induction capacity respectively, had almost identical transformation efficiencies (14.2% and 14.5%, respectively). Although the cultivars Dragana, Sonata and Rusija had exhibited considerable induction capacity, their callus tissues showed no sign of transformation. These results are in agreement with those of Pellegrineschi et al. (2002) who found that the variability of transformation was more due to the genotypic and physiological status of the donor plants than due to the efficiency of the biolistic procedure.

The above results can be interpreted in a variety of ways. First, transformation efficiency is highly

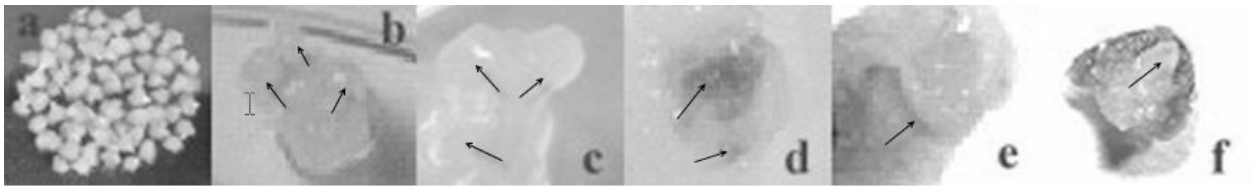


Fig. 2. Biolistic transformation of wheat and GUS expression. The arrows point to globular protuberances and blue precipitate localization; a) mature embryos derived calli after 7 days on induction medium; b) development of somatic embryos over all the surface of kanamycin resistant embryogenic calli on selection medium (arrows indicate formations of lobed structured somatic embryos) (b and c). Stable *uidA* gene expression and histochemical GUS assay of 4 weeks transformed tissue on selective medium resulted in different patterns (d, e, f).

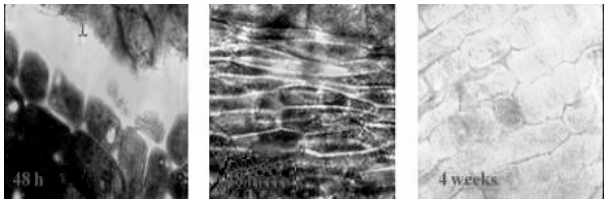


Fig. 3. Stable *gfp* expression in somatic embryos tissue after 48 h and 4 weeks on selection medium

dependent on the genotype, as claimed by Rakszegi et al. (2001). Second, transformation efficiency and induction capacity could be independently inherited characteristics. This statement is supported by a study of Pastori et al. (2001) which concluded that there was no correlation between transformation efficiency and embryogenic capacity in either one of the examined genotypes.

The scutellar tissue of all analyzed samples exhibited a vigorous GUS activity. These results are in agreement with Peters et al. (1999), who explained this phenomenon by the existence of intrinsic GUS activity in scutellar tissue similar to the GUS activity in transformed tissue, suggesting that scutellar tissue must be removed if GUS reported genes are to be used in the experiment. This was supported by our results which indicated that some embryogenic calli from the control group, which had not been bombarded (Simonida, Kantata and Rusija), exhibited the intrinsic GUS activity, although this occurrence seems to depend on the genotype.

The next pattern of expression of GUS activity is the blue precipitate which appeared as dots at the sites of microparticle impact in most of the analyzed callus tissues. This observation suggests that the GUS gene was expressed in some of the transformed cells of the host genome, which corresponds to the results of Cheng et al. (1997) dealing with transient GUS expression in freshly isolated immature embryos four days after inoculation. Also, Oktem et al. (1999) explained the same event in the cultivar Cakmak when treated by the biolistic approach.

Some callus tissues showed blue staining in rapidly dividing cells, i.e., in already generated primordial vascular tissue (Figure 2e). Identical observations were made by Clausen et al. (1998) who found a high level of GUS expression in meristematic tissue and in root tissue of a WT genotype. A combination of the last two expression patterns was also observed during GUS analysis (Figure 2d). This phenomenon was explained by Plegt and Bino (1989) and Hu et al. (1990). These authors observed high natural GUS activity in flowers and fruits of some plant species. They also found that, in the tissue of *A. thaliana*, the pH value of the GUS staining solution has a high effect on intrinsic GUS activity and that by increasing the pH value it was possible to stop or even reduce the undesirable effect of endogenous GUS activity in plant tissue.

In consideration of the above, it can be concluded that the staining of vascular tissue, in our case, indicates only the presence of intrinsic GUS activity but not the presence of transformed tissue in the analyzed calli. On the other hand, all representative callus tissues, i.e., those that show the

dotted staining pattern, including the callus tissues that combine vascular and dotted staining, indicate the presence of a stable transformation.

Young tissues (48 h) exhibited high fluorescence in the cell wall and cytosol (Figure 3a). The high fluorescence in the nucleus (Figure 3b) might indicate the introduction of transgenes into the host genome. This assumption is based on the assertion of Schenk et al. (1998) that the GFP protein lacking the sequence of transit peptide may freely accumulate in the nucleus of treated cells, thus enabling it to fluoresce. The authors added that these observations may be especially helpful in the identification of transformed cells if there exist limiting factors such as so-called fluorescent dust, microorganisms, or strong cellular autofluorescence masking the actual GFP signal.

In the layers of 4-week old tissue, complete cells, now organized into tissues, may fluoresce. Such samples may have large fluorescing areas (Figure 3c). The level of detection is low, corresponding to the weak fluorescence signal. These results are in contrast to those of Yu et al. (2000) who observed that fluorescence intensity tends to increase with callus maturation.

Although we detected a high regeneration capacity of the callus tissue induced by mature embryo culture, a low correlation between regeneration capacity and transformation efficiency was observed. Callus tissue regeneration under selective pressure attained a high level, from 10.1% to 43.0%. However, the GFP analysis showed a low fluorescence in the analyzed samples, which was an indication of low transformation efficiency. This contradiction may be due to the small number of samples analyzed by confocal microscopy.

Furthermore, molecular characterization involved only 23% of the calli (150 out of 648), i.e., those that exhibited regeneration capacity under selective pressure (50 µg/ml kanamycin) after three weeks of cultivation in the culture. The PCR did not amplify a product of appropriate size that would indicate a stable transformation.

Successful GUS expression and low detection of GFP fluorescence could be an indication of stable transformation of the wheat tissue.

Acknowledgments - The authors acknowledge Dr. Andreas Wachter (Heidelbeg Institute of Plant Science, Molecular Ecophysiology Department, Heidelberg, Germany), who kindly provided them with the transformation vector and made important scientific suggestions during the experiment, and Dr. Srbislav Denčić (Institute of Field and Vegetable Crops, Novi Sad, Serbia) who provided seed material.

The experiments described in this article were undertaken in Heidelberg Institute of Plant Science, Molecular Ecophysiology Department, Heidelberg, Germany.

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ТРАНСФОРМАЦИЈА ЗРЕЛИХ ЕМБРИОНА ПШЕНИЦЕ СА ЦИЉАНИМ СТРЕС-МОДУЛИРАНИМ АНТИОКСИДАНТНИМ ГЕНОМ

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Зрели ембриони четрнаест елитних сорти озиме пшенице су трансформисани путем биолистичке методе. Као трансген коришћен је ген који кодира γ - глутамилцистеин синтетазу (EC 6.3.2.2) у циљу постизања стабилних трансформаната отпорних на стрес суше. Коришћен је бинарни вектор pBinarUTRECS а ген је био под контролом CaMV35S промотор региона. GUS::GFP фузија гена употребљена је као репортер систем а *nptII* као селективни маркер ген. Високи капацитет регенерације калусног ткива под условима селективног притиска и успешна GUS анализа трансформисаног ткива су указали на успешну интродукцију трансгена у ткиво зрелих ембриона пшенице.