International Journal of Modern Agriculture, Volume 9, No.3, 2020
ISSN: 2305-7246

Herbicide Resistant Transgenic Wheat Plants Obtained by Microprojectile Bombardment

Mr. Arabinda Mohanta[1], Prof. Akshaya Kumar Behura[2]
[1,2]Department of Agriculture, Siksha 'O' Anusandhan (Deemed to be University), Bhubaneswar, Odisha
Email - [2]akshaybehura@soa.ac.in

Abstract

The efficient short procedure was employed for obtaining wheat (Triticum aestivum) transgenic plants from appropriate commercial cv (Oasis). First, optimum conditions were developed for the generation of embryogenic calli from immature embryos that have a high regeneration level. High speed microprojectile bombardment was used for transforming calli, using the plasmid pAHC 25 which contains the reporter gene β-glucuronidase (GUS) and the selectable BAR gene, which resists Basta herbicide. The transformations have been verified by β-glucuronidase, PCR and Southern blot testing. It was highly efficient and comparable to other reports used in the "crop" cultivar. The complex genetic modification of wheat (Triticum aestivum) and other floral organisms with a relatively low efficacy of biolistic processing and the resulting introduction of multiple copies of the genes are being added. Southern work confirmed further integration of gus and bar genes into the genomes of durum wheat. Developed by an agronomically superior durum cultivar this transformation technique will provide new opportunities for enhancing the current germplasm through biotechnology.

Key words: β-glucuronidase (GUS), Basta herbicide, Biolistic processing, Embryogenic calli, Microprojectile bombardment, Wheat (Triticum aestivum).

Introduction

Wheat (*Triticum aestivum L.*) is one of the most important food crops worldwide with annual production approaching 500MT. It is one of Argentina's main agricultural products; since the 1970s the area has gradually increased to about 6 million hectares. A complement to breeding programs aimed at improving wheat productivity is biotechnological methods. The success of plant genetic manipulation requires that functional DNA can be transmitted into the cell, that transformant tissue is regenerated and the transgenic plant is assessed. Although the first routine gene transformation process was using plant transformation *Agrobacterium tumefaciens*, it was only used successfully in dicotyledons until relatively recent times [1]. The host spectrum of A. tumefaciens was predominantly taken into account monocotyledons and, in particular, cereals. the facial tumors. Reports have, however, recently appeared on rice or wheat conversions using this process [2]. Certain devices with a varied efficiency will typically achieve monocot transformation: the protoplastic direct delivery of DNA by electrical or osmotic (polyethylene glycol) or high-speed microprojectile bombardment usually referred to as "biolistic." The last approach has provided the most persuasive and clearest results for the recovery of transgenic plants [3]. In the transformations of the biological system specific structures and reporter genes were used. Transgenic plant recovery

requires a fine tuning of the regeneration strategy and cycle. In order to improve the transformation rate, the majority of attempts were performed on explant embryogenic genotype, bombardment conditions, marker selection, selection conditions, donor plant development stage and the environment under which they are cultivated. However, biolistic transformation of wheat and significant regeneration differences and transformation levels of frequencies among varieties have occurred.

Plants are commonly used for genetic transformation with biolistic micro-projectile bombardments, including large cereal crops, such as rice (Oryza sativa), maize (Zea mays) and wheat (Triticum aestivum). Wheat has attracted significant interest in the production and optimization of efficient processing methods as one of the most important industrial cultivations [4]. The DNA coating preparedness has remained largely unchanged from the initial protocol in most of these publications based on the formation of an unstable DNA / spermidine / Ca2 + complex. In large-scale, stable transformational experiments, the reproductibility of this approach is despite some optimization. The spontaneous, multi-copy transgenic integration, which often results in unusual compositions of inverted repetitions or transgenical rearrangements, is another major concern for stable biolistic transformation [5]. For subsequent generations, this can lead to transgenic silencing, aberrant transgenes, and even transgenic loss. The problems are less affected by transgenic plants with single-copy inserts and are therefore suitable for functional genome studies or the manufacture, after genetic segregation, of markerless plants. The transformation frequency (TF) can be increased with simple integration patterns by using limited transgene cassettes instead of bombs with whole plasmid DNA. The strategy has been used in the transformation of several single-cell species like wheat, maize, pearl millet (Pennisetum glaucum) and sugar (Saccharum officinarum) [6]. Such a strategy has to date been implemented. In addition, a low number of copies of transgenes have been reversed with the amount of the DNA cassette used in the biolistic process. For efficient production of single copy events for maize and sugar cane, amounts as low as 2.50–2.73 ng of DNA cassette per shot was recommended [7].

Plant producers and cytogeneticists have been effective at transferring superior agronomical characteristics from similar wild grasses through intergeneric hybridization into both tetraploid and hexaploid wheats [8]. Although broad hybridization is efficient for the introduction of desired alien genes into wheat, it has several drawbacks, such as unwanted alien chromosome transmission and adverse genetic interactions, which contribute to sterility. Therefore, it is extremely tedious and time consuming to introduce a single desired alien gene into wheat by sexual means. Yet biotechnological techniques make it easier for desired alien genes to be inserted in plants asexually.

Microprojection genetic transformation was shown in most cereals, including spring wheat (Triticum aestivum L, 2/i = for = 42; AABBDD) [9]. Such employees have successfully used a number of embryogenic target tissues such as calluses and spring wheat processing protocols. The utility of genetic engineering in the development of germplasm of durum wheat has not been investigated (Triticum turgidum L, 2n = Ax = 28; AABB genome). The

lack of an efficient method of in vitro regeneration via somatic embryogenesis was a major constraint of durum wheat transformation [10]. We have therefore begun developing in vitro-cultural technologies and have developed a protocol for fast regeneration using isolated scutella made of four agronomically appropriate durum cultivars. A method of hard transformation using isolated scutella as target material has been developed, as well as an herbicide resistance gene, bar using this regeneration protocol.

In this paper, it has been explained the regeneration of wheat from immature calli embryos, and the high-speed microprojectile particle bombardment strategy for gene transformation with pAHC25, which includes the reportable β-glucuronidase (GUS) gene and a BAR gene that confers resistance to the herbicide Basta. With this method, transgenic plants of a commercial cultivar of wheat using resistance to Basta can be obtained and grown.

Materials and method

1. Immature embryo culture and plant regeneration:

In the greenhouse under natural illumination at 22-24℃ immature embryos have been isolated from wheat plants (Triticum aestivum, cultivar Oasis). For the establishment of callus crop, the caryopses were surface sterilized for 18 to 20 days after anthesis. The caryopses had: 70% ethanol for 2 min, 2% of commercial sodium hypochloride for 20 min, and then three sterile water shifts. Under a stereo dissecting microscopy, the immature embryo was removed aseptically, and released, with the MS medium, MS vitamin, 30 g l-1 sucrose and 9 μM (2-1 mg) acetic acid dichlorophene (2,4D), respectively. A 90 mm Petri dish with a liquid of 20 ml held 20 immature embryos. Every replicate was an explant-containing dish and the experimental size ranged from 200-400, depending on the number of scutella isolates. The pH was set to 5.7 before autoclave, all media had been solidified with 6 g l-1 agar. Crops were incubated in the dark for 15-20 days at a temperature of 22-24 ° C. In an embryogeneic medium, both MS alone and 1.71μm (0.3 mg l-1) indoleacetic acid and 4.65μM (1 mg l-1) kinetine were transmitted to the light (66 μEm2 s-1) and cultivated, until shoots developed. Plants (> 2 cm long), without plant growth regulators, were moved to MS tubes. The plants were transferred into the soil after 3-4 weeks and planted in the greenhouse under the conditions of growth of the donor plants.

2. Plasmid and leaf tissue plant DNA purification:

A researcher provided the plasmid pAHC25 (9.8 Kb) used for wheat transformation. This vector is made up of the UidA gene and the selectable BAR gene, each of which is powered by the ubiquitin Ubi1 promoter maize (Fig, 1). The UidA gene covers the β-glucuronidase enzyme (GUS reporter gene) and the selectable BAR gene codes the PAT enzyme, which inactivates the herbicide Basta active ingredient. The plasmid DNA has been isolated from alkalinelysed cells and preserved at a level of 1μg/μl. The leaf tissue was purified using the protocol described by one of the researchers.

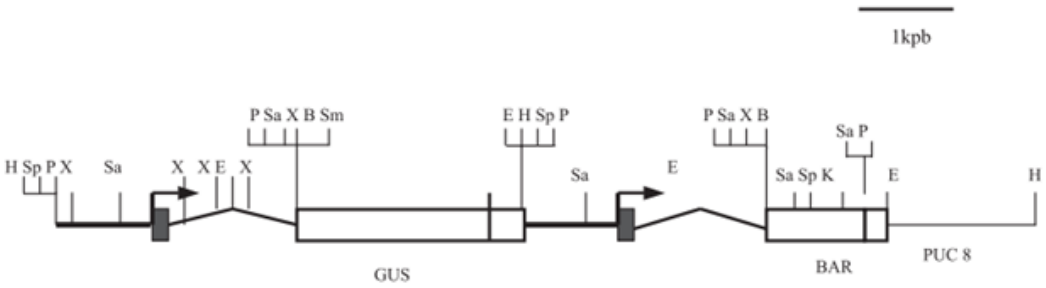

Fig.1: Schematic diagrams of pAHC25 vector.

3. Callus treatments and microprojectile bombardment conditions:

Incubated in micro-source (Solidified with 14 g l-1 agar) with 0-0,4 M sorbitol was the embryogenic calli of immature embryos (15-20 days). The calli were held for 4-6 hours before and 16 hours after the bombardment. Calli were then cultivated for two days without selection in the regeneration medium and then transported with 10 mg l-1 of filter-sterilized herbicidal Basta (22 µm Millipore) to a similitary medium. The bombardment has been conducted using a HPB device built in the laboratory following the PDS-1000/He Bio Rad model with changes. Instead of Kapton, the unit which has been used is a 12 mm thick Mylar membrane to protect temporarily the helium (He) gas-filled pressure chamber. The rupture disk was pierced by a needle releasing a powerful burst of the shock wave when the critical pressure was reached while the ruptured disk was ruptured in the case of PDS-1000/He and increased the gas pressure. A 19 mm-thick Mylar carrier has been inflated by the helium explosion, 600 psi under a vacuum pressure of 24 cm Hg. The macrocarrier had loaded, dried onto the surface of the macro-carrying machine, precipitated DNA coated (7 µl), plasmid DNA (10 µg) and adsorbed onto gold-particle suspension 50 µl (60 Mg / ml) (Bio-Rad 1 µm Gold Microcarrier).

4. Enzymatic Assays:

The leaves, flower and end of the brush have been used to test the expression of gus with the histochemical GUS trial on mature plant seeds. The samples were incubated in the X glucose solution overnight (0.05 percent w / v). The samples of GUS positive were soaked for a day before photomicrography in pure ethanol.

A silica gel thin layer chromatography technique was used to perform the Phosphinothricin acetylansferase (PAT) test. Young T leaves (one or two) were harvested, following the procedure described by one of the researchers in order to extract complete protein for the acetylation assays. On each lane of the LHP-KDF linear high-power TLC plate, a total of 5 ng of total protein has been loaded on the Whatman paper.

5. DNA isolation, PCR analysis and Southern hybridization:

DNA was extracted from leaf tissue with the qPCR freeze-dry process and the southern hybridization procedure of phenol-chloroform. A Nano-Drop spectrophotometer was used for the complete DNA quantification. Transgenic copy numbers, as described in and as confirmed by cross testing of the same people by Southern auto-radiography hybridization, were determined by real-time quantitative PCR with two reference genes, Cyclophilin and Glyceraldehyde 3-phosphate dehydrogenase. DNA samples for southern analysis have been digested with EcoRV at 37 ° C around 5 o'clock, placed on a 1.0% (v / v) agarose gel, blocked on a nylon membrane of Hybond N+ and hybridized using a transgene-specific DNA 32-plate-labeled probe. The DNA sample has been tagged with the ready-to-go DNA tag kit using the random priming process. An in-house (35S and 354bp) sample was used aiming at the backbone of the DNA fragment transformed in 4237bp. The initials for amplifying the PCR sample have been: 5'-CAACATGGGGGCACGAC-3′ and 5-GCGTCATCCTACGTGGAG-3″.

Results and discussion

1. Immature embryo culture and plant regeneration:

96-98 percent of the cultured immature embryos developed calli, which became soft and swollen under the conditions of the development of the callus, and formed calli pockets at the borders of the scutellum. The "embryogenous callus" structures had a dry, compact or nodular feature (Fig, 2a). A very low proportion of calli were friable, translucent and hyperhydrous. Sometimes in the same calli (Fig, 2b), both forms of structures have been found, which are removed and discarded. In regeneration conditions, the majority (93-95 percent) of these embryogenic calli formed little meristematic areas. Shoots were recovered in these meristematic areas as dome-shaped (Fig, 2c). It was verified by histological sections (Fig, 2d). In the median with hormones added, the rate of plant regeneration was higher (67.3%) than in the medium without hormones (43.3%). In the latter case, most structures developed were roots, probably as cytokinins equalize residual 2,4 D effects in the calli and encourage the development of other structures. In the latter case, most of the structures formed were rooted, presumably because the cytokines balance the activity of residual 2,4 D in the calli and facilitate the creation of other structures. After 30-45 days in the hormone medium, the shoots were moved to individual tubes with MS medium alone and kept under light for 2-4 weeks (Fig. 2e). Once plantlets reached a length of 5-7 cm, they have been cultivated for 4-6 weeks in Magenta boxes and then moved into sterile vermiculite pots and placed in greenhouse arrangements (Fig. 2f).

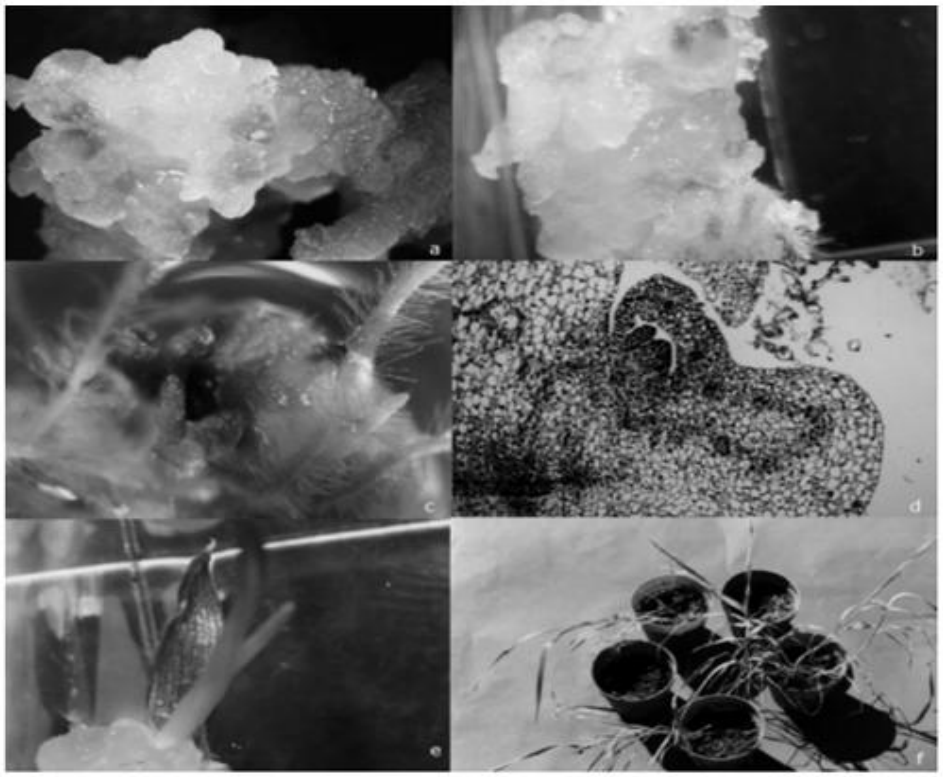

Fig.2 (a) initiation of embryogenic calli from cultured embryos, (b) Embryogenic callus culture after about 4 weeks, (c) Shoot development in regeneration media, (d) Longitudinal ultrafine section of embryogenic calli showing shoot development stained with hematoxiline-eosine, (e) Plantlet growing from embryogenic calli, (f) Wheat (Cv. Oasis) plants transferred to soil.

2. Transient transformation:

Transient GUS expression was observed in bombarded calli via histochemical activity tests of β-glucuronidase (fig. 3 a-b). GUS expression was 4-5 times higher than in untreated calli in osmotically handled calli (0.4 M sorbitol) (Fig. 3c). The partial plasmolysis of the target cells can contribute to an enhancement of osmotic therapy expression, because cells can be less likely to lose cell sap as a result of membrane damage from penetrating particles. On the other hand, the expression of the plasmid pAHC25 was also compared to a vector pGUSNco carrying the CaMV 35 S 1 β-glucuronidase gene. GUS expression was much higher with the first construction than with the latter. These findings agree with other studies that the Ubi-1 developer is more successful in monocots than 35S.

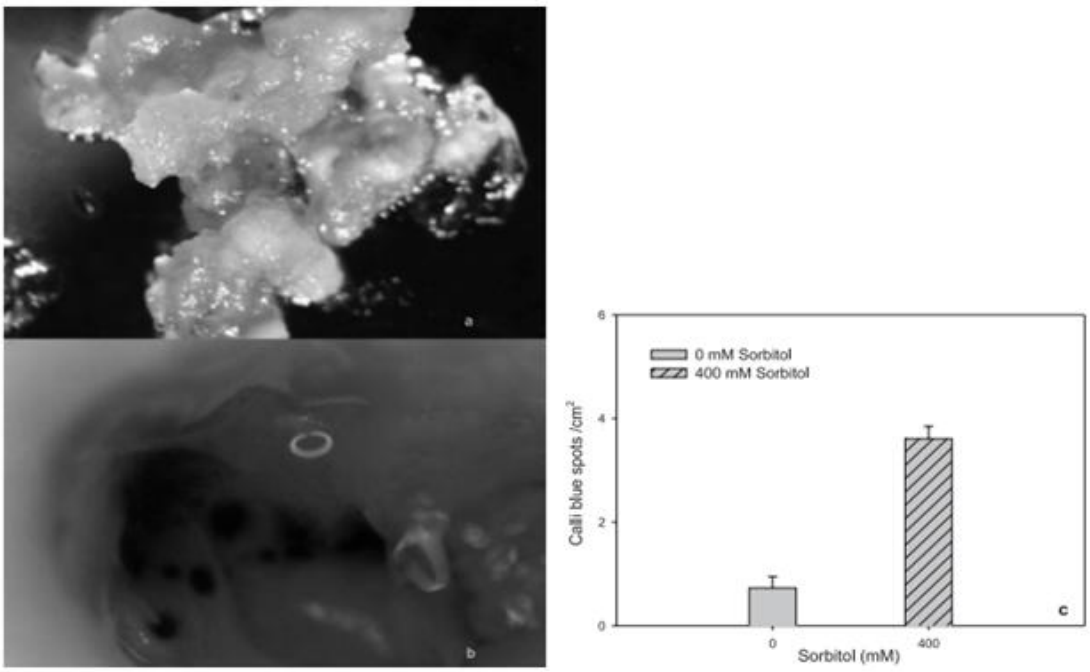

Fig.3 (a) Histochemical expression of GUS activity in callus transformed with pAHC25 plasmid, (b) Detail of GUS expression in meristematic calli zone, (c) GUS activity in osmotically treated calli with 0 and 400mM sorbitol.

3. Transgenic plants:

The calli were kept in osmotic treatment (16 h) after bombardment and then transferred to the regeneration medium. 48 hours after transformation and culture was maintained under these conditions for 2 to 4 months were selective conditions. In the first two weeks all calli were seen as green spots (4-5 per callus) and most of them had incipient leaves (2-4 mm) which were yellow-green and meristematic areas. The embryogenic potential of explants declined rapidly during the following two to four weeks, the leaves and shoots became chlorotic and died. 10-13 percent of first plantlets could be recovered at the end of the first month of cultivation under selective conditions. PCR analysis was carried out to detect the survival of plantlets in individual culture tubes and screened for the existence of marker BAR gene sequence. 64-70% of surviving plantlets were found to have positive PCR reactions, accounting for 9% of the plantlets initially. Although the selection pressure was very high, some 30% of Basta's resistant plant were exhaust events (Fig, 4). Even when it is sub-cultured in fresh medium, 55% of positive PCR plantlets died in the following weeks, suggesting that BAR expression was only transient in these plants.

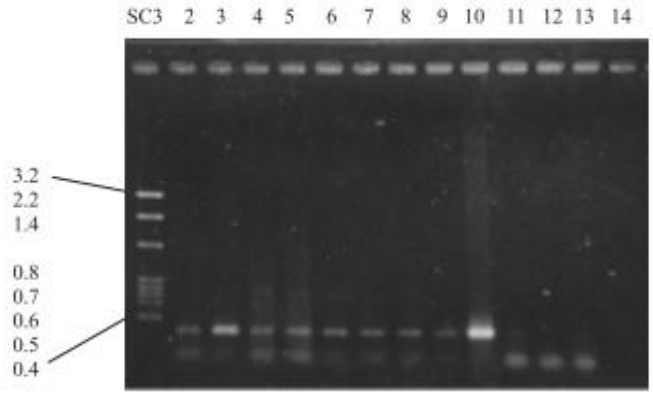

Fig.4: PCR analysis of wheat independent Basta resistant plants recovered after transformation with pAHC25. Ethidium bromide stained agarose (1.2%) gel of PCR amplification of the BAR gene.

Conclusion

Although the average efficiency of Oasis, the major commercial cultivar, in this study is higher or similar to those previously reported for "model" genotypes, although earlier reports of wheat transformation exist. The efficiency of the regenerative cultivation of calli and the short time it takes for transgenic plants to recover show that it can be used as an appropriate strategy to achieve genetically modified wheat plants. The successful processing of durum wheat, an important tetraploid cereal, was first demonstrated. For the first time, this work was carried out on a durum cultivar of agricultural importance, Medora, for which the entire process protocol was established. The researchers have produced the self-fertile transgenic hard wheat with the use of isolated scutella as the target tissue. The production of processes for agronomically superior cultlvars would open up new ways to improve the current hard germplasm using modern biotechnology instruments.

References

1. G. Schütte *et al.*, “Herbicide resistance and biodiversity: agronomic and environmental aspects of genetically modified herbicide-resistant plants,” *Environmental Sciences Europe*. 2017.
2. F. Sun *et al.*, “Whole-genome discovery of miRNAs and their targets in wheat (Triticum aestivum L.),” *BMC Plant Biol.*, 2014.
3. M. R. Abdollahi, A. Moieni, A. Mousavi, and A. H. Salmanian, “High frequency production of rapeseed transgenic plants via combination of microprojectile bombardment and secondary embryogenesis of microspore-derived embryos,” *Mol. Biol. Rep.*, 2011.
4. *Biolistic DNA Delivery*. 2013.
5. K. Zhang, J. Liu, Y. Zhang, Z. Yang, and C. Gao, “Biolistic genetic transformation of a wide range of chinese elite wheat (Triticum aestivum L.) varieties,” *Journal of Genetics and Genomics*. 2015.
6. Z. Liang *et al.*, “Genome editing of bread wheat using biolistic delivery of CRISPR/Cas9 in vitro transcripts or ribonucleoproteins,” *Nat. Protoc.*, 2018.

7. H. Wu *et al.*, "Transgene integration complexity and expression stability following biolistic or Agrobacterium-mediated transformation of sugarcane," *Vitr. Cell. Dev. Biol. - Plant*, 2015.
8. J. B. Alvarez and C. Guzmán, "Interspecific and intergeneric hybridization as a source of variation for wheat grain quality improvement," *Theoretical and Applied Genetics*. 2018.
9. S. Barampuram and Z. J. Zhang, "Recent advances in plant transformation.," *Methods Mol. Biol.*, 2011.
10. A. Horstman, M. Bemer, and K. Boutilier, "A transcriptional view on somatic embryogenesis," *Regeneration*, 2017.