# Evolution of Transgenic Technology in Cereal Crops: a Review Approach

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***Abstract***

*This review study reports a concise analysis of investigations on origin and progress of transgenic technology in major cereal crops which is the integral part of the cereals improvement programmes. Most genetic transformation approaches of cereal crops have been majorly restricted to rice, wheat, maize, barley and oats. Rice has been considered as a model system for transgenesis and also for many molecular genetic studies. Moreover, certain parameters; such as selection of target tissues for desired gene transfer, screening of competent genotypes for regeneration and transformation, modes of gene deliveries and use of suitable selection markers for screening of putative transformants have been considered as major influencing factors for the success of transgenic technology. Immature embryo in general has been proved definitely the best target tissue for cereal transformation, but the optimal size and stage of the embryos is greatly affected by vegetative and physiological status of the mother plant. Amongst various modes of gene transformation applied in cereals biotechnology; biolistic-mediated gene delivery has been used more frequently than Agrobacterium-mediated transformation techniques. In addition to conventional plant breeding techniques, the genetic transformation of cereals with agronomically important target genes is required to improve nutritional quality and quantity of cereals particularly resistance to various stresses and diseases.*

**Keywords**: Cereals, Selection Markers, Reporter gene, *Agrobacterium*, Biolistic, Transgenic.

## *INTRODUCTION*

Cereals are generally cultivated and harvested for their grains but recently have been used for energy production such as by fermentation yielding biogas or bioethanol. On global basis, cereals collectively provide approximately 40% of the energy and protein components of the human diet **[1]**. Significantly, the major cereals contribute to secure the required global food demands both at present and also in the future.

In addition to conventional breeding, biotechnology is a complementary tool to discover agronomically important genes for resistance and yield improvement. The genetic engineering approaches also support to overcome the challenges such as genotype dependent regeneration and occurrence of somaclonal variation which are very common mechanisms in case of *in vitro* regeneration of cereals **[2]**.

Transgenic technology is mainly based on the totipotency of plant cells for *in vitro* regeneration along with the delivery, stable integration and expression of transgenes in target cells followed by the transmission of transgenes to the progeny **[3]**. To begin with, in 1980s, the production of novel chimeric genes could allow to grow plant transgenic technologies **[4]**, plant expression vectors **[5]**, and systems of DNA transfer **[6]** along with plant regeneration and transformation protocols **[7].**

In recent years, transgenic technology has been utilized for the improvement of many crop plants including cereals such as maize, wheat and rice. Moreover, some of these transgenic cereals have already reached to the field for large scale cultivation **[8]**. This study mainly gives focus on how transgenic technology in major cereal crops such as rice, wheat, maize, barley and oats was initiated and could be able to evolve with time?

### *1. RICE* (*Oryza sativa* L.)

Rice has been known as one of the most competent cereal crops for *in vitro* regeneration and additionally has been considered as the leading crop in cereal gene transfer technology. Significantly, rice is the first cereal crop species for which an efficient transformation protocol mediated by *A. tumefaciens* was developed **[9]** and transgenic plants were obtained. However, the early transformation works were based on the use of rice protoplasts and the physical delivery methods, such as PEG or electroporation. In 1991, the first successful production of transgenic rice plants mediated by the biolistic method could be possible **[10]**.

***Target Tissue for Gene Transformation***

Mature seeds have always been proved to be convenient materials for the induction of embryogenic

callus in cereals in general and in rice in particular. Generally, the *indica* rice has been proved to be more recalcitrant to *in vitro* regeneration and gene transformation. Transformation of callus is still efficient only for some *japonica* varieties whereas poorly effective for *indica* cultivars. However, further studies revealed that immature embryos exhibited transgenesis much more efficiently than the calli of various genotypes of both *indica* and *japonica* varieties **[11]**.

Later, it was also observed that calli of the *indica* rice were transformed several times as efficiently as the *japonica* **[12]** whereas callus induced from the leaf-base tissues of germinating seeds was equally efficient for transformation of *indica* **[13]**. Similarly, seed-derived callus of *indica* rice was also considered as target tissue for the optimization of transformation protocols **[14]**.

***Biolistic-Mediated Transformation***

Initially, DNA-coated gold particles mediated by an electric discharge particle apparatus were introduced into immature embryos of several *japonica* and *indica* rice varieties **[10]**. Generally, bombarded tissues were cultured on media containing hygromycin or bialaphos for callus induction, selection and regeneration. Southern hybridization and progeny analysis further also confirmed that the biolistic method could be an efficient system for rice transformation. This system was gradually improved further by gradual selection at early stage through regeneration and growth of resistant tissues **[15]**.

***Agrobacterium-Mediated Transformation***

Although, biolistic-mediated rice transformation protocols were reported before the *Agrobacterium*-mediated protocols, but most of the transgenic rice could be achieved by the *Agrobacterium*-mediated transformation methods **[11]** and significantly, the first *Agrobacterium*-mediated transgenic rice was obtained in 1993 with immature embryos as explants and G418 as selection agent **[16]**.

In 1994, during *Agrobacterium*-mediated transformation, various *japonica* rice cultivars tissues such as shoot apices, root segments, immature embryos, root callus, and suspension cell cultures induced from mature seed were infected with *Agrobacterium* strains EHA101 or LBA4404 carrying either plasmid pIG121Hm (standard-binary vector) or pTOK233 (super-binary vector). In both plasmids, the *hpt* gene was incorporated as the selection marker and the *gus* gene as the reporter marker. Further, the use of various strain/vector combinations indicated that the frequency of GUS positive callus was the highest with the super-binary LBA4404 pTOK233) system. However, some of the critical factors, such as addition of acetosyringone to the co-cultivation media and incubation at 22-28°C could be the main reasons for the success of such methods **[9]**.

Moreover, mature seed-derived calli from *japonica* were also used as target explants for transformation experiments and hygromycin as selection agent to minimize the transformation time and also to avoid somaclonal variation **[17]**. Additionally, for the *Agrobacterium*-mediated-transformation of *japonica* rice, immature embryo-derived calli were infected with the *Agrobacterium strain* along with hygromycin as selection agent **[18]**. Interestingly, the frequency of transgenesis in *japonica* rice was evaluated either with the super-binary or standard-binary vectors, and only the super-binary vector system could be effective to generate transgenic *japonica* rice.

Similarly, gene transformation mediated by *Agrobacterium* was also performed for *indica* rice and calli derived from mature embryos were treated with the *Agrobacterium* (strain EHA101) along with the standard-binary vector (pIG121Hm) and the *hpt* gene as a selective marker **[19]**. However, findings further indicated that the calli derived from scutella were excellent tissues and the addition of acetosyringone (50 mM) in the co-cultivation media proved to be helpful in transformation events **[9]**.

Moreover, the mature seed-derived calli of various *indica rice* cultivars were further infected with EHA105/pCAMBIA1301 (containing the *hpt* gene as selective marker) and the transformation frequency was found to be higher than most of the previous reports **[20]**. Almost all rice varieties could be transformed with different frequencies when immature embryos were infected with the *Agrobacterium* strains LBA4404 or EHA105 carrying the *hpt* gene as selection marker. However, in case of mature seed-derived callus transformation, the time required from explants inoculation to transplantation of transgenic plants to soil was less than 2 months for the *japonica* varieties whereas 2.5 months for the *indica* rice **[11]**. Additionally, the significant improvement in transformation frequency could be made by transfer from solid co-cultivation medium to filter paper enriched with co-cultivation medium **[21]**.

Recently, in a study on mature embryo derived embryogenic callus transformation of *indica* rice, various *Agrobacterium* strains harboring variable plasmids; GV2260 (p35SGUSINT), LBA4404 (p35SGUSINT) and LBA4404 (pCAMBIA3301) were employed and transient *GUS* expression was found to be highly variable and moreover, it was observed to be maximum in callus which was infected with strain LBA4404 (p35SGUSINT). It indicates that *Agrobacteriu*m strains and vectors combinations always play a decisive role in gene transformation events **[22]**.

Besides the commonly used explants such as the immature embryos and mature seed-derived calli,

other explants, including shoot apices **[23]**, inflorescences **[24]**, and mature embryo-derived green tissues **[25]** were also investigated as target tissues for transformation experiments, but very poor response was recorded. However, high frequency of inflorescence transformation of the *japonica* rice could be possible with the strain EHA101 carrying both *gus* and *hpt* genes **[24]** whereas, calli induced from the leaf-bases of rice seedlings infected with the EHA105 (pCAMBIA1301) system exhibited poor frequency of transformation in *indica* rice **[13]**.

Hence, the high transformation potential of rice has been extensively exploited not only for establishment of transgenic technology but also to explore new technologies, such as gene targeting by homologous recombination followed by the elimination of a selectable marker gene from targeted loci without leaving footprints **[26]**.

**2. WHEAT (*Triticum aestivum* L.)**

In comparison to rice, due to the difficulties associated with recalcitrant nature of wheat during *in vitro* regeneration and poor competency of tissues for transgenes delivery, the progress in wheat genetic transformation has been restricted. However, the first transgenic wheat with herbicide resistance was obtained by using biolistic-method **[27]**. Although, the commonly used methods of genetic transformation in wheat are biolistic, *Agrobacterium*, PEG-mediated and pollen tube pathway methods, but the biolistic-mediated and *Agrobacterium*-mediated methods have been the most widely used transformation methods in wheat biotechnology.

In general, only selected tissues of wheat are suitable for *in vitro* regeneration and therefore, immature embryo is the most commonly used target tissue for gene transformation experiments. Immature embryo is the most competent tissue to form embryogenic callus and regeneration therefore, it has been considered as the most suitable target tissue for gene transformation mediated by both biolistic- and *Agrobacterium*- methods.

***Biolistic-Mediated Transformation***

The biolistic-mediated mature embryos transformation system was initially optimized and their results showed that bombardment pressure of 900 psi at a distance of 6 cm. was the optimal values for the delivery of transgene to the target tissue **[28]**. Subsequently, a shoot apical-tip transformation system was also established by biolistic method, where the optimum frequency of gene delivery was recorded at bombardment pressure of 1100 psi with distance of 9 cm. **[29]**. However, the combination of 0.6 µm gold particles, 5.5 cm target distance and 650 psi acceleration pressure exhibited equally good regeneration frequency along with the high level of *GUS* transient expression **[30]**.

Furthermore, the effects of different media composition on biolistic transformation efficiency were studied and high percentage of transgenic plantlets was obtained. The calli were initially induced on $Cu^{2+}$-containing medium and pre-cultured for 4-days before biolistic-experiments followed by transfer to regeneration medium **[31]**. Recently, biolistic-mediated transgenic wheat was also obtained for over-expressing *1Dx5* gene without selectable markers from immature embryo scutella **[32]**.

***Agrobacterium-Mediated Transformation***

Since the first *Agrobacterium*-mediated wheat transformation reported in 1997 **[33]**, it gradually became the preferred mode of wheat transformation technology. It has several advantages over biolistic method such as the ability to transfer larger segments of DNA with minimum rearrangements, insertion of few transgene copy number, minimal gene silencing events and the stable foreign gene expression in transgenic progeny.

A compared results of using immature embryos, pre-cultured immature embryos, anther culture and young spikes as explants for wheat transformation mediated by *Agrobacterium* indicated that immature embryos pre-cultured for 10-15 days exhibited the best results in terms of transient *GUS* expression and regeneration **[34]**. Further, *Agrobacterium*-mediated wheat transformation protocol was also developed with explants derived from mature embryos and the transformation efficiency was found to be very low **[35]**. However, increased acetosyringone (AS) concentration exhibited high *GUS* transient expression and efficient T-DNA delivery **[36]**. Moreover, in a comparative study, using wheat apical-meristem and tillering node as explants during *Agrobacterium*-mediated transformation, the results showed that the apical-meristem proved to exhibit higher transformation efficiency than tillering node **[37]**.

Recently, a novel non-*in vitro* protocol for direct seed transformation mediated by *Agrobacterium* strains in wheat has been reported. The *Agrobacterium* strain GV2260 (p35SGUSINT) for *T. aestivum* and strain LBA 4404 (pCAMBIA3301) for *T. durum* were employed during gene transformation experiments and moreover, various transgenic lines of *T. aestivum* and *T. durum* were obtained. Further, these transgenic lines were confirmed by *GUS* expression followed by PCR and southern blotting analysis **[38].**

**3. MAIZE (*Zea mays* L.)**

The earliest maize transformation studies could be recorded in 1966, when genomic DNA was injected directly into apical-meristems of developing maize seedlings **[39]**. Significantly, the first report on

maize transformation mediated by the biolistic-method has been documented in 1990 **[7**] whereas it is the second cereal crop in the series of establishment of an efficient transformation protocols mediated by *A. tumefaciens* in 1996 **[40]**. Moreover, the success of maize transformation system is based on following factors;

***Selection of Target Tissues***

Maize transformation generally depends on genotype of the target tissue for transgenesis and also on regeneration potential of putative transgenic tissues. Therefore, the target tissues or cells selected for transformation experiments should be competent enough for transformation and regeneration both. However, mostly abundant and readily available maize tissues, such as leaves, roots, and mature seeds are generally not suitable for *in vitro* regeneration.

***Immature Embryo and Derived Tissues***

Generally, calli and other tissues are frequently employed in rice transformation but efficient transformation has been possible mostly by using immature embryos as target tissues **[41]**. Similar to rice, maize immature embryos harvested 10-12 days after pollination are dissected and can be transformed either by particle bombardment or *Agrobacterium* infection. These treated-immature embryos are cultured in media containing selection agents and transformed cells are selected and regenerated into transgenic maize plants **[42]**.

Additionally, immature embryo-induced embryogenic cultures could be alternative tissues as source of protoplast transformation in maize. In 1990, the first fertile transgenic maize was obtained from embryogenic suspension cultures derived from immature embryos of the maize hybrids A188 X B73 or B73 X A188 **[7]**. Also, young shoot-meristem of immature embryos was bombarded with DNA carrying the visual β-glucuronidase (*gus*) and the selectable neomycin phosphotransferase II (*nptII*) marker genes. The bombarded tissues were cultured for regeneration on media containing BAP and kanamycin sulfate and high level of *GUS* activity could be observed in the bombarded tissues **[43]**.

***Seed / Seedling and Derived Tissues***

Mature seeds or seedling-derived cultures are desired alternatives tissues in absence of immature embryo tissues. In a shoot-multiplication protocol, shoot-tip tissues excised from 7-day-old seedling were bombarded with DNA carrying the gene coding for bialaphos (phosphinothricin–PPT) followed by selection and regeneration were performed on media containing of glufosinate **[44]**.

Alternatively, callus induction and plant regeneration from leaf explants excised from seedlings also have been demonstrated in maize **[45]**. Moreover, a successful leaf-based maize transformation via the biolistic method was reported in 2007 **[46]** whereas the first successful *Agrobacterium*-mediated transformation of Type- I callus induced from nodal sections of seedlings was recorded in 2006. *Agrobacterium* (strain ABI) that harbored binary vectors containing the visual marker green fluorescence protein (GFP) gene and either the *nptII* or the *bar* gene as selectable marker was employed in transformation protocols **[47]**. Further, the transformation was confirmed by southern blot and the transformation frequency depended on tissue cultivars.

***Protoplast as Target tissue***

Initially, protoplast cultures were primarily considered for gene transformation because DNA can be introduced directly into protoplasts via polyethylene glycol (PEG), electroporation, or *Agrobacterium*-mediated methods. Historically, the first fertile transgenic maize from protoplasts culture was reported in 1993, where protoplasts were isolated from embryogenic suspension cultures of maize genotype HE/89 **[48]**. However, strong genotypic dependency and difficulties in protoplasts isolation and preparation from embryogenic callus cultures could restrict to achieve stable transformation in maize.

Moreover, various factors for the maize transformation experiments, such as strains of *A. tumefaciens*, types of vectors and duration of co-cultivation of maize tissues with *A. tumefaciens* were also studied. Transformants were screened for numerous desired characteristics, such as single copy integration, absence of vector-backbone sequences and other unnecessary foreign DNA segments, adequate level of transgene expression, absence of alteration of non-target traits and other aspects of transformants **[49]**.

***Pollen as Target Tissue***

Maize pollen could also be employed as an alternative target tissue for transgenesis. The first maize pollen transformation experiments were conducted by mixing maize pollens with DNA isolated from a phenotypically different donor plant to generate seeds **[50, 51]**. Simultaneously, the biolistic-method was also employed in maize pollen transformation but unfortunately, however, concrete molecular confirmation and progeny data analysis couldn't be established. In general, the low frequencies or lack of reproducibility have prevented pollen tissues for treating as targeting tissues for transformation experiments in maize **[52]**.

***Effects of Genotype***

Genotype dependence of the transformation technology seems to be greater in maize than in rice and therefore, two types of embryogenic callus cultures have been successfully used for maize transformation. Type- II callus is more friable than Type-I callus in the culture morphology and is more suitable for selection,

regeneration, and culture suspension **[53]**. Moreover, the Hi-II genotype that forms Type-II callus is one of the most successful genotypes for maize transformation **[54, 42]**. Significantly, inbred A188 is the first genotype that was transformed efficiently by *A. tumefaciens* **[40]** followed by the transformation of the genotype Hi-II. However, in other genotypes of maize, transformation could be recorded either only at a very low frequency or not at all.

***Transgene Delivery System***

For maize transformation, the main DNA delivery systems can be classified into direct DNA transfer and *Agrobacterium*-mediated methods. The major methods of direct DNA transfer include electroporation **[55]**, PEG- incubation **[56]**, silicon carbide whiskers **[57]** and particle bombardment **[7]**.

***Biolistic-Mediated Transformation***

The most effective direct DNA delivery system is the particle bombardment and, moreover, the first transgenic maize plants were generated by means of a gene gun PDS1000/He **[7]**. Recently, the gene gun has been also utilized to deliver chemicals and proteins loaded with nanoparticles to plant cells **[58, 59]**.

***Agrobacterium-Mediated Transformation***

Till 1996, *Agrobacterium*-mediated gene delivery system was not functional where extra copies of the *Agrobacterium* virulence (*vir*) genes- *virB*, *virC*, and *virG* were incorporated in super-binary vector system, **[40]**. Later, standard binary vectors (non-super-binary) had been used to transform both the Hi-II genotype **[42, 60]** and the inbred B104 **[61]**. The major advantage of the *Agrobacterium*-mediated gene delivery system is that it typically inserts fewer transgene copy numbers than the biolistic gun method, thus minimizes the probability of gene silencing because of insertions of multiple transgene copies **[62]**. Currently, the biolistic gun and *Agrobacterium*-mediated methods are the two most efficient and popular methods for transgenic technologyof maize.

***Selection Markers***

A selectable marker gene is being introduced to differentiate transformed cells from a population of untransformed cells and is typically co-transformed with the gene of interest. Most selectable markers are antibiotics, such as hygromycin and kanamycin, or herbicides, such as glyphosate and PPT. The first transgenic maize was achieved with PPT selection marker **[7]** that is considered as efficient and is a widely used selection system **[40, 54, 61, 63]**. The PPT selection system contains genes coding either for bialaphos (*bar*), PPT acetyltransferase (*pat*) or glufosinate (an ammonium salt of PPT). Among the three selective agents, bialaphos has been the most efficient to transform the hybrid Hi-II and some other inbred lines **[64]**.

Another herbicide selection system is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)/glyphosate. EPSPS is a key enzyme that synthesizes chorismate-derived aromatic amino acids in plants and can be inhibited by glyphosate, the active ingredient in the herbicide. A mutated EPSPS-encoding gene of maize producing a modified enzyme resistant to glyphosate inhibition was reported to be an efficient selectable marker for the production of fertile transgenic maize plants **[65]**.

The kanamycin selection system consists of the *nptII* gene and the antibiotic kanamycin (or its analogs geneticin G418 or paromomycin) has been used in early maize transformation assays **[66, 43]**. Compared to kanamycin, maize is more sensitive to hygromycin and this selection system (with the hygromycin phosphotransferase- *hpt* gene and hygromycin as selection agent) has been applied for both particle bombardment and *Agrobacterium*-mediated maize transformation **[67, 40]**.

Alternatively, selectable marker butafenacil can be also considered as an herbicide and butafenacil-resistant plants can be generated by selecting resistant mutants or by overexpressing native plant protoporphyrinogen genes. A mutated protoporphyrinogen gene of *Arabidopsis thaliana* that was highly tolerant to butafenacil has been identified and successfully employed as a selectable marker for *Agrobacterium*-mediated maize transformation **[68]**. Another well-tested selection system utilizes the phosphomannose isomerase (*pmi*) gene as selectable marker and mannose as selection agent **[69]**. The *pmi* gene converts mannose-6-phosphate to fructose-6-phosphate and, hence, only the transformed cells can utilize mannose as sugar source in the medium whereas non-transformed cells will be failed to grow on mannose sugar supplemented medium.

Significantly, the most productive and stable technologies are required to improve the transformation efficiency of maize and, moreover, some of them have been currently tested. For example, backbone-free, low-copy-transgene maize plants were generated by delivering T-DNA from the *pica* locus of the chromosome of *A. tumefaciens* **[70]**. Furthermore, targeted mutagenesis also could be performed in maize by zinc-finger nuclease **[71]**, mega-nuclease **[72]** and by TALENs and CRISPR/Cas9 **[73]**.

## 4. BARLEY (*Hordeum vulgare* L.)

As for many other cereal crops, transgenic barley was first achieved through the biolistic delivery method and the initial works on barley transformation involved a number of targeting explants such as; immature embryos, immature embryo-derived callus, and microspore-derived embryos.

***Biolistic-Mediated Transformation***

The targeting explants; immature embryo-derived callus and microspore-derived embryos of spring barley were bombarded with the BioRad PDS1000/He delivery system and subsequently, self-fertile transgenic barley lines were also produced by means of the *bar*/bialaphos selection system **[74]**. However, the loss of regenerability and occurrence of albinism during the *in vitro* culture process was recognized as the essential limiting factors in the barley **[75, 76]**. Interestingly, addition of BAP and copper to the media was found to be effective to support induction of more regenerable callus with green shoots **[77]**.

***Agrobacterium-mediated Transformation***

The transformation success in rice and maize further encouraged the establishment of *Agrobacterium*-mediated transgenesis protocol in barley. Immature embryos were used as target tissues and were infected with the *Agrobacterium*-standard-binary vector system carrying the *bar* gene and subsequently selected on bialaphos selection medium. Moreover, multiple-independent transformants were generated from single embryo explants to calculate the transformation frequency **[78]**.

In the first successful *Agrobacterium*-mediated transformation of Australian elite barley cultivars, immature embryos were infected with an *Agrobacterium* -strain carrying the standard-binary vector system and the *hpt*/hygromycin selection system **[79]**. The presence of hygromycin in the media seemed to be able to suppress the *Agrobacterium* overgrowth in addition to its function as selective agent, thus supporting the vigorous callus growth in barley transformation. Moreover, a breakthrough transformation of immature embryos could be achieved in the barley cv. Golden Promise via the *Agrobacterium* strain of the pBract serial vectors that contain the *hpt* gene as selection marker and the luciferase (*luc*) gene as reporter gene **[80]**.

Significantly, addition of copper in the callus induction medium and inclusion of an intermediate culture step (transition medium) prior to pursue for regeneration, increased the transformation efficiency and moreover, half of the transgenic lines exhibited single-copy insertions.

***Other Target Tissues for Transformation***

In transgenic technology of barley, other alternative tissues, such as shoot meristematic cultures **[81]**, androgenetic pollen cultures **[82, 83]**, *in vitro* cultured ovules **[84]**, and mature embryos **[85]**, were also employed as target tissues to deliver desired genes.

***Shoot-Meristem/Mature Embryo Transformation***

Shoot-meristem cultures from germinating seedlings of barley have been used for the biolistic delivery of the *bar/nptII* and *gus* genes. However, the frequency of fertile transgenic barley plants with stable expression was found to be very low **[81]**. Further, since mature embryos are generally competent tissues for both callus induction and regeneration indicates that these tissues may contain high transformation potential **[85]**.

***Microspore Transformation***

Microspores have been considered as one of the most reliable and efficient regeneration systems in barley. Transformation of microspore has the potential to generate homozygous $T_0$ transgenic plants when the transgenic di-haploid can be either simultaneous or chemically induced **[86]**. Bombardments of winter barley microspores with basta as selective agent yielded fertile and homozygous transgenic lines **[87]**; moreover, the frequency was very low and the generated transgenic barley plants were reported to be diploid and fertile **[82]**.

Pollen transformation has also been achieved by means of the *Agrobacterium* super-binary vector system and hygromycin as selection agent. Although, acetosyringone (0.5mM) could limit the *Agrobacterium* overgrowth in the culture, but encouraged its transformation activity as indicated by the frequency and intensity of *GUS* expression. Hygromycin was more effective than bialaphos as selection marker, suggesting that the transformation frequency can be influenced also by other factors, such as *Agrobacterium* strain/vector, medium pH and $CaCl_2$/glutamine concentrations **[83]**.

***Ovule Transformation***

The use of young ovules isolated soon after pollination has been also investigated in barley cv. Golden Promise and transformants could be achieved by the *Agrobacterium* standard-binary vector with hygromycin selection system. Because of the reduced *in vitro* culture time, transgenic plants produced from this system were of high quality **[88]** and these findings indicate that genotype- independent *Agrobacterium*-mediated transformation of ovules may be achieved in barley **[84]**.

In general, the frequency of transformation in barley was initially not very high even from immature embryos tissues, but gradually increased and now has been well established **[89, 90]**. Moreover, it is realized that the gene transformation in barley may be less efficient than in rice, as efficient as in wheat, but more efficient than in maize and other cereals **[91]**.

**5. OATS (*Avena sativa* L.)**

The protocol of genetic transformation of oat has been mainly based on bombardment techniques. The first transgenic oat plants were generated by bombardment of immature embryo-derived callus with a plasmid carrying both the *bar* and *gus* genes **[92]**.

***Selection Markers***

To select the putative transgenic plants, various selection markers were employed and with kanamycin selection, both neither the *npt*II/kanamycin nor *npt*II/G418 combination was successful **[92]**, however, the *npt* II/paromomycin combination was more effective **[93]**. Almost all the paromomycin-resistant lines exhibited the production of the NPTII protein as detected by enzyme-linked immunosorbent assay (ELISA) and also contained the transgenes as indicated by Southern hybridization. The transgenic lines were more regenerable compared with that obtained with PPT as selective agent **[92]**.

Furthermore, investigation of hygromycin as a selective agent revealed that immature embryo-derived callus from oat was less sensitive to hygromycin than to PPT **[94]**. Additionally, the application of only visual markers, such as the *gfp* gene could be also effective as selection marker for putative transgenic selection and therefore, embryogenic callus of the oat was bombarded with a plasmid containing only the *gfp* gene. Moreover, the regenerated fertile transgenic lines of oats were further selected visually only with *gfp* expression **[95]**.

***Biolistic–Mediated Transformation***

In addition to immature embryo-derived callus tissues, explants such as; mature embryos **[95, 96]**, leaf-base segments **[97]**, and shoot-meristematic cultures **[81]**, have also been employed for biolistic transformation. Plasmid DNA carrying the *npt*II and *gus* genes was bombarded into mature embryo-derived callus cultures followed by selection and regeneration of bombarded calli on paromomycin (50 mg/L)-containing medium **[96]**. Moreover, a similar result has been also recorded when mature embryos were used as target tissues for transformation of oats **[95]**.

***Leaf-base Transformation***

Oat is the first cereal crop that could be transformed by using leaf-base segments as explants. The freshly isolated leaf- base segments were bombarded with a plasmid carrying both the *gus* and *pat* genes. The bombarded tissues were cultured and the embryogenic calli were selected on PPT-supplemented shoot-inducing medium. Significantly, this system involves only 10-12 weeks from leaf-base isolation to transfer of transgenic plants to soil compared to over 12 months and 6 months needed for immature and mature embryo-derived callus as explants, respectively **[97]**.

***Shoot-Meristem Transformation***

An efficient regeneration system was established for oat shoot-meristematic cultures **[98]** with the successful generation of transgenic oat plants **[81**]. Shoot-meristemetic cultures from the vegetative shoots of germinating seedlings of oats were bombarded and selected with bialaphos-supplemented selection medium **[99]**. The transformation frequency was found to be higher than that obtained from embryogenic callus and leaf-base segments. The high regenerability potential of fertile plants may be due to the fact that the shoot-meristemetic cultures don't go through callus or dedifferentiation stages that usually decrease the regenerability and increase the somatic variations **[81]**.

Moreover, an osmotic stress-resistant gene (the late embryogenesis abundant *hva1* gene) was also delivered biolistically into the shoot-meristemetic cultures and bialaphos was used as selective agent. Resulted transgenic oat progenies could show stable transgenes expression and their osmotic stress tolerance had significantly increased **[100]**. Generally, shoot-meristemetic cultures are considered superior because of their low genotype dependence in tissue culture responses, enhanced regeneration potentials, high fertility rate, and less somatic variations in transgenic lines **[81, 101]**; therefore, shoot-meristemetic cultures have the potential to replace other explants for biolistic gene delivery in oats.

***Agrobacterium-Mediated Transformation***

In case of *Agrobacterium*-mediated gene transformation in oats, only limited studies have been reported so far. Mainly two types of explants (immature embryos and leaf-base segments) from oat cultivars have been evaluated with the super-binary system LBA4404 (pTOK233) and kanamycin as selective agent and the standard-binary system AGL1 (pGreen) with PPT selection agent. The immature embryos seemed to be more efficient and competent for *Agrobacterium* infection and regeneration than the differentiated leaf-base segments. A high transformation frequency was obtained with the LBA4404/pTOK233 system and kanamycin selection agent. Moreover, southern analysis on selected lines exhibited that 1-3 transgene copies occurred in the immature embryo-derived transgenic plants **[102]**.

***CONCLUSION***

The progress made so far in cereal transformation has been remarkable and the major cereals are now quite efficiently transformed by *A. tumefaciens*. Technology for the major cereals has reached the point where specific genes may be tested insufficient numbers of transgenic events in basic and applied studies. Immature embryos are definitely the best target tissue for cereal transformation, but the quality and quantity of the embryos is greatly affected by vegetative and physiological conditions of the mother plant. However, calli could be a more convenient alternative target tissue for desired gene transformation to achieve transgenic cereals. Significantly, rice could play a pivotal role as a model to develop new transformation protocols. The advantage of rice is in the small genome size compared to maize and sorghum and therefore, it could be a

convenient approach to test genes first in rice and then optimize them in other cereal crops.

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