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An over view of genetic transformation methods for stress adaption in Wheat

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Abstract
Abiotic stresses especially drought and heat are major environmental factors that limit wheat production worldwide. To ensure food security for ever increasing world population, improving wheat yield under such stress conditions is important. In this study, transgenic events were analysed with different conditions for genetic transformation and their effect on wheat, changes during different *in-vitro* developmental stages after transformation. Previous reports from various researchers indicated that drought stress at early reproductive stage and heat stress at grain filling stage had severe impact on wheat development. In this study a total of 3500 wheat calli were used, including five genotypes that responded best for *in-vitro* tissue culture. The stress may also hamper many critical processes including floral development, photosynthetic activity and stomatal movement. Furthermore, the validation of best genetic transformation method within minimum time period is effectively used in future and confirmed their responsiveness to abiotic stress under different genetic backgrounds. This study not only improved our understanding of wheat changes during transgenic development but also provided useful information to manipulate stress tolerant gene in wheat by transgenic technology. The comparison of two methods *Agrobacterium* mediated and particle bombardment can be useful in development of transgenic wheat for various abiotic and biotic stresses in future. Further verification of transformation conditions, chemicals and concentration of growth hormones in this study will shed light in this area in future for other related crops.

Keywords: wheat, drought stress, heat stress, genetic transformation

Introduction
Agriculture has been and will continue to be the lifeline of the Indian economy. On a global basis, drought in conjunction with high temperature and radiation possess the most important environmental constrain to plant survival and to crop productivity. Wheat plant is widely exposed to drought and it adversely affects productivity of cereal crops. Bread wheat (*Triticum aestivum* L.) is an annual herb which belongs to the family Gramineae or Poaceae. It has undergone hybridization and genome duplication events to generate its hexaploid genome (2n = 6x = 42, AABBDD), in past. Despite the considerable efforts of the international research community, development of wheat genetic engineering lags behind other key agricultural crops like rice and maize. According to FAO global wheat production falls down from last year from 95.8 million tonnes to 92.0 million tonnes in 2015. The average wheat productivity of India is very low as compared to global averages. Global climate change is expected to exacerbate these problems [1]. Hence, to blasting the yield barrier and enhancing the abiotic stress tolerance are important goals of wheat improvement programmes. Abiotic stress adversely affect growth and productivity of plants causing average yield loss every year for major crops and prompt a series of morphological, physiological, biochemical and molecular changes. Among the various abiotic stress drought, high temperature and salinity are the most common encountered by plants. Therefore, genetically engineered crops developed by the introduction &/or over expression of selected genes seems to be a viable option hasten the breeding of "improved" plants [2].

The first successful wheat transformation was reported by Vasil *et al.,* (1992) [3], by using microprojectile bombardment of embryogenic callus tissue and Cheng *et al.,* (1997) [4] by using *agrobacterium*-mediated transformation technique. However, despite these first successful experiments, wheat transformation efficiency remained low [5] (Moghaieb *et al.,* 2010). According to Sparks and Jones (2009) [6] *Agrobacterium* mediated transformation is limited to specific wheat genotypes whereas biolistic methods are applicable to a much wider range. Immature embryo is the most commonly used explant for transformation in wheat, although embryogenic callus has also been used successfully [7] (Wu *et al.,* 2009). However, availability of immature embryos is limited to a narrow window of growth. Therefore, mature

embryos are preferred alternative for callus and somatic embryo formation, and transformation [8] Wheat is a recalcitrant cereal crop, which offers only a few explant tissues suitable for *in vitro* regeneration and crop improvement through gene transfer method is very challenging approach. The most common target tissue used is the scutellum surface of the mature seed embryo, which are amenable to DNA uptake *via* both biolistic and *Agrobacterium* and readily forms embryogenic calli. Transformation of cereal crops is a powerful tool for gene discovery and function to investigate genetically controlled traits and is fast becoming a key element in the process of varietal improvement. Wheat was among the last of the major crops to be transformed with the first fertile transgenic plants being reported using particle bombardment little over a decade ago. Advances in the design of micro-projectile devices, choice of explants, media composition, selection systems and transformation methods has enabled the application of wheat transformation to study the role of specific genes in a wide range of agronomical important traits. The objective of present investigation was to compare two most widely used transformation protocols of wheat.

Material and Method

Five local wheat (*Triticum aestivum* L.) cultivars were tested for their performance during genetic transformation and *in vitro* response after using transformation methods specially callus maintenance and regeneration. Mature embryo was the system of tissue culture used in the present study. Mature embryos of the five cultivars were developed by using protocol given in Salari *et al.*, (2013) [8]. Seeds were surface sterilized with 2 % Sodium hypochlorite supplemented with three drops of Tween 20, and then washed five times with sterile double distilled H_2O. Mature embryos of each cultivar were aseptically isolated. Around 3500 mature embryos were cultured with the scutellum side up onto the callus induction medium, for wheat cell culture containing MS [9]) salts (Himedia), supplemented with growth hormones (Himedia). Calli were maintained in dark at 25ºC, subcultured onto a fresh medium after a week intervals and were maintained by subculture for maximum fifteen days for transformation experiments. After one to two weeks from culturing, the calli were used for genetic transformation by particle bombardment method and *Agrobacterium*-mediated method for comparison of transformation conditions for five genotypes. Calli were maintained at 16 hr. photoperiod of about 40-50 µE $m^{-2}s^{-1}$ provided by daylight cool fluorescent lamplight at 25ºC temperature. Data obtained *i.e.* number of calli cultured, number of days for shoot formation, average number of shoots and survival percentage; were exposed to the proper statistical analysis of completely randomized design. Plant transformation was done by using two methods *ie.* particle bombardment method and *Agrobacterium*-mediated transformation. GUS histochemical staining method given by Jefferson *et al.* (1987) [10] was used to stain callus to confirm the success of experiment. Biolistic PDS-1000 /He Gene Gun for biolistic method of transformation from BIORAD Inc. California, USA Consumables and accessories for biolistic method of transformation was obtained from BIORAD Inc. California, USA.

Results and Discussion

Bread wheat is one of the important food crops of the world and second most important crop of India next to rice. Regeneration of cultured tissues into full plants is essential for crop improvement through biotechnological approaches. Immature embryos have been used frequently as an explant source in wheat tissue culture experiments because of high regeneration efficiency, but the limitation to obtain immature embryo throughout the year restrict it for culture. Therefore, mature embryos which are readily available at all times are choice of explants for callus induction and regeneration [8] (Salari *et al.*, 2013) [8].

High escape frequencies 76, 90, 50, and 95% have been reported in past years during wheat genetic transformation [11, 12] (and hence standardization of an optimized protocol with less escapes was challenge in this experiment. The method described an initial incubation of wheat mature embryos in a liquid culture of *Agrobacterium tumefaciens* strain (LBA4404). Following the initial inoculation with the *Agrobacterium*, the embryos were co-cultivated for 3 hours after which the *Agrobacterium* is selectively destroyed using an antibiotic. Tissue culture of the embryos on regeneration media with a balance hormonal concentration allows embryogenic callus formation followed by regeneration of plantlets [13], and in the later stages of tissue culture a selectable marker (herbicide) is included to minimize the incidence of non-transformed plants. This protocol has been used successfully used to generate transformed plants of a wide range of wheat varieties in bread wheat (*T. aestivum* L.).

Presently, biolistics and *Agrobacterium*-mediated transformation using mature embryos as explants remain the main method for genetic engineering of wheat. Each method has its own advantages and drawbacks. The main advantages of *Agrobacterium* transformation are the relatively high ratio of single copy gene inserts and relative simplicity of the transformation procedure. In contrast, biolistics offer benefits in their capacity to transform organelles and deliver RNA, proteins, nanoparticles, dyes, and complexes into cells. Utilization of Expression Cassettes (MECs) in biolistic transformation enables the production of plants carrying much simpler patterns of transgene integration compared to plasmid bombardment, with a higher proportion of single copy inserts [14, 15] in contrast to *Agrobacterium*, does not introduce vector backbone DNA or repetitive border sequences flanking the T-DNA into the transformed plant cells. A simplified method for DNA/tungsten coating was described by Sanford *et al*., (1987) [16] for the high throughput biolistic production of single copy transgenic wheat. This method involves the application of 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine free base in coating solution for the particle preparation used in the procedure. Biolistics allow for the transfer into wheat of relatively large DNA fragments, conducted successful transformation of wheat. In recent years several groups have reported efficient *Agrobacterium* transformation of a number of wheat cultivars [17]. The protocol of Medvecka and Harwood, 2015 using Bobwhite SH98-56, allows production of transformants at a transformation frequency of 2.2%. Supartana *et al*., (2006), reported Agrobacterium mediated transformation of wheat *cv*. Shiranekomugi using seeds soaked overnight in water. This method used the *Agrobacterium* strains LBA4404 and an M-21 mutant strain, and no tissue culture steps were used at any stage. The plants obtained were analysed for antibiotic resistance, and plasmid rescue to confirm their transgenic status. Zhao *et al*. (2006) [18] produced *Agrobacterium*-mediated transgenic wheat by adding inoculums to an incision made at the base of wheat seedlings.

Although there has been significant progress in developing suitable genetic transformation system in wheat for improved

agronomic traits, some challenges still persists which need to be addressed for ensuring easy market adoption and building public confidence on GM crops. One such challenge is to develop wheat transgenic varieties without selectable markers [19]. Strategies followed to develop marker-free transgenic plants include, usage of markers not based on antibiotic or herbicide resistance genes, to excise or segregate marker genes from the host genome after regeneration of transgenic plants and co-transformation. A very few attempts were made to develop marker free transgenics in wheat through co-bombardment. Altpeter *et al*. (1996) [20] mixed the plasmid pAHC25 (Christensen *et al*., 1996) [21] with other potential useful genes in a 1:1 molar ratio, but the results in relation to the integration and expression patterns were not analysed. Transgenic wheat plants without the selectable marker gene were obtained during the transformation protocol. The optimization of wheat co-transformation procedures with gene cassettes in an improvement in transformation frequency.

In the present study, 2:1 for gene construct: *BAR* was selected because of the difference in their size as 6.5 and 8.95 kb, to maintain a equal number of molecules to coat the tungsten to established an efficient *in-vitro* regeneration system of wheat. A comparative *in-vitro* regeneration and genetic transformation efficiency of different wheat genotypes from mature embryos was standardized.

The comparison of two transformation methods was done successfully with five genotypes HD2894, HD2833, HD2932, HD2329 and GW365, to cope up with highly changed climatic condition as earlier reported by Varshney *et al*., (2011) [22]. Figure 1 depicted the mean value of total number of calli survived after transformation. As shown in fig. 1 particle bombardment method was found better than Agrobacterium mediated transformation in all five wheat genotypes used in the present study. Genotype GW365 was reported highest survival percentage followed by HD2894, HD 2833, HD2329 and lowest percentage was recorded in HD2932. In *Agrobacterium* transformation genotype HD2894 and HD2833 were reported highest survival percentage followed by GW365, lowest percentage was reported in HD2932 and HD2329.

Figure 2 depicted the mean of no. of days for shooting after transformation event. Genotype GW365 and HD2833 were recorded least days for shoot formation after bombardment which were followed by HD2894, HD2329 and the maximum days to shoot formation was recorded in HD2932. In *Agrobacterium* transformation genotype HD2894 recorded minimum days to shoot formation followed by HD2833, HD2932 and HD2329, maximum days to shoot formation was recorded in GW365, which indicates the conditions and chemical stress on these two genotype during transformation.

Figure 3 depicted average numbers of shoots proliferate after transformation experiment. In particle bombardment transformation maximum number of shoots were recorded in GW365 which was followed by HD2894, HD 2833 and HD2932. Minimum number of shoots were recorded in HD2329. In *Agrobacterium* transformation maximum number of shoots was recorded in GW365 which was followed by HD2329, HD2932 and HD2833. The minimum number of shoots was recorded in HD2894.

In fig. 4 depicted the comparison of survival percentage after *Agrobacterium* and particle bombardment transformation in Indian wheat genotypes. Genotype GW365 was reported highest survival percentage followed by HD2932, HD2894 and HD2833 and lowest percentage was recorded in HD2329. In *Agrobacterium* transformation genotype GW365 and HD2329 were reported highest survival percentage followed by HD2894 and HD2932, lowest percentage was reported in HD2833.

In our study it was observed that the positive correlation between conditions of bombardment (DNA coating, distance of explants) and regeneration after transformation. The results are genotype dependent and quality of seed may also affect the performances of genotype in transformation experiment. Marker gene has also played a major role in gene transformation like phosphinothricin acetyl transferase (PAT or BAR) successfully used in cereal crops, but results indicates that it mainly depend on the species in transformation experiments. Genes for PAT/BAR were the best marker for maize and wheat [23]. It was concluded from the past research [24] (Kansara *et al*., 2013) [24] that the cytokines positively involve to established higher rate and good quality of shoots in media. Seed health play an important role for successful cultivation and yield exploitation of a crop species and seed borne pathogens of wheat are responsible to cause variation in plant morphology [25].

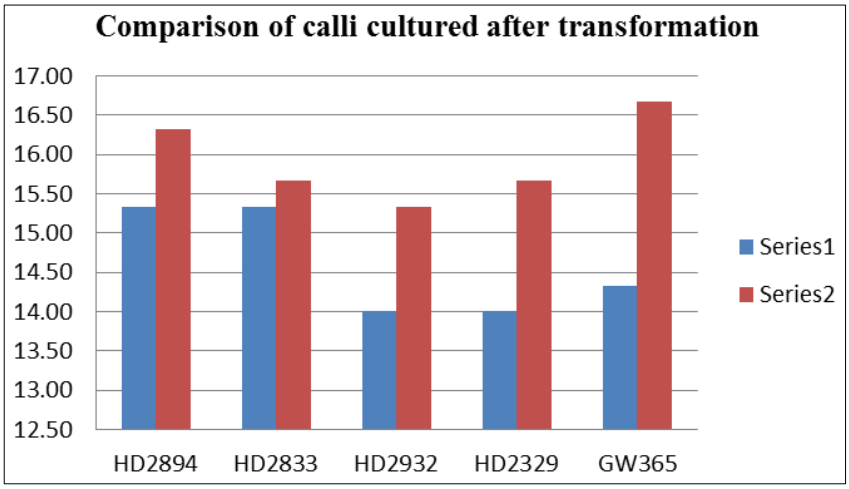

Fig 1: Comparison of calli after *Agrobacterium* mediated and Particle Bombardment transformation for regeneration characteristics and effect of chemicals in Indian wheat genotypes. Series 1: Number of calli cultured/plate after co-cultivation in *Agrobacterium* method; Series 2: Number of calli cultured/plate after Particle Bombardment transformation.

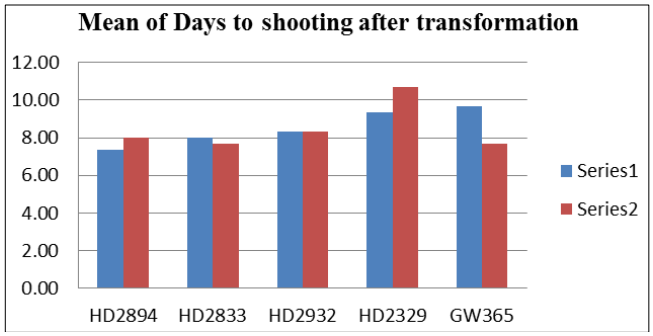

Fig 2: Mean of No. of days to shooting after *Agrobacterium* and Particle Bombardment transformation in Indian wheat genotypes. Series 1: Days to shooting after *Agrobacterium* transformation; Series 2: Days to shooting after Particle Bombardment transformation.

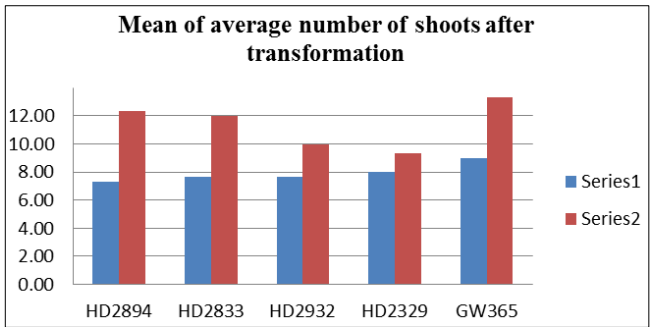

Fig 3: Mean of average number of shoots after *Agrobacterium* and Particle Bombardment transformation in Indian wheat genotypes. Series 1: Average no. of shoots after *Agrobacterium* transformation; Series 2: Average no. of shoots after Particle Bombardment transformation.

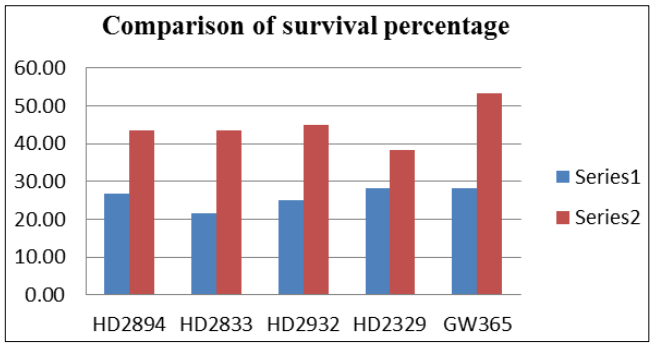

Fig 4: Comparison of survival percentage after *Agrobacterium* and Particle Bombardment transformation in Indian wheat genotypes. Series 1: Survival percentage after *Agrobacterium* transformation; Series 2: Survival percentage after Particle Bombardment transformation.

Conclusion

Considering that population is expected to reach 9 billion by 2050 and changes in the environmental conditions as a result of global warming, there is a clear need to sustain and even accelerate the rate of improvement in crop productivity, simply to be able to feed, cloth, provide energy and building materials for such a large population. Enhancing intrinsic yield and plant stress tolerance through genetic engineering will be a critical part of this effort, adding feather in the cap on the achievements of conventional methods. Genetic modification provides a very exciting future for plant breeders, farmers, and society with very substantial opportunities in improving crop production. There is an opportunity to reduce our dependence on chemical inputs and to improve crops in dynamic ways. However, the acceptance of transgenic crops in the long term is not simply a matter of science and market forces. It is important that as a global society, we develop our vision for the future of agriculture, food and environment security. We need to be sensitive to the desires of society for choice and to find mechanisms to provide that choice. Above all, we need to provide crops that are of clear benefit to the mankind (e.g., price, health and environment) without hampering to our environment and on human health. In this field, the genetic transformation methodologies employed are identical for bread wheat, thus opening the possibility of extending this system to other genotypes as well. The present efforts are encouraging and further in-depth analysis of the integration patterns of transgenes in *T. aestivum*, will pave way for the possibilities of engineering Indian bread wheat with genes of agronomic importance. The present study is an attempt to understand two transformation methods and their comparison in different wheat genotypes for developing transgenic wheat with enhanced tolerance for abiotic stress.

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