Regeneration from mature and immature embryos and transient gene expression
via Agrobacterium-mediated transformation in emmer wheat
(Triticum dicoccum Schuble)

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The present study establishes a regeneration protocol and optimizes conditions for Agrobacterium-mediated transformation of the tetraploid emmer wheat, Triticum dicoccum. Regeneration from mature and immature embryos was accomplished as a two-step process involving callus induction in the presence of 2,4-D followed by regeneration on a 2,4-D free, cytokinin-containing medium (RM1). Higher concentrations of 2,4-D (4 mg/l) though conducive for callusing (89.39% in mature embryos and 96% in immature embryos) proved detrimental for further regeneration. At lower 2,4-D (1 mg/ml) although callusing was suboptimal, (56.8% and 84% from mature and immature embryos, respectively) the regeneration response was the highest on RM1 medium (64.4% and 56.6% from mature and immature embryos, respectively). Overall, the regeneration response of immature embryos was lower than the mature embryos by 10-12%. Due to the ease of availability of mature embryos the mature embryo-derived calli were chosen as the target tissue for Agrobacterium-mediated transformation in the two Indian varieties DDK1001 and DDK1009. Histochemical GUS expression revealed the suitability of the mature embryo-derived calli for such investigations. Of the CaMV35S and Act1 promoters employed, the monocot promoter Act1 displayed higher GUS gene activity in the mature embryo derived calli when co-cultivated with LBA4404 (pB7101::Act1).

Since the onset of human civilization, wheat has been an important crop for world food production. Hulled wheats (Triticum monococcum, T. dicoccum and T. spelta) were amongst the most ancient cereals cultivated by humankind and were gradually replaced by the modern, high yielding, unhusked varieties (T. durum and T. aestivum). Last few years have witnessed the refocusing of attention on natural foods and cereal biotechnologists have been attracted to the group of hulled wheats, T. dicoccum in particular, owing to its distinct but underutilized features when compared with the present day cultivated bread wheat.

Triticum dicoccum is an annual tetraploid wheat, popularly referred to as emmer wheat. This wheat along with barley was found on sites, including the Pyramids, over the Near East and Europe from the earliest times. Emmer wheat spread throughout the Neolithic age from the Near East to become the primary wheat crop of Europe. During the Bronze age, it was gradually replaced by spelt wheat (T. spelta) in many places, and still lingers as a relic in parts of Europe and southwest Asia, especially Iran and India. In India, emmer wheat is grown in the Nilgiri hills and the neighboring areas of Tamil Nadu, Karnataka, Maharashtra and Andhra Pradesh. Known as ‘farro’ in Italy, ‘escanda’ in Spain, ‘gernik’ in Turkey and ‘khapli/samba’ in India, emmer is consumed in a variety of forms ranging from bread, cakes, biscuits to fresh pasta and the Indian suji. The increased popularity and demand of emmer has provoked attention on the improvement of its nutritional and genetic aspects. The tetraploid wheat possess many superior traits over the commonly cultivated bread wheat, such as, high protein content, lowered gluten content, resistance towards various biotic and abiotic stresses, etc. The species is known to be well adapted to poor light soils and to warm, dry climates.

With these advantages of emmer wheat, the present investigation explored the development of regeneration protocol from the embryogenic explants which can be further employed in developing suitable transformation strategies. In bread and pasta wheat, mature

Abbreviations: Act1, actin 1 promoter; BAP, benzylaminopurine; CaMV 35S, 35S promoter of cauliflower mosaic virus; CCM, cocultivation medium; CIM, callus induction medium; 2,4-D, 2,4-dichlorophenoxyacetic acid ; GUS, β-glucuronidase gene; NAA, α-naphthaleneacetic acid; up11, neomycin phosphotransferase gene; RM, regeneration medium; Ubil, ubiquitin 1 promoter; X-Gluc, 5-bromo-4-chloro-3-indolyl β-D glucuronide.
and immature embryos have been often considered as potential explants for obtaining high regeneration and exploited for efficient transformation\textsuperscript{15}. Although a number of reports of regeneration from mature embryos have been published in \textit{T. aestivum}\textsuperscript{8–12}, there exists a single report of regeneration from \textit{T. dicoccum} by Eapen and Rao\textsuperscript{13}.

Regeneration frequency determines the ability to incorporate foreign genes into plants and is often the key to the success of any genetic manipulation endeavor. One of the efficient gene transfer methods of plant genetic engineering relies on the expertise of the soil phytopathogen \textit{Agrobacterium tumefaciens} to transfer and stably integrate part of its extrachromosomal DNA in the nuclear genome of the host plant\textsuperscript{14}. The monocots in general have remained refractive to \textit{A. tumefaciens} infectivity\textsuperscript{15} but rigorous research in this area during the past decade has resulted in \textit{Agrobacterium}-mediated transformation of important crop plants like rice, wheat, maize and barley\textsuperscript{2,16–21}.

In the present investigation on \textit{T. dicoccum}, regeneration from the mature and immature embryos was optimized. The mature embryos and the embryo-derived calli were employed to study the effect of various parameters influencing \textit{Agrobacterium}-mediated gene delivery using \textit{GUS} as the reporter gene.

\textbf{Materials and Methods}

\textbf{Mature embryos}

Husked seeds of the tetraploid emmer wheat, \textit{Triticum dicoccum} var DDK1001, DDK1009 were obtained from the Directorate of Wheat Research, Karnal. The seeds were dehusked manually and treated with 0.2% Teepol (Reckett and Colman, India), washed in running tap water and kept for imbibition for at least 45 min. Subsequently, the seeds were surface sterilized with absolute ethanol for 30 sec, followed by a 4% sodium hypochlorite treatment for 30 min. The disinfected seeds were washed four times with sterile distilled water prior to inoculation.

\textbf{Immature embryos}

Immature seeds were harvested at the milky stage, and surface sterilized using 4% v/v hypochlorite for 10 min followed by washing with sterile water at least four times prior to embryo isolation.

\textbf{Culture medium and conditions}

For all studies, MS medium\textsuperscript{22} containing 3% sucrose as a carbon source and 0.8% agar as a gelling agent (Himedia, India) was used. The hormones, viz. auxins (2,4-D, NAA dissolved in 70% ethanol) and cytokinins (Kinetic, BAP dissolved in 0.1N NaOH) were added to the medium prior to autoclaving. Coconut milk and antibiotics were filter sterilized with a 0.22 \textmu m membrane filter (Millipore Corp., USA) and added to the molten medium (50-60°C) in the required concentrations. All aseptic procedures were carried out under sterile conditions in laminar flow hood (Kartos, India). Cultures were maintained under a daily photoperiodic regime of 16:8 hr light/dark cycle. Four cool white fluorescent tubes (Philips, TL40W/54) provided the light intensity of 65 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1} at 26 ± 2°C.

\textbf{In vitro regeneration}

For callus induction, the embryonal portion of the disinfected seeds was sliced aseptically and placed on the callus induction medium (CIM) composed of MS medium supplemented with varying concentrations of 2,4-D. In case of immature embryo culture, the embryos were dissected from the sterilized immature seeds using a pair of sterilized needles. In both types of cultures, after two weeks of culture in dark, the explants producing callus were selected, calli excised, and placed on fresh CIM for another two weeks in dark. The four-week-old calli were then placed on different media (RM1-4) to study their regeneration potential (Table 1). The regeneration response was calculated as number of calli regenerating two or more shoots/the total number of calli plated × 100. The data represent average of minimum three experimental sets with 30-40 explants/experiment.

\textbf{Bacterial culture}

Two different plasmid constructs were introduced in the host \textit{Agrobacterium} strain LBA4404; plasmid 35SGUSINT carried CaMV35S driven \textit{GUS} reporter gene (interrupted with an intron)\textsuperscript{23}, while in pBI101 the \textit{GUS} gene was driven by the monocot promoter \textit{Actl} from rice. Both, binary vectors carry \textit{nptII} selection marker under the control of \textit{nos} promoter and terminator. The plasmids were introduced into host \textit{Agrobacterium} strain LBA4404 by triparental mating\textsuperscript{24,25}

The agrobacterial cultures were raised by inoculating 50 \textmu l glycerol stock of LBA4404 (p35SGUSINT) or LBA4404 (pBI101::Actl) in LB medium (tryptone 10 g/l, yeast extract 5 g/l and sodium chloride 10 g/l, pH 7.0) supplemented with 0.1% glucose, antibiotics (50 mg/l kanamycin and 50 mg/l rifampicin) and
200 μM acetoxyringone. The bacterial cultures were kept at 200 rpm at 28°C for 2 days.

Co-cultivation

Bacterial culture in the log phase was harvested by centrifuging at 4000 rpm at 4°C for 10 min. The pellet was washed with liquid medium (MS + 1 mg/l 2,4-D) and resuspended in 1 ml medium. Bacterial cell density was adjusted to 0.1 after taking the absorbance at 600 nm. The explants (mature embryos and embryo-derived calli) were submerged in *Agrobacterium* culture supplemented with 100 μM acetoxyringone (AS) for 45 min. The explants were then either placed on MS medium supplemented with 200 μM AS along-with 1 mg/l 2,4-D (CCM1) or auxin free basal medium (CCM2) and co-cultivated in dark for 5 days. In addition, mature embryos were also placed on auxin free MS medium for co-cultivation.

Selection

Explants were blotted dry on sterile Whatman 3 filter paper and placed on selection medium (MS with 1 mg/l 2,4-D) containing 50 mg/l paromomycin as the selection agent and 500 mg/l carbenicillin as the bacteriostatic agent (Sigma Co., St. Louis, USA). Further culture conditions were as described earlier.

GUS activity

The GUS expression studies were carried out using histochemical assay. The co-cultivated explants (after 3 days of selection) were incubated in the GUS buffer (100 mM phosphate buffer, pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 50 mM EDTA, 0.15% Triton X-100, 1 mM X-gluc) at 37°C for 24 hr. The tissue was decolorized by treating with a mixture of ethanol and acetonitrile (3:1) for at least 6 hr prior to observation under binocular stereozoom microscope (Nikon-SMZU, Japan).

Results and Discussion

A well-established regeneration protocol is an essential step towards developing an efficient transformation strategy for any plant system. The endeavor in the present investigation was thus initially directed towards establishing a regeneration protocol in *T. dicoccum* employing mature and immature embryos as explants. The mature embryos and the embryo-derived calli were further selected for investigating various aspects of *Agrobacterium*-mediated transformation employing the GUS reporter gene for transient expression studies.

**In vitro regeneration**

**Callusing**—Towards optimization of a regeneration protocol, a significant step is to obtain proliferating calli from the explants. In cereals, presence of auxins like 2,4-D is known to play a crucial role in callus induction as well as proliferation. This was found true in the present study also where the effect of various levels of 2,4-D on the callusing potential of mature and immature embryos was undertaken.

**Fig. 1—**Effect of 2,4-D on callusing and regeneration response on RM1 (Table 1) from mature and immature embryos of *T. dicoccum* var DDK1001.
Excised mature embryos of *T. dicoccum* var DDK1001 when cultured on MS medium supplemented with varying concentrations of 2,4-D exhibited callusing within three-four days of culture (Fig. 1). At lower concentrations of 2,4-D, embryos germinated into seedlings and only 40-45% cultures showed callus formation at the shoot-root junction. Callusing response of embryo explants increased with increasing 2,4-D concentration. By the end of four weeks, vigorously proliferating calli were obtained at higher concentrations. Maximum callusing (-90%) was observed at 4 mg/l 2,4-D (Fig. 1A). Immature embryos exhibited an over all higher callusing than the mature embryos (7-15%) and the maximum callusing response (up to 96%) was observed at 4 mg/ml 2,4-D (Fig. 1B).

In both mature and immature embryo cultures, calli consisted of nodular white sectors at lower concentrations of 2,4-D, whereas the calli raised above 2 mg/l 2,4-D were vitrified and pale yellow in appearance. These observations are further corroborated by previous reports that describe the regenerative cultures of the Gramineae family as compact and white to off-white in colour.

**Somatic embryogenesis**

In many cereals including wheat, auxins are known to trigger somatic embryogenesis and the expression is manifested either upon lowering the auxin level or its total elimination. In the present investigation also, regeneration from mature and immature embryos was successfully achieved by eliminating 2,4-D. In both mature and immature embryo cultures, four-week-old callus on RM1 medium (Table 1) showed greening and differentiation of globular embryos. However, this differentiation was discernible only in calli raised on lower concentrations of 2,4-D. Embryos exhibited germination within ten days and well developed shoots were discernible in three- to four-weeks (Fig. 3). A maximum of 64% regeneration was obtained from the calli raised on 1 mg/l 2,4-D (Fig. 2). Root formation was observed on the same medium after a period of two weeks. Immature embryo calli raised on 1 mg/ml also responded fairly well on RM1 showing 53% regeneration (Fig. 2). Comparatively, the regeneration ability of immature embryo culture was lower than the mature embryos (-10%) though the explants exhibited better callusing response at the concentrations of 2,4-D tried (Figs 1 and 2). High regeneration efficiency using immature embryos has been reported earlier by several workers in bread

<table>
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<th>Table 1—Composition of various media employed to evaluate the regeneration potential of the mature embryo derived calli of <em>T. dicoccum</em> var DDK1001</th>
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<tr>
<td><strong>Regeneration medium</strong></td>
</tr>
<tr>
<td>RM1</td>
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<td>RM2</td>
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<td>RM3</td>
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<td>RM4</td>
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![Fig. 2—Effect of preculturing media (1 or 1.5 mg/l 2,4-D) on regeneration efficiency of the mature and immature embryo calli (see Table 1 for the composition of various regeneration media employed).](image-url)
wheat. The inability of calli raised from either explants on 3 or 4 mg/l 2,4-D to regenerate shoots, confirms the adverse effect of higher concentrations of 2,4-D. Transfer of regenerating plantlets to half-strength MS medium led to shoot elongation and profuse rooting (Fig. 3). Nearly 80% of the acclimatized plantlets were successfully transferred to pots for maturity.

Improvement in the regeneration capacity from the two explants sources was explored by transferring the calli to medium with different hormones. The calli raised on 1.0 and 1.5 mg/l 2,4-D showed maximum regeneration response on RMI (64% and 50% respectively, for mature embryos and 54% and 42% regeneration, respectively, for immature embryos), and the initiation of shoots was also early in RMI as compared to the other media (Fig. 2). Culture of calli on medium containing coconut milk (RM3) resulted in 32-42% regeneration, which was similar to that observed by Eapen and Rao. Calli initiated on 1mg/l 2,4-D was more regenerative than that initiated on 2 mg/l. There was no major morphological difference in the plantlets regenerated on the various media or from the explants employed. The regenerated plants, however, exhibited reduced height and lower number of tillers than the seed derived plants.

Agrobacterium-mediated transformation

Genetic transformation is an important tool not only to gain insight into basic gene function but also to insert useful genes in commercially important crop plants. Recognition of the ability of the soil bacterium Agrobacterium tumefaciens to transfer a portion of its DNA has been perhaps one of the most important milestones in plant biotechnology. Many factors are attributed for the relative refractoriness of Agrobacterium towards monocots, e.g., absence of vir inducing factors, fewer competent cells, a different cell wall composition, etc. Nonetheless, persistent efforts have led researchers to successfully engineer many cereals of prime economic importance by Agrobacterium.

In the present study on emmer wheat, an attempt has been made to assess the transformation efficiency of LBA4404 harboring two different binary con-

Table 2—The effect of various parameters—promoters, explants, media and genotype on the transient GUS expression in T. dicoccon upon co-cultivation with Agrobacterium tumefaciens strain LBA4404

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% GUS expression</th>
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<tr>
<td></td>
<td>DDK1001</td>
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<tr>
<td>Explant and medium*</td>
<td></td>
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<tr>
<td>a. Mature Embryos on</td>
<td></td>
</tr>
<tr>
<td>CCM1</td>
<td>83</td>
</tr>
<tr>
<td>CCM2</td>
<td>42</td>
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<tr>
<td>b. Mature-Embryo derived calli on</td>
<td></td>
</tr>
<tr>
<td>CCM1</td>
<td>100</td>
</tr>
<tr>
<td>CCM2</td>
<td>36</td>
</tr>
<tr>
<td>Promoter strength</td>
<td></td>
</tr>
<tr>
<td>a. p355GUSINT</td>
<td>17</td>
</tr>
<tr>
<td>b. pBI101::Act1</td>
<td>85</td>
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</tbody>
</table>

*The explants were co-cultivated with Agrobacterium tumefaciens strain LBA4404 carrying pBI101::Act1 for three days and the GUS expression as in other experiments was carried out after three days of washing.

Fig. 3—Regeneration of plantlets from mature (a-c) and immature embryo derived calli (d-f). a and d, Initiation of embryogenic shoots on RMI; b and e, Shoot formation by profuse rooting; c and f, Regenerated plantlets (with inflorescence) after transfer to pots for six weeks.
structs, p35SGUSINT and pBI101:: Act1; the influence of the co-cultivation medium; and the response of mature embryos and embryo-derived calli, by assaying GUS enzyme activity. Though immature embryos have been extensively used as the target tissue to introduce transgene(s) in *T. aestivum* and *T. durum*, in *T. dicoccum* since these explant cultures exhibited lower regeneration response transformation studies were confined to the mature embryos only. Moreover, the mature embryo explants are available throughout the year unlike the immature embryos which have a seasonal availability.

Potential of various explants and influence of cocultivation medium

The physiological status of the explants is well known to play an important role in influencing the host–bacteria interaction. Meristematic tissues, seedling explants, and actively dividing cells are generally chosen for Agrobacterium-mediated transformation. The present study revealed higher GUS activity (100%) in mature embryo-derived calli in both the varieties than the mature embryos. Embryogenic calli derived from immature embryos and mature embryos have been successfully used for *Agrobacterium-

![Fig. 4](image)

Fig. 4—Comparative study of Act1 and CaMV35S promoters on transient GUS expression in mature embryos (a, b) and mature embryo-derived calli (c, d) of *T. dicoccum* var DDK1001. The explants were co-cultivated on CCM1 with *Agrobacterium tumefaciens* LBA4404 carrying either pBI101:: Act1 or p35SGUSINT for three days. GUS histochemical assay was performed after three days of washing.
mediated transformation of *T. aestivum*\(^2\). Amongst the two varieties of *T. dicoccum* employed DDK1001 was more responsive (85%) than DDK1009 (50%) (Table 2). Such genotypic differences are not uncommon and have also been reported earlier in wheat\(^5,12,13,34\).

Various explants display differential susceptibility towards *Agrobacterium*. Higher transformation efficiency of LBA4404 (pB1101:: Act\(1\)) was decisive in pursuing further experiments with this construct and subsequent standardization of other parameters, viz. composition of the co-cultivation medium and type of explants. Co-cultivation of mature embryos on medium supplemented either with or without 2,4-D (CCM1, CCM2, respectively) exhibited variation both in terms of percentage GUS expression and the intensity of blue spots (Table 2). Maximum expression was observed when the explants were co-cultivated on CCM1. Explants co-cultivated on CCM2 showed a lower percentage response and intensity (Table 2). Similar results have been reported for wheat\(^35\) and rice\(^36\) when co-cultivated with *Agrobacterium*. Higher GUS activity of explants cultured on 2,4-D could probably be due to the presence of actively dividing cells thereby increasing the chances of infectivity. Also, use of one-tenth MS salts during co-cultivation of wheat immature embryos has been reported to result in a high transient GUS expression than when full strength MS salts are employed\(^3\).

**Comparative study of promoters**

Compatibility of the promoter with the targeted plant system is a highly desirable factor for ensuring the survival as well as successful expression of the foreign gene. The 35S promoter of cauliflower mosaic virus\(^36\) is one of the most frequently used constitutive promoters in plant transformation strategies. Other monocot promoters like Act\(1\) from rice and Ub\(1\) from maize are also known to enhance the transformation efficiency by increasing the transgene expression\(^37,38\).

Freshly excised mature embryos of *Triticum dicoccum* var DDK1001 and DDK1009 were co-cultivated on CCM1 medium (MS+1 mg/l 2,4-D) with *Agrobacterium* strain LBA4404 carrying two different constructs, p35SGUSINT and pB1101:: Act\(1\) for five days. After three days of selection, histochemical analysis revealed 85% GUS expression with pB1101:: Act\(1\) with blue spots spread uniformly over the explants testing positive, while GUS expression was 17% in the explants co-cultivated with LBA4404 (p35SGUSINT) (see Table 2 and Fig. 4). Higher efficiency of the construct carrying GUS gene driven by Act\(1\) promoter\(^38\) reaffirms the need for expression cassettes for development of efficient transformation procedures in the monocots\(^39\). This is especially important since Cauliflower mosaic virus exhibits relatively low activity in the agriculturally significant graminaceous plants such as wheat\(^40,43\), rice\(^39\) and maize\(^44\). Thus, it is not surprising that the requirement of some degree of homology between the promoters and host cells have been supported by a number of other transformation studies\(^45,47\).

To conclude, an efficient protocol for *in vitro* regeneration of *T. dicoccum* from zygotic embryos has been established which can now be effectively employed for developing a suitable transformation strategy for improvement of this ancient crop known for its richer protein content. Emmer wheat is also well adapted to poor light soils and to warm, dry climates. Other traits such as tolerance to low temperature and resistance to stripe rust further makes it an attractive target for genetic manipulation studies.

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**References**


