Front. Agric. China 2008, 2(3): 262–265
DOI 10.1007/s11703-008-0038-y

RESEARCH ARTICLE

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Primary studies on tissue culture from mature embryos in diploid and tetraploid wheat

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Abstract Using mature embryos (MEs) as the explants, the callus induction, embryogenic callus differentiation, plantlet regeneration and culture efficiency in diploid and tetraploid wheat of four genotypes were studied. The tested four genotypes wheat included cultivable emmer wheat (*Triticum dicoccum* Schuble), durum wheat (*Triticum durum* Desf.) and the common wheat progenitors *Triticum dicoccoides* and *Triticum aegilopides*. Results indicated that there were significant differences in the efficiency of callus induction, callus differentiation and plant regeneration among the tested genotypes. The efficiency of differentiation and regeneration shows strong genotype dependence. The rates of callus induction, embryogenic callus differentiation, plantlet regeneration and culture efficiency respectively were 95.00%, 90.00%, 32.40%, and 27.70% in cultivable emmer wheat, which were significantly higher than other tested genotypes. Therefore, this study has provided a basis for genetic transformation, gene cloning and molecular plant breeding in wheat and other related species.

Keywords diploid wheat, tetraploid wheat, mature embryo, tissue culture

1 Introduction

Currently, it is still very difficult to clone important functional genes from wheat (*Triticum aestivum* L.) owing to its large and repeated sequences in genome. Compared with hexaploid species, the genomes of diploid and tetraploid wheat are smaller. Thus, diploid and tetraploid wheat could be used as the elite experimental materials for insertional mutagenesis, separation and cloning of wheat genes. The discovery of the genotypes of diploid and tetraploid wheat with high efficiency of tissue culture, by which to establish a fast, simple, efficient plant regeneration and genetic transformation system is becoming one of the research hotspots in wheat molecular breeding.

Although the calli derived from immature embryo and immature inflorescences of *Triticum* have higher ability of differentiation and regeneration than those from other explant tissues. The preparation is strictly restricted by the plant growth stage (Yu et al., 1999; Qin and He, 2001). If the mature embryos (MEs) can be used as the explants for tissue culture, there are many remarkable advantages. For example, the dry seeds are available for isolating MEs in large quantity with no seasonal influence the whole year round. The physiological states of the MEs are similar and the dry seeds are easy to manipulate in tissue culture. In the past years, some studies had regenerated plants from the MEs (Liu et al., 1996; Özgen et al., 1998; Tang et al., 2004; Bi et al., 2007). Thin mature embryo fragments (Delporte et al., 2001), and meristematic shoot segments (Sharma et al., 2005) of wheat (*Triticum aestivum* L.) have been reported. Up to date, there were still few reports on tissue cultures of diploid and tetraploid wheat in which the MEs to be the explants in our study, the capabilities of callus induction, differentiation, and plant generation from the MEs of diploid and tetraploid wheat were evaluated.

The objective of our study was to identify suitable genotypes for tissue culture of diploid and tetraploid and to provide a theoretical basis for establishment of genetic transformation system, functional analysis of the wheat genes by an insertional mutagenesis method and the molecular breeding research of wheat in the future.

2 Materials and methods

2.1 Plant materials

2.1.1 Plant materials

Mature dry seeds of tetraploid wheat viz. cultivable emmer wheat (*Triticum dicoccum* Schuble), durum wheat

Translated from *Molecular Plant Breeding*, 2007, 5(4): 480–484
[译自: 分子植物育种]

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(*Triticum durum* Desf.), *Triticum dicoccoides* and diploid wheat (*Triticum aegilopides*) were used as the sources of MEs. The seeds of these genotypes were provided by the Tai'an Subcenter of National Wheat Improvement Center in China.

2.1.2 Cultural media

The basal medium for tissue culture was MS culture medium supplemented with 500 $mg\cdot L^{-1}$ casein hydrolysate, 200 $mg\cdot L^{-1}$ glutamine, 150 $mg\cdot L^{-1}$ asparagine, 30 $g\cdot L^{-1}$ sucrose, and 8 $g\cdot L^{-1}$ agar. The callus induction medium was the basal medium supplemented with 2.0 $mg\cdot L^{-1}$ 2,4-dichlorophenoxy acetic acid (2,4-D), 0.5 $mg\cdot L^{-1}$ α-naphthalene acetic acid (NAA), 0.1 $mg\cdot L^{-1}$ abscisic acid (ABA). The callus sub-cultivation medium was the basal medium supplemented with 2.0 $mg\cdot L^{-1}$ 2,4-dichlorophenoxy acetic acid (2,4-D), 0.5 $mg\cdot L^{-1}$ α-naphthalene acetic acid (NAA), 0.5 $mg\cdot L^{-1}$ abscisic acid (ABA). The differentiation medium was the basal medium supplemented with 5.0 $mg\cdot L^{-1}$ kinetin (KT). The rooting medium was 1/2 basal MS medium supplemented with 20 $g\cdot L^{-1}$ sucrose, 8 $g\cdot L^{-1}$ agar.

All media components except the plant growth regulators were dissolved and adjusted to pH 5.8 and then autoclaved at 121°C for 20 min. The filter-sterilized plant growth regulators were added to the media when its temperature dropped to 40–50°C.

2.2 Methods

2.2.1 Surface-sterilization of dry seeds

Dry plump seeds with consistent color and size were selected from the four tested wheat species. The seeds were surface-sterilized as follows: first treated with 70% ethanol (v/v) for 5 min, rinsed 2–3 times with sterile distilled water, then sterilized with 0.1% mercuric chloride ($HgCl_2$) (w/v) for 20 min. After the sterilization, the seeds were rinsed 6–8 times with sterile distilled water and soaked in sterile water at (26 ± 1)°C for 18–20 h under darkness.

2.2.2 Induction and sub-cultivation of callus

The mature embryos (MEs) were dissected from the endosperms with slightly damaging the plumule and radicle after pre-cultivation as the mentioned above. The MEs were then transferred onto the callus induction medium for a 20-day culture at (26 ± 1)°C in darkness. Embryogenic callus was selected for further sub-cultivation in the novel induction medium every 2–3 weeks.

2.2.3 Differentiation and plant regeneration of embryogenic callus

After three or four times of induction in the medium, the embryogenic callus was transferred onto the differentiation media. The callus was firstly cultivated for 10 days at (26 ± 1)°C in dark, followed by 30 days under 40 w cool-white fluorescent light (40 $\mu mol\cdot m^{-2}\cdot s^{-1}$) under a 16/8 h (light/dark) photoperiod. After 40 days of culture for differentiation, with once of renewed the culture medium every 10 days, the green shoot primordial was formed from the calli. When the regenerated shoots were elongated to 2–3 cm, they were transferred onto the rooting medium for rooting for two weeks. Except the changes of the medium, the culture conditions were the same as those of differentiation during the rooting process.

2.2.4 Vernalization and transplantation of regenerated plants

After being grown to 8–10 cm high, the regenerated plantlets with healthy roots were vernalized for 6–8 weeks at 4°C under a 16/8 h (light/dark) photoperiod with cool-white fluorescent light (40 $\mu mol\cdot m^{-2}\cdot s^{-1}$) to be the light source. Before the transplantation, the plantlets were kept in the greenhouse for 7 days with the cover openness of the culture bottle and then transferred to pots filled peat/soil mixture to grow to maturity under the temperature of (26 ± 2)°C, 16/8 h photoperiod with cool-white fluorescent light of 80 $\mu mol\cdot m^{-2}\cdot s^{-1}$.

2.2.5 Observation and record of callus

After 15 days cultivation on the induction medium, the callus induction quality and type were evaluated according to the descriptions of Bi et al. (2007). The symbol "+" numbers were used to score the quality of callus. Among them, "+" represents low callus quality in which the callus was of 50–100 mg weight, a diameter of 1–3 mm, pale or brown, even dead, watery and soft. "++" represents moderate callus quality in which the callus was of 150–200 mg weight, 3–4 mm in diameter, yellowish or pale, irregular shaped and compact. "+++" represents high callus quality in which the callus was of 250 mg or more in weight, 4–5 mm in diameter, light yellow, irregular, granular shaped and compact.

2.2.6 Data analysis

The frequency of callus induction was calculated by the ratio between the MEs that induced callus to the total MEs that pre-cultured. The frequency of callus differentiation was calculated based on the number of calli with green primordial shoots out of the total number of calli plated on the differentiation medium. The frequency of plantlet regeneration was assessed by the number of

regenerated plantlet (with roots and shoots) and the total number of calli plated on the differentiation medium.

The statistical analyses including a homogeneity test for variance, one-factor analysis of variance and Duncan multiple range test (at the 0.05 probability level) were all performed using the Data Processing System (DPS) software.

3 Results and analysis

3.1 Preliminary experiments

During the tissue culture process in plants, the tissue growth and development were affected by many factors. In the preliminary studies in which the MEs of wheat (*Triticum aestivum* L.) to be the explants, we found that the light quantity, quality, temperature, and concentration of 2,4-D in induction medium all could be the factors to affect MEs induction (data not shown). The better quality of induced calli, the higher frequencies of callus induction and the subsequent differentiation rate were obtained in darkness at 26°C with the induction medium supplemented with 2 mg·L^{-1} 2,4-D. Therefore, the above factors were adopted for the callus induction of MEs in diploid and tetraploid wheat in this study.

3.2 Response of MEs to tissue culture in diploid and tetraploid wheat

MEs of diploid and tetraploid wheat were taken out from seeds and placed on the induction medium. After 2 days, the MEs began to be intumescent. After 4 days, the yellowy calli began to be initiated form the MEs. The time of callus initiation in cultivable emmer wheat and durum wheat were similar and a little earlier than that in *Triticum dicoccoides* and diploid wheat. During the callus induction, the callus derived from cultivable emmer wheat had better quality showing a faster growing speed and a relative large volume, light yellowy color, irregular and granular shape and are compact. After 20 days of induction, its callus diameter was about 4–6 mm (Fig. 1a). The callus of durum wheat and *Triticum dicoccoides* was similar to that derived from cultivable emmer wheat but callus diameter was about 3–5 mm. The callus quality of diploid wheat was worse showing a slower growth speed, small volume, pale or brown color and is watery and soft in texture. The frequency of callus induction evaluated after 20 days of callus induction was listed in Table 1. Results show that there were significantly differences ($P < 0.05$) on the callus initiation, the callus quality and the callus induction frequency among the tested genotypes. Among these genotypes, the MEs from the cultivable emmer wheat were shown to be the elite explants in tissue culture because of their earlier initiation time of callus, better quality of the callus induced and the higher frequency of callus induction (95.00%).

Embryogenic calli of tested genotypes were transferred onto the differentiation medium after 20 days of callus induction. 8–10 days later, the green shoot primordia could be initiated in the MEs from cultivable emmer wheat and further differentiated into green shoots with

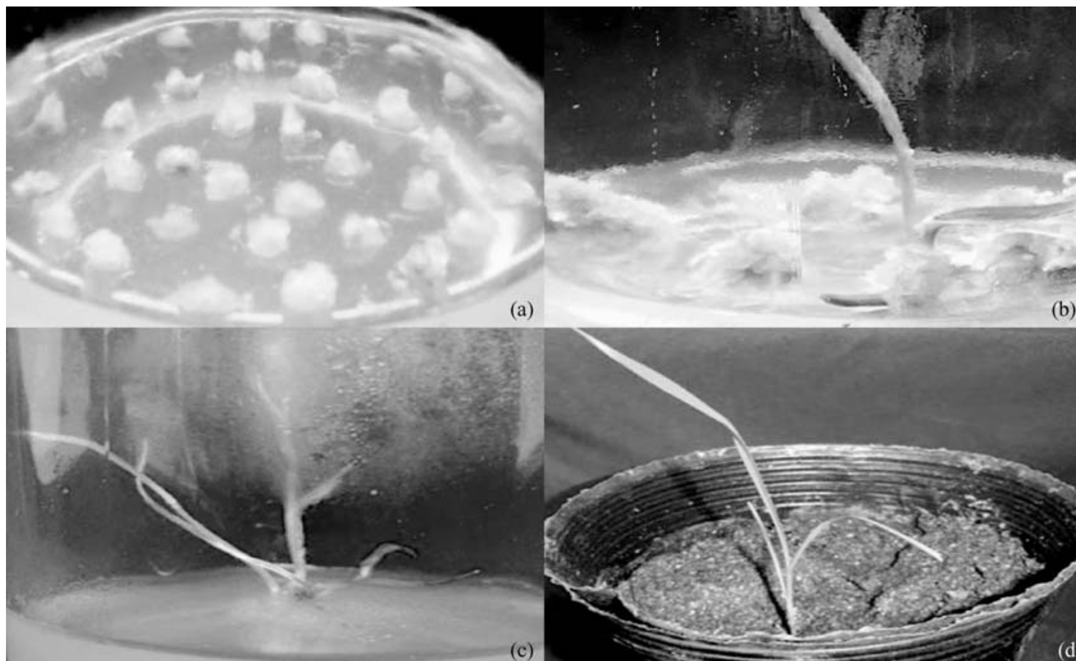

Fig. 1 Plant regeneration derived from *Triticum dicoccum* with the MEs to be the culture explants
Note: (a) calli from MEs; (b) the differentiated green primordia and green bud from embryogenic calli; (c) regenerated plant; (d) regenerated plant growing in plastic pot.

Table 1 Comparisons on induction, differentiation and regeneration of callus derived from MEs in four tested genotypes from diploid and tetraploid wheat species

genotype	genome	origin	quality of callus	frequency of induction/%	frequency of differentiated/%	frequency of regeneration/%	culture efficiency/%
T. durum	AB	Canada	+ +	96.20 a	33.33 b	2.17 c	0.70
T. dicoccum	AB	unclear	+ + +	95.00 a	90.00 a	32.40 a	27.70
T. dicoccoides	AB	Anti-Lebanon	+ +	73.86 b	40.91 b	8.18 b	2.47
T. aegilopides	A	Macedonia	+	68.75 bc	27.27 c	3.19 c	0.60
P				0.0244^{*}	0.0017^{**}	0.0064^{**}	

Note: The values of percentages listed in Table 1 were the average from three independent experiments. The significance test was carried out according to the variance analysis and Duncan multiple range test at 0.05 level of probability; *P* was the result of variance analysis; +: Callus quality was low; + +: Callus quality was moderate; + + +: Callus quality was high.

another 15 days (Fig. 1b). The initiation time of green shoots in other genotypes was posterior to the cultivable emmer wheat. The frequency of callus differentiated was evaluated after 40 days of callus differentiation (Table 1). Green differentiated shoots were transferred onto rooting medium (Fig. 1c). The frequency of callus regeneration after 20 days of rooting culture is listed in Table 1. Finally, the regenerated plantlets with healthy roots were vernalized and transplanted into flowerpots to maturity in the greenhouse (Fig. 1d). In this study, it is found that there were significant differences ($P < 0.01$) on the frequencies of differentiation and regeneration among tested genotypes. The MEs from cultivable emmer wheat, with 90.00% of differentiation rate and 32.40% regeneration rate, respectively, was the highest among the genotypes.

Taken together, the culture efficiency of cultivable emmer wheat was the highest, with the regeneration rate of 27.70%. This indicated that MEs derived from the cultivable emmer wheat had high differentiation capability and could be used as the better potential explants in tissue culture in the diploid and tetraploid wheat species.

4 Discussion

Previous results indicated that the callus induction frequency from MEs among the genotypes in common wheat (*Triticum aestivum* L.) did not have a marked difference, but the frequencies of differentiation and regeneration were markedly changed (Qin and He, 2001; Ren et al., 2003; Yu et al., 2003). In this study, we also obtained similar results (Table 1). Though the callus induction frequencies in four tested genotypes from diploid and tetraploid wheat were all over 68%, the frequencies of differentiation and regeneration were strongly genotype-dependent. Therefore, it is very important to select the suitable genotypes in tissue cultures of diploid and tetraploid wheat of the MEs to be the explants which will result in high tissue culture efficiency. The factors affecting the callus differentiation in tissue culture and plant regeneration of diploid and tetraploid wheats need to be further studied.

Acknowledgements This study was supported by the Key Projects in the National Science & Technology Pillar Program in the Eleventh Five-year Plan Period (No. 2006BAD01A02).

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