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Plant Regeneration from Sliced Mature Embryo Fragments of Wheat Cultivars

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ABSTRACT: Mature embryos were aseptically excised with a scalpel and sliced in fragments measuring 0.5 mm in diameter (sliced mature embryo fragment; 4 ~ 5 fragments/one embryo). Sliced mature embryo fragments of six wheat cultivars were cultured to develop an efficient method of callus induction and plant regeneration. Callus derived from sliced mature embryo fragments showed a good capacity to embryogenesis and regeneration. Furthermore sliced mature embryo fragments decreased contamination from fungi and bacteria. The high efficiency of callus induction were obtained Keumkangmil and Bobwhite. For plant regeneration, selected embryogenic calli were transferred to two types regeneration media. An average number of green spots per callus was 4 to 5 in regeneration media after about one week. Percentage of plant regeneration showed high in regeneration medium containing 0.1 mg/l 2,4-D and 5 mg/l zeatin. Especially, Keumkangmil (27.5%) and Bobwhite (33.3%) showed high regeneration efficiency. This regeneration system from sliced mature embryo fragments may provide an effective and convenient explant for plant transformation studies.

Keywords: wheat, sliced mature embryo fragments, callus induction, plant regeneration

The success in plants genetic engineering-based improvement depend on efficient *in vitro* regeneration system. Most of published transformation systems rely on the recovery of transgenic plants from embryogenic callus derived from immature embryos, mature embryos, and protoplasts. Wheat has been extensively investigated with respect to plant regeneration from *in vitro* culture. La Rue (1949) raised the first successful tissue culture in cereal from endosperm. Gamborg & Eleveigh (1968) succeeded in producing suspension cultures of wheat using a defined medium consisting of mineral salts containing sucrose, B vitamin and 2,4-D. Shimada *et al.* (1969) reported callus formation and single cell culture in wheat.

Regeneration of wheat achieved via organogenesis as well as embryogenesis from various explants sources such as anthers (Chu & Gill, 1988; Last & Brettell, 1990; Zhou *et al.*, 1991; Orshinsky & Sadasivaiah, 1997; Zheng & Konzak, 1999; Brisibe *et al.*, 2000), immature embryos (Ozias-Akins & Vasil, 1982, 1983; Eapen & Rao, 1985; Vasil *et al.*, 1990; Li *et al.*, 1992 a, b; He *et al.*, 1992; Varshney *et al.*, 1996; Ben Amer & Borner, 1997; Hess & Carman, 1998; Machii *et al.*, 1998; Moon *et al.*, 2003), inflorescences (Ozias-Akins & Vesil, 1982; Maddock *et al.*, 1983; Sharma *et al.*, 1995; Barro *et al.*, 1999), isolated microspores (Mejza *et al.*, 1993; Hu & Kasha, 1997; Liu *et al.*, 2002), and shoot tips (Viertel & Hess, 1996). These explants vary in their ability to regenerate whole plants. The high rates of callus induction and plant regeneration of wheat have been obtained from the culture of immature embryos (Ozias-Akins & Vasil, 1982, 1983; Maddock *et al.*, 1983; Redway *et al.*, 1990; Varshney *et al.*, 1996). Successful genetic transformation reports have been used immature embryos initiated regeneration system (Vasil *et al.*, 1992, 1993, Becker *et al.*, 1994; Nehra *et al.*, 1994; Altpeter *et al.*, 1996). However, a limiting factor in the use of immature embryos as explants is that donor plants must be grown year-round for a continuous supply, requiring greenhouse space and demanding extra labor and expense. Winter wheat research requires the additional input of controlled low temperature chambers to satisfy the vernalization requirements for floral induction prior to embryo collection.

Mature embryos are a potentially useful alternative to immature embryos because they can be stored in the form of dried seeds and are available at all times. Plant regeneration has previously been achieved from callus cultures derived from mature embryos (Lazar *et al.*, 1983; Heyser *et al.*, 1985; Mohmand & Nabors, 1990; Özgen *et al.*, 1996; Özgen *et al.*, 2001; Moon *et al.*, 2003). Mature embryos from seeds can be used directly in tissue culture (Ozias-Akins & Vasil, 1983; Özgen *et al.*, 1998; Delporte *et al.*, 2001). Mc Kinnon *et al.* (1987) determined that 2 mg/l 2,4-D was optimal for calli induction from mature embryo explants. Elena & Ginzo (1988) observed that the regeneration of shoot was influenced by a reduced auxin content. Generally, high concentration of auxins and low cytokinins

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in the medium promote abundant cell proliferation with the formation of callus. Shoot regeneration is better on hormone-free medium or 2,4-D at low concentration than on a medium supplemented with IAA and BAP (Chawala & Wenzel, 1987). Regeneration occurs either by somatic embryogenesis or adiventitious bud and shoot development with subsequent rooting (Bhaskaran & Smith, 1990), while sometimes it may occur through direct organogenesis (Li *et al*., 1992).

There is another regeneration system based upon initiation of embryogenic calli from mature embryos. Delporte *et al.* (2001) show that calli derived from crushed mature embryos (nylon mesh: approximately 600 μm porosity) have a good aptitude to embryogenesis and regeneration. Here we suggest their potential use for production of transformed tissue and subsequent transgenic plant regeneration. The purposes of this study were to develop an easy method of callus induction and plant regeneration from sliced mature embryo fragments and to evaluate the culture procedure to make good and sufficient materials for an efficient genetic transformation.

MATERIALS AND METHODS

Plant materials

Mature seeds of wheat genotypes were kindly provided by the National Crop Experimental Station in Suwon. Six cultivars of wheat (Gobunmil, Jaeraeneumil, Keumkangmil, Topdongmil, Urimil, and Bobwhite) were used as target tissues to improve an efficient method of callus induction and regeneration.

Mature seeds surface-sterilization

Wheat mature seeds were washed several times in soapy water, rinsed in distilled water, surface-sterilized in 70% ethanol for 5 min, soaked in 60% clorox containing two drops of tween 20 for 50 min with vigorous shaking and finally washed three times in sterile distilled water. And then mature seeds were imbibed for 48 h in sterile water at room temperature.

Tissue culture media

The basic nutrient medium was the Murashige & Skoog's (1962) medium (MS) with vitamins, supplemented with glutamine, proline, asparagine, 3% maltose, and 0.7% phyto agar. For callus induction, 2 mg/l 2,4-dichlorophenoxyacetic acid and 2.2 mg/l picloram were added. Regeneration media were supplemented with 2,4-D, zeatin, IAA, and BA (Table 1).

Aseptic embryo isolation and plant regeneration

Mature embryos were aseptically excised with a scalpel and sliced in fragments measuring 0.5 mm in diameter. The embryo fragments (sliced mature embryo fragments; 4 ~ 5 fragment/one embryo) were washed two times in MS basal liquid medium and individually placed on callus induction medium. Sliced mature embryo fragments were culture for 5 ~ 6 weeks at 25°C in the dark and subcultured on the same medium every 2 weeks. After 5 ~ 6 weeks, calli were transferred to the regeneration media. Calli were maintained at 25°C in 16 h/8 h light/dark provided fluorescent light (3000 lux) and subcultured on the same medium every two week. Plant regeneration frequency was calculated as the number of callus showing plant regeneration out of the total number of callus.

RESULTS AND DISCUSSION

Callus induction from sliced mature embryo fragments

Callus formation and embryogenesis were observed from sliced mature embryo fragments cultured on callus induction medium supplemented with 2,4-D and picloram. Eight days later, the callus induction rate reached 82% (Fig. 1A). Some organization emerged as a smooth part where cells lined up on the same plane. Keumkangmil and Bobwhite showed high callus induction rates 88% and 87% (Table 2). Plant material from the field is often more contaminated as compared to greenhouse or growth chamber-grown plant material. Especially, mature seed is more contaminated as compared with immature embryo. There are pollutants on the surface and in small crevices. In this study, sliced mature

Table 1. Media composition of callus induction and plant regeneration from sliced mature embryo fragments.

Medium type		Composition
Callus induction		MS salts plus vitamins, 500 mg/l glutamine, 100 mg/l proline, 100 mg/l asparagine, 30 g/l maltose, 2 mg/l 2,4-D, 2.2 mg/l picloram
Regeneration	RI	MS salts plus B5 vitamins, 30 g/l sucrose, 0.1 mg/l 2,4-D, 5 mg/l zeatin
	R2	MS salts plus B5 vitamins, 30 g/l sucrose, 0.5 mg/l IAA, 1 mg/l BA

embryo fragments decreased contaminants from fungi and bacteria. After 28 days, embryogenic calli are white to yellow-white and compact. Non-embryogenic calli are white-limpid, watery, friable structures formed. Six weeks later, the size of the calli form from sliced mature embryo fragments were 6 ~ 9 mm.

The effectiveness of 2,4-D as auxin for callus induction and growth has been demonstrated for wheat (Abdrabou & Moustafa, 1993). An excellent callus growth was obtained within two weeks when the calli were sub-cultured for proliferation on MS medium supplemented with 2 mg/l 2,4-D in combination with 0.5 mg/l BAP reported by Mohammad *et al.* (2003). Delporte *et al.* (2001) observed that 2,4-D induces good callogenesis from small mature embryo fragments (nylon mesh: approximately 600 μm porosity), but it needs to be replaced with IAA after 3 weeks of culture to induce a greater number of embryogenic calli. Media supplemented with picloram were the most effective callus formation from mature embryos of 'Bobwhite' followed by dicamba and then by 2,4-D (Mendoza & Kaeppler, 2002). In this study, the embryogenic calli were obtained when 2 mg/l 2,4-D and 2.2 mg/l picloram was added for 5 ~ 6 weeks.

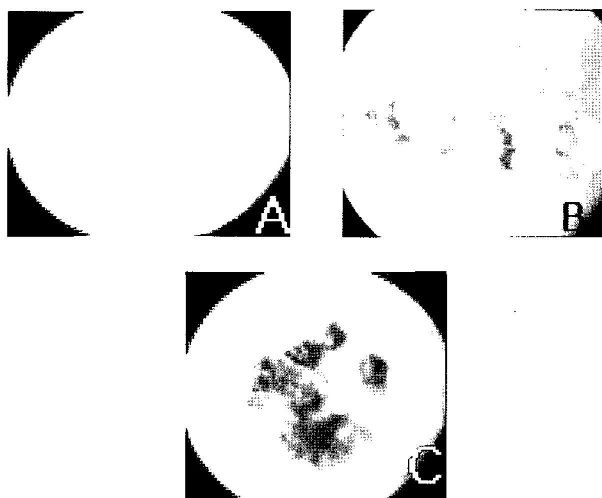

Fig. 1. Callus maintenance and plant regeneration from sliced mature embryos of wheat cultivars. A: Eight-day-old callus on 2 mg/l 2,4-D and 2.2 mg/l picloram, B: Embryogenic callus from sliced mature embryo frag-ments, C: Plantlet development on wheat callus on MS containing 0.1 mg/l 2,4-D and 5 mg/l zeatin after about 3 weeks of culture.

Plant regeneration

Selected embryogenic calli from callus induction medium were transferred to two types of regeneration media (R1: 0.1 mg/l 2,4-D and 5 mg/l zeatin, R2: 0.5 mg/l IAA and 1 mg/l BA). In sliced mature embryo fragments, green spots (average number : 4 ~ 5) from calli in regeneration media were observed after about one week (Fig. 1B). Especially, green spots from Keumkangmil and Bobwhite were appeared the highest numbers (5 ~ 7). Plantlet regeneration was observed on MS medium supplemented with 0.1 mg/l 2,4-D and 5 mg/l zeatin within 10 days. Percentage of plant regeneration showed high in regeneration medium containing 0.1 mg/l 2,4-D and 5 mg/l zeatin (Fig. 1C). In two genotypes, Keumkangmil (27.5%) and Bobwhite (33.3%) were observed high regeneration efficiency compared with other cultivars (Table 2). On the basis of the analysis of two replicates from sliced mature embryos we observed a good reproducibility. It could be valuable to evaluate the effect of the fragment size on the calli induction and regeneration efficiency. An average regeneration rate of 19.9% from the embryogenic calli were appeared. This result is similar to preview report that performed with mature embryo of Keumkangmil (Moon *et al.*, 2003). Delporte *et al.* (2001) show that calli derived from crushed mature embryos (nylon mesh: approximately 600 μm porosity) have a good capability to embryogenesis and regeneration, but, an average of 11% from the embryogenic calli were regenerated.

The positive effect of various medium growth regulator

Table 2. Sliced mature embryo fragments culture response of six wheat cultivars.

Cultivar	Sliced mature embryo fragments			
	Embryogenic callus induction (%)†	No. of green spots per callus	Regeneration efficiency (%)‡	
			2,4-D + zeatin	IAA + BA
Gobunmil	78.8	4	18.3	15.8
Jaeraeneulmil	71.5	4	24.4	17.9
Keumkangmil	88.1	6	27.5	20.6
Topdongmil	84.6	5	16.9	10.0
Urimil	73.3	3	19.7	18.3
Bobwhite	86.8	6	33.3	26.6

†Percentage of callus induction was calculated as the number of embryos inducing callus out of the total number of embryos.
‡Percentage of plant regeneration was calculated as the number of embryos showing plant regeneration out of the total number of callus.

contents and combinations on somatic embryo induction and germination has been demonstrated (Ozias-Akins & Vasil, 1982; Fennell *et al.*, 1996; Viertel & Hess, 1996). Irrespective of the explant being cultured, 2,4-D is the most widely used growth regulator for wheat calli induction and maintenance (Ozias-Akins & Vasil, 1982, 1983; Mc Kinnon *et al.*, 1987; Li *et al.*, 1992 a, b; Viertel & Hess, 1996). Although 2,4-D has been found to elicit rapid cell proliferation and callus formation, the reduction or removal of 2,4-D from regeneration medium is essential for plant development from calli (Marsolais & Kasha, 1985; Liang *et al.*, 1987; Ball *et al.*, 1993). The reduction of 2,4-D concentration upon completion of the induction phase may be essential for optimal plant regeneration, since new gene products are needed for the transition from callus and development to plant regeneration reported by Zheng & Konzak (1999).

In conclusion, these studies indicated beneficial methods from sliced mature embryo fragments to calli induction and plant regeneration, we provided to develop a regeneration system based upon mature embryos. The number of calli induced from mature embryos available for sliced fragments (400 ~ 500 fragments/100 embryos) is higher than those obtained by other explants systems (Delporte *et al.*, 2001: nylon mesh; approximately 600 μm porosity-500 ~ 600 explants/100 embryos). This culture system may be provide an appropriate target to gene transfer method.

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