High frequency plant regeneration from mature embryos of an elite barley cultivar (*Hordeum vulgare* L. cv Baegdong)

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Abstract

An efficient plant regeneration system was developed for *Hordeum vulgare* L. cv Baegdong - an important Korean cultivar. The protocol was based on a series of experiments involving the sizes of mature embryos and the culture media. The embryo size is found to be critical for the establishment of embryogenic callus. Embryos of 1.1-1.5 mm size showed a much higher ability to produce embryogenic callus capable of regenerating green plants. The auxins picloram and dicamba proved effective in inducing callus from mature embryos. 2.5 mg l^{-1} dicamba and 4.0 mg l^{-1} picloram in Murashige and Skoog's (MS) medium was optimum for the induction of primary callus. The induced primary callus was loose and friable which ultimately developed into creamy white and compact callus after transferring into the fresh medium. Multiple shoots were induced in the MS medium supplemented with 6.0 g l^{-1} maltose, 20 mg l^{-1} sorbitol, 0.5 mg l^{-1} 2, 4-D and 1.0 mg l^{-1} kinetin and the rate was 6.5 shoots per embryo. Regenerated plants were hardy and developed roots rapidly in the medium containing 0.2 l^{-1} IBA. This efficient plant regeneration system provides a foundation for generating transgenic plants of this important barley cultivar.

Keywords:Plant regeneration,Barleyembryo,Embryogenic callus,Embryo size

Introduction

Barley (*Hordeum vulgare*L.) is an important cereal crop and a model plant genetic system. It is used for malting, brewing, distilling and as an animal as well asa human food. Barley, a diploid, has been the subject of extensive genomics research and is considered a model for the Triticeae. Genetic transformation is a key tool for genetics research and can be of agronomic importance. Low recovery of green plants from barley callus cultures is a major problem limiting the efficiency of generating transgenic barley. Although both biolistics and *Agrobacterium*-mediated methods have been successfully applied to barley genetic transformation, most work has been performed on a single model barley cultivar, 'Golden Promise' (Wan and Lemaux 1994 Tingay et al. 1997 Trifonova et al. 2001). Immature embryos are presently being used as explant (Bregitzer et al 1998, Wan and Lemaux 1994) and many related factors such as the size of the embryo, and growth regulator combinations involved in successful regeneration (Chang et al. 2002) have been studied in detail. Immature embryos are suitable explants for genetic transformation, but this requires a consistent supply of stock barley plants in growth chambers or greenhouses, which can be expensive, time consuming and labour intensive. However the protocol has poor success rate when applied to other barley cultivars. Selecting proper stage of maturity may be one of the limiting factors when using immature embryos. On the other hand, use of mature embryos from dry seeds has several advantages. They are available in bulk quantities year round and easy to handle. There are records of successful shoot and bud induction from calli derived from mature embryos in other cereals such as oat (Torbet et al 1998), rice (Khanna and Raina 1997) and Wheat (Heyser et al 1985). Although there are reports on the use of mature embryos for regeneration of barley (Lupotto 1984; Rengal 1987, Akula et al. 1999) the success rate was low. The barley regeneration through mature embryo derived callus is cultivar specific (Akula et al 1999). The purpose of this study was to optimize conditions for regenerating plants from mature embryos of 'Baegdong' barley, and to develop an efficient regeneration protocol that can be used for genetic transformation and other fundamental studies. The effect of the size of mature embryos, basal medium and plant growth regulators on callus multiplication, maintenance and regeneration of green plants were studied.

Materials and methods

Seeds were soaked in water for 1 hour and then surface sterilized with 10% bleach for 20with gentle shaking and then with 70% alcohol for 1 each followed by three successive washings in

sterile distilled water. Embryos were dissected and were cut along the longitudinal axis before being transferred into callus initiation medium (CIM) for 24at 25C in 16 hour day. Unless otherwise stated, all the culture media were prepared with 3.0% sucrose, 0.8% agar and the pHwas adjusted to 5.75 before autoclaving. Dicamba and picloram was filter sterilized before adding to the autoclaved medium.

Effects of basal media and plant growth regulators on callus induction

Three types of basal media (MS, B5 and N6) were used with different concentrations and types of plant growth regulators to examine callus induction from mature embryos. Embryos were cultured on either MS, B5 or N6 medium supplemented with or without 2.0 mg l^{-1} 2,4-D and picloram (0, 2.5 or 5.0 mg l^{-1}) and dicamba (0, 2.0 or 4.0mg l^{-1}) (Table 1) for 4weeks and then sub cultured into same fresh medium. Each callus was divided into 23diameter pieces during transfer. There were 30 replicates in each treatment.

Plant regeneration

After 8 weeks of subculture to the same fresh medium for secondary callus initiation, they were transferred into four regeneration (R1, R2, R3 and R4) media described by Akula et al. 1999. There were 30 replicates. After 6 weeks of incubation, morphogenic changes in callus were observed using light and scanning electron (SE) microscope.

- R1 medium - N6 + 5 mg l^{-1} Thiamin-HCl + 5 mg l^{-1} pyridoxine + 5 mg l^{-1} nicotinic acid + 500 mg l-1 myo-inositol +1 g l-1 casein hydrolysate + 3% sucrose + 2.0 mg l-1 2,4 D and 80 mg l-1 proline
- R2 medium N6 + 5 mg l-1 Thiamin-HCl + 5 mg l-1 pyridoxine + 5 mg l-1 nicotinic acid + 500 mg l^{-1} myo-inositol +1 g l^{-1}casein hydrolysate + 3% sucrose + 0.5 mg l^{-1} 2,4 D, 1.0 mg l^{-1} kinetin
- R3 medium - MS + 6g l^{-1} maltose + 20 g l^{-1} sorbitol + 0.5 mg l^{-1} 2,4 D and 1.0 mg l^{-1} kinetin
- R4 medium - MS + 6 g l^{-1} maltose + 20 g l^{-1} sorbitol + 0.5 mg l^{-1} 2,4 D and 1.0 mg l^{-1} kinetin

The effects of embryo size on callus inductionand regeneration

The effects of the size of the embryo (0.51.0, 1.11.5, 1.62.0 and 2.1-2.5 mm) on callus induction and plant regeneration were studied. Embryos were sorted randomly and there were 30

replicates for each size class. The embryos of each size class were cultured on CIM (MS + 2.5 mg l^{-1} dicamba + 4.0 mg l^{-1} picloram) for 10weeks, and then transferred to regeneration medium (R4) incubated at 25C under fluorescent light at approximately $40E^{-2}s^{-1}$ in a 16 hour day. The regeneration data were collected after 4

Rooting of regenerated plants

Regenerated shoots were transferred to rooting medium when the leaves were about 1in length. Rooting medium consisted of half-strength MS medium supplemented with 0.2 mg l-1 Indole butyric acid (IBA), 2% sucrose, and 0.6% agar. After 34plantlets with well-developed roots were transferred to soil.

Statistical analysis

Completely randomise design was used in all experiments. An analysis of variance (ANOVA) was performed for each experiment. The least significance difference test (LSD at P=0.05) was used for multiple mean comparisons.

Results and discussion

Effects of basal media and plant growth regulators on callus induction

Both 2, 4-D, dicamba and picloram induced vigorously growing, friable callus (primary callus). Subsequently milky white, compact and nodular shape callus (secondary callus) formed on the surface of the primary callus. Akula et al (1999) observed the same type of primary and secondary callus in some of the barley cultivars tested. SEM indicated that, cells in the primary callus are globular shaped and compact (Fig. 2b.) while the cells from secondary callus are elongated (Fig. 2c) Tips of this elongated cells form a globular shape sack at the stage of shoot initiation. All treatments in MS basal medium showed high amount of callus formation (0.5-1.3 mm). The callus quality and quantity were significantly different in all three basal media used. Low concentration of dicamba (2.5 mg l^{-1}) and high concentration of picloram (4.0 mg l^{-1}) found to be the best combination for callus induction in both MS and N6 media. B5 basal salts showed lower response in callus production from mature embryos (Figure 1). There was a significant

difference between the basal media and among the concentrations of 2, 4-D, dicamba and picloram combinations on callus induction. Choi et al (2001) reported that, higher concentrations of growth regulators might result in a greater possibility of somatic mutations. Trifonova et al (2001) reported that plant regeneration from barley transgenic callus was difficult to obtain when 2, 4-D was used, so use of dicamba and picloram may be a better choice for transgenic barley plant regeneration and the optimal concentrations would be 2.5 mg l^{-1} dicamba and 4.0 mg l^{-1} picloram for Baegdong variety.

Plant regeneration

Some of the calli transferred into R1 and R2 where the basal medium was N6 degenerated and turned into yellowish brown after 2 weeks and the electron micrographs indicated the cells are deformed. Low concentration of 2, 4-D (0.5 mg l^{-1}) and kinetin (1.0 mg l^{-1}) found to be favourable in both root and shoot initiation (R2, R3 and R4) but the composition of the basal medium have a significant effect over shoot initiation (2% in N6, 14% in 1/2MS and 75% in MS which represents R2, R3 and R4 respectively). Mean number of shoots per callus was high (6.5) in R4 (Table 2). Akula (1999) also reported that 2,4-D and kinetin in 1/2MS medium significantly improve the regeneration efficiency from mature embryos of barley but the concentrations required are cultivar specific.

The effects of embryo size on callus induction and regeneration

When embryos were cultured on CIM, callus growth was initiated in about 5days. There were significant differences in the callus quality and quantity among size classes (PSmaller embryos (less than 2.0produced significantly high percentage of compact callus than larger embryos (larger than 2.1). Embryos of 1.1-1.5 mm size produced the highest percentage of secondary callus (93.5%) and also generated the highest number of green plants per embryo (6.4) (Table 3). Although most of the larger embryos (>2.1produced callus (88.5%), only 3040% of them formed compact secondary callus. This callus became very soft and did not regenerate well (0.8per embryo). The callus derived from the 0.52.0mm embryos was very soft and white or light colour during the first week and then grew quickly and gradually became more compact and yellowish (Fig. 2a). More than 80% became compact within 3This callus initiated shoots (Fig. 2d) in 23In this experiment, we observed that the embryos of 1.01.5 mm had the highest regeneration potential. Some cereal crops regenerate from callus derived from mature embryos,

and the optimal size of embryos varies with plant genotype or species (Ward and Jordan 2001). Developmental stage of the immature embryos is reported to be a crucial factor for in vitro regeneration in other cereal crops (Lu et al. 1984 Bebeli et al. 1988 Castillo et al. 1998 Sahrawat and Chand 2001).

Rooting of regenerated plants

When multiple shoots grown on regeneration medium were divided and transferred to rooting medium (half-strength MS basal medium with 0.3mg l^{-1} IBA), thick white roots developed in about 1 (Fig.2e). Plantlets with well-developed roots were successfully transferred to soil. No obvious morphological differences were observed.

This study reveals that there is an effect of the size of the mature embryo on in vitro regeneration of Baegdong barley and picloram and dicamba play an important role in callus induction. Shoot induction form callus was favoured by changing the carbohydrate source to maltose and reducing the concentration of basal salt mixture. IBA found to be the best treatment for root initiation.

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Tables:

Table 1: Different growth regulator combinations used with three basal media used.

Treatment code	*2,4 D*	Dicamba	Picloram
T1	2	0	0
T2	2	0	2
T3	2	0	4
T4	0	2.5	0
T5	0	2.5	2
T6	0	2.5	4
T7	0	5.0	0
T8	0	5.0	2
T9	0	5.0	4
T10	0	0	2

Table 2: Morphogenic changes in the secondary calli on culture media R1-R4. Observations after 6 weeks of incubation.

Medium	*Callus degeneration*	*Rooting*	*Shoot initiation*	*Mean no. of shoots/callus*
R1	20%	10%	-	-
R2	30%	12%	2%	1
R3	-	8%	14%	1.5
R4	-	8%	75%	6.5

Table 3: Effects of the size of mature embryos on the regeneration frequency of Beakdong barley(data obtained after 4 weeks in regeneration med:um) i

Size of embryos (mm)	*Percentage primary callus formation*	*Percentage secondary callus formation*	*No. of shoots/embryo*
0.5-1.0	100	83.8	4.8
1.1-1.5	100	93.5	6.4
1.6-2.0	100	86.4	2.6
2.1-2.5	88.5	36.9	0.8

Figures 1:

Figure 1: Callus production from mature embryos in different growth regulaters after 5 weeks