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BRIEF COMMUNICATION

An efficient regeneration system of barley cultivars from leaf base segments

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Abstract

A simple and reliable regeneration system from leaf bases of barley (*Hordeum vulgare* L.) has been developed. The *in vitro* regeneration frequencies of seven commercial barley genotypes were compared using segments from the first leaves of 5-d-old seedlings. The regeneration frequency ranged from 31.56 to 72.22 % among the barley genotypes. Murashige and Skoog medium supplemented with 6-benzyladenine (0.5 mg dm^{-3}) and kinetin (0.5 mg dm^{-3}) was optimum for the regeneration. Longitudinal cut of the segments or the removal of coleoptiles further increased plantlet regeneration frequency.

Additional key words: abscisic acid, benzyladenine, explants, *Hordeum vulgare*, kinetin.

Barley is one of the most important crop plants in the world and has received considerable attention regarding *in vitro* regeneration system that is prerequisite for producing transgenic plants. Different barley regeneration protocols have been developed, mostly based on immature embryos (Dahleen and Bregitzer 2002). However, the growth of mature donor plants for a regular supply of immature embryos requires a long time. Furthermore, the limited success in establishing highly reproducible *in vitro* cultures has mostly been restricted to a few cultivars. Development of another efficient regeneration system in barley would facilitate *in vitro* breeding or barley transformation.

Young seedlings can be grown *in vitro* and used as donor plants for fast, frequent supply of explants (Chung *et al*. 2007, Sujatha and Kumar 2007). Leaf segments derived from seedlings are easily available and require a short time for culture (Haliloglu 2006, Orban *et al*. 2007, Mingozzi and Morini 2009). They can serve as ideal target for genetic transformation (Gless *et al*. 1998a). Rengel and Jelaska (1986) used the basal part of barley seedlings containing the scutellum, the apical meristem and the coleoptiles as explants for callus induction and regeneration. However, Pasternak *et al.* (1999) reported an efficient production of embryogenic callus and plantlet regeneration from leaf segments devoid of apical meristem. This is different from what has been reported for oat (Gless *et al*. 1998b) and wheat (Wang and Wei 2004) and there is an apparent need for further investigation into *in vitro* culture of leaf bases by using a group of different barley genotypes. The specific objectives in this study were *1*) to ascertain whether apical meristems of barley seedlings are necessary for efficient plantlet regeneration, *2*) to find out the role of coleoptiles associated with leaf base segments in callus induction and regeneration, and *3*) to establish a highly efficient reproducible regeneration system from leaf base segments of commercial barley cultivars.

Mature seeds of seven different semi-winter, commercial cultivars of barley (*Hordeum vulgare* L.) E32380, Hua 30, Epi 2, Yangpi 2, Zaoshu 3, Hua 2162 and Guangfeng 3 were de-husked, surface-sterilized with 70 % ethanol for 3 min and 0.1 % mercuric chloride for 15 min, and rinse three times with sterilized water. Mature embryos from seeds imbibed for 5 h were isolated using a dissection microscope and incubated scutellum side up on Murashige and Skoog (1962; MS) medium at at 25 °C under irradiance of 100 μmol m^{-2} s^{-1} for 5 d. Five leaf segments from leaf base to tip (referred as L0, L1, L2, L3 and L4) were dissected from 5-d-old seedlings after cutting

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Abbreviations: ABA - abscisic acid; KIN - kinetin; BA - 6-benzyladenine; MS - Murashige and Skoog; 2,4-D - dichlorophenoxyacetic acid.

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away leaves and roots. The first basal segments (L0) consisting of the leaf base, coleoptile base, primordial leaves and shoot apical meristem, were either incubated intact on medium or longitudinally cut into two halves before culture. All leaf base segments were cultured on callus induction medium D2 (MS medium supplemented with 2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid, 2,4-D) in dark at 25 °C for two to four weeks. The induced compact calli were separated from shoot meristem and leaf base segments and transferred onto fresh induction medium for a further 2 weeks of culture.

Embryogenic calli were transferred onto shoot regeneration medium and were incubated under 16-h photoperiod (100 μmol m^{-2} s^{-1}) at 25 °C for 2 - 3 weeks. Three regeneration media, BK, DK and MS, were used. BK medium contained MS medium plus 0.5 mg dm^{-3} of 6-benzyladenine (BA) and 0.5 mg dm^{-3} of kinetin (KIN) while DK medium consisted of MS medium supplemented with 0.2 mg dm^{-3} of 2,4-D and 1.0 mg dm^{-3} of KIN and no hormone was present in MS medium. The regenerated plantlets were cultured on root regeneration medium (1/2 MS medium) for 2 weeks and then vernalized at 4 °C for two weeks before growing in soil. Frequencies of embryogenic callus formation from different segments were calculated after one month of culture. In each treatment 100 initial explants were used and two to three replicates were made in all experiments. Frequencies for induction and regeneration were expressed by calculating the callus formation percentage of the initial leaf base segments, and regenerated plantlet percentage of the calluses used, respectively. The data were analyzed by Duncan's multiple range test (DMRT) using the *SAS* programme version *6.12* (SAS Institute, Cary, NC, USA).

Leaf base segments L0 from cultivar Zaoshu 3 (1 mm) dissected from 5-d-old seedlings (Fig. 1*A*) were incubated on D2 medium (Fig. 1*B*). The induction of callus occurred at the cut ends 3 d after culture. The callus tissue initially appeared to be soft and translucent and after two weeks developed into large, compact embryogenic structures (Fig. 1*C-D*). These calluses were suitable for plantlet regeneration *via* somatic embryogenesis. After four weeks of culture, calluses were transferred into MS shoot induction medium and somatic embryos started to germinate and form shoot and roots. Barley callus can readily produce shoots and roots on regeneration medium (Fig. 1*E*) and the regenerants were morphologically normal and set up viable seeds (Fig. 1*F*).

Seven different barley genotypes, E32380, Hua 30, Epi 2, Yangpi 2, Zaoshu 3, Hua 2162 and Guangfeng 3 were compared for their ability to regenerate plants from leaf base segments L0. All the genotypes could produce embryogenic calluses at 100 % frequency on D2 medium (Table 1), suggesting that D2 medium appeared to be universal for callus induction of barley independent of genotype. However, plantlet regeneration frequencies varied significantly among the genotypes on MS medium

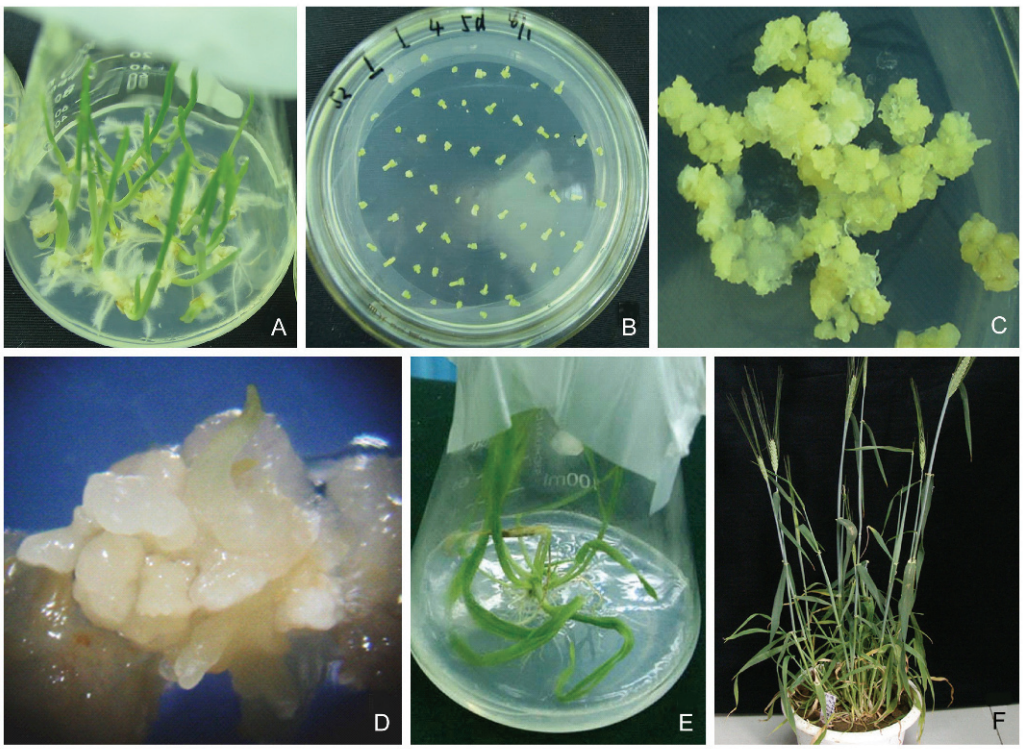

Fig. 1. Embryogenic callus induction and plantlet regeneration from leaf base segments L0 of barley seedlings. *A* - seedlings germinated on hormone-free medium, *B* - freshly isolated basal leaf segments from seedlings, *C* - callus formation on callus initiation medium, *D* - formation of embryogenic callus from the first basal segment, *E* - shoot regeneration and elongation of regenerated plants on regeneration medium, *F* - regenerated plants in the soil.

Table 1. Effect of genotype on embryogenic callus induction and plantlet regeneration. Means ± SE from three repeated experiments. Each treatment consisted of 100 explants. The means with the same letters are not significantly different at $P = 0.05$ according to DMRT.

Response [%]	E32380	Zaoshu3	Hua30	Epi2	Yangpi2	Hua2162	Guangfenf 3
Callus	100	100	100	100	100	100	100
Plantlet	72.22±2.37a	52.63±1.68b	52.5±1.85b	41.54±1.45c	40±1.63c	31.56±1.23d	27.27±0.87d

Table 2. Effect of different media on plantlet regeneration. MS - MS medium; DK - MS medium supplemented with 0.2 mg dm^{-3} 2,4-D and 1.0 mg dm^{-3} KIN; BK - MS medium supplemented with 0.5 mg dm^{-3} 6-BA and 0.5 mg dm^{-3} KIN. Means ± SE from three repeated experiments. Each treatment consisted of 100 explants. The means with the same letters are not significant different at $P = 0.05$ according to DMRT.

Plantlets [%]	MS	DK	BK
Zaoshu3	52.63±2.23c	52.16±2.45c	56.56±2.89b
Epi2	41.54±1.87d	66.36±3.21b	77.79±3.68a

(Table 1). E32380 displayed a frequency of 72.22 % whereas Guangfeng 3 generated only 27.27 % of plantlets. The remaining cultivars had regeneration frequencies ranging from 31.56 to 52.63 %. Thus, plantlet regeneration from leaf base segments L0 was dependent of barley genotypes. Two cultivars, Epi 2 and Zaoshu 3, had intermediate regeneration frequencies, 41.54 and 52.63 %, respectively, and were selected for subsequent studies for the optimization of culture conditions.

To reduce germination and favour plantlet regeneration, each of the segments L0 from Epi 2 and Zaoshu 3 was cut longitudinally into two halves, and assayed for their ability to regenerate plantlets. The cut and non-cut segments were initially incubated on D2 induction medium and then transferred into MS medium to compare their regeneration frequency. The cutting significantly increased regeneration frequency for Epi 2 (41.54 and 74.78 % for the non-cut and cut segments, respectively) and for Zaoshu 3 (52.63 and 61.34 % for the non-cut and cut segments, respectively). It is conceivable that cutting mechanically damaged the apex integrity of the segments, whereby translocation of hormone was apparently affected, consequently resulting in inhibiting germination and favouring plantlet regeneration. A similar phenomenon was recently observed in the shoot culture of sorghum segments (Maheswari *et al*. 2006).

Comparison of three different regeneration media, MS, DK and BK revealed that BK was the best medium for plantlet regeneration (Table 2). In addition different cultivars appeared to have a different response to plant growth regulators. Zaoshu 3 produced comparable regenerants in MS and DK media, suggesting no effect of 2,4-D and KIN on regeneration of this genotype. A significant increase of regeneration frequency for this genotype was only seen in BK medium containing BA and KIN. However, Epi 2 was very sensitive to plant growth regulators. Addition of 2,4-D and KIN (DK medium) or BA and KIN (BK medium) significantly enhanced plantlet regeneration. These results indicated a direct interaction between genotype and plant growth regulators in leaf base culture of barley.

Different leaf base segments, L1, L2, L3 and L4 derived from Epi 2 and Zaoshu 3, were tested for their ability to produce calluses and plantlets in the presence or absence of coleoptiles. All the segments were incubated on D2 medium for the first two weeks and then each of them were divided into two parts, one with coleoptiles and another one without coleoptiles, when transferred into fresh D2 medium for continuous culture. For the segments from Zaoshu 3 with coleoptiles, callus induction frequencies are 100 (L1), 93.55 (L2), 92.32 (L3) and 74.19 % (L4), respectively. The removal of coleoptiles significantly reduced callus induction of L3 (70.73 %) and L4 (53.66 %) but had no effect on the induction of L1 and L2. All four segments from Epi 2 with or without coleoptiles displayed comparable capability to produce callus, ranging from 95.46 to 100 %.

However, the removal of coleoptiles from the segments L1 had a remarkable effect on plantlet regeneration. More than three-fold increase of regeneration frequency was observed for Zaoshu 3 after the removal of coleoptiles (23 %) compared to the non-removal control (5.41 %). About 49 % increase was obtained for Epi 2 (17.29 and 25.71% for non-removal and removal of coleoptiles, respectively) under the same conditions. No effect was seen for the segments L2, L3 and L4 from the two genotypes since no plantlets were generated from them. These results suggested that apical meristem appeared to be essential for plantlet regeneration as only the segments L0 had a high frequency of plantlet regeneration (Table 1). The present study showed a high frequency for callus induction and plantlet regeneration from the leaf base segments of commercial barley cultivars, which would serve as the foundation for the efficient generation of transgenic barley plants.

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