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Somatic Embryogenesis and Plant Regeneration in Barley (*Hordeum vulgare* L.)

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Abstract

Commercial cultivars and elite germplasms of barely (*Hordeum vulgare L*) are still recalcitrant to genetic transformation because of the lack of an efficient regeneration system. In this study, we established an efficient plant regeneration procedure from embryogenic calli derived from mature embryos. Callus induction from germinated mature embryos was best as over 95% in CIM medium (CI medium containing 2.5 ㎎/ℓ dicamba) under dark incubation. Development of embryogenic callus was highest as over 50% in CI3D medium (EC medium supplemented with 3 ㎎/ℓ 2,4-D). The highest regeneration of plants from embryogenic callus (40%) was obtained with CIS medium (SI + 1 mg/ℓ IAA and 2 mg/ℓ BA). These plant regeneration conditions could be useful in improving barley transformation efficiency.

Introduction

Barley (*Hordeum vulgare L.*) is one of the major cereal crops grown in the world, ranking fourth in total grain behind maize, wheat, and rice. Genetic engineering of barley is likely to play an increasingly important role in improving agronomic traits such as quality and disease resistance in the future. However, the development of techniques for the genetic modification of barley has, in general, been slower than other cereals (Harwood et al. 2000).

Barley is considered recalcitrant to tissue culture and the morphogenic responses have been found to depend on several factors including genotype, size and physiological state of explant, media constitution and the plant growth regulator used (Jahne-Gartner and Loerz, 1996). The pattern of morphogenesis is also not determined. Since the first report of barley tissue culture (Norstog, 1970), regeneration has been achieved from various explants, including mature and immature embryos (Luppoto, 1984, Bregitzer et al. 2000), immature inflorescence (Astwood and Hill, 1995), leaf (Mohanty and Ghosh, 1988), apical meristem (Weigel and Hughes, 1985), seedling explants (Becher et al. 1992), and coleoptile (Toyoda et al., 1990). Immature embryos have been used successfully for suspension and protoplast cultures and for genetic transformation in barley (Lazzeri and Loerz, 1990, Wan and Lemaux, 1994, Lemaux et al. 1995, 1999, Tingay et al. 1997, Nobre et al. 2000). However, extensive care and expenses are required to prepare immature embryo explants. Alternatively, mature embryos are readily available with little limitations.

Thus, an efficient plant regeneration system by somatic embryogenesis from the embryonic axes of mature barley embryos could be useful in improving barley transformation system. The objective of this study was to develop an efficient plant regeneration system using mature embryos for the ultimate use in a transformation system.

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Materials and Methods

Primary Callus Induction

Two barley varieties, Dooweonchapssalbori and Igri, were grown in the field of Honam Agricultural Research Institute (HARI), NICS, Korea, and were used for callus induction. Mature seeds were successively surface-sterilized with 70% ethanol for 2 min and 5% sodium hypochlorite (NaOCl)

solution for 40 min, followed by thorough rinse with sterile water. Sterilized mature seeds were placed on moist filter paper at 23℃ in the dark for 1 day. The embryos were dissected from the seed and the embryonic axis was removed from each embryo. For callus induction, the embryos were then placed scutellum-side up on CI medium (Wan and Lemaux 1994). CI medium is composed of modified MS (Murashige and Skoog 1962) salts and Gamborg B5 vitamins containing 250 mg/ℓ myo-inositol, 690 mg/ℓ proline, 1 mg/l thiamine-HCl, and 1 g/ℓ casein hydrolysate, supplemented with 30 g/ℓ maltose, and solidified with 3.5 g/ℓ gelrite, pH 5.7 in petri dishes. In the preliminary experiments, 2,4-dichlorophenoxyacetic acid (2,4-D) and 3,6-dichloro-o-anisic acid (dicamba) were selected as the most effective growth regulators for callus induction and they were used in the subsequent experiments. Effect of other auxins on the callus induction was examined on CI medium supplemented with different concentrations (0.5, 1, 2, 3, 4 mg/ℓ) of 2,4-D or dicamba. The embryos were cultured at 25℃ under dark condition for 3 weeks. All experiments were repeated more than three times, and data were statistically analyzed and presented as mean values.

Embryogenic Callus Formation and Maintenance

For embryogenesis, primary callus was transferred to embryogenic callus induction medium (EC) containing 50 g/ℓ maltose, and solidified with 4 g/ℓ phytagel, pH 5.7 (Table 1). Effect of other auxins on the maintenance of embryogenic callus was examined on EC medium supplemented with different concentrations (0.5, 1, 2, 3, 4 mg/ℓ) of 2,4-D or dicamba. Cultures were incubated at 25℃ for 2 weeks in the dark. All experiments were repeated more than three times, and data were statistically analyzed and presented as mean values.

Plant Regeneration

After 3 weeks on EC medium, embryogenic callus was transferred to shoot induction medium (SI) containing 3 g/l sucrose, combined with 0, 1, 2, 3 mg/ℓ IAA and BA respectively, and solidified with 4 g/ℓ phytagel, pH 5.7 (Table 1). Cultures were incubated under the light intensity of 20 $\mu molm^{-2}s^{-1}$, 16 hours photoperiod at 26℃ for 7-8 weeks and subcultured every 2 weeks. For root formation, well-developed

Table 1. Compositions of the media used for barley somatic embryogenesis (mg/ℓ).

Media	CI (callus induction)	EC (embryogenic callus)	SI (shoot induction)	PR (plant regeneration)
MS salts	1x	-	-	1x
B5 salts	1x	-	-	1x
$(NH_4)NO_3$	-	165	165	-
KNO_3	-	1,900	1,900	-
KH_2PO_4	-	170	170	-
$MgSO_4 \cdot 7H_2O$	-	370	370	-
$CaCl_2 \cdot 2H_2O$	-	440	440	-
$MnSO_4 \cdot 4H_2O$	-	16.9	16.9	-
$ZnSO_4 \cdot 7H_2O$	-	8.6	8.6	-
H_3BO_3	-	6.2	6.2	-
KI	-	0.82	0.82	-
$CuSO_4 \cdot 5H_2O$	-	0.025	0.025	-
$Na_2MoO_4 \cdot 2H_2O$	-	0.025	0.025	-
Pyridoxine HCl	-	2	2	-
Nicotinic acid	-	2	2	-
Ca panthothenate	-	2	2	-
Biotin	-	0.02	0.02	-
Na pyruvate	-	10	10	-
$FeNa_2EDTA \cdot 2H_2O$	-	40	40	-
Thyamine HCl	1	0.4	0.4	-
Citric acid	-	10	10	-
Casein hydrolysate	1,000	300	300	-
Myo Inositol	250	2,000	2,000	-
L Glutamine	-	256	256	-
L proline	690	250	250	-

shoots/plantlets (3-4 ㎝ in length) were transferred to plant regeneration medium (PR) containing 3 g/ℓ sucrose, no plant growth regulators, and solidified with 4 g/ℓ phytagel, pH 5.7 (Table 1) in culture bottles (13 cm, Φ 7) under the light intensity of 38 μmolm-2s-1, 16 hours photoperiod at 26℃.

Acclimation System

Plantlets were regenerated from embryonic callus induced from mature embryos of the two barley cultivars, and were transplanted to a pot (8 × 8 × 7.5 ㎝) filled with vermiculite without aeration. The pots were placed in plastic containers filled with Yoshida's nutrient solution [NH_4NO_3 11.4 ㎎/ℓ, $NaH_2PO_4{\cdot}2H_2O$ 50.4 ㎎/ℓ, KNO_3 89.3 ㎎/ℓ, $CaCl_2$ 110.8 ㎎/ℓ, $MgSO_4{\cdot}7H_2O$ 405 ㎎/ℓ, $MnCl_2{\cdot}4H_2O$ 1.9㎎/ℓ, H_3BO_3 1.2 ㎎/ℓ, $CuSO_4{\cdot}5H_2O$ 0.04 ㎎/ℓ, $(NH_4)_6{\cdot}MO_7O_{24}{\cdot}4H_2O$ 0.09 ㎎/ℓ, $ZnSO_4$ 0.04 ㎎/ℓ $FeSO_4$ 9.6 ㎎/ℓ, and citric acid (monohydrate) 14.9 ㎎/ℓ (Yoshida et al. 1976)] at pH 5.6 and kept at 27℃ under 16/8 hr photoperiod with light intensity of about 150 $\mu molm^{-2}s^{-1}$ for 2 weeks. The nutrient solution was refilled to avoid nutrient depletion and exchanged every three days to maintain pH at 5.6. The top of each pot was closed with a polyethylene bag to make a room to maintain humidity for the aerial part of the plantlets. The closed polyethylene bag was gradually opened to harden plantlets off. After hardening off, the rooted plantlets were transferred into soil in pots and grown in a greenhouse until flowering and seed set.

Results and Discussion

Effect of Plant Growth Regulators on Callus Induction from Mature Embryos and Embryogenic Callus Development

Embryos were dissected from the mature seed and inoculated on the callus induction medium (CIM) containing plant growth regulators. After 3-4 weeks of culture, calli were induced from 80-95% of the embryos of the two varieties, Dooweonchapssalbori and Igri. Effects of the two auxin type growth regulators, 2,4-D and dicamba, on callus induction from mature embryo were examined. The amount and texture of callus induced was similar in 2,4-D and dicamba after one week of incubation. However, after 3 weeks of incubation, induction of primary callus was significantly higher in dicamba than in 2,4-D, with highest callus induction at 2-3 ㎎/l of dicamba (Figure 1). The plant growth regulator 2,4-D, has most widely been used for callus induction from mature and immature barley embryos at a concentration of 2.5-10 mg/l (Chang et al. 2003, Cho et al. 1998, Sharma et al. 2005). Dicamba, another plant growth regulator and herbicide that shares similarities in structure and activity to 2,4-D, has also been used for callus induction from mature and immature barley embryos at a concentration of 2.5-10 mg/l dicamba (Chang et al. 2003, Sharma et al. 2005, Wan and Lemaux, 1994). The callus formation in plants is affected greatly by many factors such as genotype, tissue type, and media regime. Thus, the different responses of callus induction to 2,4-D and dicamba between experiments could be attributed to one or more of these factors.

Primary calli induced in CIM medium were transferred to EC medium to develop embryogenic callus. After 3 weeks of incubation, embryonic tissue formation rate was about 30-50% and it was about 5 to 10% higher in Dooweonchapssalbori than in Igri. Development of embryogenic calli was about 5 to 10% higher in dicamba than in 2,4-D, with highest embryonic callus formation at 2-3 ㎎/l of dicamba. The EC medium supplemented with 3 ㎎/l 2,4-D (CI3D) showed the highest formation of embryogenic calli. Embryogenic calli were developed well from the shiny, compact, slightly nodular and slightly brown-colored calli. However, embryogenic calli were developed poorly from the soft, friable and white calli (Figure 2). Halamkova et al. (2004) reported that dicamba was more suitable for somatic

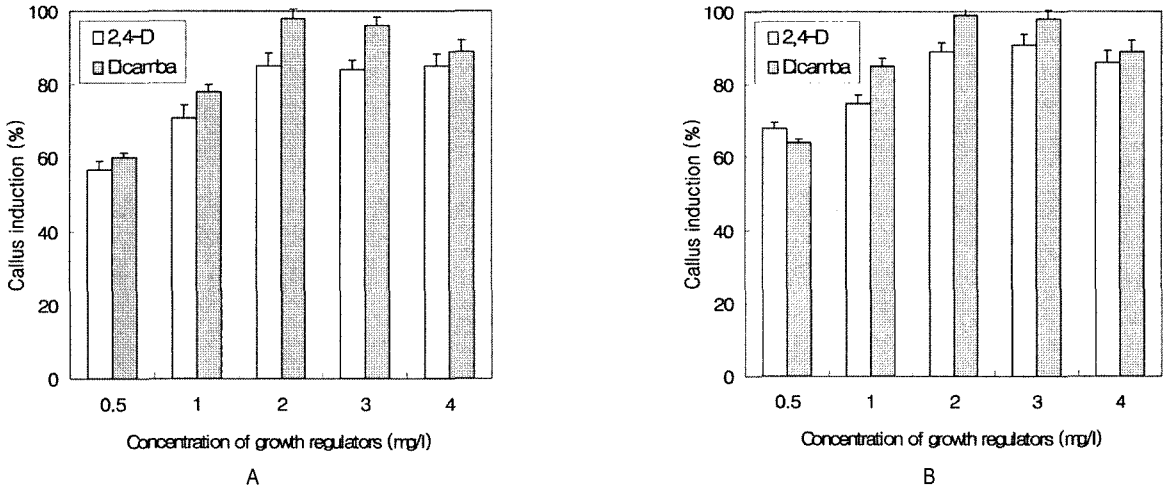

Figure 1. Effect of 2,4-D and dicamba on the responsiveness of callus induction on MS basal medium in the two barley varieties, Dooweonchapssalbori (A) and Igri (B).

Table 2. Composition of the media with plant growth regulators used for barley transformation (mg/ℓ).

Media	CIM	CI3D	CIS	CIR
Basal medium	CI	EC	SI	PR
Maltose	30,000	50,000	-	-
Sucrose	-	-	3,000	3,000
Dicamba	2.5	-	-	-
2,4-D	-	3	-	-
IAA	-	-	1	-
BA	-	-	2	-
pH	5.7	5.7~5.8	5.7~5.8	5.7
Gelrite	3,500	-	-	-
Phytagel	-	4,000	4,000	4,000

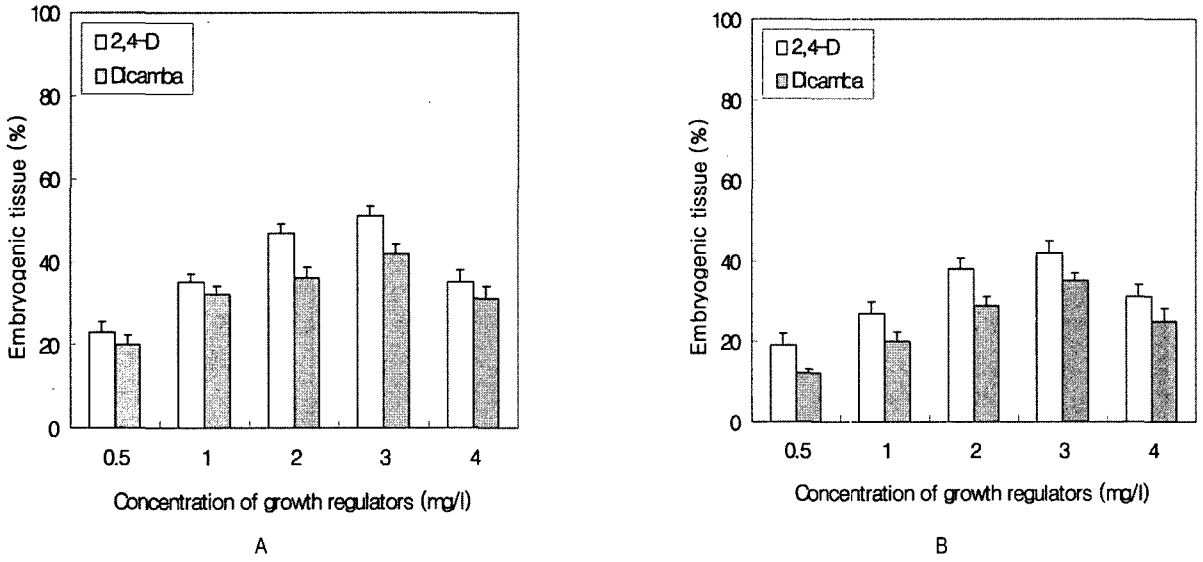

Figure 2. Effect of 2,4-D and dicamba on the development of embryogenic tissues from mature embryos the two barley varieties, Dooweonchapssalbori (A) and Igri (B), on CI3D medium after 3 weeks of culture.

embryogenesis induction from barley immature embryos and exhibited a higher frequency of regenerants than 2,4-D did. Contrary to the results from this experiments, however, the induction of primary callus and embryogenesis from barley mature embryos were higher with 2,4-D than with dicamba (Sharma et al. 2005).

Effects of Plant Growth Regulators on Plant Regeneration

Effects of plant growth regulators on plant regeneration from embryogenic tissues were examined with different concentration and combinations of auxin and cytokinin type growth regulators. The plant regeneration ratio was significantly different among the concentration combinations, and the ratio was higher in the 1-2 mg/ℓ IAA and 1-2 mg/ℓ BA combination as 25-50% after 7 weeks of culture (Figure 3).

Regeneration of barley plants from callus tissues has been reported in different hormone combinations with different genotypes, tissue explants, and media compositions.

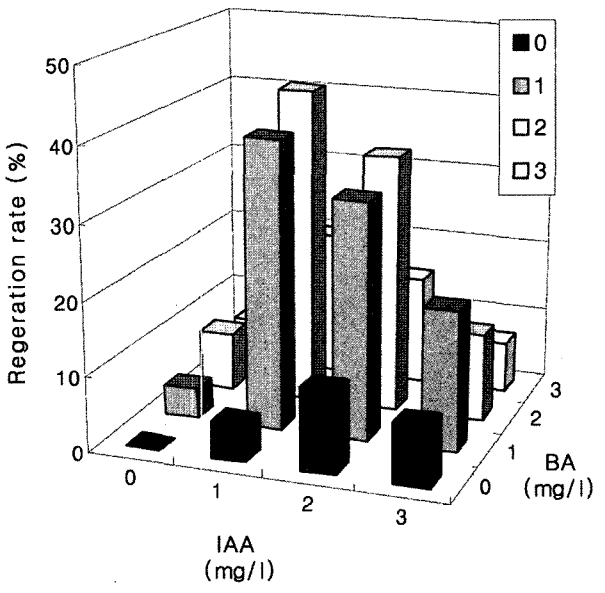

Figure 3. Effects of the combinations of IAA and BA on plant regeneration from embryogenic calli on CIR medium after 7 weeks of culture (cv. Dooweonchapssalbori).

Efficient shoot regeneration from callus induced from immature embryo was reported on modified MS medium containing 0.5-1.0 mg/ℓ BA (Chang et al. 2003), and FGH medium (Hunter, 1988) containing 1 mg/ℓ BA and 10 mg/ℓ phenylacetic acid (Choi et al. 2000). Cho et al. (1998) showed that the optimal combinations of 2,4-D and BA help to maintain the regeneration potential of green plantlets. Efficient regeneration of plants from embryonic tissues derived from mature barley embryos was achieved with the combinations of 1 mg/l 2,4-D and 0.1 mg/ℓ of BA or thidiazuron (Sharma et al. 2005). Ten-fold lower concentration of BA reported to be most effective for plant regeneration by Sharma et al. (2005) than this experiment could be related to differences in genotypes and medium compositions used in the two experiments.

Acclamation of plantlets employing a hydroponic system was highly effective. Maintaining pH of the nutrient solution and humidity for the aerial part of the plantlets are the factors most important during acclamation. Hardened off plants were transferred into soil in pots and grown in a greenhouse until flowering. Seeds were set and developed normally.

In summary, optimal conditions for plant regeneration via embryogenesis using mature embryos of elite varieties were established by adjusting media compositions and hormonal concentrations and combinations. These plant regeneration conditions could be applied to improve genetic transformation of commercially important barley varieties.

Acknowledgements

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