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Full Length Research Paper

Establishment of a highly efficient *in vitro* regeneration system for Chinese kale (*Brassica oleracea* L. var. *alboglabra*)

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The effects of different combinations and concentrations of plant growth regulators on differentiation frequency were studied using hypocotyls, cotyledons, and flamingo-bills as explants to establish an effective *in vitro* regeneration system for Chinese kale. The results showed that hypocotyls on a culture medium consisting of Murashige and Skoog (MS) medium + 1% sucrose + 2.0 mg/L BAP + 7.0 mg/L $AgNO_3$ achieved a differentiation frequency of 86.7%. The differentiation frequency of cotyledons on MS + 3% sucrose + 2.0 mg/L BAP + 0.40 mg/L NAA + 5.0 mg/L $AgNO_3$ was 94.0%. Flamingo-bills produced shoots on MS + 3% sucrose + 2 mg/L BAP, and the average number of shoots obtained per explant was 4.5. Adventitious shoots produced roots when inoculated on MS + 0.2 mg/L NAA.

Key words: Chinese kale, regeneration, differentiation, hypocotyl, cotyledon, flamingo-bill.

INTRODUCTION

The rapid development of genetic engineering of plants has opened new pathways for the genetic improvement of crops. Several studies on the successful genetic transformation of Chinese kale have been conducted through *Agrobacterium*-mediated transformation using hypocotyls and cotyledons as explants (Chen et al., 2006; Li et al., 2006; Huang et al., 2007). Successful transformation depends on several factors, including the establishment of an efficient plant regeneration system. Easy differentiation of explants is necessary to ensure that a large number of regenerated plants can be obtained for screening, which could yield transgenic plants with normal expression of exogenous genes.

Some studies on Chinese kale tissue culture using different plant parts, including hypocotyls and cotyledons (Zee and Hui, 1977), root segments (Wong and Loh, 1988), stems (Pua et al., 1989), and axillary buds (Huang et al., 1999) have been conducted. In recent years, hypocotyls (Huang et al., 2004) and cotyledons (Hu et al., 2006) have been used as explants for selecting the optimal culture medium for differentiation, and they yielded good results. However, the repeatability of these results is not high and some findings do not meet the requirements of *Agrobacterium* transformation. Flamingo-bill, a new type of explant from tomato and pepper (Pozueta-Romero et al., 2001), was excised upon the removal of one cotyledon and the primary and secondary meristems from seedlings, leaving the radical, hypocotyl, and one cotyledon as an explant for culture on MS medium. The results showed that flamingo-bill explants easily regenerated multiple shoots and were amenable to *Agrobacterium*-mediated transformation. Therefore, their findings on flamingo-bill could yield high reference values

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Abbreviations: BAP, 6-Benzylaminopurine; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **MS,** Murashige and Skoog medium; **NAA,** α-naphthalene acetic acid.

for other dicotyledonous plants. A literature survey shows that none of the investigations conducted thus far have focused on the use of flamingo-bill explants in Chinese kale and other related plants.

In this study, cotyledons, hypocotyls, and flamingo-bills of Chinese kale were used as explants to investigate the effects of growth regulators on plant differentiation and regeneration with the aim of improving the differentiation frequency, and establishing an efficient regeneration system for the genetic transformation of kale.

MATERIALS AND METHODS

Plant materials

Seeds of Chinese kale (*Brassica oleracea* L. var. *alboglabra* cv. Chenghai) were provided by College of Horticulture of South China Agricultural University.

Culture of aseptic seedlings

Seeds were surface sterilized with 70% ethanol for 30 s followed by 0.1% mercuric chloride for 10 min, and washed 4 to 5 times in sterile distilled water. Then, the seeds were dried by blotting on sterile filter paper, and placed in culture flasks containing MS medium.

Explants, culture media and culture conditions

Hypocotyls were cut into segments of 0.5 to 0.8 cm and cotyledons (with attached petioles) were excised from the seedlings when the cotyledons of aseptic seedlings began to flatten (5 to 6-day-old seedlings); these were used as explants. Hypocotyl explants were inoculated horizontally on culture media containing MS medium + 1% sucrose (w/v) + 2.0 mg/L BAP + 7.0 mg/L $AgNO_3$ + 0.8% agar and various concentrations of NAA (0, 0.03, 0.06, 0.10, and 0.20 mg/L) (w/v). Petioles of cotyledons were inserted into MS medium + 3% sucrose + 2 mg/L BAP + 5 mg/L $AgNO_3$ and various concentrations of NAA (0.05, 0.10, 0.20, 0.30, 0.40, 0.50, and 1.00 mg/L). Differentiation frequency was investigated in hypocotyl and cotyledon explants cultured for 30 days.

Flamingo-bill explants were excised from seedlings after removal of one cotyledon and the apical and axillary meristems when the leaf primordia of aseptic seedlings became visible. Flamingo-bills were inoculated on media containing the following: MS medium + sucrose 3% + 0.8% agar and various concentrations of BAP (0, 0.5, 1.0, 2.0, and 3.0 mg/L). Percentages of explants producing one shoot and multiple shoots, average number of shoots, and average shoot length were investigated 15 days after inoculation. All media were adjusted to pH 5.8 prior to autoclaving for 15 min at 121°C and 1.05 kg/cm^2. Cultures were incubated in a growth room at 25±1°C, with a photoperiod of 16 /8 h (day/night) and light intensity of 40 µmol/m^2·s.

Rooting of shoots and transplanting

Regenerated adventitious shoots were cut from the base when they reached a length of 2 cm and cultured on MS medium + 0.2 mg/L NAA, MS medium + 0.2 mg/L IAA, and MS medium + 0.2 mg/L IBA; 36 shoots were inoculated per medium. Rooting frequency was investigated 15 days after inoculation. Plantlets with well-developed roots were washed with water to remove the agar that adhered to the roots after 2 to 3 weeks of rooting culture, transferred to sterilized mixed substrates containing peat, vermiculite, and perlite (3:1:1 v/v/v), and then covered with plastic bags. Plantlets with significant growth after hardening were transferred to soil in the greenhouse.

Data analysis

Each treatment was inoculated with 30 explants for hypocotyls, 25 explants for cotyledons and 16 explants for flamingo-bills, respectively, and was repeated three times. The data were analyzed using analysis of variance (ANOVA) to compare the means of the treatments using the Duncan's multiple range test at 5% level of significance (P<0.05). The means in a column followed by different letters are significantly different at the 5% level.

RESULTS

Regeneration of hypocotyls

Hypocotyls began to elongate 1 day after inoculation; some hypocotyls began to regenerate approximately one to five unequal single or multiple shoots from incision 12 days after inoculation. Shoots were subsequently regenerated from explants (Figure 1B). The differentiation frequencies of hypocotyls varied among culture media containing different concentrations of NAA combined with 2.0 mg/L BAP (Table 1). The differentiation frequency was higher in the medium without NAA and decreased as the concentration of NAA increased. Hypocotyls did not form calli, and direct shoot formation on the medium without NAA was observed. In contrast, hypocotyl incisions de-differentiated into more calli, which slowly re-differentiated into shoots on media with increasing NAA concentrations. While the differentiation frequency of hypocotyls could exceed 80%, shoots on the Petri dishes were easily vitrified. Inoculating the hypocotyls into a culture flask with a ventilated stopper improved the differentiation frequency (90%) and reduced vitrification (data not shown).

Regeneration of cotyledons

Cotyledons increased in size soon after inoculation, and calli were gradually induced on the incisions 7 days after inoculation. Calli of the explants increased significantly as the concentration of NAA increased. A small number of explants began to regenerate shoots from the calluses 12 days after inoculation. An increasing number of explants regenerated over time (Figure 1A). Table 2 shows that the differentiation frequency of cotyledons initially increased and then decreased as the concentration of NAA in the seven treatments increased. The differentiation frequency increased (94.0%) as the NAA concentration reached 0.40 mg/L. Lower differentiation frequencies were observed at 0.05 and 1.00 mg/L NAA. An average of 3.6 to 5.2 shoots per explant with no

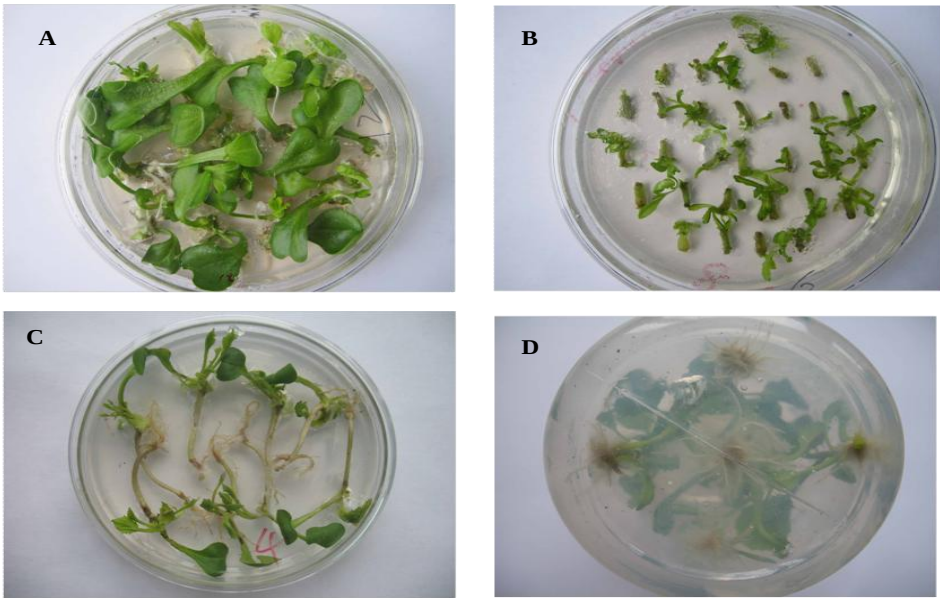

Figure 1. *In vitro* regeneration of Chinese kale from (A) cotyledons, (B) hypocotyls, and (C) the cut surface of flamingo-bill explants, (D) rooted shoots.

Table 1. Effects of different NAA concentrations on the differentiation frequency of hypocotyls.

Treatments	BAP concentration (mg/L)	NAA concentration (mg/L)	Average differentiation frequency (%)
1	2.0	0	86.7[a]
2	2.0	0.03	70.0[ab]
3	2.0	0.06	50.0[bc]
4	2.0	0.10	63.4[ab]
5	2.0	0.20	28.4[c]

Each treatment was replicated three times with 30 hypocotyls per replicate.

Table 2. Effects of different NAA concentrations on the differentiation frequency of cotyledons.

Treatments	BAP concentration (mg/L)	NAA concentration (mg/L)	Average differentiation frequency (%)
1	2.0	0.05	79.0[b]
2	2.0	0.10	80.0[b]
3	2.0	0.20	89.9[a]
4	2.0	0.30	87.8[a]
5	2.0	0.40	94.0[a]
6	2.0	0.50	88.0[a]
7	2.0	1.00	76.0[b]

Each treatment was replicated three times with 25 cotyledons per replicate.

significant difference among the treatments was observed.

Regeneration of flamingo-bill explants

Small shoots developed in flamingo-bill incisions after 3 days of culture. The number of adventitious shoots gradually increased as the shoots elongated (Figure 1C). The flamingo-bills exhibited 100% shoot formation on the various culture media after 15 days of culture; however, the average number of shoots per explant and the average shoot length showed significant differences (Table 3). Most of the flamingo-bill incisions formed single

Table 3. Effects of different BAP concentrations on flamingo-bill explants differentiation.

BAP concentration (mg/L)	Percentage of explants producing multiple shoots	Average number of shoots per explant	Average shoot length
0	18.8[b]	1.4[b]	2.10[a]
0.5	87.5[a]	3.5[ab]	0.86[b]
1.0	81.3[a]	4.1[a]	1.05[b]
2.0	93.8[a]	4.5[a]	0.79[b]
3.0	87.5[a]	4.0[a]	0.81[b]

Each treatment was replicated three times with 16 flamingo-bills per replicate.

shoots, although others could form two to three shoots on MS medium. The percentage of explants that produced multiple shoots exceeded 80% on MS medium supplemented with BAP. A BAP concentration of 2.0 mg/L yielded better results in both percentage of explants producing multiple shoots and average number of shoots produced.

Rooting culture

Adventitious shoots (2 cm long) were cut from the explants and transferred to MS medium supplemented with 0.2 mg/L NAA, IAA, or IBA as a rooting medium. Some shoots began to take root 6 days after inoculation. The rooting frequency on the medium with MS medium + 0.2 mg/L NAA was 100% 15 days after inoculation. The average number of roots was above 20, and roots, although shorter than those on MS supplemented with IAA or IBA, were the thickest on MS medium containing 0.2 mg/L NAA. Hence, MS medium + 0.2 mg/L NAA was a suitable rooting medium for adventitious shoots. Over 80% of the plantlets could survive and develop into morphologically normal and fertile plants after transplantation and hardening.

DISCUSSION

Regeneration of hypocotyls and cotyledons

Several factors affect plant regeneration, including the appropriate type of explant (such as donor cells and their physiological state) and the composition and concentration of the medium used. These factors are the most important because they directly determine the degree and pattern of organ differentiation. Cotyledons and hypocotyls are commonly used as explants in *in vitro* plant culture because of their higher differentiation frequency compared with other explants. Also, cotyledons and hypocotyls may be obtained from aseptic seedlings without seasonal restrictions. In this study, we selected two plant growth regulators most commonly used in the plant tissue culture of cabbage and its related species: NAA and BAP. Concentrations of NAA and BAP were also selected according to previously described methods of tissue culture of Chinese kale and related plants. Effects on the differentiation frequency were compared on MS medium with BAP as the common concentration and different concentrations of NAA to enhance the pertinence and lessen the workload. The differentiation frequency of hypocotyls was 86.7% when 2 mg/L of BAP was used without other growth regulators; the differentiation frequency of cotyledons was 94% on the medium supplemented with 2.0 mg/L of BAP and 0.40 mg/L of NAA.

Huang et al. (2004) demonstrated that the hypocotyl was the optimum regeneration explant of Chinese kale on an optimum culture medium containing MS medium + 2.0 mg/L BAP + 0.03 mg/L NAA + 1% sucrose + 7.0 mg/L $AgNO_3$ + 0.8% agar. In our experiment, the differentiation frequency of hypocotyls was 70% on the same culture medium, which may be attributed to differences in genotypes. The highest regeneration frequency obtained was only 48.72% when cotyledons were used as explants in their study. Hu et al. (2006) used cotyledons as explants cultured on MS medium + 0.05 mg/L NAA + 2.0 mg/L BAP + 30 g/L sucrose + 8 g/L agar and obtained a shoot differentiation frequency of 93.75%, which was approximately equal to the differentiation frequency (94.0%) obtained on MS medium + 3% sucrose + 2.0 mg/L BAP +0.40 mg/L NAA + 5.0 mg/L $AgNO_3$ in our study. However, our media were supplemented with $AgNO_3$. The differentiation frequency of cotyledons without $AgNO_3$ was only 52.0% in our study. Most studies on the application of $AgNO_3$ in plant tissue cultures illustrate that a certain concentration of $AgNO_3$ plays a catalytic role in shoot regeneration (Palmer, 1992; Eapen and George, 1997). Furthermore, 3% sucrose is commonly used in culture media; however, the differentiation frequency (54%) on media with 3% sucrose was lower than that with 1% sucrose for Chinese kale hypocotyls, agreeing well with the results of previous studies (He et al., 1998; Huang et al., 2004).

Regeneration pathway of flamingo-bill explants

Pozueta-Romero et al. (2001) reported that flamingo-bill explants regenerated multiple shoots (2.9 to 5.3 shoots

per explant for tomato and 1.2 to 2.2 for bell pepper cultivars) on the cut surface after 14 days, effectively improving the regeneration frequency. Then they used this type of explant to transform tomato with *Agrobacterium tumefaciens*, showing that 47% of the tomato explants produced transformed meristems differentiating into plants. Southern blots and analysis of inheritance of the foreign genes indicated that T-DNA was stably integrated into the plant genome. Later, Loskutov et al. (2008) and Chen et al. (2008) successfully used flamingo-bill explants to transform and obtain transgenic celery and pepper, respectively. Mendi et al. (2009) reported that flamingo-bill explants exhibit better shoot formation than proximal explants of bottle gourd. Furthermore, flamingo-bill explants can regenerate more shoots over shorter regeneration periods, indicating that they can be potentially applied in other dicotyledonous plants, particularly those with less efficient regeneration. In this *in vitro* culture study, flamingo-bills of Chinese kale were used as explants. The percentage of explants producing shoots was 100%, and formation of numerous shoots and rapid shoot growth superior to those of cotyledons and hypocotyls in many aspects were observed. Most flamingo-bill explants regenerated only a single shoot when on MS medium without any plant growth regulator, whereas most explants regenerated multiple shoots on MS medium supplemented with BAP. Increasing numbers of shoots tended to induce nutrient competition among multiple shoots, resulting in mutual inhibition of and slow growth and shorter multiple shoots compared with single shoots. Further experiments were carried out to study the effects of MS medium supplemented with various combinations of 1.0, 2.0, and 3.0 mg/L IAA and 2.0 mg/L BAP on flamingo-bills producing shoots. No significant differences were observed compared with using 2.0 mg/L BAP alone.

In summary, we determined suitable culture media for three different types of explants, and an *in vitro* regeneration system for Chinese kale was established. In this system, a differentiation frequency of over 80% was obtained, meeting the requirements of *in vitro* propagation and genetic transformation.

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