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BRIEF COMMUNICATION

High frequency plant regeneration from cotyledon and hypocotyl explants of ornamental kale

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Abstract

A high frequency shoot regeneration system for ornamental kale [*Brassica oleracea* L. var. *acephala* (D.C.) Alef.] was firstly established from seedling cotyledon and hypocotyl explants. The ability of cotyledon and hypocotyl to produce adventitious shoots varied depending upon genotype, seedling age and culture medium. The maximum shoot regeneration frequency was obtained when the explants from cv. Nagoya 4-d-old seedlings were cultured on Murashige and Skoog (MS) medium supplemented with 3 mg dm^{-3} 6-benzylaminopurine (BA) and 0.1 mg dm^{-3} naphthaleneacetic acid (NAA). The frequency of shoot regeneration was 65.0 % for cotyledons, 76.1 % for hypocotyls; and the number of shoots per explant was 4.3 for cotyledons, 8.2 for hypocotyls. Hypocotyl explants were found to be more responsive for regeneration when compared with cotyledons. Among the 4 cultivars tested, Nagoya showed the best shoot regeneration response. The addition of 3.0 mg dm^{-3} $AgNO_3$ was beneficial to shoot regeneration. Roots were formed on the base of the shoots when cultured on half-strength MS medium.

Additional key words: $AgNO_3$, auxin, *Brassica oleracea* var. *acephala,* cytokinin, genotype, rooting, shoot regeneration.

Ornamental kale is an important ornamental foliage plant, which is widely used as an ornamental potted plant or as a cut flower in winter (Dai *et al.* 2009). In past years, genetic improvement of ornamental kale has been mainly achieved by conventional breeding methods. Recently, genetic engineering opened a new avenue for plant improvement (Hansen and Wright 1999). The success of plant genetic engineering is determined by several factors, among which an efficient tissue culture system, in which plants can be regenerated from cultured cells and tissues at high frequencies, is thought to be a crucial one (Karami *et al*. 2009). In present study, we firstly established efficient and stable shoot regeneration from cotyledon and hypocotyl explants of ornamental kale. The effects of genotype, explant type, age of explant, the combination of plant growth regulators (PGRs), and $AgNO_3$ on shoot regeneration were studied in order to establish an optimal regeneration system for use in genetic transformation.

The seeds of 4 ornamental kale [*Brassica oleracea* L. var. *acephala* (D.C.) Alef.] cultivars (Nagoya, White Coral, Tokyo and Sunset), purchased from Zhejiang Hongyue Flower Company (Hangzhou, China), were surface-sterilized in 70 % (v/v) ethanol for 30 s, followed by 0.1 % (m/v) mercuric chloride solution for 8 min, and then rinsed three times with sterile distilled water (5 min every time). Twenty seeds were placed on half-strength Murashige and Skoog (1962; MS) medium without PGR. The cotyledons including 1 - 2 mm petioles and the 5 - 10 mm long hypocotyl segments excised from 3 to 8-d-old seedlings were inoculated on shoot regeneration medium. To optimize the concentrations of PGR for shoot regeneration, we cultured 4-d-old cotyledons and hypocotyls of cv. Nagoya on MS medium supplemented with different combinations of 6-benzylaminopurine (BA) (2.0, 3.0, 4.0 and 5.0 mg dm^{-3}) and naphthaleneacetic acid (NAA) (0.02, 0.05, 0.1, 0.2 and 0.5 mg dm^{-3}) (Table 1). For genotype screening, 4-d-old explants of the 4 genotypes were cultured on MS medium supplemented with

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Abbreviations: BA - 6-benzylaminopurine; MS - Murashige and Skoog medium; NAA - naphthaleneacetic acid; PGR - plant growth regulator.

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3.0 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA (Table 2). The effect of seedling age was studied by culturing explants of cv. Nagoya derived from seedlings of different ages (3, 4, 5, 6, 7 and 8 d) on MS medium containing 3.0 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA (Table 2). To investigate the effect of $AgNO_3$ on shoot regeneration, 4-d-old explants of cv. Nagoya were cultured on MS medium containing 3.0 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA with different concentrations of $AgNO_3$ (0, 1, 2, 3, 4 and 5 mg dm^{-3}) (Table 2). The frequency of shoot regeneration and number of shoots per explant were counted after 4 weeks of culture. Regenerated shoots (about 2 cm in length) were transferred to half-strength MS medium without PGR for rooting. MS basal medium in this study contained 3 % (m/v) sucrose, 0.75 % (m/v) agar, at pH 5.8. All cultures were incubated at 25 °C and a 16-h photoperiod with irradiance of 30 μmol m^{-2} s^{-1} (cool-white fluorescent tubes). Rooted plantlets were transferred to small pots containing 1:2 (v/v) soil-*Perlite* mixture, which were covered with plastic cups and kept under high humidity for acclimatization (7 d) before being exposed to greenhouse conditions. Each treatment consisted of 50 cultures and all the experiments were repeated three times. Means and standard deviations (SD) were calculated. An analysis of variance (*ANOVA*) using Duncan's multiple range test with a 95 % confidence interval was used to compare the means of all treatments.

Shoots were visually observed on both cotyledon and hypocotyl explants of ornamental kale in all treatments after 4 weeks. Both cotyledons and hypocotyls expanded, and shoots were formed on the cut ends of the explants (Fig. 1*A*,*B*,*C*). Cotyledon explants produced shoots on the ends of cotyledonary petioles, while for the hypocotyl explants, the upper segments of hypocotyl produced shoots earlier than the middle and lower segments, but all positions of hypocotyl explants did not show significant differences in shoot regeneration after 4 weeks of culture. Zhang and Bhalla (2004), and Qin *et al*. (2007) also observed the same results in *B. napus* and *B. oleracea* var. *italica*, respectively. But Khehra and Mathias (1992) reported that different positions of hypocotyl explants had different regeneration responses. We consider that the regeneration potentials of different positions of hypocotyl explants are related to species. In addition, more than 90 % of the shoots were mainly formed on the proximal ends (morphological lower ends) of the hypocotyl segments, only a few of which were formed on both ends of segments (Fig. 1*D*). This was contrary to the reports of Yang *et al*. (1991). We deduce that different regeneration potentials of the two ends of the hypocotyl segments are related to polarity, which needs further study. When compared regeneration potential of cotyledon and hypocotyl explants, hypocotyl explants were found to be more responsive for regeneration in almost all experiments of this study (Tables 1, 2). Bhalla and Smith (1998), Hou and Jia (2005) also reported that hypocotyl explants had a higher regeneration potential than cotyledon explants of *B. oleracea* var. *botrytis* and *Perilla frutescens*, but there were opposite conclusions that cotyledon had been found to be more responsive for shoot regeneration in studies of *B. napus* (Kamal *et al*. 2007) and *B. oleracea* var. *italica* (Qin *et al*. 2007). Zhang *et al*. (2005) suggested that the

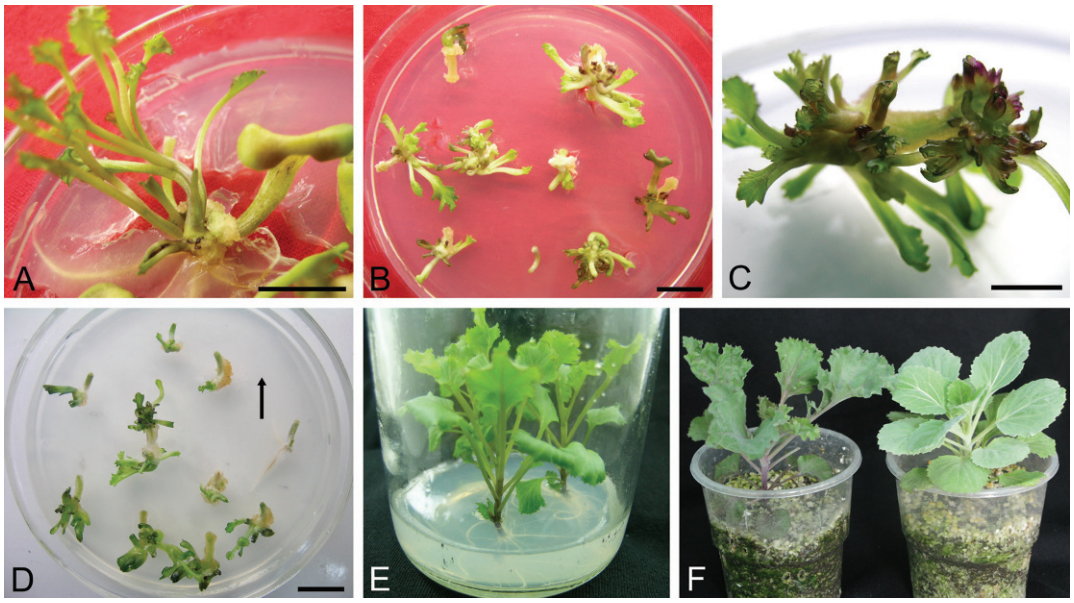

Fig. 1. Regeneration of shoots from the cotyledon and hypocotyl explants of ornamental kale. *A* - Regeneration of shoots from a cotyledon explant (*bar* = 1 cm). *B* - Shoots regenerated from the hypocotyl explants cultured for 4 weeks on MS medium with 3.0 mg dm^{-3} BA, 0.1 mg dm^{-3} NAA and 3.0 mg dm^{-3} $AgNO_3$ (*bar* = 1 cm). *C* - Shoots regenerated from a hypocotyl explant on regeneration medium (*bar* = 0.5 cm). *D* - Shoots initiated from proximal ends of hypocotyls, and most of the shoots were formed on the morphological lower ends of the hypocotyl segments. *Black arrow* indicates the morphological growth orientation, the direction of *arrowhead* shows the morphological upper ends (*bar* = 1 cm). *E* - Regenerated shoots in rooting medium. *F* - Acclimatized plantlets were growing in pots containing soil and *Perlite* (1:2).

Table 1. Shoot regeneration frequency and number of shoots from 4-d-old explants of ornamental kale cv. Nagoya on MS medium containing various combinations of BA and NAA. Means ± SD based on three replicates, 50 explants per replicate. Means followed by the same letters within a column were not significantly different at the 5 % level according to Duncan's multiple range test.

BA [mg dm^{-3}]	NAA [mg dm^{-3}]	Shoot regeneration frequency [%] cotyledon	hypocotyl	Number of shoots [explant^{-1}] cotyledon	hypocotyl
2.0	0.02	23.7 ± 5.1 hij	48.7 ± 4.2 fgh	2.6 ± 0.2 c	4.9 ± 0.5 d
2.0	0.05	41.3 ± 3.1 def	74.1 ± 8.7 a	2.6 ± 0.2 c	5.3 ± 1.0 c
2.0	0.10	42.1 ± 5.4 def	56.7 ± 3.1 defg	2.6 ± 0.3 c	3.9 ± 4.2 de
2.0	0.20	61.3 ± 4.2 ab	51.7 ± 9.1 fgh	2.5 ± 0.1 cd	3.4 ± 0.2 ef
2.0	0.50	51.0 ± 3.6 cd	32.7 ± 3.1 jk	1.9 ± 0.3 e	2.8 ± 0.3 ef
3.0	0.02	16.7 ± 5.0 j	64.2 ± 9.5 bcde	2.2 ± 0.3 cde	3.5 ± 0.5 def
3.0	0.05	40.7 ± 3.1 def	70.9 ± 6.4 ab	4.4 ± 0.4 a	6.7 ± 1.0 b
3.0	0.10	65.0 ± 7.6 a	76.1 ± 9.2 a	4.3 ± 0.5 a	8.2 ± 2.3 a
3.0	0.20	64.6 ± 6.0 a	69.3 ± 7.0 abc	3.3 ± 0.4 b	6.9 ± 1.5 b
3.0	0.50	55.9 ± 4.0 bc	31.1 ± 7.4 jk	3.6 ± 0.7 b	3.5 ± 0.7 ef
4.0	0.02	21.8 ± 5.5 j	46.3 ± 2.8 gh	2.1 ± 0.5 de	2.9 ± 0.5 ef
4.0	0.05	29.3 ± 4.2 hi	55.8 ± 4.7 defg	1.7 ± 0.1 e	3.2 ± 0.4 ef
4.0	0.10	32.4 ± 2.4 gh	38.8 ± 3.0 ij	2.5 ± 0.2 cd	2.7 ± 0.6 ef
4.0	0.20	40.2 ± 2.8 ef	44.6 ± 6.1 hi	2.1 ± 0.1 cde	2.7 ± 0.3 ef
4.0	0.50	36.4 ± 6.7 efg	30.8 ± 9.5 jk	1.7 ± 0.1 e	2.4 ± 0.1 f
5.0	0.02	23.1 ± 2.7 ij	56.1 ± 3.3 defg	2.2 ± 0.1 cde	3.6 ± 0.4 def
5.0	0.05	36.2 ± 2.0 fg	66.2 ±12.8 abcd	2.5 ± 0.1 cd	3.6 ± 0.2 def
5.0	0.10	49.8 ± 6.0 cd	60.0 ± 9.2 cdef	2.3 ± 0.1 cde	2.7 ± 0.6 ef
5.0	0.20	52.1 ± 8.8 cd	53.3 ± 5.0 efg	2.1 ± 0.2 cde	2.4 ± 0.3 ef
5.0	0.50	45.1 ± 10.3 de	24.3 ± 2.1 k	1.8 ± 0.2 e	2.2 ± 0.3 f

Table 2. Influence of genotype, seedlings age and $AgNO_3$ concentration on regeneration frequency and number of shoots from cotyledonary and hypocotylar explants of ornamental kale. Means ± SD based on three replicates, 50 explants per replicate. Means followed by the same letters within a column were not significantly different at the 5 % level according to Duncan's Multiple Range Test.

Factors		Shoot regeneration frequency [%] cotyledon	hypocotyl	Number of shoots [explant^{-1}] cotyledon	hypocotyl
Genotype	Nagoya	63.7 ± 3.7 a	75.7 ± 8.5 a	4.3 ± 0.6 a	8.1 ± 2.7 a
	White coral	48.5 ± 6.4 b	55.2 ± 3.2 b	2.8 ± 0.6 b	4.5 ± 0.7 b
	Tokyo	26.0 ± 2.0 c	30.0 ± 5.3 c	1.9 ± 0.3 c	3.2 ± 0.2 c
	Sunset	61.5 ± 8.1 a	51.3 ± 6.4 b	3.1 ± 0.1 b	5.2 ± 0.3 b
Seedling age [d]	3	10.6 ± 0.4 d	21.3 ± 1.6 d	1.4 ± 0.1 b	2.6 ± 0.3 c
	4	65.1 ± 3.4 a	77.8 ± 8.7 a	4.6 ± 0.8 a	7.8 ± 0.5 a
	5	61.2 ± 5.3 a	75.6 ± 6.8 a	4.4 ± 1.2 a	7.3 ± 0.4 a
	6	42.6 ± 6.2 b	52.6 ± 4.3 b	2.5 ± 0.5 b	4.2 ± 0.8 b
	7	35.2 ± 3.5 b	27.6 ± 3.8 c	1.3 ± 0.2 b	3.3 ± 0.3 c
	8	21.5 ± 2.3 c	17.3 ± 4.2 d	1.0 ± 0.1 b	1.3 ± 0.1 d
$AgNO_3$ conc. [mg dm^{-3}]	0	63.1 ± 8.2 c	75.5 ± 11.8 b	4.3 ± 0.9 d	8.1 ± 2.2 c
	1	65.3 ± 11.0 c	84.1 ± 7.8 ab	5.1 ± 1.3 cd	8.8 ± 0.8 c
	2	78.9 ± 6.4 ab	86.9 ± 4.7 ab	6.8 ± 0.4 bc	10.6 ± 0.5 ab
	3	81.8 ± 7.3 a	94.1 ± 5.8 a	7.7 ± 2.1 a	12.8 ± 1.4 a
	4	76.4 ± 2.3 ab	84.5 ± 5.5 ab	7.3 ± 0.4 ab	11.7 ± 0.3 a
	5	70.9 ± 7.1 bc	79.6 ± 8.6 b	5.6 ± 0.1 cd	9.5 ± 0.3 bc

difference of regeneration potential between cotyledon and hypocotyl explants was influenced by combination of PGRs, and the difference may be contributed to the different endogenous phytohormone content between cotyledons and hypocotyls. When the shoots were transferred to the rooting medium, healthy and vigorous roots were formed directly at the base of shoots after cultured for 4 weeks (Fig. 1*E*). A high rooting ability was

shown in ornamental kale, and there were no differences among the 4 genotypes. Almost all the regenerated plantlets survived after transferred to glasshouse (Fig. 1*F*). The plants presented usual colour after 6 weeks of cold treatment and effloresced normally in April.

In all of the 20 PGR combinations, adventitious shoots were observed from cotyledons and hypocotyls after 4 weeks of culture. The maximum frequency of shoot regeneration was obtained on the medium containing 3.0 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA. The frequency of shoot regeneration was 65.0 % for cotyledons, 76.1 % for hypocotyls; and the number of shoots per explant was 4.3 for cotyledons, 8.2 for hypocotyls (Table 1). The combination of 3.0 mg dm^{-3} BA and 0.2 mg dm^{-3} NAA also showed high shoot regeneration frequency, but the shoots were feeble and the number of shoots per explant was fewer. When the concentration of BA was fixed, with the rise of NAA concentration, shoot regeneration efficiency increased initially, but when NAA concentration was higher than 2.0 mg dm^{-3}, the frequency of shoot regeneration decreased and increasing number of roots formed instead. We deduce that shoot regeneration of ornamental kale depends on the ratio and concentrations of cytokinin and auxin in the nutrient medium, which have been observed in other studies (Jonoubi *et al*. 2005, Reda *et al*. 2006). No shoot regeneration was observed in the absence of BA, and there was low frequency of shoot regeneration in the absence of NAA (data not shown). Based on these results, the PGR combination with 3.0 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA was chosen for investigating the effects of genotype, $AgNO_3$ and seedling age on shoot regeneration.

Genotype effect was one of the most important factors on shoot regeneration of ornamental kale. Shoot regeneration efficiency was significantly affected by genotypes studied ($P < 0.05$). The highest frequency of shoot regeneration and the highest number of shoots per explant were obtained in cv. Nagoya, which was chosen for investigating the effects of other factors on shoot regeneration. Cv. Tokyo showed the lowest response for shoot regeneration with both cotyledon and hypocotyl explants. The age of seedlings was another factor that influenced shoot regeneration in ornamental kale (Table 2). 4-d-old explants obtained the highest shoot regeneration frequency and number of shoots per explant. 3-d-old explants showed the lowest response for shoot regeneration. No significant differences of shoot regeneration efficiency were shown between 4 and 5-d-old seedlings with both cotyledon and hypocotyl explants, but there was a steady decrease in shoot regeneration frequency and number of shoots per explant from 6 to 8-d-old seedlings. Ono *et al*. (1994) and Tang *et al*. (2003) also reported that 4-d-old explants showed the highest frequency of shoot regeneration in *B. napus*, and the shoot regeneration frequency decreased with the increase of seedling age.

The addition of $AgNO_3$ was beneficial for shoot regeneration in ornamental kale. The shoot regeneration efficiency increased significantly when explants were cultured on the medium containing different concentrations of $AgNO_3$ (1, 2, 3, 4 and 5 mg dm^{-3}). The highest shoot regeneration frequency and number of shoots per explant from both cotyledon (81.8 % and 8.3, respectively) and hypocotyl (94.1 % and 12.8, respectively) explants were achieved on MS medium containing 3.0 mg dm^{-3} $AgNO_3$ (Table 2). The shoot regeneration efficiency decreased gradually with higher concentrations of $AgNO_3$. The positive effect of $AgNO_3$ was consistent with previous results from the cotyledon explants of *B. campestris* (Zhang *et al*. 1998) and *B. napus* (Tang *et al*. 2003), and hypocotyls of *B. juncea* (Pua and Chi 1993), *B. napus* (Khan *et al*. 2003). $AgNO_3$ is a potent inhibitor of ethylene action, and ethylene is considered to suppress *in vitro* shoot formation. Zhang *et al*. (1998) claimed that the increase of shoot regeneration frequency by $AgNO_3$ is caused by the interruption of an ethylene signal transduction pathway. The actual mechanism of ethylene on *in vitro* shoot regeneration is not yet elucidated. However, the relationship between ethylene and polyamines, which promote organogenesis and embryogenesis (Pua *et al*. 1999), is known, and both of them compete for the same precursor, s-adenosylmethionine.

In conclusion, an efficient plant regeneration system from seedling cotyledon and hypocotyl explants of ornamental kale (*B. oleracea* var. *acephala*) has been successfully established for the first time, which can provide the foundation for genetic transformation of ornamental kale in the future.

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