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High-frequency embryogenesis, regeneration of broccoli (*Brassica oleracea* var. *italica*) and analysis of genetic stability by RAPD

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Abstract

High-frequency somatic embryogenesis and shoot regeneration of broccoli (*Brassica oleracea* var. *italica*) were achieved. Cotyledon and hypocotyl explants from four varieties of broccoli were cultured on MS and modified MS media (mMS, supplemented with PG-96 organic components) with different combinations of growth regulator. The effects of genotypes, different explants, growth regulator combinations, organic components and $AgNO_3$ on induction of calli and shoots were evaluated. The optimal media for inducting calli/shoots and roots were mMS medium containing 3% (w/v) sucrose and 0.8% (w/v) agar supplemented with NAA at 0.5 mg l^{-1}, 6-BA at 3.0 mg l^{-1}, $AgNO_3$ at 4.0 mg l^{-1} and MS medium containing 3% sucrose and 0.8% (w/v) agar supplemented with NAA at 0.2 mg l^{-1}, respectively. The callus induction percentages were over 90% in all four varieties; shoot induction percentage was 92.5% and the average number of shoot per explant was 4.1 from cotyledon explant in variety Bishan. In this study, we established high-efficient embryogenesis and shoot regeneration system of broccoli and analyzed genetic stability of regenerants at DNA level using RAPD molecular marker. Out of 62 arbitrary primers screened using PCR amplification, 79 polymorphic bands were amplified from 20 primers. The results demonstrated the genetic stability of regenerants from the same variety.

Keywords: Broccoli; Explant; Somatic Embryogenesis; Regeneration; Genetic stability; RAPD marker

1. Introduction

Broccoli (*Brassica oleracea* var. *italica*) is a vegetable cole crop deserving great breeding attention by seed companies due to the increasing area of production in recent years and anticarcinogenic properties detected in some cultivars (Wrage and Writer, 1994). Transgenes are likely to be used widely for the development of new plant varieties in *Brassica* for agriculture (Dale et al., 1998). The genetic transformation methods have been used to introduce new traits into commercially important plants, thereby producing combinations of features which could not have been achieved by traditional breeding programs (Hansen and Wright, 1999). Genetic engineering relies on the development of efficient methods for regeneration of viable shoots from cultured tissues, and the successful application of transformation techniques (Zhang and Bhalla, 2004). For *Brassica* crops, *in vitro* plant regeneration is essential for developing genetic transformation technology. A variety of plant tissues of broccoli have been used for regeneration, including peduncle explants (Christey and Earle, 1991), hypocotyls (Metz et al., 1995; Puddephat et al., 2001), leaf tissues (Cao and Earle, 2003), flowering stalk and hypocotyl petiole (Metz et al., 1995). The average regeneration rates of more than 75% were obtained from peduncle explants in a regeneration study of broccoli (Christey and Earle, 1991). According to Sparrow (Sparrow et al., 2004), transgenic plants were successfully obtained from genotypes that regenerated multiple shoots via a distinct swelling or callus phase in *B. oleracea*. Mode of regeneration was found to be the most significant factor in transformation. Transgenic shoots were obtained from genotypes that regenerated via an indirect callus mode, even when susceptibility to *Agrobacterium* was low. So extensive screening of genotypes and tissue culture conditions has greatly improved the frequency of shoot regeneration for most *Brassica* species.

However, it has been reported that *in vitro* cultured plantlets might exhibit somaclonal variation. This variation is often heritable and it therefore unwanted in somatic clones. Thus, it

Abbreviations: 6-BA, 6-benzylaminopurine; NAA, 1-naphthaleneacetic acid; MS, Murashige and Skoog medium; RAPD, random amplified polymorphic DNA

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would be very important to be able to detect this variation quite early in the life of a plant to avoid economically disastrous. Many workers have tried to assess these variations in *Brassica* species through morphological analysis, isozyme and ISSR (King, 1995; Lazaro and Aguinagalde, 1998; Lim et al., 1998; Leroy et al., 2000). We have employed random amplified polymorphic DNA (RAPD) method for the analysis of genetic stability among regenerants. RAPD is technically simple, quick to perform, requires very little plant material, and yields true genetic markers and have been used widely in genetic analysis of *Brassica* species (Divaret and Thomas, 1998; Crockett et al., 2000; Zhang et al., 2003; Chen et al., 2005) and have been used in identifying broccoli and cauliflower cultivars (Hu and Quiros, 1991; Astarini et al., 2004). Their results showed that RAPD markers could be used to detect genetic similarities or dissimilarities in regenerants.

The study reported here was aimed at developing an efficient *in vitro* regeneration protocol for broccoli plants to enable shoot regeneration and subsequent genetic and molecular studies from different explants. To our knowledge, there is no report on genetic stability studies on somatic clones of *B. oleracea* var. *italica*, and the study presented here is the first assessment of genetic variation at DNA level in somatic clones of *B. oleracea* var. *italica*.

2. Materials and methods

2.1. Plant materials

Four commercial varieties of *B. oleracea* var. *italica* including Beilu, Meilu 118, Bishan and Mantuolu were analyzed for somatic embryogenesis and shoot regeneration. Seeds were soaked in Tween 20-enriched tap water (two drops/50 ml water) for 6 min, surface sterilized with 2% (w/v) sodium hypochlorite solution for 8 min and then rinsed three times in sterile distilled water and aseptically germinated on MS medium containing 2% (w/v) sucrose, solidified with 0.8% (w/v) agar (Sangon, China), at pH 5.7.

2.2. Cotyledon and hypocotyl culture

Two-week-old hypocotyls were excised into sections of 8 mm in length and cotyledons were cut into halves used as explants for all *in vitro* experiments. Eight explants were inoculated on the surface of the medium. Different combinations of 6-BA and NAA were tested for their effects on callus and shoot induction. The culture media were MS medium and mMS medium which supplemented with PG-96 (Guo et al., 1999) organic components, containing 1.0, 2.0 and 3.0 mg l^{-1} of 6-BA and combination with NAA at 0.0, 0.5 and 1.0 mg l^{-1}. The cultured cotyledon and hypocotyl explants were subcultured every 2–3 weeks. The experiments were conducted at 25 °C under a 16-h photoperiod regime of cool white fluorescent light (40 μmE m^{-2} s^{-1}). Basal media containing 3% (w/v) sucrose, solidified with 0.8% (w/v) agar (Sangon, China) were autoclaved (121 °C/20 min).

2.3. Plant regeneration, vernalization and pollen viability test

Before rooting culture, regenerated shoots were transplanted into a growth regulator-free MS medium and cultured for 2 weeks to allow elongation of shoots and eliminate the effects of growth regulators in the previous experiment on rooting. Shoots with a size of more than one-centimeter were separated and transferred to root medium containing NAA at 0.2 mg l^{-1}. Five-centimeter plantlets were then potted and placed in a greenhouse under plastic covering in order to maintain a high humidity. Three to 4 weeks later, well developed plants were vernalized at 6 °C in dim light for 5 weeks. Mature pollen was stained with aceto-carmine to examine pollen fertility.

2.4. DNA extraction and RAPD amplification

DNA extraction from young shoots (100 mg) following a cetyltrimethylammonium bromide (CTAB) protocol was described by Guo et al. (2003). A total of 62 arbitrary 10-mer primers (Sangon, China) were screened for RAPD amplification in the broccoli genome DNA. A single primer was used in each PCR reaction, which was carried out in a total volume of 20 μl containing 2 μl of genomic DNA (30 ng), 2.0 μl of 10× buffer, 2.0 μl $MgCl_2$ (2.5 mM), 10 μm primer, 2 μl of 100 mM dNTPs and 1.5 unit of Taq polymerase (Sbsbio, China). PCR amplifications were performed on a TC-312 (Techne, UK) under the following conditions: a hot start at 94 °C for 5 min, followed by 40 cycles of three steps: denaturation at 94 °C for 1 min, annealing at 36 °C for 1 min, extension at 72 °C for 2 min, and a final extension for 10 min at 72 °C. The RAPD amplification products were mixed with loading buffer (0.005% each of bromophenol blue, as tracking dyes), run for 2 h on 2% agarose gels at a constant voltage (100 V) and detected using ethidium bromide. The molecular-weight size ladder Smartladder (Sangon, China) was used for band sizing.

3. Results and discussion

3.1. Genotypes and different explant types effect on callus and shoot formation

Explant response (i.e. swelling of explants) was observed within 1 week on MS and mMS media. Callus appeared within 2 weeks in culture (Fig. 1A). Over 90% of the explants formed callus in all four varieties (Table 1). No significant difference in callus induction among different varieties. Shooting appeared within 3–4 weeks of culture. In contrast to callus induction, the percentages of explant with shoot formation varied greatly among the varieties; the highest percentage of explant (cotyledon) with shoot was 92.5% from variety Bishan (Table 1; Fig. 1A and B). Beilu and Meilu 118 did not show statistical difference in their shoot regeneration capacity. Variety Mantuolu showed the lowest response to shoot regeneration with both cotyledon and hypocotyl explants (Table 1). Our results indicated that genotype plays an

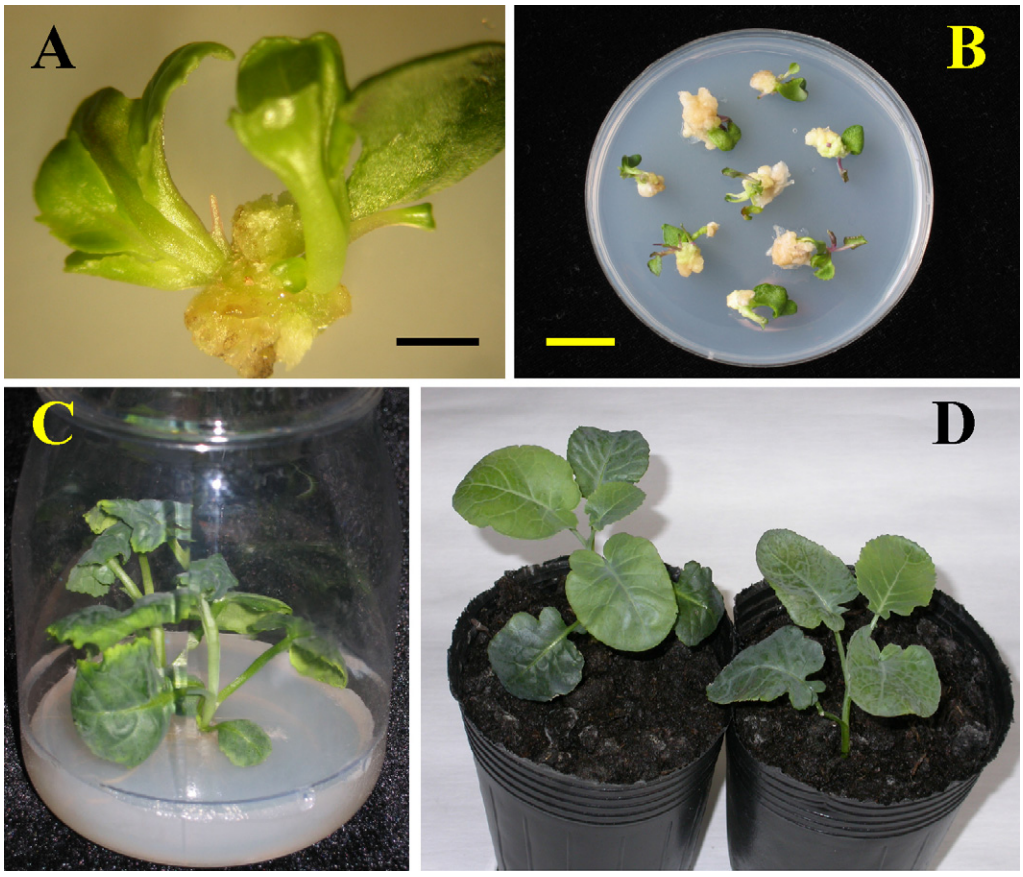

Fig. 1. Shoot differentiation from cotyledon explants and plant regeneration in broccoli var. Bishan. (A) Callus formation and shoots differentiation from a cotyledon explant, scale bar 1.5 mm. (B) Shoots differentiated from cotyledon explants in a Petri dish, scale bar 18 mm. (C) Regenerated shoots in rooting medium. (D) Regenerated Bishan plants in a greenhouse.

important role in callus and shoot formation. Of *in vitro* regeneration of *B. oleracea*, genotype effect was one of the most important factors, the shooting efficiency was genotype dependent (Bhalla and Smith, 1998; Zhang and Bhalla, 2004).

We compared regeneration potential based on the average shoot number per explant using cotyledon and hypocotyl explants from four broccoli varieties, the results revealed that, shoot formation from cotyledon explant was easier than that from hypocotyl explant in four studied varieties (Table 1). The average number of shoots from cotyledon explant varied from 1.6 to 4.1; the average number of shoots from hypocotyl explant varied from 1.0 to 3.3 (Table 1). Cotyledon explants were found to be more responsive to regeneration in current test.

Meanwhile the experiments suggested that subtype of hypocotyls explant (upper, middle, and lower position of hypocotyls segments) did not show any differences in shoot regeneration (data not shown). This result was agreed with the study of *B. napus* (Bhalla and Smith, 1998; Zhang and Bhalla,

Table 1
Shoot regeneration from broccoli seedling explants of four varieties on mMS medium (mMS supplemented with NAA at 0.5 mg l^{-1}, BA at 3.0 mg l^{-1}, $AgNO_3$ at 4.0 mg l^{-1})

Varieties	Explant type	Explant no.	Explant with callus		Explant with shoot		Total shoot no.	Shoot no./ explant ± S.D.[a]
Bishan	Cot.	160	160	100.0%	148	92.5%	656	4.1 ± 1.6 A
	Hyp.	160	158	98.8%	135	84.4%	527	3.3 ± 1.3 a
Beilu	Cot.	160	148	92.5%	132	82.5%	512	3.2 ± 0.9 A
	Hyp.	160	146	91.3%	124	77.5%	320	2.0 ± 0.6 ab
Meilu118	Cot.	160	152	95.0%	129	80.6%	464	2.9 ± 1.0 AB
	Hyp.	160	144	90.0%	116	72.5%	288	1.8 ± 0.5 ab
Mantuolu	Cot.	160	145	90.6%	119	74.4%	256	1.6 ± 0.6 B
	Hyp.	160	144	90.0%	97	60.6%	160	1.0 ± 0.4 b

[a] Means followed by the same letters are not significantly different according to the LSD test at the 5% level of significance. Capital letters refer to cotyledon; small letters refer to hypocotyl.

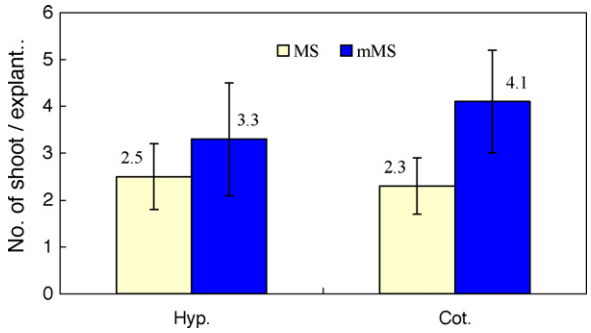

Fig. 2. Effect of induction medium and type of explant on broccoli shoot differentiation. Means and standard deviation are from eight replicates.

2004), but in contrast to Khehra and Mathias (1992) who reported that *B. napus* explant type accounted for 44–95% of the regeneration response.

3.2. Medium, growth regulator and $AgNO_3$ on callus initiation and shoot formation

MS medium and mMS medium were used as callus induction and shoot regeneration medium. The effects of different media were tested in this study. The results revealed that there is no universal calli/shoots induction culture medium for all of varieties tested. Compared with MS, mMS gave better results, especially in variety Bishan. The average number of shoot per cotyledon explant was 4.1 on mMS, 2.3 on MS; the average number of shoots per hypocotyl explant was 3.3 on mMS, 2.5 on MS, respectively (Fig. 2).

In the present study, mMS medium supplemented with PG-96 (Guo et al., 1999) organic components was used as the callus and shoot induction medium. The PG-96 medium was composed of relatively complex organic acids, amino acids and vitamin compounds. In previous studies, modified PG-96 induction medium promoted androgenic embryogenesis and regeneration in timothy, rye and festulolium (Guo et al., 1999, 2005; Guo and Pulli, 2000).

Auxin and cytokinin as a whole are essential in callus induction and shoot formation. In this broccoli somatic embryogenesis and direct shoot regeneration study, low level of NAA (0.0, 0.5 and 1.0 mg l^{-1}) was used in callus initiation and shoot formation, combined with 6-BA at 1.0, 2.0 and 3.0 mg l^{-1}. When 6-BA was used alone in the induction medium, shoots were produced from explants, but the higher percentage of shoot formation and more shoots per explant were achieved when combined with NAA. The highest frequency of shoot formation was achieved on medium containing 3.0 mg l^{-1} 6-BA combined with 0.5 mg l^{-1} NAA. The number of shoots increased as the 6-BA concentration was raised from 1.0 to 3.0 mg l^{-1}. On the other hand, increasing the concentration of NAA did not visibly reduce the frequency of shoot formation (Table 2) but promote the root formation (data not shown). This result agreed with Cao and Earle (2003) in a broccoli *in vitro* propagation and transformation study.

The presence of $AgNO_3$ showed a remarkable effect for shoot regeneration in this study (Fig. 3). The frequencies of shoot formation increased significantly when explants were cultured on the medium containing $AgNO_3$. The highest percentage of shoot formation from both cotyledon and hypocotyl explants (variety Bishan was used) were 92.5% and 84.4%, respectively, on the mMS medium containing $AgNO_3$ at 4.0 mg l^{-1} (Fig. 3). There was a distinct difference in appearance of explants grown on callus induction medium supplemented with or without $AgNO_3$. Explants cultured on the medium with $AgNO_3$ remained green for 3 weeks; however,

Table 2
Effect of combinations of growth regulators on differentiation of broccoli (mMS supplemented with $AgNO_3$ at 4.0 mg l^{-1})

BA (mg l^{-1})	NAA (mg l^{-1})	Explant type	Explant no.	Explant with callus		Explant with shoot		Total shoot no.	Shoot no./explant ± S.D.[a]
1.0	0.0	Cot.	80	59	73.8%	55	68.8%	160	2.0 ± 0.5 B
		Hyp.	80	49	61.3%	37	46.3%	81	1.0 ± 0.2 c
	0.5	Cot.	80	64	80.0%	60	75.0%	176	2.2 ± 0.7 B
		Hyp.	80	50	62.5%	42	52.5%	112	1.4 ± 0.4 bc
	1.0	Cot.	80	69	86.3%	55	68.8%	168	2.1 ± 0.4 B
		Hyp.	80	67	83.8%	52	65.0%	96	1.2 ± 0.4 bc
2.0	0.0	Cot.	80	72	90.0%	57	71.3%	256	3.2 ± 0.8 AB
		Hyp.	80	71	88.8%	57	71.3%	160	2.0 ± 0.7 ab
	0.5	Cot.	80	76	95.0%	68	85.0%	264	3.3 ± 1.4 AB
		Hyp.	80	74	92.5%	59	73.8%	200	2.5 ± 0.8 ab
	1.0	Cot.	80	77	96.3%	69	86.3%	267	3.3 ± 1.2 AB
		Hyp.	80	76	95.0%	57	71.3%	192	2.4 ± 1.0 ab
3.0	0.0	Cot.	80	80	100.0%	72	90.0%	279	3.5 ± 0.9 A
		Hyp.	80	77	96.3%	64	80.0%	288	3.6 ± 1.0 a
	0.5	Cot.	80	80	100.0%	80	100 %	331	4.1 ± 1.3 A
		Hyp.	80	78	97.5%	67	83.8%	279	3.5 ± 1.4 a
	1.0	Cot.	80	80	100.0%	73	91.3%	294	3.7 ± 1.1 A
		Hyp.	80	80	100.0%	65	81.3%	238	3.0 ± 1.1 a

[a] Means followed by the same letters are not significantly different according to the LSD test at the 5% level of significance. Capital letters refer to cotyledon; small letters refer to hypocotyl (variety Bishan was used).

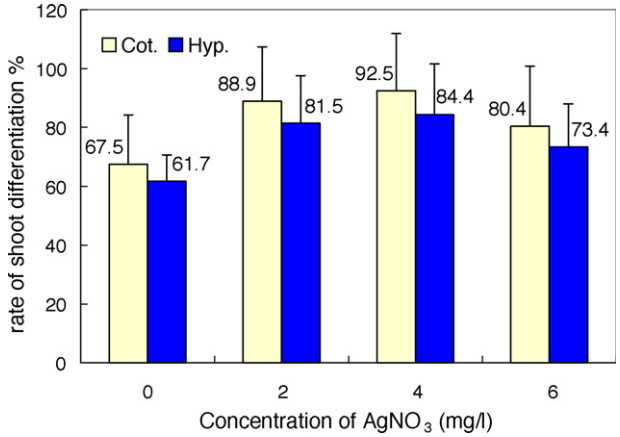

Fig. 3. Effect of $AgNO_3$ concentration on shoot generation from the explants of broccoli var. Bishan. Average values were calculated from 10 replicates.

explants on the medium without $AgNO_3$ turned brown or yellow in color and finally died. $AgNO_3$ promoted callus induction and shoot formation in present study. This result agreed with that of Akasaka-Kennedy et al. (2005) reported in rapeseed. However, when the concentration of $AgNO_3$ reached 6.0 mg l^{-1}, the frequencies of shoot formation reduced (Fig. 3).

3.3. Plant regeneration

Healthy and vigorous roots were formed directly at the based of shoots on the rooting medium (MS containing 0.2 mg l^{-1} NAA) after 2–3 weeks. The result showed that broccoli had a high rooting ability, and there was no difference among four varieties. Approximately 98% regenerated plantlets (Fig. 1C and D) survived when transferred under glasshouse conditions. There was no difference observed in the morphology of these regenerated plants compared with seed-derived control plants under glasshouse conditions. The plants grew normally and developed tight curds after 5 weeks of cold treatment. Pollen fertility of the regenerated plants was over 85% based on acetocarmine staining.

3.4. Analysis of genetic stability by RAPD

Molecular markers are considered to be reliable in monitoring variability in the DNA sequences of plants. The RAPD technique was used in purity analysis of hybrid broccoli and found it very efficient and reliable (Crockett et al., 2002). Munthali et al. (1996) compared the results obtained from RAPD analysis with those obtained with restriction fragment length polymorphism (RFLP) and isozymes and found no difference in their results in beet study. Using the ISSR technique, Leroy has reported the absence of genetic variation in somatic *in vitro* culture of cauliflower (Leroy et al., 2000).

In our study, a total of 62 arbitrary 10-mer primers were screened, ten random-selected regenerants from each variety were employed in RAPD amplification. Of these 62 primers, 20 primers gave polymorphic and clearly identifiable bands and these were therefore used further in PCRs (Table 3). These 20 primers included four primers from Stipic and Campion (1997) that were used for genetic diversity in microspore derived progeny in broccoli.

Three sets of PCRs were carried out for RAPD fingerprinting of each sample. Only bands reproducible on all runs were considered for analysis. Each primer generated a unique set of

Table 3
Primers used in RAPD analysis of genetic stability in broccoli regenerants and number of scoreable bands for each primer

Primer	Primer sequence (5′–3′)	Bands amplified	MW size range (bp)
S22	TGCCGAGCTG	4	350–1600
S24	AATCGGGCTG	3	400–1500
S39	CAAACGTCGG	4	350–1400
S126	GGGAATTCGG	4	500–1600
S134	TGCTGCAGGT	4	300–1600
S265	GGCGGATAAG	4	400–1700
S349	TGAGCCTCAC	5	300–1700
S350	AAGCCCGAGG	4	400–1600
S354	CACCCGGATG	4	500–1500
S362	GTCCCGTGGT	3	600–1200
S366	CACCTTTCCC	4	400–1400
S375	CTCCTGCCAA	4	450–1200
S422	ACCAGGGGCA	3	600–1500
S427	CAGCCCAGAG	4	500–1700
S435	CAGCGACTGT	5	400–1600
S440	GGTGCTCCGT	2	400–1800
S1067	CTTGGGGGAC	5	500–1600
S1069	AGGTCGGCGT	5	300–1400
S1289	GAGGTCGTAC	5	300–1700
S1300	TCATGCGCAC	3	500–1600

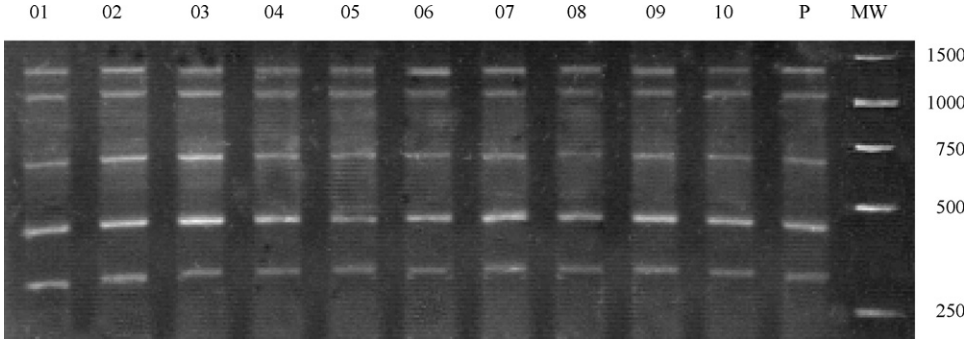

Fig. 4. RAPD profile of plantlets regenerated from broccoli var. Bishan obtained with the primer S1069. Lanes (1–10): regenerated plantlets, (P): broccoli var. Bishan. MW is DNA molecular size marker.

amplification products ranging in size from 300 bp in S134, S349, S1289 and S1069 to 1800 bp in S440. The number of bands for each primer varied from two in S440 to five in S435, S1067, S1069, S1289 and S1300 (Table 3). The 20 primers used in this analysis yielded 79 scoreable bands with an average of 3.95 bands per primer. These 79 markers were monomorphic across all shoots. Fig. 4 showed the RAPD amplification using primer S1069 in variety Bishan, the results demonstrated the genetic stability of regenerants from the mother plant. The RAPD amplifications in other three varieties were also absence of genetic variation (data not shown). However, some results in *B. oleracea* pointed out that genetic variations may take place during the callus step, such variations may, indeed, result from changes in either the hormonal composition of the media used or an undifferentiated cell step. Another proposed explanation is that the plants regenerated from unorganized callus vary more than those from organized callus, whereas no or very little variation occurs when plants are regenerated directly without an intermediate callus phase (Leroy et al., 2001). Ozeki et al. (1997) reported that insertion of a transposable element is one of the mechanisms that can cause variation of plant cell cultures during repeated subculture.

In the present study, we have developed an effect method for viable shoot regeneration from seeding explants of four broccoli varieties. The cotyledon explants had higher regeneration potential than that of hypocotyl explants, and could be the preferred explants for genetic transformation experiments. Analyzed by RAPD, we conclude that high-efficient embryogenesis and regeneration system for broccoli can be carried out for a considerable length of time without much risk of genetic instability.

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References

Akasaka-Kennedy, Y., Yoshida, H., Takahata, Y., 2005. Efficient plant regeneration from leaves of rapeseed (*Brassica napus* L.): the influence of $AgNO_3$ and genotype. Plant Cell Rep. 24, 649–654.

Astarini, I.A., Plummer, J.A., Lancaster, R.A., Yan, G.J., 2004. Fingerprinting of cauliflower cultivars using RAPD markers. Aust. J. Agric. Res. 55, 117–124.

Bhalla, P.L., Smith, N., 1998. Comparison of shoot regeneration potential from seedling explants of Australian cauliflowers (*Brassica oleracea* var. *botrytis*) varieties. Aust. J. Agric. Res. 49, 1261–1266.

Cao, J., Earle, E.D., 2003. Transgene expression in broccoli (*Brassica oleracea* var. *italica*) clones propagated in vitro via leaf explants. Plant Cell Rep. 21, 789–796.

Chen, L.P., Zhang, M.F., Li, C.S., Hirata, Y., 2005. Production of interspecific somatic hybrids between tuber mustard (*Brassica juncea*) and red cabbage (*Brassica oleracea*). Plant Cell Tissue Organ Cult. 80, 305–311.

Christey, M.C., Earle, E.D., 1991. Regeneration of *Brassica oleracea* from peduncle explants. Horticult. Sci. 26, 1069–1072.

Crockett, P.A., Bhalla, P.L., Lee, C.K., Singh, M.B., 2000. RAPD analysis of seed purity in a commercial hybrid cabbage (*Brassica oleracea* var. *capitata*) cultivar. Genome 43, 317–321.

Crockett, P.A., Singh, M.B., Lee, C.K., Bhalla, P.L., 2002. Genetic purity analysis of hybrid broccoli (*Brassica oleracea* var. *italica*) seeds using RAPD PCR. Aust. J. Agric. Res. 53, 51–54.

Dale, P., Al-Kaff, N., Bavage, A., 1998. Transgene expression and stability in *Brassica*. Acta Horticult. 459, 167–171.

Divaret, I., Thomas, G., 1998. Use of RAPD markers to analyze the genetic variability of a collection of *Brassica oleracea* L. Acta Horticult. 459, 255–262.

Guo, Y.D., Sewón, P., Pulli, S., 1999. Improved embryogenesis from anther culture and plant regeneration in timothy. Plant Cell Tissue Organ Cult. 57, 85–93.

Guo, Y.D., Pulli, S., 2000. Isolated microspore culture and plant regeneration in rye (*Secale cereale* L.). Plant Cell Rep. 19, 875–880.

Guo, Y.D., Yli-Mattila, T., Pulli, S., 2003. Analysis of timothy (*Phleum pratense* L.) genetic variation with RAPD and UP-PCR. Hereditas 138, 101–113.

Guo, Y.D., Mizukami, Y., Yamada, T., 2005. Genetic characterization of androgenic progeny derived from *Lolium perenne* x *Festuca pratensis* cultivars. New Phytol. 166, 455–464.

Hansen, G., Wright, M.S., 1999. Recent advances in the transformation of plants. Trends Plant Sci. 4, 226–231.

Hu, J., Quiros, C.F., 1991. Identification of broccoli and cauliflower cultivars with RAPD markers. Plant Cell Rep. 10, 501–505.

Khehra, G.S., Mathias, R.J., 1992. The interaction of genotype, explant and media on the regeneration of shoots from complexes explants of *Brassica napus* L. J. Exp. Bot. 43, 115–120.

King, G.J., 1995. Morphological development in *Brassica oleracea* is modulated by in vivo treatment with 5-azacytidine. J. Horticult. Sci. 70, 333–342.

Lazaro, A., Aguinagalde, I., 1998. Genetic diversity in *Brassica oleracea* L. (Cruciferae) and wild relatives ($2n = 18$) using isozymes. Ann. Bot. 82, 821–828.

Leroy, X.J., Leon, K., Charles, G., Branchard, M., 2000. Cauliflower somatic embryogenesis and analysis of regenerant stability by ISSRs. Plant Cell Rep. 19, 1102–1107.

Leroy, X.J., Leon, K., Hily, J.M., Chaumeil, P., Branchard, M., 2001. Detection of in vitro culture-induced instability through inter-simple sequence repeat analysis. Theor. Appl. Genet. 102, 885–891.

Lim, H.T., You, Y.S., Park, E.J., Song, Y.N., Park, H.K., 1998. High plant regeneration, genetic stability of regenerants, and genetic transformation of herbicide resistance gene (bar) in Chinese cabbage (*Brassica campestris* ssp. *pekinensis*). Acta Horticult. 459, 199–208.

Metz, T.D., Dixit, R., Earle, E.D., 1995. Agrobacterium tumefaciens-mediated transformation of broccoli (*Brassica oleracea* var. *italica*) and cabbage (*B. oleracea* var. *capitata*). Plant Cell Rep. 15, 287–292.

Munthali, M.T., Newbury, H.J., Ford-Lloyd, B.V., 1996. The detection of somaclonal variants of beet using RAPD. Plant Cell Rep. 15, 474–478.

Puddephat, I.J., Robinson, H.T., Fenning, T.M., Barbara, D.J., Morton, A., Pink, D.A.C., 2001. Recovery of phenotypically normal transgenic plants of *Brassica oleracea* upon *Agrobacterium rhizogenes*-mediated co-transformation and selection of transformed hairy roots by GUS assay. Mol. Breed. 7, 229–242.

Ozeki, Y., Davies, E., Takeda, J., 1997. Somatic variation during long term subculturing of plant cells caused by insertion of a transposable element in a phenylalanine ammonia-lyase (PAL) gene. Mol. Gen. Genet. 28, 407–416.

Sparrow, P.A.C., Dale, P.J., Irwin, J.A., 2004. The use of phenotypic markers to identify *Brassica oleracea* genotypes for routine high-throughput *Agrobacterium*-mediated transformation. Plant Cell Rep. 23, 64–70.

Stipic, M., Campion, B., 1997. An improved protocol for androgenesis in cauliflowers (*Brassica oleracea* var. *botrytis*). Plant Breed. 116, 153–157.

Wrage, K., Writer, S., 1994. A new era for value-added vegetables? Bringing anti-cancer compounds to the produce shelves. Seed Crop Ind. 45, 4–15.

Zhang, F.L., Aoki, S., Takahata, Y., 2003. RAPD markers linked to microspore embryogenic ability in *Brassica* crops. Euphytica 131, 207–213.

Zhang, Y., Bhalla, P.L., 2004. In vitro shoot regeneration from commercial cultivars of Australian canola (*Brassica napus* L.). Aust. J. Agric. Res. 55, 753–756.