RESEARCH ARTICLE

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Variation of plants derived from indirect somatic embryogenesis in cotyledon explants of papaya

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ABSTRACT: Somatic embryogenesis in papaya (*Carica papaya*) cv. Kaekdum was induced from embryogenic callus derived from cotyledon explants by culturing on modified half-strength MS basal medium supplemented with 15 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 400 mg/l glutamine, 80 mg/l adenine sulphate, 170 mg/l $NaH_2PO_4 \cdot H_2O$, 15% (w/v) coconut water, and 60 g/l (w/v) sucrose. The somatic embryos were grown on modified half-strength MS basal medium supplemented with 2 mg/l 2,4-D and 60 g/l sucrose. Upon transferring to MS basal medium, somatic embryo germination and plantlet conversion were observed. Variations at the morphological and molecular level were examined in the somatic embryo-derived plants which, after acclimatization, were grown under field conditions. Three types of primers (S-02, S-03, and S-07) were used for random amplified polymorphic DNA analysis. The results from DNA fingerprints revealed both monomorphic and polymorphic bands with sizes ranging from approximately 190–7120, 120–4990, and 280–3600 base pairs, respectively. The size differences might be related to morphological variations observed in the field-grown papaya plants derived from somatic embryogenesis. These morphological variations include plant height, number of flowers, floral length, number of fruits, fruit dimension, and fruit shape. Since these plants produced only hermaphrodite flowers, it is likely that the somaclonal variation could occur in field-grown papaya plants derived from indirect somatic embryogenesis.

KEYWORDS: fruit plants, hermaphroditic flowers, *Carica papaya*, RAPD analysis, somaclonal variation

INTRODUCTION

Among the papaya cultivars grown and sold in Thailand, Kaekdum is one of the most popular to capture a share of the commercial food and fruit markets. This is because the edible pulpy mass can be consumed raw as papaya salad or as sweet fruit when ripened. Like other cultivars, the flowers of Kaekdum exhibit different sexual forms, i.e., male, female, or hermaphrodite[1]. Most farmers prefer growing the hermaphrodite plants as they have both stamen and pistil in one flower and hence there is a better prospect of obtaining a higher yield than from plants bearing only male or female flowers. To determine the genetic relationship at the molecular level and evaluate the hermaphrodite trait among papaya cultivars, random amplified polymorphic DNA (RAPD) analysis was recently applied[2,3].

Propagation of the papaya cultivar can be achieved from seeds, but an alternative and competitive way would be via somatic embryogenesis. In the past two decades, somatic embryogenesis of papaya has been reported[4–8], but little is known about the morphological variations, if any, of papaya plants derived from somatic embryos grown under field conditions. We report here our study using the RAPD approach to investigate the morphological variations of field-grown papaya plants (cv. Kaekdum) generated from indirect somatic embryogenesis.

MATERIALS AND METHODS

Somatic embryo induction

Seeds were taken from ripe fruit of papaya (*Carica papaya* L. cv. Kaekdum) purchased from a local market in Khon Kaen province, Thailand. After cleaning with tap water and laboratory detergent, they were soaked under running water for 1 h. Subsequently, the seeds were immersed in 70% (v/v) ethanol for 1 min and their surface sterilized twice with Clorox (a commercial bleach solution containing 5.25% (w/w) sodium hypochlorite as available chlorine) for 15 min

each, first with 10% (v/v) and then 5% (v/v), followed by addition of 2–3 drops of Tween-20. The seeds were then rinsed 3 times (5 mins each time) with sterile distilled water. The seed coat of the surface-sterilized seeds were removed and the embryos were placed onto a Murashige and Skoog (MS) basal medium[9] containing 0.1 mg/l 1-naphthaleneacetic acid and 0.1 mg/l benzyladenine. After a period of 4 weeks, the somatic embryogenic callus was induced, followed by the excision and culturing of cotyledons on the induction and proliferation medium comprising of modified half-strength MS basal medium supplemented with 15 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 400 mg/l glutamine, 80 mg/l adenine sulphate, 170 mg/l $NaH_2PO_4 \cdot H_2O$, 15% coconut water, and 60 g/l sucrose. All media in this study were adjusted to pH 5.8, gelled with 0.8% (w/v) agar, and autoclaved at 121 °C and 15 psi for 20 min. All cultures were kept under 16 h of illumination with white fluorescent lamps (13.5 $\mu mol\, m^{-2}\, s^{-1}$ light intensity) and 8 h of darkness in a growth room at 25 ± 2 °C.

Somatic embryo maturation, conversion and plantlet acclimatization

Following initiation, the embryogenic callus was first transferred to the maturation medium which consisted of modified half-strength MS basal medium supplemented with 2 mg/l 2,4-D and 60 g/l sucrose for 4 weeks. Then the callus containing mature somatic embryos was transferred to MS basal medium (germination medium) for another 4 weeks. When complete plantlets had been obtained, they were separated from the callus and grown in sand on a plastic tray with a lid during acclimatization to ex vitro conditions.

Field trial and morphological studies

After 4 weeks of acclimatization, the lid of the plastic tray was removed. The seedlings were then grown in pots (one seedling per pot, 13 cm high × 12 cm diameter pot size) with a soil mixture (soil:manure:peanut hull in a 2:1:2 ratio) for 6 weeks. Twenty four plants were randomly selected and then transferred to the field (4 rows, 6 plants per row, and 180 cm × 180 cm separation distance) at the Kor village, Muang district, Khon Kaen province, Thailand. Survival percentage of these plants and their morphological variations, if any, such as plant height, number of flowers, floral length, sexual form of flowers, number of fruits, fruit dimension and fruit shape, were studied for 8 months.

Genetic variations at the molecular level

Genomic DNA was isolated from 1 g of young leaf tissues of 4-month-old plants grown in the field using the method of Kikuchi et al[10]. The DNA concentration was quantified spectrophotometrically at wavelengths of 260 and 280 nm[11]. DNA was amplified by the RAPD method in a total volume of 20 µl of reaction mixture containing 9.675 µl of double distilled water, 2 µl of 10× PCR buffer (Promega), 1.2 µl of 25 mM $MgCl_2$, 2 µl of 2.5 mM dNTP, 2 µl of a primer pair, 3.0 µl of template DNA, and 0.125 µl of Taq DNA Polymerase (Eurotag, UK). Three arbitrary primers (S-02, S-03 and S-07) with sequences 5′-CCTCTGACTG-3′, 5′-CAGAGGTCCC-3′, and 5′-TCCGATGCTG-3′, respectively, were used for DNA amplification. The amplification cycles were run in a thermal cycler (Hybaid) using the following sequence of steps: 1 cycle of pre-denaturation at 95 °C for 3 min, 45 cycles of denaturation at 93 °C for 1 min, annealing at 36 °C for 2 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 73 min. The PCR products were examined by electrophoresis (50 V, 90 min) using 1.5% agarose gel (Seakem, UK) and 1× TBE (Tris borate/EDTA) buffer containing 1 µl ethidium bromide (10 mg/ml). DNA profiles were visualized with the aid of a UV transilluminator.

RESULTS

Somatic embryo induction and maturation

When excised cotyledon explants from surface-sterilized papaya seeds were placed on the induction and proliferation medium, embryogenic callus was induced after 1–2 weeks. Somatic embryos started to develop on this friable callus 3–4 weeks later (Fig. 1).

Maturation of somatic embryos occurred after the embryogenic callus was transferred to the maturation medium for 4 weeks (Fig. 2).

Fig. 1 Formation of somatic embryos in callus initiated from excised cotyledon explants of papaya (cv. Kaekdum) on induction and proliferation medium (scale = 1 mm).

Table 1 Survival percentage and a summary of the morphological changes of 24 papaya (cv. Kaekdum) plants derived from indirect somatic embryogenesis. Results ($\pm$ SE) were obtained after 2–8 months of growing the plants in the field.

Characteristic	months			
	2	4	6	8
survival percentage	100	100	95.83	95.83
average of plant height (cm)	42.02 ± 9.78	76.71 ± 3.97	85.46 ± 4.95	110.04 ± 7.39
average number of flowers	0	5.08 ± 1.32	4.57 ± 1.32	10.26 ± 2.81
average floral length (cm)	-	0.68 ± 0.17	0.85 ± 0.22	1.22 ± 0.19
sexual form of flowers	-	hermaphrodite	hermaphrodite	hermaphrodite
average number of fruits	0	0.04 ± 0.04	0.83 ± 0.43	5.04 ± 1.36
average fruit length (cm)	-	0.24 ± 0.24	1.61 ± 0.81	6.14 ± 1.46
average fruit perimeter (cm)	-	0.13 ± 0.13	1.39 ± 0.66	6.96 ± 1.63
average number of elongate fruits	0	0.04 ± 0.04	0.26 ± 0.16	4.09 ± 1.15
average number of five-furrowed fruits	0	0	0.30 ± 0.17	0.39 ± 0.12
average number of irregular-shaped fruits	0	0	0.26 ± 0.13	0.52 ± 0.16

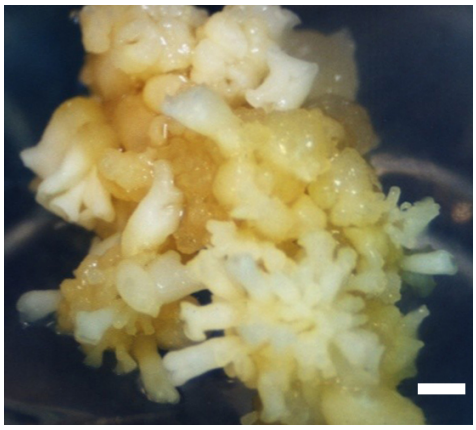

Fig. 2 Mature somatic embryos of papaya (cv. Kaekdum) developed on maturation medium (scale = 1 mm).

Fig. 3 Germination of papaya (cv. Kaekdum) somatic embryos and plantlet conversion on germination medium.

Somatic embryo conversion, plantlet acclimatization and morphological study

Complete plantlets were obtained following transferring of mature somatic embryos to the germination medium (Fig. 3). Sixty percent of the plantlets were found to survive after being acclimatized in sand. When the acclimatized plants were grown under field conditions, 100% of the plants survived for 5 months (Table 1). The survival percentage decreased to 95.83 after 6 months as one of the 24 plants died. Flowering first occurred 3 months after the plants were grown in the field. Detailed morphological observations on the plants after growing in the field for 8 months are shown in Table 2. Not all the plants could produce flowers and there were some differences in plant height, number of flowers, and floral length among the tissue-culture-derived plants grown in the field. However, it is most notable that the plants produced only hermaphrodite flowers (Table 1). These flowers could be classified into three types: elongata, pentandria, and intermedia or capelloid[1,12]. Fruit number, dimension, and shape exhibited some variations as well (Table 2). Fruits developed from the hermaphrodite flowers could be classified into 3 different categories according to the type of flower: an elongate fruit from an elongata flower, a five-furrowed fruit from a pentandria flower, and a ridged or irregular-shaped fruit from an intermedia or capelloid flower (Fig. 4).

Study of genetic variation in field-grown plants

Isolated genomic DNA from young leaf tissues of the 24 plants derived from somatic embryos was used as

Table 2 Morphological variation of papaya (cv. Kaekdum) plants (plant number, *P*) derived from indirect somatic embryogenesis. Results were obtained after the plants were grown under field conditions for 8 months.

P	plant height (cm)	number of flowers	average floral length (cm)	number of fruits	average fruit length (cm)	average fruit perimeter (cm)	number of elongate fruits	number of five-furrowed fruits	number of irregular-shaped fruits
1	153	17	2.32	13	13.47	13.81	11	1	1
2	78	0	-	0	-	-	0	0	0
3	101	51	1.79	10	10.35	11.62	9	0	1
4	62	0	-	0	-	-	0	0	0
5	90	3	0.93	0	-	-	0	0	0
6	150	13	2.07	8	18.96	18.25	5	2	1
7	59	0	-	0	-	-	0	0	0
8	130	20	1.55	6	10.5	13.28	3	1	2
9	190	43	1.42	20	7.88	8.99	17	1	2
10	87	3	3.06	0	-	-	0	0	0
11	93	5	2.12	0	-	-	0	0	0
12	101.5	3	0.93	0	-	-	0	0	0
13	107	2	0.3	0	-	-	0	0	0
14	98	0	-	0	-	-	0	0	0
15	64	3	2.16	0	-	-	0	0	0
16	62	0	-	0	-	-	0	0	0
17	135	17	1.52	6	11.62	12.53	4	0	2
18	151	23	1.53	20	14	15.18	16	2	2
19	92.5	6	1.41	3	7.07	11.47	2	0	1
20	112	0	-	0	-	-	0	0	0
22	154.5	11	1.71	12	14.66	17.21	11	1	0
23	124.5	5	1.8	9	16.83	21.01	8	1	0
24	136	11	1.48	9	15.84	16.62	8	1	0

Fig. 4 Fruits developed from the hermaphrodite flowers of a papaya (cv. Kaekdum) plant derived from indirect somatic embryogenesis.

the templates for amplification of DNA in RAPD-PCR. The amount of extracted DNA ranged from 1.05 to 14.6 mg/ml. The number of reproducible bands amplified with the 3 primers is shown in Table 3. The S-03 primer gave the best result in producing the highest number of total fragments amplified from the 24 plants (Table 4, Fig. 5).

Table 3 Arbitary primers used for amplification of DNA and summary of the RAPD products generated from the DNA templates of 24 plants derived from indirect somatic embryogenesis using the 3 primers.

Type of primer	Sequence of RAPD from 5′ to 3′	Number of total DNA bands	Approximate size of DNA bands (bp)
S-02	CCTCTGACTG	66	190–7120
S-03	CAGAGGTCCC	120	120–4980
S-07	TCCGATGCTG	77	280–3600

DISCUSSION

Somatic embryogenesis of papaya could be achieved either via direct[5,8] or indirect[4,6,7] pathway. In this study, indirect somatic embryogenesis was induced from excised cotyledons of surface-sterilized seeds.

Table 4 Number of DNA bands (N) generated using the S-03 primer and their approximate sizes from 24 different papaya (cv. Kaekdum) plants (plant number, P) derived from indirect somatic embryogenesis. Control (C) was DNA from germinated seeds of the same cultivar.

P	N	Approximate size of DNA bands (bp)
C	5	260, 380, 530, 930, 1210
1	8	240, 380, 530, 800, 930, 1210, 2450, 3970
5	6	270, 390, 550, 840, 990, 1290
6	7	270, 390, 550, 840, 1000, 1290, 3230
7	4	270, 390, 560, 850
8	8	280, 390, 550, 850, 1000, 1290, 3010, 4830
9	8	270, 390, 550, 850, 1000, 1290, 3120, 4990
10	8	270, 390, 550, 850, 1000, 1300, 3120, 4990
11	8	270, 390, 550, 850, 990, 1300, 3230, 4990
12	3	390, 550, 990
15	7	150, 190, 270, 370, 520, 790, 1160
16	8	150, 190, 270, 360, 520, 800, 920, 1160
17	8	120, 250, 360, 520, 800, 920, 1160, 2580
18	10	140, 180, 270, 360, 520, 590, 800, 930, 1170, 2580
19	6	140, 190, 290, 360, 450, 520
20	8	200, 240, 360, 520, 670, 810, 890, 1170
21	1	270
23	7	170, 270, 360, 450, 520, 820, 1170

Plant numbers 2, 3, 4, 13, 14, 22, 24 gave non-reproducible results

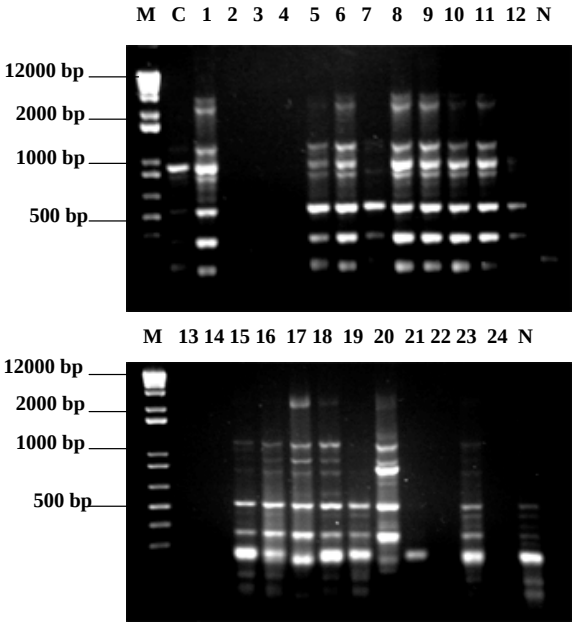

Fig. 5 Patterns of DNA bands amplified using the S-03 primer with the template DNA of papaya (cv. Kaekdum) plants derived from indirect somatic embryogenesis. M: standard DNA (100 bp plus DNA ladder, Life technology), C: control DNA (from germinated seeds of the same cultivar), 1–24: DNA bands from plant number 1–24 (each derived from a somatic embryo).

This type of explant and culture conditions for embryogenic callus induction differ from those used in other previous studies. In the present study, it appears that auxin concentration played an important role in somatic embryo induction and development from cotyledon explants of papaya cv. Kaekdum. Somatic embryos were initiated and proliferated well (Fig. 1) on the induction and proliferation medium containing high auxin concentration (15 mg/l 2,4-D) under 16 h light condition. These somatic embryos developed further on the maturation medium (Fig. 2) in which the concentration of 2,4-D was reduced to 2 mg/l. However, auxin was unnecessary in the germination medium as successful plantlet conversion took place in the medium that lacks auxin (Fig. 3). In comparison to previous studies of indirect somatic embryo formation in papaya[4,6,7], it is likely that different sources of papaya explants may require different concentrations and types of plant growth regulators.

Morphological variation in plants derived from somatic embryogenesis have been examined in many plant species such as barley[13], black spruce, and white spruce[14,15]. Recently Rimberia et al[16] reported that one difference among the papaya plants regenerated via somatic embryogenesis was the height of the plant, which could then be employed to group the plants into 3 groups (dwarf, semi dwarf, and tall). The study of Rimberia et al used plants that were derived from another culture and that produced only female flowers. The present result is different from that reported previously. We demonstrate that the plants derived from indirect somatic embryogenesis bore only hermaphrodite flowers. This seems to be the only major morphological difference between the somatic embryo-derived papaya plants in comparison to those grown from seed of the same cultivar in the same field[17].

Random amplified polymorphic DNA markers have been used to diagnose morphological variations in regenerated plants derived from somatic embryogenesis in previous studies[18–20]. The DNA fingerprints from this method could reveal the relationship, at the molecular level, of the regenerated plants. In papaya, RAPD analysis was used to examine genetic relationship[2,21] and perform sex determination[3,22]. In this study, RAPD analysis was employed to investigate molecular variation in papaya (cv. Kaekdum) plants derived from indirect somatic embryogenesis (Table 3, Table 4, Fig. 5). The use of S-02, S-03, and S-

07 primers generated DNA bands of sizes ranging from approximately 190–7120, 120–4990, and 280–3600 bp, respectively. The resulting DNA fingerprints which displayed both monomorphic and polymorphic bands suggested that these differences might be associated with some morphological variations such as plant height, number of flowers, number of fruits, fruit dimension, or fruit shape in the acclimatized plants grown in the field. In conclusion, this is the first report on somaclonal variation observed in field-grown plants derived from indirect somatic embryogenesis of papaya cv. Kaekdum.

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