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Short Communication: In vitro response of papaya (*Carica papaya*) to plant growth regulators

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Abstract. *Teixeira da Silva JA. 2016. In vitro response of papaya (*Carica papaya*) to multiple plant growth regulators*. *Nusantara Bioscience 8*: *77-82*. The use of plant growth regulators (PGRs) in papaya (*Carica papaya* L.) tissue culture is essential for tissue and organ culture in vitro. In this study, in a bid to expand the information available on the response to PGRs, a wide range of PGRs, roughly divided into four groups (auxins, cytokinins, alternative PGRs, growth inhibitors, and retardants) was tested. Among them, the auxins 2,4-D, dicamba, and picloram formed most callus (hard and soft). Callus inductions by chitosan and coconut water are novel results for papaya. Shoots only formed in response to BA and TDZ, but TDZ-induced shoots were fascinated and/or hyperhydric. These results provide novel perspectives for papaya researchers who may have recalcitrant genotypes or tissues that are unresponsive in vitro.

Keywords: 2,4-D, dicamba, PGR, picloram, thidiazuron

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; 2iP, N^6–[Δ2-isopentenyl] adenine (syn. 6 (γ,γ-dimethylallylamino)purine); ABA, (±)-*cis*,*trans*-abscisic acid; AC, activated charcoal; Ads, adenine hemisulphate; BA, 6-benzyladenine (syn. BAP, 6-benzylaminopurine); BNOA, β-naphthoxyacetic acid; BSSAA, benzoselenienyl-3-acetic acid; 4-CPPU, *N*-(chloro-4-pyridyl)-*N*′-phenylurea (or forchlorfenuron); dicamba, 3,6-dichloro-2-methoxybenzoic acid (syn. 3,6-dichloro-*o*-anisic acid); GA_3, gibberellic acid; JA, (±)-jasmonic acid; Kin, kinetin; MeJa, methyl jasmonate; PGR, plant growth regulator; picloram, 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid; PPFD, photosynthetic photon flux density; SA, salicylic acid; TCL, thin cell layer; TDZ, *N*-phenyl-*N*'-1,2,3-thiadiazol-5-yl-urea or thidiazuron; TIBA, 2,3,5-triiodobenzoic acid; ZR, zeatin riboside (*syn*. 9-(β-D-ribofuranosyl)-*trans*-zeatin or N^6- (*trans*-4-hydroxy-3-methyl-2-buten-1-yl)adenosine)

INTRODUCTION

Papaya (*Carica papaya* L.; Caricaceae) is a popular tropical fruit, but also contains important secondary metabolites (Canini et al. 2007). This plant is usually propagated by seed (Teixeira da Silva et al. 2007; Jiménez et al. 2014). Somatic embryos are a stable form of clonal propagation and are useful for the establishment of suspension cultures (Anandan et al. 2012). Consequently, somatic embryogenesis has been well explored in papaya (Teixeira da Silva et al. 2007) and to achieve this, several plant growth regulators (PGRs) have been employed throughout the literature. This will be elaborated upon in more detail in the discussion. A robust regeneration system allows for a greater chance of recovering transgenic plants in vitro (Tennant 2010). By adjusting the ratios of red: blue light-emitting diodes (LEDs), papaya plant growth can be manipulated, and photoautotrophic micropropagation is an excellent means to increase plantlet biomass or to alter plant architecture (Teixeira da Silva 2014a). Teixeira da Silva (2013) noted that cotyledonary tissue exposed to five auxins (2,4,5-trichlorophenoxyacetic acid, 2,4,5-T; indole-3-acetic acid, IAA; indole-3-butyric acid, IBA; NAA; β-naphthoxyacetic acid, BNOA) could form axillary roots. A previous study (Teixeira da Silva 2014b) compared the response of six previously established protocols. In that study, friable callus or embryogenic callus was induced from cotyledons that were then encapsulated as synthetic seed (synseed). This synseed were then subjected to low-temperature storage and cryopreservation, which are two important techniques for preservation of important horticultural germplasm (Cha-um and Kirdmanee 2007; Engelmann 2011; Sharma et al. 2013).

This study exposed two explants (leaves and stem thin cell layers, or TCLs) of a papaya cultivar to a wide range of PGRs, many of which have not yet been tested in papaya tissue culture, to assess developmental responses. TCLs were selected as they are sensitive tissues useful for plant tissue culture (Teixeira da Silva and Dobránszki 2013a, 2015; Teixeira da Silva et al. 2015).

MATERIALS AND METHODS

All chemicals and reagents were purchased from either Sigma-Aldrich (St. Louis, USA), Wako Chemical Co. (Osaka, Japan), or Nacalai Tesque (Kyoto, Japan).

Seeds of hybrid papaya (*Carica papaya* L. cv. 'Sunrise Solo') cultivar were surface sterilized and germinated using the protocol of Giang et al. (2011) and Teixeira da Silva (2013, 2014a, 2014b). In brief, seeds were soaked for 48 h then washed in running tap water with scrubbing to remove the sarcotesta. Seeds that passed a floatation test (viable seeds) were surface sterilized in 0.1% $HgCl_2$ + 2-3 drops of

Tween-20 for 5 min, rinsed 3 times in sterilized distilled water (SDW), sprayed with 80% ethanol for 1 min then rinsed 3 times in SDW. Surface-sterilized seeds were slightly embedded (5/Petri dish; As-One, Osaka, Japan) in autoclaved (100 KPa; 21 min) full-strength (macro- and micronutrients) PGR-free Murashige and Skoog (1962) (MS) basal medium at pH 5.8 (adjusted with 1N NaOH or HCl) containing 3% sucrose and 2 g/L gellan gum (Gelrite®, Merck, USA). Petri dishes were sealed with Parafilm® (Pechiney Plastic Packaging Co., Chicago, USA) and placed at 25°C in the dark for 7 days. Seedlings then transferred to 250-ml Erlenmeyer flasks (5 plantlets per flask) containing 50 ml of Hyponex® (N:P:K = 6.5 : 6 : 19; Hyponex Japan Corp., Tokyo, Japan), 3% sucrose and 2 g/L Gelrite® and placed under a 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 45 µmol m^{-2} s^{-1} provided by plant growth fluorescent lamps (Plant Lux, Toshiba Co., Japan). Young leaves of one-month-old rooted seedlings established in vitro were used for the experiments that follow. Transverse thin cell layers (tTCLs), which are useful explants for better control of plant organogenesis in vitro (Teixeira da Silva and Dobránszki 2013a, 2014), were prepared from seedling stems, 1 mm thick and 1-2 mm in diameter.

The base of young leaves of two-month-old established in vitro rooted seedlings, including mid-vein tissue, as well as stem tTCLs, were exposed to four groups of PGRs in the light (16-h photoperiod; PPFD = 45 µmol m^{-2} s^{-1}) and in the dark. The rationale was to assess whether a dose of any single PGR could induce organogenesis. Four categories of PGRs were tested. Group 1, auxins (3,6-dichloro-2-methoxy benzoic acid (syn. 3,6-dichloro-*o*-anisic acid)), dicamba (pesticide that mimics an auxin); 2,4-D (pesticide that mimics an auxin); 4-amino-3,5,6-trichloro-2-pyridine carboxylic acid, picloram (a herbicide)). Group 2, cytokinins (adenine hemisulphate, Ads; 6-benzyladenine, BA (syn. 6-benzylaminopurine; see notes in Teixeira da Silva 2012); benzoselenienyl-3-acetic acid, BSSAA; *N*-(chloro-4-pyridyl)-*N*′-phenylurea (or forchlorfenuron), 4-CPPU; kinetin, Kin; *N*-phenyl-N'-1,2,3-thiadiazol-5-yl-urea or thidiazuron, TDZ (cytokinin-like); zeatin riboside (syn. 9-(β-D-ribofuranosyl)-*trans*-zeatin or N^6-(*trans*-4-hydroxy-3-methyl-2-buten-1-yl)adenosine), ZR; N^6–[Δ2-isopentenyl] adenine (syn. 6 (γ,γ-dimethylallylamino) purine), 2iP). Group 3, other PGRs (chitosan (a fungal elicitor); coconut water (CW); gibberellic acid, GA_3; jasmonic acid, JA; methyl jasmonate, MeJA; salicylic acid (SA); 1-triacontanol (TRIA; syn. melissyl alcohol, myricyl alcohol). Group 4, growth inhibitors and retardants ((±)-*cis*,*trans*-abscisic acid, ABA; ancymidol; chlormequat; mepiquat; paclobutrazol; uniconazole; 2,3,5-triiodobenzoic acid, TIBA). Four concentrations (1, 2, 4 and 8 mg/L) of each PGR was tested separately, except for CW, which was tested at 1, 2, 4 and 8% (v/v). Coconuts were purchased fresh in a green state from Osaka fruit market, transported to Kagawa under cooled conditions. A hole was drilled using aseptically treated tools and CW was poured into a sterile plastic bottle and frozen until needed, or used fresh immediately after extraction. The control was PGR-free MS medium.

The formation of shoots or callus was assessed with the naked eye and by light microscopy and quantified after 60 days. The amount of hard callus (HC), soft, friable callus (SFC) and shoots that formed was not quantified. More discussion about HC and SFC may be found in Teixeira da Silva (2104b).

Experiments were organized according to a randomized complete block design with three blocks of 10 replicates per treatment. All initial trials were repeated once. Only those treatments that showed an organogenic response (Table 1) were repeated three times.

RESULTS AND DISCUSSION

A wide range of PGRs and growth retardants on the organogenesis of papaya in vitro was tested since several of these PGRs have not yet been used in papaya tissue culture. The most popular PGR used in papaya somatic embryogenesis, for example, is 2,4-D, given its success across a wide range of cultivars. The in vitro responses of papaya explants cultured on MS media supplemented with different PGR are given in Table 1. No growth or explant response was observed for any of the group 4 PGRs (growth retardants). The most responsive group was the auxins in group 1, particularly 2,4-D, picloram, and dicamba. Other auxins had already been tested in Teixeira da Silva (2013). Both leaf explants and tTCLs were responsive to 2,4-D in the light and in the dark, forming both HC (Figure 1E, F, G, H) and SFC (Figure 1D). HC formed in response to dicamba and picloram, both in the dark, but only in tTCLs in the latter and in leaves and tTCLs in the former while SFC showed a similar trend with respect to explant receptivity as dicamba, but in the light. In group 2, Kin and TDZ formed SFC, but TDZ showed a wider response for both explants and both in the dark and light while 2iP showed a limited formation of HC by leaves in the dark. In group 3, chitosan and GA_3 formed HC, independent of the dark or light conditions, but only tTCLs were responsive for chitosan. CW formed some SFC from tTCLs in both the light and dark. TRIA showed limited HC formation from tTCLs in the dark. Shoots only formed in the presence of BA or TDZ, but those from TDZ tended to be fascinated and/or hyperhydric (Figure 1C). As expected, none of the growth retardants (group 4) induced any response (callus or shoots).

Even though somatic embryogenesis was widely studied in papaya (see below), no clear somatic embryos or somatic embryo-like structures were observed in this study, even within the wide range of PGRs tested. One possible reason may be that this study only tested individual PGRs or a single medium step, while most other studies employed PGR combinations or transfer to more than one medium. Therefore, it is possible that the HC or SFC observed in this study could develop somatic embryos had they been transferred to alternative PGR-containing media for somatic embryo induction. Another reason may be the range of concentrations tested (1-8 mg/L). For example, de Almeida et al. (2001) required a higher concentration of 2,4-D (10 mg/L) to induce friable callus and then, after

Table 1. Effect of plant growth regulators on organogenesis (HC, SFC or shoots) from two-month-old leaves or stem tTCLs of papaya (*Carica papaya* L.) 'Sunrise Solo' in the light and dark, assessed after 60 days in culture. All plant growth regulator (PGRs) and concentrations not displayed did not show any growth or organogenic response (even if the fresh weight may have increased).

		HC						SFC						Shoots					
		Light			Dark			Light			Dark			Light			Dark		
	PGR	Leaf	Stem	tTCL	Leaf	Stem	tTCL	Leaf	Stem	tTCL	Leaf	Stem	tTCL	Leaf	Stem	tTCL	Leaf	Stem	tTCL
Control	No PGRs																		
Group 1	2,4-D	1-8	1-8		1-8	1-8		1-8	1-8		1-8	1-8							
	Dicamba				1, 2	1, 2		1	1										
	Picloram					1			1										
Group 2	AdS																		
	BA													1, 2	1, 2, 4				
	BSSAA																		
	4-CPPU																		
	Kin								1										
	TDZ							2, 4	1-8		2	1-8					1-8	1-8	
	ZR																		
	2iP	1			1														
Group 3	Chitosan		1, 2			1, 2													
	CW								1			1							
	GA_3	1	1		1, 2	1, 2													
	JA																		
	MeJA																		
	SA																		
	TRIA					1													
Group 4	ABA																		
	Ancymidol																		
	Chlormequat																		
	Mepiquat																		
	Paclobutrazol																		
	Uniconazole																		
	TIBA																		

Callus types: HC, hard callus; SFC, soft, friable callus. Shading key: no shading = no response or only explant expansion; yellow = SFC; orange = HC; green = shoots. Numbers in table boxes: no numbers = no response; all other numbers correspond to either 1, 2, 4 or 8 mg/L of the PGRs, or 1, 2, 4 or 8% of CW. See text for PGR abbreviations

transfer to another medium, somatic embryos.

Apparently lower levels of organogenesis observed in tTCLs relative to conventional leaf explants does not necessarily reflect a poorer performance of tTCLs since organogenesis is restricted by the actual explant size, surface area and volume (Teixeira da Silva 2013; Teixeira da Silva and Dobránszki 2013b). To correct for this difference between the organogenic outcome of a TCL and a conventional explant, the Plant Growth Correction Factor (Teixeira da Silva and Dobránszki 2011, 2014) takes three aspects (explant size, surface area, and volume) into consideration, allowing the organogenic outcome to be directly compared. Another possibility may relate to the timing of sampling and the developmental state of the tissues, both factors playing a considerable outcome on the organogenic response (Teixeira da Silva and Dobránszki 2013b). It is thus a possibility that the observed results may have been different (qualitative and quantitative) had the explants been sampled at 30 or 90 days, instead of 60 days. This timing of sampling effect was also observed in papaya by Heringer et al. (2013).

PGRs are an important determinant of the success of papaya tissue culture. Except for seed germination, which can take place on PGR-free basal medium, most organogenic experiments in papaya have employed PGRs. This is not an exhaustive exploration of the literature, but serves to highlight some of the diversity of PGRs explored to date. Phloridzin (a dihydrochalcone) at 3 g/L improved somatic embryo germination in 'Maradol' (Ascencio-Cabral et al. 2008). Auxins can induce rooting in in vitro explants while phloroglucinol, an auxin-like substance (Teixeira da Silva et al. 2013), improved rooting of established in vitro papaya plants (Teixeira da Silva 2013). An overview of the papaya literature, primarily from 2000 onwards, reveals that 2,4-D is the most commonly used PGR for inducing somatic embryogenesis, but the requirement for light and darkness appears to be not only genotype-dependent but also dependent on other factors such as medium constituents and explant. In fact, very early studies such as Litz and Conover (1981, 1982, 1983), Fitch and Manshardt (1990) or Fitch (1993) already indicated the importance of 2,4-D and CW for somatic embryogenesis

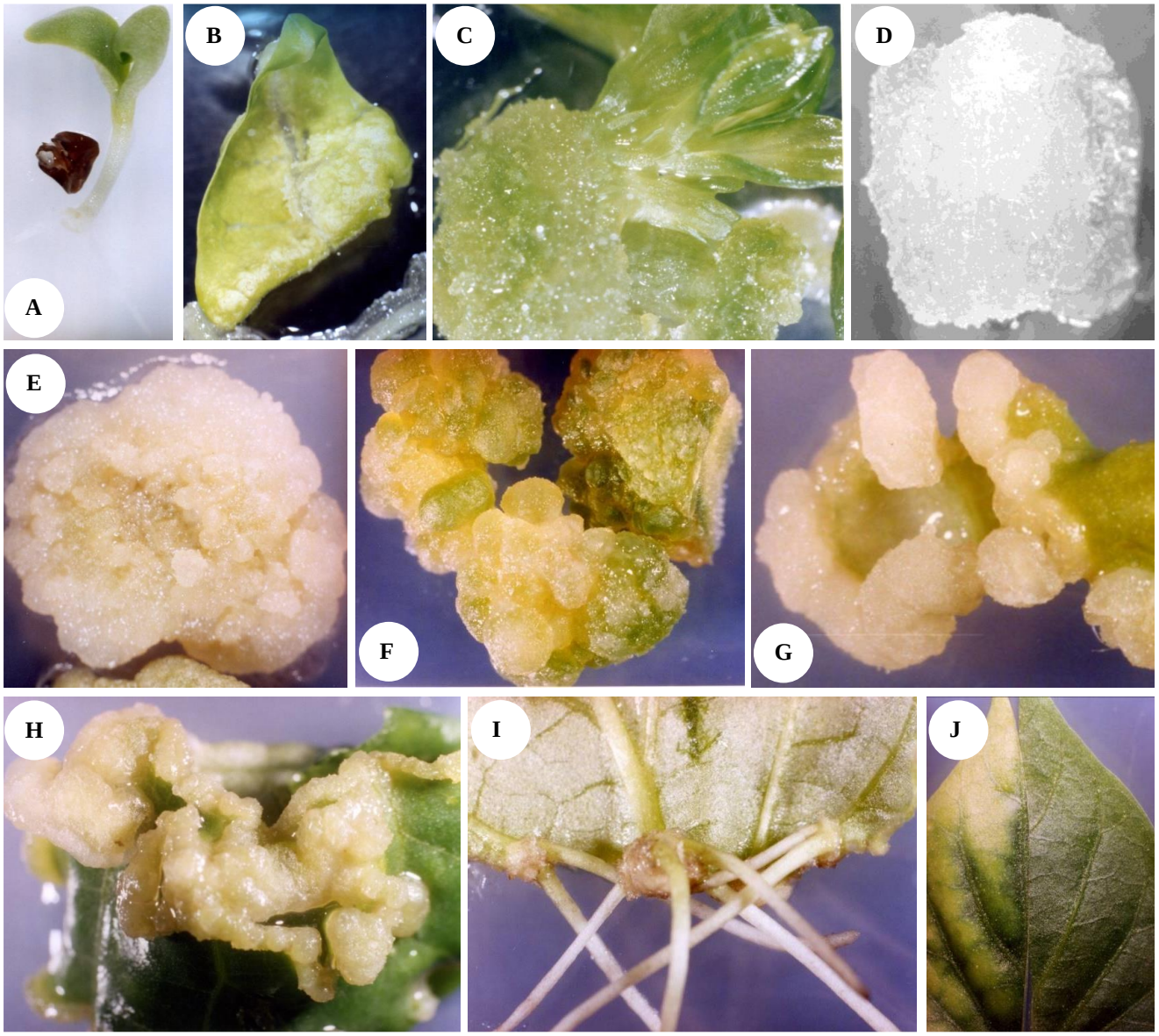

Figure 1. The response of papaya (*Carica papaya* L.) 'Sunrise Solo' to single doses of select plant growth regulators. A. Seed germination. B. Leaf explant. C. Fasciated and/or hyperhydric shoots in response to 8 mg/L TDZ. D. SFC in response to 1 mg/L picloram. E. HC formation in response to 1 mg/L dicamba. F. HC formation in response to 2 mg/L GA_3. G. HC formation in response to 2 mg/L chitosan. H. HC formation in response to 4 mg/L 2,4-D. I. Root formation in response to 8% v/v. CW. J. Leaves of developed adult plantlet: left = TDZ-derived; right = control. A-D = light; E, F = darkness. Leaf explants = G, H, I; stem tTCLs = C, D, E, F. HC = hard callus; SFC = soft, friable callus.

while Chen et al. (1987) noted that a combination of 1.0 mg/L NAA, 0.5 mg/L Kin and 1.0 mg/L GA_3 was essential for embryogenic callus induction. Khatoon and Sultana (1994) found that petiole segments and epidermal or longitudinal TCLs of 'Malir' could form callus and shoots in the presence of 2.5 mg/L NAA + 0.5 mg/L 2iP, and 1 mg/L NAA + 1 or 5 mg/L 2iP, respectively, all media requiring 15% (v/v) CW. Cabrera-Ponce et al. (1996) could propagate transgenic plantlets in the presence of 0.2 mg/L BA and 0.1 mg/L Kin. Fernando et al. (2001) found that 'Sunrise Solo' mature zygotic embryos could form somatic embryos in the presence of 2 mg/L 2,4-D. Usman et al. (2002) claimed that leaves with mid-rib were much more receptive to somatic embryogenesis than petioles or leaves without mid-rib, as was also found in this study. Shoots of 'Honey Dew', 'Washington' and 'Co2' could be induced either with 2.25 µM TDZ or 4.4 µM BA + 0.5 µM NAA, elongated with 4.7 µM GA_3 and rooted with 14.7 µM IBA (Bhattacharya et al. 2003). Farzana et al. (2008) induced embryogenic callus in the presence of 3 mg/L NAA when 'Rathna' zygotic embryos and hypocotyls were used while 1 mg/L 2,4-D was optimal for young leaf explants. In that

study, NAA at 0.02 mg/L and 0.5 mg/L BA were best for somatic embryo maturation. Jattana et al. (2008) found that CW was important in the induction of embryogenic callus of 'Kaekdum' cotyledons, while somatic embryos could form in the presence of 2 mg/L 2,4-D and 4 mg/L Zea. Renukdas et al. (2010) found that several PGRs could be ranked in terms of their ability to form somatic embryos: 4 μM picloram > 20 μM dicamba > 10 μM 2,4-D > 4 μM 2,4,5-T; moreover, somatic embryos could mature in the presence of 1 μM spermidine (a polyamine), 0.1 μM ABA or 0.5 μM $AgNO_3$ (an ethylene inhibitor). Malabadi et al. (2011) claimed successful somatic embryogenesis for 11 papaya cultivars in the presence of 4.52 μM 2,4-D and 2.27 μM TDZ. Mahadevamma et al. (2012) could induce friable callus from gamma ray-irradiated shoot tips using 6 mg/L BA and 2 mg/L IAA or 0.1 mg/L NAA and 0.6 mg/L BA. Wu et al. (2012) used 0.25 mg/L BA + 1 mg/L GA_3 to elongate shoots from somatic embryos of hermaphroditic 'Meizhonghong'. Heringer et al. (2013) claimed that polyethylene glycol is important for the maturation of hybrid UENF/CALIMAN 01 somatic embryos.

The response of papaya in vitro leaf tissues to a wide range of PGRs was tested. Several of these PGRs have not yet been used in papaya tissue culture, in part because of the wide success of somatic embryogenesis in response to 2,4-D, or shoot production in the presence of BA, with or without NAA. Papaya leaves respond sensitively to inductive agents such as PGRs in vitro, and thus serves as a useful model tropical fruit species. The ability to chemically control organogenesis, such as rhizogenesis (Teixeira da Silva 2013; Fig 1I), or the responsiveness of papaya tissue cultures to LEDs and variation in the light spectrum (Teixeira da Silva JA 2014a) provide exciting prospects for papaya biotechnology. Reliable and flexible (i.e., alternative pathways) to achieve the same organogenic outcome serve well for genetic transformation experiments or studies on development, including those using TCLs (Teixeira da Silva et al. 2015).

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