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Plantlet Regeneration via Organogenesis from Immature Seeds of *Rosa rugosa* Thunb. ex. Murray

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ABSTRACT

Rosa rugosa Thunb. ex. Murray possesses unique and value traits including: large, fragrant flowers, a long flowering period, disease resistance, and winter hardiness. An improved regeneration system is essential to exploit the potential of this species utilizing transgenic applications. The development of an efficient plantlet regeneration system was explored using different combinations and rates of 2,4-dichlorophenoxy acetic acid (2,4-D) and *trans*-zeatin (ZT) during the shoot induction stage. Immature seeds (14-28 days after pollination) were cut transversely in half and placed onto a medium containing Murashige and Skoog (MS) basal salts plus vitamins, 0.25% (w/v) Gellan gum, and varying concentrations and combinations of 2,4-D and ZT for 33 to 49 days. The treatment producing the highest frequency of explants with calli containing shoot primordia (14.4%) contained 2.0 mg/l 2,4-D and 1.5 mg/l ZT. Explants were transferred to the same medium without phytohormones for 56 to 67 days, each differentiating ~12 adventitious shoots. The shoots were easily separated and rooted after 28 days on medium without phytohormones or medium with 0.1 mg/l 1-naphthaleneacetic acid (NAA).

Keywords: Murashige and Skoog, phytohormones, regenerated shoots, Rosaceae

INTRODUCTION

Rosa rugosa Thunb. ex. Murray is distributed in the northern part of Japan and has contributed to the development of some modern commercial roses including hybrid rugosas, hybrid kordesii, and shrub roses (Cairns 2000). This species possesses desirable characteristics including disease resistance, cold hardiness, fragrance, ornamental fruit, and recurrent flowering during the growing season (Svejda 1974, 1977). It is one of the few recurrently flowering species, which has been critically important to establish its widespread use in the ornamental trade.

Previously, seven MADS-box genes, *MASAKO C1*, *D1*, *BP*, *B3*, *euB3*, *S1* and *S3* were isolated from *R. rugosa* to determine the molecular mechanisms of flower morphology and development within roses (Kitahara and Matsumoto 2000; Kitahara *et al*. 2001; Matsumoto and Kitahara 2005; Hibino *et al*. 2006). Their function was further investigated by expression and transgenic analyses using *Arabidopsis thaliana* (L.) Heynh. and *Torenia fournieri* Lind. (Kitahara and Matsumoto 2000; Kitahara *et al*. 2001, 2004; Matsumoto and Kitahara 2005; Hibino *et al*. 2006). However, to understand the function of these genes in rose, it is essential to conduct loss and gain of function experiments using transgenic roses. To introduce genes into *R. rugosa*, an efficient plantlet regeneration system must be established. To date, plant regeneration through somatic embryogenesis and organogenesis has been reported for modern and wild roses (de Wit *et al*. 1990; Noriega and Sondahļ 1991; Rout *et al*. 1991; Ibrahim and Debergh 2001; Asano and Tanimoto 2002; Wen and Deng 2005), and plantlet regeneration has also been reported for *R. rugosa* via somatic embryogenesis (Kunitake *et al*. 1993). Murashige and Skoog (MS) medium (Murashige and Skoog 1962) was the most common basal medium within these regeneration systems, and efficient regeneration was possible with the use of both cytokinin and auxin, except for embryogenic callus where the induction phase occurred without phytohormones (Kunitake *et al*. 1993; Asano and Tanimoto 2002). Embryogenic and organogenic regeneration systems generally share a two-stage process: the first involving embryogenic callus or adventitious bud induction and then a second regeneration step typically involving modified hormonal combinations to promote shoot elongation and development. Using a two-stage process with *R. rugosa*, Kunitake *et al*. (1993) induced embryogenic calli from 5.9% of immature seeds with their best treatment. Under the best subsequent treatments 20.6 somatic embryos were produced per callus and 3% of the somatic embryos continued to develop normally, corresponding to ~3.65% of the original immature seeds leading to a plantlet. As the frequency of somatic embryo germination and plantlet recovery was quite low, it is difficult to apply the method to the genetic transformation of *R. rugosa*.

The effects of variable combinations and concentrations of the phytohormones 2,4-dichlorophenoxy acetic acid (2,4-D) and *trans*-zeatin (ZT) during the induction phase was explored in order to identify an efficient plantlet regeneration system (embryogenic or organogenic) for *R. rugosa* applicable to genetic transformation for multiple objectives, including insertion and continued study of the *MASAKO* genes.

MATERIALS AND METHODS

Immature hips (hypanthium tissue surrounding the achenes which are individually seeded fruits) from reciprocal crosses between a genotype of each *R. rugosa* and *R. rugosa* var. *alba* were harvested at the experimental farm of Gifu University 14–28 days after pollination. Hips were surface-sterilized for 10 min with 70% (v/v) ethanol and washed three times with sterile distilled water. Two or three surface-sterilized hips were dissected aseptically and immature achenes (average number of achenes in one hip was 88 ± 2.1, mean ± SE) were cut transversely in half. They were cultured on 0.25% (w/v) Gellan Gum-solidified Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing vitamins and various concentrations of 2,4-D and/or ZT, namely 2 mg/l 2,4-D, 1.5 mg/l ZT; 2 mg/l 2,4-D and 0.75 mg/l ZT; 2 mg/l 2,4-D and 1.5 mg/l ZT; and 2 mg/l 2,4-D and 3 mg/l ZT. Additionally, MS

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Short Communication

medium without phytohormones was used as a control. Sucrose (3%) was added in all MS medium as carbohydrates. Cultures were maintained at 25°C under a 16:8 hr (light: dark) photoperiod using cool white fluorescent light (*ca*. 4000 lux) for 33 to 49 days. Induced calli were transferred to the same medium without phytohormones, and then cultured under identical conditions. Finally, I used MS medium containing 0.1 mg/l NAA for root induction of regenerated shoots without roots. The experiments were repeated four or five times with 14 to 31 seeds per replication over time placed in a plastic Petri dish (15 × 100 mm).

RESULTS AND DISCUSSION

Initially, the induction of embryogenic calli of *R. rugosa* according to Kunitake *et al*. (1993) was attempted, but their method failed to form calli of any kind from the immature seeds of *R. rugosa* on MS medium without phytohormones in this study (**Table 1, Fig. 1A**). This might be the result of using different *R. rugosa* genotypes. Since Noriega and Sondahl (1991) claimed that the combination of 2,4-D (2 mg/l) and ZT (1.5 mg/l) was efficient for the induction of globular embryos of modern roses, these phytohormones were employed in this study. The effects of varying concentrations and combinations of 2,4-D and ZT on callus induction are shown in **Table 1**. No embryogenic callus was produced in this study, and organogenic callus formed between day 9 and 15 and also 19 and 28, following transfer to phytohormone-free medium. In order to induce callus, cutting the immature seeds in half was essential regardless of the conditions. As callus frequently develops from wounded surfaces, obtaining callus from intact seeds might prove to be difficult.

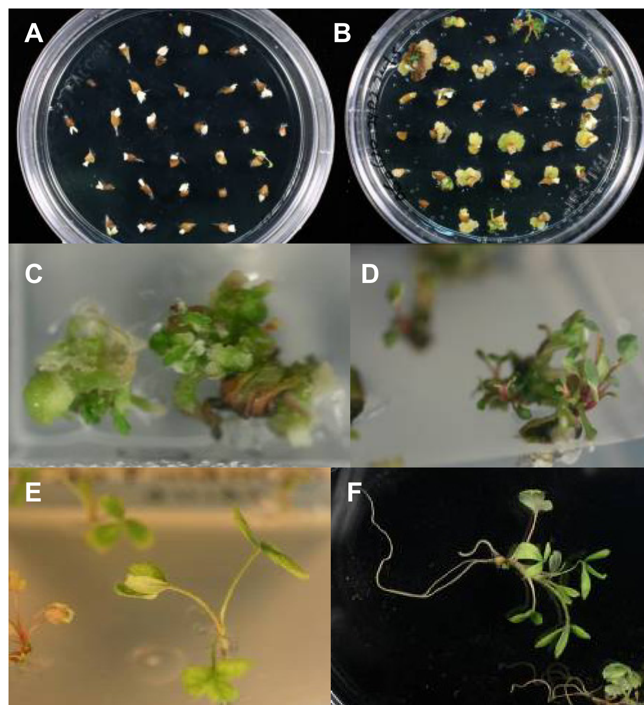

Fig. 1 Plantlet regeneration from *Rosa rugosa* immature seeds via organogenesis. (**A**) Immature seeds placed onto MS phytohormone-free medium after 27 days. (**B**) Immature seeds placed onto MS medium containing 2 mg/l 2, 4-dichlorophenoxy-acetic acid (2,4-D), and 1.5 mg/l *trans*-zeatin (ZT) after 27 days. Such callus with shoot primordia was transferred to MS phytohormone-free medium after 33 days. (**C**) Primordium of regenerated shoots from induction medium with 2 mg/l 2,4-D and 1.5 mg/l ZT at 37 days, followed by (**D**) shoots 115 days and (**E**) 150 days after transfer to phytohormone-free medium, respectively. (**F**) Regenerated shoots with roots at 178 days after transfer.

Table 1 Frequency of callus and callus plus shoot regeneration from immature seeds of *Rosa rugosa* having phytohormones applied during the callus induction stage.

Phytohormone (mg/l)	No. of seeds (mean ± S.E)	No. of seeds producing callus (mean ± S.E, %[a])	No. of seeds producing callus with ≥1 shoot primordium (mean ± S.E, %[b])
No phytohormone(s)	28	0	0
	30	0	0
	14	0	0
	26	0	0
	28	0	0
	(25.2 ± 2.9)	(0, 0)	(0, 0)
2,4-D[c] (2)	28	2	0
	30	10	0
	28	2	0
	27	2	0
	28	0	1
	(28.2 ± 0.5)	(3.2 ± 1.7, 11.3)	(0.2 ± 0.2, 0.01)
ZT[d] (1.5)	28	2	5
	30	1	8
	28	1	5
	27	1	3
	28	0	4
	(28.2 ± 0.5)	(1.0 ± 0.3, 3.5)	(5.0 ± 0.8, 17.7)
2,4-D (2), ZT (0.75)	28	16	4
	31	10	12
	28	14	8
	28	6	7
	(28.8 ± 0.8)	(11.5 ± 2.2, 39.9)	(7.8 ± 1.7, 27.1)
2,4-D (2), ZT (1.5)	28	19	3
	31	15	5
	18	9	5
	27	13	6
	28	15	0
	(26.4 ± 2.2)	(14.2 ± 1.6, 53.8)	(3.8 ± 1.1, 14.4)
2,4-D (2), ZT (3)	28	8	11
	31	6	21
	28	7	7
	27	8	6
	28	7	8
	(28.4 ± 0.7)	(7.2 ± 0.4, 25.4)	(10.6 ± 2.7, 37.3)

[a] No. of callus / No. of seeds × 100
[b] No. of callus with a shoot primordium / No. of seeds × 100
[c] 2,4-dichlorophenoxy-acetic acid
[d] *trans*-zeatin

As shown in **Table 1**, 2,4-D alone, except in one instance, induced undifferentiated calli rather than calli with at least one shoot primordium, whereas ZT alone generally induced calli with at least one shoot primordium rather than undifferentiated calli. For the explants treated with only ZT, induced shoots had only small (<1 mm) calli resulting in minimal amounts of cells available for dedifferentiation into shoots. To obtain transgenic rose shoots at a high frequency, it is desirable that callus grows enough of a degree that many undifferentiated cells are present.

The combination of 2,4-D (2 mg) and ZT (1.5 mg) was selected as the optimal shoot induction treatment for transformation studies. Regenerated shoots from induction medium with 2,4-D (2 mg) and ZT (0.75 mg) showed a higher level of undeveloped shoots, while those from 2,4-D (2 mg) and ZT (3 mg) exhibited shoot development with minimal initial callus development. The frequency of immature seeds with callus growth and at least one shoot primordium for the chosen treatment was 14.4% (19 shoot primordia per callus under ideal conditions) (**Table 1**). As shown in **Fig. 1**, several regenerated shoots were obtained from one callus cluster (12 shoots were obtained from 19 shoot primordia) and were easily separated (**Fig. 1B-E**). This value is 47.3 times higher for shoot recovery per explant than the embryogenic callus regeneration system in a previous report (Kunitake *et al.* 1993). Many of the shoots developed roots spontaneously on phytohormone-free MS medium, while the remainder without roots also successfully developed roots on MS medium with 0.1 mg/l NAA. The plants were

morphologically normal and were transferred into soil. Plastic wrap was used to elevate the humidity around the plantlets. The first plants withered and died, and we are currently exploring methods to optimize acclimatization. The reduction of humidity and/or elevation of light intensity might be too early. The introduction of *MASAKO* genes into *R. rugosa* using this system is also currently in progress.

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REFERENCES

Asano G, Tanimoto S (2002) Plant regeneration from embryogenic calli derived from immature seeds in miniature rose cultivar. 'Shortcake': somaclonal variation, cytological study and RAPD analysis. *Plant Biotechnology* **19**, 271-275

Cairns T (2000) *Modern Roses XI, The World Encyclopedia of Roses*, Academic Press, San Diego, 576 pp

de Wit JC, Esendam HF, Honkanen, JJ, Tuominen U (1990) Somatic embryogenesis and regeneration of flowering plants in rose. *Plant Cell Reports* **9**, 456-458

Hibino Y, Kitahara K, Hirai S, Matsumoto S (2006) Structural and functional analysis of rose class B MADS-box genes '*MASAKO BP*, *euB3*, and *B3*': paleo-type *AP3* homologue '*MASAKO B3*' association with p*etal* development. *Plant Science* **170**, 778-785

Ibrahim R, Debergh PC (2001) Factors controlling high efficiency adventitious bud formation and plant regeneration from in vitro leaf explants of roses (*Rosa hybrida* L.). *Scientia Horticulturae* **88**, 41-57

Kitahara K, Matsumoto S (2000) Rose MADS-box genes '*MASAKO C1* and *D1*' homologous to class C floral identity genes. *Plant Science* **151**, 121-134

Kitahara K, Hirai S, Fukui H, Matsumoto S (2001) Rose MADS-box genes '*MASAKO BP* and *B3*' homologous to class B floral identity genes. *Plant Science* **161**, 549-557

Kitahara K, Hibino Y, Aida R, Matsumoto S (2004) Ectopic expression of the rose *AGAMOUS*-like MADS-box genes '*MASAKO C1* and *D1*' causes similar homeotic transformation of sepal and p*etal* in *Arabidopsis* and sepal in *Torenia*. *Plant Science* **166**, 1245-1252

Kunitake H, Imamizo H, Mii M (1993) Somatic embryogenesis and plant regeneration from immature seed-derived calli of rugosa rose (*Rosa rugosa* Thunb.). *Plant Science* **90**, 187-194

Matsumoto S, Kitahara K (2005) MADS-box genes in rose: expression analyses of *AGAMOUS*, *PISTILLATA*, *APETALA3* and *SEPALLATA* homologue genes in the green rose. *Acta Horticulturae* **690**, 203-210

Murahige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Noriega C, Sondahl MR (1991) Somatic embryogenesis in hybrid tea roses. *Bio/Technology* **9**, 991-993

Rout GR, Debata BK, Das P (1991) Somatic embryogenesis in callus cultures of *Rosa hybrida* L. cv. Landora. *Plant Cell, Tissue and Organ Culture* **27**, 65-69

Svejda F (1974) Reproductive capacity of F_1 hybrids from *Rosa rugosa* and *chinensis* cultivars. *Euphytica* **23**, 665-669

Svejda F (1977) Breeding for improvement of flowering attributes of winter-hardy *Rosa rugosa* hybrids. *Euphytica* **26**, 697-701

Wen XP, Deng XX (2005) Plant regeneration from immature zygotic embryos of chestnut rose (*Rosa roxburghii* Tratt) through organogenesis. *Journal of Horticultural Science and Biotechnology* **80**, 643-649