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Induction of Meristematic Nodular Calli from Various Explants of *Lilium* spp. and Long Term Stability in Plant Regeneration Ability and Ploidy Level of the Calli.

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Abstract

Meristematic nodular calli (NOD) were successfully induced from various plant parts such as seeds, flower organs, shoot apices, stem segments and bulb scales of *Lilium* spp. on media containing 1 mg/ℓ picloram alone or in combination with 1 mg/ℓ cytokinin. In *L. longiflorum*, green friable callus (FC) was also induced from bulb scales. Suspension cultures established from both NOD and FC showed high proliferation rate. NOD retained high regeneration ability as well as stable ploidy level during the culture for more than 4 years, while the suspension culture of FC became tetraploid. Protoplasts were efficiently isolated from NOD in some species and cultivars after long term subculture in suspension cultures and the plants were successfully regenerated from the protoplasts of *L.* x *formolongi*.

1. Introduction

The genus *Lilium* involves many ornamental species and the lily cultivars have been economically important for cut flowers, potted plants and garden flowers [1]. The lily cultivars have commercially been propagated vegetatively by bulb scale cutting because of their heterozygous nature. As tissues excised from various parts of lily plant have been shown to possess regeneration ability, numerous studies have been conducted to examine the propagation potential of various tissues of *Lilium* spp. and hybrids *in vitro*. Consequently, tissue culture techniques are now widely used for producing commercially high quality lily plants [2-4].

For the breeding of lilies, cut-style pollination and embryo rescue techniques have been successfully used for producing interspecific hybrids [5, 6]. However, there are still some limitations in the application of these techniques for interspecific hybridization because of the sexual barriers existing between some combinations of the species. To overcome the limitations, somatic hybridization and genetic transformation are now considered to be useful. For the application of these biotechnologies, one of the useful approaches is the use of protoplast-plant system, by which somatic hybridization and direct gene transfer through electroporation or polyethylene glycol treatment will be achieved. Therefore, it is necessary to establish a plant regeneration system from protoplasts. In lilies, however, plant regeneration from protoplasts isolated from various sources, such as mesophyll cells, friable callus cultures and cell suspension cultures has been difficult [7, 8]. Recently, plant regeneration from protoplasts in *L.* x *formolongi* was sucessfully achieved by using meristematic nodular calli derived from seeds [8] and shoot apices [9] as donor sources of protoplasts. Therefore, NOD can be considered as a possible donor source of protoplasts with plant regeneration ability in many lily species and cultivars.

In the present study, we examined the necessary factors for the induction of NOD from various somatic tissues and seeds in various *Lilium* species and cultivars. Plant regeneration ability, ploidy level and efficiency of protoplast isolation of NOD were also examined after long term subculture.

2. Materials and Methods

2. 1 Plant materials

Various explants collected from the species and cultivars shown in **Table 2, 3** and **4** were used as materials for callus induction. Flower buds of *L.* x *formolongi* 'Akasu', *L. longiflorum* 'Georgia' and *L.* 'Gran Paradiso' were collected from the plants grown in a green house just before anthesis. After sterilization with 70% ethanol for 5 min, filaments, ovary and petals were carefully separated from the flower buds in a sterile chamber. Before plating to culture medium, the filaments were cut into 5 mm long-seg-

ments and the ovaries were also cut horizontally into about 1 mm thick slices. One-fourth long petal segments were excised from the base of the petals, sterilized with sodium hypochlorite (NaClO solution, 1% active chlorine) for 15 min and rinsed in sterile distilled water as described above. The petal base explants were further divided into 1×1 cm segments just prior to culture.

Seeds collected in the fields and purchased from the market were surface-disinfected with NaClO solution (1% active chlorine) containing a few drops of Tween 20 for 30 min, followed by washing twice with sterile distilled water.

In *L.* x *formolongi* 'Akasu', shoot apices of 0.5 cm long and stem segments of 1 cm long with node were excised from *in vitro* grown plants after removing the expanded leaves. Bulb scales were excised from *in vitro*-produced bulblets. Each bulb scale was horizontally placed on culture medium with abaxial side down.

2.2 *Media and culture conditions*

Basal medium used for callus induction and plant regeneration was 0.2% (w/v) gellan gum (Kelco, Division of Merck & Co.Inc., San Diego, CA)-solidified Murashige and Skoog (MS) medium [10] containing 3% (w/v) sucrose, to which PGRs were added. The pH of culture media was adjusted to 5.7 - 5.8 prior to autoclaving at 1.1 kg/cm^2 and 120°C for 20 min. The cultures were incubated under continuous illumination with daylight fluorescent lamps (40 μmol•m^{-2}s^{-1}) or dark condition at 20±1°C.

2.3 *Induction of meristematic noduiar callus*

Explants obtained from various sources were cultured on MS medium containing different kinds and concentrations of auxin (α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) or 4-amino-3,4,6-trichloropicolinic acid (picloram)) alone or in combination with cytokinin (N^6-benzylaminopurine (BA), kinetin N-(1,2,3-thiadiazol-5-yl) - N'- phenylurea (thidiazuron, TDZ)). After 2 months of culture, callus formation and characteristics of the calli were recorded. Compact calli with creamy-white or yellow coloration were selected as NOD. In addition, NOD-induction efficiency was compared among donor explants and genotypes.

2.4 *Establishment of suspension cultures of NOD and FC*

NOD were induced and multiplied for 2 months on basal medium containing 1 mg/ℓ picloram by subculturing monthly. Clusters of NOD showing vigorous growth were selected from plates and sliced into 3-5 mm in size. Then, aliquots of 2 g fresh weight (FW) of calli were transferred into 125 mℓ flasks each containing 40 mℓ of sterile-liquid basal medium with 1 mg/ℓ picloram. Cultures were kept on a rotary shaker (90-100 rev•min^{-1}) under continuous illumination with day light fluorescent lamps (40 μmol•m^{-2}s^{-1}) at 25°C. Subculture at 3 week-intervals was done by transferring 2 g FW of the calli to 40 mℓ of the same medium.

FC produced in *L. longiflorum* 'Georgia' were also transferred into liquid medium with the same composition and cultured under the same conditions as used for NOD, but without slicing. Suspension culture of FC was occasionally filtered with 1×1 mm-pore size metal mesh during the subculture period.

2.5 *Protoplast isolation from NOD and FC*

Protoplasts were isolated from 3 year-old NOD suspension culture of *L.* x *formolongi* and cultured according to the protocol reported by Mii *et al.* [8] and Godo *et al.* [9], with some modifications. NOD suspension cultures which had been subcultured in liquid basal medium supplemented with 1 mg/ℓ picloram at 3 week-intervals were used for protoplast isolation. One gram FW of calli was enzymatically digested with 10 mℓ filter-sterilized enzyme solution containing 2% Cellulase Onozuka RS, 0.5% Macerozyme R-10, 0.05% Pectolyase Y-23, 0.5 M glucose, 5 mM MES, 5 mM $CaCl_2$•$2H_2O$ and 1/2 MS medium (MS medium containing half-strength inorganic elements). After purification, protoplasts were cultured by embedding in 0.1% gellan gum-solidified 1/2 MS medium containing 0.5 M glucose and 1 mg/ℓ picloram at a density of 2×10^5 protoplasts/mℓ. The culture dishes each containing 2 mℓ aliquot of medium with protoplasts were sealed with Parafilm and incubated under dim light (1.2 μmol•m^{-2}s^{-1}) at 25°C. In addition, the yields and viability of protoplasts isolated from suspension culture of NOD or FC were also compared in *L.* x *formolongi* 'Akasu', and 'Raizan', *L. henryi* and *L. longiflorum* 'Georgia'.

2.6 *Plant Regeneration*

For the plant regeneration, NOD of several species and cultivars were transferred to 20×90 mm-Petri dishes containing 0.2% gellan gum-solidified MS media, in which strength of inorganic salts, concentration of sucrose, and the kind and concentration of plant growth regulator (PGR) were varied.

2.7 *Cytological study of NOD and NOD-derived plant*

Chromosome number of the plantlets regenerated from NOD was investigated in *L.* x *formolongi*. Root segments with root tips were excised from the plantlets and treated with 2 mM 8-hydroxyquinoline for 3

h, washed with water and hydrolyzed in 1 N HCl for 3 min at 60°C. After staining with 1% aceto-orcein, root tips were excised and squashed in a drop of 45% acetic acid. Chromosome number was counted under a microscope.

Ploidy level of the calli and the plants regenerated from the calli were tested by flow cytometry. Leaves of plants regenerated from NOD, FC, and protoplast-derived calli were chopped and stained with DAPI solution (10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 4 μg/mℓ 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI)). The solution containing nuclei was passed through a nylon mesh (40 μm) to remove the large cell debris and analyzed for DNA content using a flow cytometer (Partec CA II®, Germany). In order to examine the ploidy level of the calli after long term maintenance in suspension culture, suspension cultures of NOD of *L.* x *formolongi* 'Akasu' and FC of *L. longiflorum* 'Georgia', which had continuously been subcultured more than 3 years, were also analyzed to assess ploidy level.

3. Results

3. 1 Induction of NOD from various plant parts of Lilium

Efficiency of NOD formation was initially compared among various explants of *L.* x *formolongi*. As shown in **Table 1**, NOD could be induced from seeds, shoot apices, stem segments and bulb scales on media supplemented with auxin. In seed explants, NOD were formed from both the root and cotyledon part of the seedling after germination. For the other explants, NOD were induced from the cut end of explants that attached with culture medium. Among the auxins tested, picloram and 2,4-D gave the higher effect for inducing NOD than IBA and NAA. Picloram at 1-5 mg/ℓ was most suitable for NOD induction. Among the explants tested, those from flower organs showed poor response to any kind of auxins. Especially, ovary explants did not produce any calli in any of the media tested. However, efficiency of NOD induction in flower organs was increased by combining picloram with cytokinins at 1 mg/ℓ in *L.* x *formolongi* as well as *L. longiflorum* and *L.* 'Gran Paradiso' (**Table 2**). The percentages of explants inducing NOD in medium containing TDZ were slightly higher than those in media containing other cytokinins. By adding these cytokinins, shoot regeneration from the explants was also induced in these 3 genotypes.

The percentage of NOD induction from seeds on MS medium containing 1 mg/ℓ picloram was varied among the species (**Table 3**), although NOD were obtained in all of the species examined. NOD produced from seeds could successfully be multiplied on the same medium except for some species such as *L. medeoloides* and *L. pyrenaicum*, in which NOD proliferated poorly in the early period of culture and regenerated plantlets even on medium containing picloram. However, NOD of these 2 species started to proliferate after 3 months of culture with continu-

Table 1. Induction of meristematic nodular calli (NOD) from differentkinds of explants in *L.* x *formolongi* in MS medium containing various kinds and concentrations of auxin after 1 month of culture.

Auxin (mg/ℓ)		Percentage of explant producing NOD*1						
		seed	filament	ovary (slice)	petal base	shoot apex	stem segment	bulb scale
		(20)*2	(64)	(120)	(68)	(20)	(48)	(45)
IBA	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	5	0.0	0.0	0.0	0.0	0.0	4.2	0.0
	10	10.0	0.0(P)	0.0	0.0(P)	10.0	8.3	6.7
NAA	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	5	0.0	0.0	0.0	0.0(P)	0.0	0.0	0.0
	10	10.0	0.0(P)	0.0	0.0(P)	0.0(P)	8.3	13.3
2,4-D	1	15.0	0.0	0.0	13.2	10.0	10.4	17.7
	5	25.0	0.0	0.0	0.0	15.0	16.7	17.7
	10	10.0	0.0	0.0	0.0	0.0	4.2	6.7
picloram	1	40.0	12.5	0.0	16.2	20.0	37.5	44.4
	5	40.0	0.0	0.0	20.6	30.0	39.5	35.6
	10	25.0	0.0	0.0	0.0	20.0	29.2	11.1

*1 Most of the explants were obtained from 'Akasu', except seeds-explants which were obtained from 'Asama' and the data were expressed as the averages.

*2 No. of explants per treatment.

(P) : plant regeneration.

Table 2. Effect of cytokinins on the induction of meristematic nodular calli (NOD) and shoots from flower organs of three lilies.*1

Species	Explant	No. of explants per treatment	% Induction of NOD (shoot*2)			
			Cytokinin (1 mg/ℓ)			
			none	BA	Kinetin	TDZ
L. x *formolongi* 'Akasu'	filament	25	4(0)	20(40)	20(60)	36(40)
	ovary	60	0(0)	5(20)	5(50)	10(30)
	petal base	25	0(8)	8(50)	4(88)	12(60)
L. longiflorum 'Georgia'	filament	40	0(7.5)	5(60)	5(60)	5(50)
	ovary	60	0(0)	0(20)	0(30)	5(20)
	petal base	20	0(10)	5(30)	5(20)	10(20)
L. 'Gran Paradiso'	filament	25	0(0)	16(12)	16(36)	32(12)
	ovary	60	0(0)	10(40)	0(45)	10(30)
	petal base	25	0(0)	20(32)	24(50)	20(32)

*1 MS medium containing 3% sucrose and 1 mg/*l* picloram was used as a basal medium.

*2 Numbers in parentheses show the percentage of explants inducing shoots of 2 repeated experiments.

Table 3. Induction of meristematic nodular calli (NOD) from *Lilium* seeds after 2 months of culture on MS medium supplemented with 1 mg/ℓ picloram and 3% sucrose.

Species	No. of seeds tested	Induction of NOD (%)*
L. auratum	80	11.3
L. carniolicum	42	9.5
L. dauricum	29	20.7
L. formosanum	45	26.7
L. leichtlinii	30	10.0
L. martagon	35	11.4
L. medeoloides	28	14.3
L. pumilum	35	34.3
L. pyrenaicum	22	18.2
L. szovitsianum	24	38.1
L. willmottiae	30	40.0
L. x *formolongi*	60	40.0

* (No. of seeds with NOD/No. of seeds tested) ×100

ous subculture on picloram-containing medium at 1 month-intervals.

On MS medium containing 1 mg/ℓ picloram, bulb scales also produced NOD in most species and cultivars except for *L. auratum*, *L. japonicum*, *L. rubellum* and *L.* 'Star Gazer', which predominantly produced shoot primordia (**Table 4**). In these 4 lilies, shoot primordia eventually formed shoots, roots and bulblets even when the concentration of picloram was increased up to 10 mg/ℓ. The recalcitrant NOD induction in these species could be overcome by culturing the explants in the dark on medium supplemented with picloram at 5-10 mg/ℓ (**Table 5**).

NOD induced from various kinds of explants could further be multiplied on medium containing picloram at 1-5 mg/ℓ (**Fig. 1A**). Although NOD could be induced by 2,4-D in some genotypes of lilies, they showed less proliferation than NOD induced on picloram-containing medium. NOD suspension culture was well established after continuous subculture in liquid medium supplemented with 1 mg/ℓ picloram for 2 months (**Fig. 1B**).

3. 2 Plant regneration from NOD

NOD of all the genotypes examined showed almost 100% plant regeneration ability on the basal medium without supplementation of PGR in the early period after callus induction. In these cultures, each nodule had the ability to regenerate shoots and roots simultaneously. Plant regeneration was also achieved from NOD which had been subcultured for more than 2 years in all the lilies examined (**Table 6**). However, these NOD showed altered step of regeneration, *i.e.* shoot regeneration occured first and then roots were produced at the base of shoots. In these lilies, sucrose concentration and medium strength affected the plant regeneration from NOD and almost all of NOD regenerated plantlets on 1/2 MS medium containing 1.5% sucrose. High frequency plant regeneration of *L. longiflorum* was also obtained from FC which had been maintained as a suspension culture for 3 years (data not shown).

Yield of protoplasts was affected by the type of calli in *L. longiflorum* 'Georgia' (**Table 7**). The use of FC for protoplast isolation resulted in the low yield due to the breakage during enzyme treatment, whereas high yield and healthy protoplasts were obtained from NOD suspension culture (**Table 7**). High yield of protoplasts from NOD was also confirmed in *L.* x *formolongi* and *L. henryi*. However, the successful colony formation was only achieved in *L.* x *formolongi* 'Raizan' (**Fig. 2**), although both cell division and plating efficiency gradually decreased during subculture for 3 years (data not shown). These proto-

Table 4. Induction of meristematic nodular calli, green friable calli and shoot primordia from *in vitro* grown bulb-scales of *Lilium* spp. on MS medium supplemented with 1 mg/ℓ picloram and 3% sucrose.

Species or cultivar	No. of explants	Development of bulb-scale explants		
		NOD (%)	FC (%)	SP (%)
L. auratum	60	0.0	0.0	65.0
L. 'Corte d' Azur'	60	45.0	0.0	10.0
L. 'Gran Paradiso'	45	51.1	0.0	15.5
L. henryi	50	70.0	5.0	64.0
L. japonicum	40	0.0	10.0	70.0
L. longiflorum				
'Georgia'	65	0.0	69.2	10.8
'Gelria'	50	4.0	50.0	6.0
L. rubellum	60	5.0	10.0	15.0
L. 'Star Gazer'	45	0.0	0.0	46.6

Note: NOD: meristrmatic nodular calli, FC: green friable calli, SP: calli with shoot primordia.

Table 5. Effect of light condition on the induction of NOD from bulb scales in the recalcitrant species and cultivar of lily[*1].

Concentration of picloram (mg/ℓ)	No. of explants	Number of explants producing NOD					
		Light			Dark		
Species or cultivar		1	5	10	1	5	10
L. auratum	80	0.0 c[*2]	0.0 c	6.3 c	53.8 ab	70.0 a	43.8 b
L. japonicum	60	0.0 c	0.0 c	0.0 c	6.7 c	21.7 b	33.3 a
L. longiflorum	60	0.0 c	11.7 bc	41.6 a	6.7 c	23.3 b	36.7 a
L. rubellum	40	5.0 c	42.5 b	30.0 bc	20.0 bc	45.0 b	25.0 bc
L. 'Star Gazer'	50	0.0 c	0.0 c	0.0 c	22.0 bc	46.0 a	36.0 b

[*1] MS medium containing 3% sucrose and 0.2% gellan gum was used as a basal medium and the cultures were incubated under continuous light condition (22 μmol·m^{-2}·S^{-1}) or complete darkness.

[*2] Different letters within the same row show significant difference by Duncan's multiple range test at $p=0.05$.

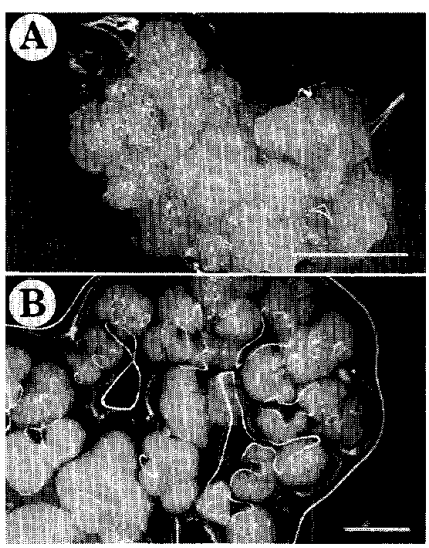

Fig. 1 Characteristics of meristematic nodular callus (NOD) and NOD suspension cultures of *Lilium* x *formolongi* 'Akasu'. (A) NOD induced from bulb scales on gellan gum-solidified MS medium supplemented with 1 mg/ℓ picloram; (B) NOD-suspension culture after continuous subculture in liquid medium with the same composition, bar in (A) and (B) = 0.5 cm.

Table 6. Effect of mineral nutrient strength and sucrose concentration on plant regeneration from NOD derived from various sources of lilies after 2 months of culture.

Species or cultivar	Age and source of NOD*2	Plant regeneration ability of NOD (%)					
		Medium composition*1					
		MS			1/2 MS		
		1.5%	3%	6%	1.5%	3%	6%
L. auratum	2 years (seed)	100.0	100.0	95.3	100.0	100.0	60.0
L. rubellum	3 years (seed)	90.3	89.3	98.5	100.0	90.3	45.5
L. x *formolongi* 'Raizan'	4 years suspension (shoot apex)	89.5	80.3	44.5	100.0	95.3	50.2
L. 'Gran Paradiso'	1.5 years (bulb scale)	82.5	80.5	35.8	100.0	90.5	46.5
L. henryi	2 years suspension (bulb scale)	90.5	70.5	38.2	98.5	92.8	65.4
L. longiflorum 'Georgia'	3 years (bulb scale)	100.0	98.5	45.0	100.0	98.8	70.0

*1 MS=MS basal medium, 1/2 MS=MS containing half-strength inorganic nutrients. Both media were supplemented with 1.5, 3 or 6% sucrose.

*2 NOD of 2×2 mm in diameter were used as the explants and at 125 to 150 explants per treatment.

Table 7. Protoplast isolation from suspension cultures of NOD and FC in four lilies.

Callus clone	Age of suspension culture before isolating protoplasts	Protoplast Yields ($\times 10^5$)
L. longiflorum 'Georgia'		
NOD	6 months	10.8±0.1
FC	5 months	0.1±0.1
NOD of *L.* x *formolongi* 'Raizan'	3 years	15.4±1.4*
NOD of *L.* x *formolongi* 'Akasu'	2 years	12.6±0.1
NOD of *L. henryi*	1 year	17.0±0.1

Protplasts were isolated from suspension culture 10 days after subculture, by treating 1 g calli with 10 m*l* of filter-sterilized enzyme solution containing 2% Cellulase Onozuka RS, 0.5% Macerozyme R-10, 0.05% Pectolyase Y-23, 0.6 M glucose, and half strength MS mineral salts for 5 h befor purifying protoplasts by centrifugation.

* Visible colonies were obtained.

plast-derived calli still retained high ability for plant regeneration (**Fig. 2**).

3.3 *Ploidy level of the suspension culture of NOD, FC and the plants regenerated from long term-subcultured NOD*

Cytological study on root tip cells in several plantlets regenerated from 4 year-old NOD suspension culture of *L.* x *formolongi* 'Raizan' revealed that the original chromosome number ($2n=24$) was maintained in these plantlets (**Fig. 3**). Results on the flow cytometric analysis showed that no polyploids were observed in these plants, including those of other species regenerated from NOD and from protoplasts of *L.* x *formolongi* (data not shown).

NOD cultures and NOD-derived plantlets of all the species examined had the same ploidy level as original plants. The stability in ploidy level of NOD suspension culture of *L.* x *formolongi* 'Akasu' was still maintained after 3.5 years. However, 3 year-old FC in suspension culture of *L. longiflorum* 'Georgia' showed tetraploidy (**Fig. 4**).

4. Discussion

In this study, 2 types of callus cultures with high plant regeneration ability, NOD and FC, were established from various parts of lily explants. The induction and multiplicaion of NOD, which is considered to be an efficient source of protoplasts in lily [8, 9], was mainly achieved on medium containing picloram. In the previous reports, other auxins such as IAA, NAA and 2,4-D have been used for callus induction of lilies in combination with cytokinin such as kinetin and BA

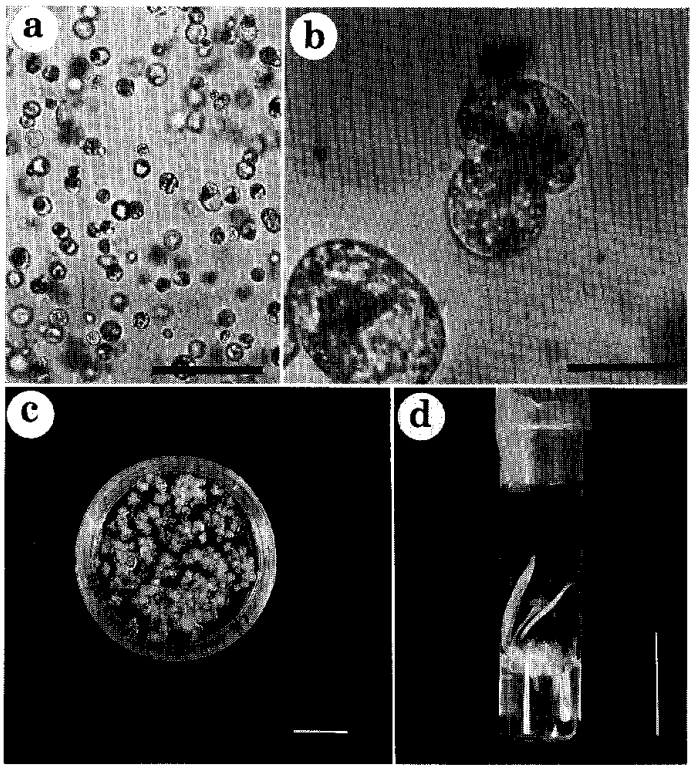

Fig. 2 Culture of protoplasts which were isolated from 3 year-old NOD suspension culture of *L.* x *formolongi* 'Raizan'. (a) Freshly isolated protoplasts, bar=350 μm; (b) Cell division of protoplasts 14 days after isolation and emmbedded in 0.1% gellan gum-solidified 1/2MS medium with 0.5M glucose and 1 mg/ℓ picloram, bar=110 μm; (c) Protoplasts-derived colonies after 3 months of culture, bar=1 cm; (d) Plant regeneration from protoplast -derived callus after 6 months of culture, bar=3 cm.

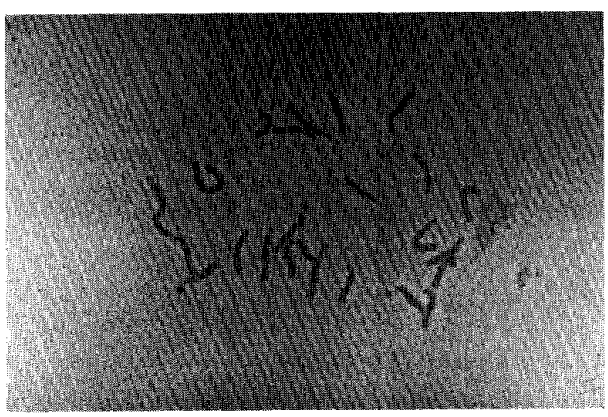

Fig. 3 Normal diploid ($2n=24$) chromosome number of *L.* x *formolongi* 'Raizan'plant regenerated from 4 year-old suspension culture after transferring the callus to MS medium with no supplement of plant growth regulator.

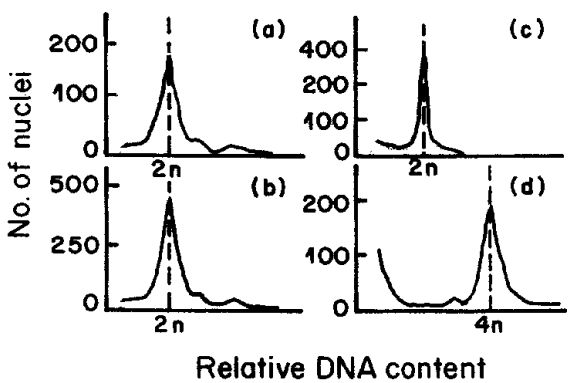

Fig. 4 Flow cytometric analysis on ploidy levels of plantlets and long term-subcultured NOD and FC suspension cultures; (a) *In vitro* plants of *L.* x *formolongi* 'Akasu' which were propagated by division (control); (b) NOD suspension culture of *L.* x *formolongi* 'Akasu' after 3.5 years; (c) *In vitro* bulb scale-propagated plants of *L. longiflorum* 'Georgia' (control); (d) FC of *L. longiflorum* 'Georgia' after maintaining for 3 years in suspension cultures.

[11-14]. The present study demonstrates the advantage of picloram over other auxins for callus induction in various species of lily. There also is the report of picloram-induced embryogenic calli in a related species of *Liliaceae* [15]. A relatively low concentration (1 mg/ℓ) of picloram was effective for the induction of NOD under light condition and for the maintenance of the calli without changing the characteristics during the subcultures. On the other hand, in species such as *L. auratum*, *L. japonicum*, and *L. rubellum*, which have been used as the genetic sources for producing 'Oriental hybrids', it was difficult to induce NOD under light condition and required dark condition for NOD induction.

In the present study, high plant regeneration ability of NOD without any change in ploidy level was confirmed even after subculture for several years. The plant regeneration was easily induced on simple medium containing low concentration of sucrose as suggested previously in *L.* x *formolongi* [9], although it was necessary to add cytokinin to the medium for some species such as *L. henryi* to promote vigorous shoot growth (data not shown). As NOD could successfully be multiplied in suspension cultures without loosing their high totipotency and chromosome stability, it is expected that the NOD culture system could be efficiently used for micropropagation and germplasm preservation of various lily species and cultivars if somaclonal variations are not detected in the regenerated plants. On the other hand, suspension culture of FC showed tetraploid level of DNA after 3 years of subculture, suggesting the instability of chromosome number in this type of callus even though it still retained high plant regeneration ability.

As Langeveld *et al.* [16] reported, lilies may have a possibility to be transformed with *Agrobacterium*. Although they used stem buds of *L.* 'Harmony' as a target for the transformation, NOD cultures will be more efficiently used than the stem buds for the genetic transformation with *Agrobacterium* as well as direct gene transfer by electroporation, particle bombardment, *etc.* because of the highly totipotent and meristematic nature. Plant regeneration ability of NOD-derived protoplasts of *L.* x *formolongi*, which was reported previously [8, 9], was also confirmed in the present study by using long term-subcultured NOD suspension culture. Therefore, NOD system will not only afford the propagation method but can also be used for somatic hybridization and genetic transformation. Plant regeneration from protoplasts derived from NOD culture in other lily species is now under investigation.

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