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Efficient Plantlet Regeneration via Callus Formation from Leaf Segment of *Lilium* Oriental Hybrid 'Casa Blanca'

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Abstract

Callus induction from a leaf explant has been achieved in *Lilium* Oriental hybrid 'Casa Blanca'. The highest frequency of callus induction was obtained on MS medium supplemented with 0.5 mg/L BA and 2.0 mg/L NAA after 2 months of culture. The cultures maintained continuously without change in color and type of callus when they cultured in the dark. Plantlet regeneration with a high frequency was achieved from induced calli on the same medium. A number of shoots are formed from one cluster of callus, and bulblets developed into intact plantlets after transfer to hormone-free MS medium. No phenotypic variations were observed among regenerants. Enhancement in plantlet regeneration via callus formation would be expected to facilitate the efficiency of transformation of this Oriental hybrid 'Casa Blanca'.

Key words: bulblet, lily, scale, shoot regeneration

Introduction

Lily constitutes a large share of the worldwide market for ornamental plants as one of the three major bulbous crops along with the gladiolus and tulip due to its large and attractive flowers (Tribulato et al. 1997). Moreover, its commercial applications are increasing recently.

There is growing interest in further improving some of its specific characteristics, such as flower color, disease or herbicide resistance, using gene transfer techniques. In conventional breeding, the lily is hampered by its heterozygous state and self-incompatibility among the species of the different *Lilium* groups (van Tuyl et al. 1990). The introduction of useful genes into lily has recently been reported by several researchers (Masahiro et al. 1993; Watad et al. 1998; Park and Park 2002). Most of the works had been done by biolistics system. Until this time, in case of *Lilium*, the genetic transformation using Agrobacterium is not routine system yet. A prerequisite for *Agrobacterium*-mediated transformation for the introduction of a useful gene into lily is the establishment of a regeneration system at a high frequency (Watad et al. 1998) using any type of explant. It is difficult to obtain a transgenic plant via organogenesis from a bulblet, because the extent of *Agrobacteria* in penetration into scales is limited and the frequency is too low for the integration of a foreign gene into the genomic DNA. And also, bulb scale propagation also makes it difficult to obtain a large number of regenerated bulblets in a short time (Arzate-Fernandez et al. 1997). It is well known that the lily can be regenerated via either direct bulblets formation from scale explants or regeneration from callus. Plant regeneration from bulb scales has been reported by a number of researchers (Niimi 1984; Nhut 1998; Nhut et al. 2001). Bulb scale propagation also makes it difficult to obtain a large number of bulblets from disease-free stocks in a short time (Arzate-Fernandez et al. 1997). Several researchers have reported successful plant regeneration from callus. Arzate-Fernandez et al. (1997) reported on efficient callus induction and plant regeneration from filaments with an anther in *Lilium longiflorum*. Also plantlet regeneration could be accomplished from callus which induced from the leaves in *L. longiflorum* (Stemberg et al. 1977), *L. leichtlinii* var. maximowiczii (Kato and Yoshinori 1977), *L. rubellum* (Niimi 1984). Adventitious shoot regeneration from leaf explants and stem nodes of *Lilium* spp. was recently

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reported by Bacchetta et al. (2003). These studies verified the effects of several combinations of growth regulators and various media under several culture conditions on callus induction using different explants. However, most of their works are cultivar-dependent, so it is needed optimal condition for regeneration from leaf explants of *L.* Oriental hybrid 'Casa Blanca' which is still the most popular in the world.

In this paper, we report on callus induction and shoot regeneration from leaf explants of L. Oriental hybrid 'Casa Blanca'. A convenient and routine system for plant regeneration from leaf explants will be useful for *Agrobacterium*-mediated transformation.

Materials and Methods

Leaf explants culture

Pathogen-free scales were detached from *L.* Oriental hybrid 'Casa Blanca' bulbs and washed with tap water to remove primary contaminants. The detached scales were surface-sterilized first in 70% ethanol for 30 s and then in a 10% sodium hypochlorite solution (NaClO) for 10 min, followed by three washes with sterilized distilled water. The sterilized scales were placed on MS medium (Murashige and Skoog 1962) supplemented with 30 g/L of sucrose, 100 g/L of inositol, 0.8% (w/v) agar for solidification, and adjusted to pH 5.8 with 1 N NaOH before autoclaving. The medium was dispersed in a petridish after autoclaving for 20 min at 121℃. The explants were maintained in a growth chamber at 25±1℃, with a 16 hr photoperiod and PPF (Photosynthetic Photon Flux) 320 μm$m^{-2}s^{-1}$. A number of bulblets were formed at the basal part of the scales after 2 months of culture. Each blublet was detached from the base of the scales and then transferred to MS medium containing 9% sucrose for bulblet enlargement. The cultures were maintained in growth chamber at 17±2℃ in the dark. After 4-5 months of culture, the bulblets (about 1.5-2.0 g in fresh weight) were transferred to fresh medium that was identical to the proliferation medium for induction of *in vitro* leaves. The explants were maintained in a growth chamber at 25± 1℃, with a 16 hr photoperiod and 320 μmm-2s-1 PPFD in the presence of a florescent lamp (Osrams). After 4 weeks of culture, the bulblets produced new leaves. Fully expanded leaves were cut into small pieces about 0.5 cm × 0.5 cm in size and used as plant material for plant regeneration through callus induction.

Callus formation and plantlet regeneration

The leaf segments were placed with the abaxial side touching the medium in the following different media. For induction of callus from leaf segments, the MS supplemented with 1.5 mg · L^{-1} BA plus 2.0 mg/L NAA, 2.0 mg/L zeatin plus 0.01 mg/L NAA plus 0.1 mg/L GA_3, 0.5 mg/L kinetin plus 0.1 mg/L NAA, 4.0 mg/L 2, 4-D, and N6 (Chu et al. 1975) supplemented with 2.0 mg/L 2,4-D media were used. The pH of the medium was adjusted to 5.8 prior to autoclave sterilization at 121℃ for 15 min. NAA and 2,4-D were added to the medium before autoclaving, while the other growth regulators were added to filter sterilizing the cooling autoclaved medium.

In a following experiment, leaves of oriental hybrid 'Casa Blanca' were cultured on MS medium supplemented with combination of 0.1, 0.5, 1.0, 1.5, 2.0 mg/L BA and 0.1, 0.5, 1.0, 1.5, 2.0 mg/L NAA. All media combinations ware tested in dark and light conditions with a 16 hr photoperiod and PPF 320 μm m^{-2} s^{-1} at 25℃. Fifty leaf explants were placed in each Petri-dish and three replicates were prepared for each treatment. Bulblets regenerated from callus were separated and transferred to MS medium containing 9% sucrose for enlargement.

Results and Discussion

The response of the explants to various media was variable, especially 2 months after culture initiation, as shown Fig. 1. The highest percentage of explants inducing callus was obtained on MS medium supplemented with 1.5

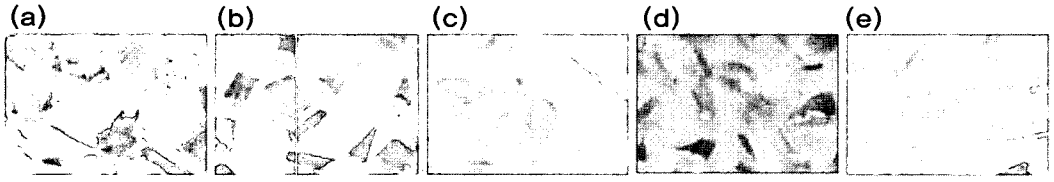

Figure 1. Callus induction from leaf explant on various media as follows; (a) MS medium supplemented with 1.5 mg/L BA and 2.0 mg/L NAA, (b) MS medium supplemented with 2.0 mg/L zeatin, 0.01 mg/L NAA and 0.1 mg/L GA_3, (c) MS medium supplemented with 0.5 mg/L kinetin and 0.1 mg/L NAA, (d) MS medium supplemented with 4.0 mg/L 2,4-D, (e) N6 medium supplemented with 2.0 mg/L 2,4-D. The leaves were cut into 0.5 x 0.5 cm^2 and the cut leaves were placed with the abaxial side touching the medium.

mg/L BA and 2.0 mg/L NAA in the dark (Fig. 1a). The explants on MS medium supplemented with 4.0 mg/L 2,4-D showed a frequency of 15.7% (Fig. 1d). In combinations of 2.0 mg/L zeatin, 0.01 mg/L NAA and 0.1 mg/L GA3, the mean value of induced callus from leaf explants was 2.5% (Fig. 1b). MS medium supplemented with 0.5 mg/L kinetin plus 0.1 mg/L NAA (Fig. 1c) and N6 medium supplemented with 4.0 mg/L 2,4-D (Fig. 1e) were used, but no callus formation occurred after either 2 or 4 months culture.

After 1 month, most of calli were typically formed on the edge of a leaf segment and the other part of surface that did not form callus turned a dark brown color, and subsequently necrosis. There are some cases of direct bulblet formation without callus formation. Otherwise, leaf explants in which non-formed calli turned a brown color after about a week and then withered. This result is in agreement with findings reported by Bacchetta et al. (2003) who reported that explants of 'Star Gazer' (Oriental cv.) appeared to be more responsive in a media containing auxins, especially in combination with BA, than those with only cytokinins. Niimi and Tsuyoshi (1979) also reported similar results for *L. rubellum*. Also, Nguyen et al. (2003) reported that leaf explants failed to form callus on any of the media tested on *Alocasia micholitiana*, otherwise petiole explants showed the earliest symptom of callus formation. It means that callus induction from different organs of same plant is very variable.

Morphological variations of the induced calli were observed and were categorized, as suggested by Nguyen et al. (2003). Type I calli were soft, brown in color with a smooth, wet appearing surface, finally becoming brown and necrotic. They usually were formed on medium supplemented with 2,4-D or kinetin (Fig. 2C). Type II calli were compact, nodular, white, bearing a high component of shoot regeneration (Fig. 2A, B). They were induced well on the medium with BA and NAA. Induced calli of type II were transferred to medium with same combinations of BA and NAA, and incubated in the dark or light to investigate their potential for the continuous propagation of the same calli. They continuously propagated with no change the color and callus type when cultured in the dark. New white calli also developed from the callus mass (Fig. 2A). Under light conditions, the calli developed a light green color within 2 weeks and became dark or blue green in the following weeks of culture (Fig. 2B). Similar results were reported for some other monocots. In the case of *Alocasia*, the frequency of compact callus explants initiating shoots is low and a wider range of auxins and cytokinins can be used (Nguyen et al. 2003). Tribulato et al. (1997) reported that most callus formed was compact on media containing NAA or 2,4-D at low concentrations and friable callus was obtained on media containing only low concentrations of dicamba or picloram. Whitish friable callus has been previously reported for *L. longiflorum* (Loffler et al. 1990) and *L. japonicum* (Mizuguchi et al. 1994) on NAA- plus BA-containing media. Otherwise, compact yellow callus formation has been reported to occur using picloram as a plant growth regulator in *L. formolong* (Mii et al. 1994; Godo et al. 1996), *L. longiflorum*, Oriental hybrids and Asiatic hybrids (Famelaer et al. 1996). Plant regeneration in the Oriental hybrid 'Casa Blanca' from a leaf segment proved to be controlled by the concentration of cytokinin/auxin in the medium. Our results show that a medium supplemented with optimal ratio induced highest frequency of callus and bulblet formation from a whitish friable callus.

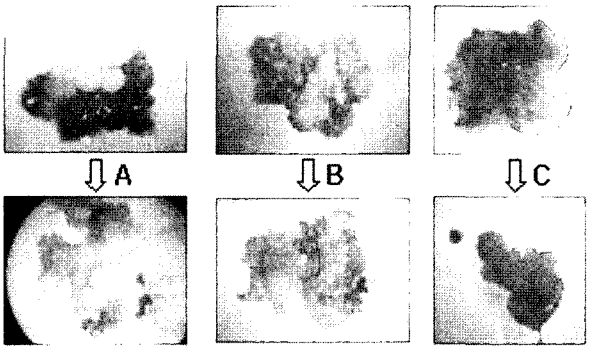

Figure 2. Different types of callus induced from leaf segments. Compact and friable callus was continuously propagated in the dark (A). Otherwise, under light conditions, the callus turned a light green color (B). The callus has a smooth and wet looking surface, non-embryogenic callus, became brown and necrotic (C).

Our results show that the optimal concentration of the combination of BA and NAA in MS medium is the most effective for callus induction from leaf segment of Oriental hybrids 'Casa Blanca' (Fig. 3). An auxin/cytokinin combination is also a prerequisite for leaf regeneration in many other species, such as Rubus (Cousineau and Donnelly 1991), Gentiana (Hosokawa et al. 1996) and Paulownia spp. (Dimps et al. 1996; Lo 1997), Gypsophila (Zucker et al. 1997) and *Lilium* (Bacchetta et al. 2003). To improve the frequency of shoot induction from callus, in the following experiment, leaf segments of 'Casa Blanca' were cultured on MS medium supplemented with the combination of 0.1, 0.5, 1.0, 1.5, 2.0 mg/L NAA and 0.1, 0.5, 1.0, 1.5, 2.0 mg/L BA. When concentrations lower than 0.5 mg/L NAA with no BA presents the frequency of callus formation was nil or poor. For example, in combinations of 0.1 mg/L NAA and 0.1, 1.0, 1.5 mg/L BA did not lead to callus or shoot production. We also did not observe any shoot regeneration

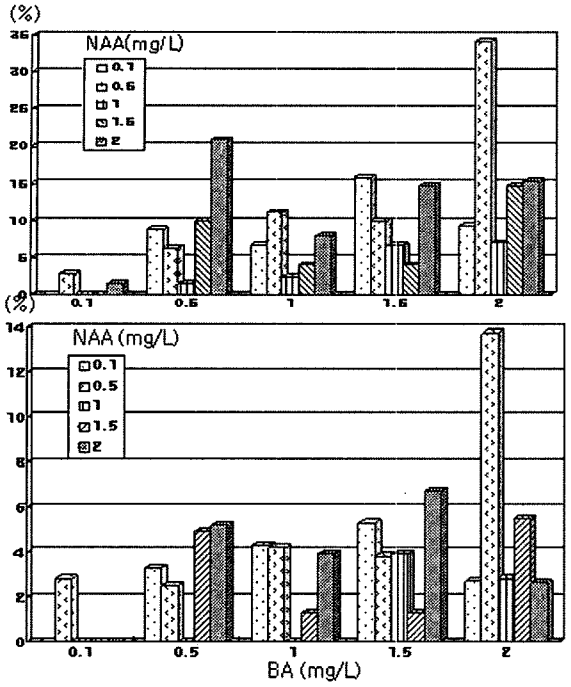

Figure 3. Effect of BA and NAA on callus induction (upper) and shoot regeneration (lower) from leaf of *L.* Oriental Hybrid 'Casa Blanca'.

via direct differentiation in that medium. The percentage of callus induction of L. Oriental hybrid 'Casa Blanca' leaf ranged from 37.0% to 1.4%, and shoot regeneration from callus ranged from 19.2% to 2.4%. Based on Baccetta et al. (2003) reported, the percentage of shoot regenerating explants of 'Star Gazer' ranged from 83.3% to 12.5% on half-strength MS medium with 0.1 mg/L BA and 1.0 mg/L IAA. Otherwise, in the case of leaf explants of cv. 'Pollyanna', the highest number of shoots was observed on a medium supplemented with 1.0 mg/L BA or TDZ, and the number of shoots was 4.8% and 4.2 %, respectively (Bacchetta et al. 2003). It is indicated that responses to callus formation, shoot regeneration are variable on cultivars within *Lilium* spp.

Figure 4 shows each stage of development from callus induction to bulblet formation through shoot regeneration in MS medium containing 0.5 mg/L BA and 2.0 mg/l L NAA. About after 2 months, excess of shoots are formed from the one cluster of callus, approximately 10 shoots. After that, shoots were isolated to a single one and were then transferred to a hormone-free MS medium. Each shoot developed into a completely regenerated plantlet, 2 months after transfer to the hormone-free medium. All of the shoots successfully rooted and formed their fresh leaves, resulting in an intact plant.

Plants regenerated from callus did not differ phenotypically from *in vitro* cultured Oriental hybrid 'Casa Blanca' plantlets (data not shown). Arzate-Fernandez et al. (1997) also reported that no morphological and genetic variation was found among filamentous derived callus-regenerated plantlets as the mother plant. The lily is known to be genetically stable, since there are no reports describing any variations among regenerated plantlets and Qu et al. (1988) also pointed out that genetic variations arising in somatic tissues are not easily observed in lily.

The transformation of monocot plants is now feasible using both biolistic and *Agrobacterium*-based methods (Tingay et al. 1997; Horvath et al. 2000; Trifonova et al. 2001). Liau et al. (2003) presented a protocol for establishing a routine transformation via *Agrobacterium tumefaciens* for an important *Oncidium* Orchid cultivar, and Murray et al. (2003) reported the production of transgenic barley plants following the infection of immature embryos with *Agrobacterium tumefaciens.* They obtained transformed callus using hygromycin resistance as a selectable marker and either GFP or GUS as a reporter.

During a genetic transformation it is essential that the regeneration occur at the wounded site at a high frequency (Watad et al. 1998). Plant regeneration through leaf-derived callus is easier, compared to bulbs or anther-derived callus. Stemberg et al. (1977) obtained plant regeneration from the leaves of *L. longiflorum*, Niimi and Tsuyoshi (1979) and Niimi (1984) reported the induction of adventitious buds from leaf explants in *L. rubellum.* Recently, Bacchetta et al. (2003) identified the explants and established cultural

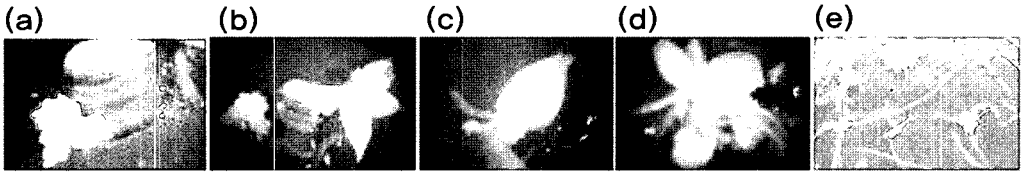

Figure 4. A series of cultures for the development of a regeneration system. (a) Callus induction from leaf explant, (b) regeneration of bulblets from callus cluster, (c) Isolation of a single bulblet, (d) roots developing from a regenerated bulblet, (e) A well developed plantlet 2 months after transfer to a hormone-free MS medium.

conditions for shoot formation from leaves and stem nodes.

In this study, calli were successfully induced from leaf explants of the Oriental hybrid 'Casa Blanca' and a procedure for plant regeneration from the induced calli has been developed. Based on result, future experiments will be directed at transforming the useful gene into the leaf explants of L. Oriental hybrid 'Casa Blanca' using *Agrobacterium*-mediated transformation.

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References

Arzate-Fernandez AM, Nakazaki T, Okumoto Y, Tanisaka T (1997) Efficient callus induction and plant regeneration from filaments with anther in lily (*Lilium longiflorom* Thunb.). Plant Cell Rep 16: 836-840

Bacchetta L, Remotti PC, Bernardini C, Saccardo F (2003) Adventitious shoot regeneration from leaf explants and stem nodes of *Lilium*. Plant Cell Tiss Org Cult 74: 37-44

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18: 659-668

Cousineau JC, Donnelly DJ (1991) Adventitious shoot regeneration from leaf explants of tissue cultured and greenhouse-grown raspberry. Plant Cell Tiss Org Cult 27: 249-255

Dimps RC,Chong-Jin G, Prakash P, Kumar (1996) High frequency adventitious shoot regeneration from excised leaves of *Paulownia* spp. cultured *in vitro*. Plant Cell Rep 16:204-209

Famelaer I, Ennik E, van Tuyl JM, Meijer H, Creemers-Molenaar C (1996) The establishment of suspension and meristem cultures for the development of a protoplasts regeneration and fusion in lily. Acta Hort 414: 161-168

Godo T, Matsui K, Kida T, Mii. M (1996) Effect of sugar type on plant regeneration from protoplasts isolated from shoot tip-derived meristematic nodular cell clumps of *Lilium* x. *formologi* hort. Plant Cell Rep 15:401-404

Horvath H, Huang JT, Wong O, Kohl E, Okita T, Kannangara CG, von Wettstein D (2000) The production of recombinant proteins in transgenic barley grains. Proc Natl Acad Sci USA 97: 1914-1919

Hosokawa K, Nakano M, Oikawa Y, Yamamura S (1996) Adventitious shoot regeneration from leaf, stem and root explants of commercial cultivars of *Gentiana*. Plant Cell Rep 15:578-581

Holme IB, Krogstrup P, Hansen J (1997) Embryogenic callus formation, growth and regeneration in callus and suspension cultures of *Miscanthus* x *ogiformis* Honda Giganteus' as affected by praline. Plant Cell Tiss Org Cult 50: 203-210

Kato Y, Yoshinori Y (1977) Plantlet formation and differentiation of epidermal tissue in green callus culture from excised leaves of *Lilium*. Phytomorphology 27: 390-396

Lo KH, Giles KL, Sawhney (1997) Acquisition of competence for shoot regeneration in leaf discs of *Saintpaulia ionantha* x *confusa* hybrids (African violet) cultured in vitro. Plant Cell Rep 16: 416-420

Loffler HJM, Mouris JR, van Harmelen MJ (1990) In vitro selection for resistant against *Fusarium oxysporum* in lily; prospects, Lily Yearb N Am Lily Soc 43: 56-60

Mii M, Yuzawa Y, Suetomi H, Motegi T, Godo T (1994) Fertile plant regeneration from protoplasts of a seed propagated cultivar of *Lilium* x *formolongi* by utilizing meristematic nodular cell clumps. Plant Sci 100: 221-226

Mizuguchi S, Ohkawa M, Ikekawa T (1994) Effects of naphthaleneacetic acid and benzyladenine on the growth of white callus and formation of bulblets from callus induced from mother-scale of *Lilium japonicum* Thunb. J Jpn Soc Hort Sci 63: 131-137

Murashige T, Skoog F (1962) A revised medium for rapid and bioassay with tobacco tissue culture. Physiol Plant 15:473-497

Murray F, Brettell R, Matthews P, Bishop D, Jacobson J (2004) Comparison of Agrobacterium-mediated transformation of four barley cultivars using the GFP and GUS reporter genes. Plant Cell Rep22: 397-402

Nguyen TPT, Yukio O, Hiroshi O (2003) Callus induction and plantlet regeneration in ornamental *Alocasia micholitziana*. Plant Cell Tiss Org Cult 73: 285-289

Nishihara M, Ito M, Tanaka I, Kyo M, Ono K, Irifune K, Morikawa H (1993) Expression of the β-glucuronidase gene in pollen, nicotiana rustica, and poeny by particle bombardment. Plant Physiol 102: 357-361

Niimi Y, Tsuyoshi O (1979) In vitro bulblet formation from leaf segments of lilies, especially *Lilium rubellum* Baker. Sci Hortic 11: 379-389

Niimi Y (1984) Bulblet productivity of explants from scales, leaves, stems and tepals of *Lilium rubellum* Baker. Sci Hort 22: 391-394

Nhut DT (1998) Micropropagation of lily (*Lilium longiflorum*) via in vitro stem node and pseudo-bulblet culture. Plant Cell Rep 17:913-916

Nhut DT, Fukai BVS, Tanaka M, Van TT (2001) Effects of activated charcoal, explant size, explant position and sucrose concentration on plant and shoot regeneration of *Lilium longiflorum* via young stem. Plant Growth Regul 33: 59-65

Park IH, Park HS (2002) Lily pollen growth in vitro and

Agrobacterium-mediated GUS gene transformation via vacuum-infiltration. J Plant Biotech 4:151-154

Qu Y, Mok MC, Mok DWS, Stang JR (1988) Phenotypic and cytological variation among plants derived from anther cultures of *Lilium longiflorum* Thumb. Proc SD Acad Sci 56: 152-158

Robinson K, Firoozabady E (1993) Transformation of floriculture crops. Sci Hortic 55: 83-99

Stemberg NG, Chen CH, Ross JG (1977) Regeneration of plantlets from leaf cultures of *Lilium longiflorum* Thunb. Proc SD Acad Sci 56: 152-158

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S, Brettell R (1997) *Agrobacterium tumefaciens*-mediated barley transformation. Plant J 11: 1369-1376

Tribulato A, Remotti PC, Loffler HJM, van Tuyl JM (1997) Somatic embryogenesis and plant regeneration in *Lilium longiflorum* Thunb. Plant Cell Rep 17: 113-118

Trifonova A, Madsen S, Olesen A (2001) *Agrobacterium*-mediated transgene delivery and integration into barley under a range of in vitro culture conditions. Plant Sci 161: 871-880

van Tuyl JM, van De Sande K, van Dien R, van Holsteijin HMC (1990) Overcoming interspecific crossing barriers in *Lilium* by ovary and embryo. Acta Hort 266: 317-322

Watad AA, Yun DJ, Matsumoto, Niu X, Wu Y, Kononowicz AK, Bressan RA, Hasegawa PM (1998) Microprojectile bombardment-mediated transformation of *Lilium longiflorum*. Plant Cell Rep 17: 262-267

Zucker A, Ahroni A, Shejtman H, Vainstei A (1997) Adventitious shoot regeneration from leaf explants of *Gipsophila paniculata* L. Plant Cell Rep 16: 775-778