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Thin Cell Layers: Developmental Building Blocks in Ornamental Biotechnology

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ABSTRACT

The capacity to control developmental and morphogenetic processes *in vitro* has always been the fascination and primary focus of many ornamental tissue culture studies. Thin cell layers or TCLs were first used to control the development of flowers, roots, shoots and somatic embryos in tobacco pedicels. Since those studies over 30 years ago, TCLs have been successfully used in the micropropagation of many ornamental plant species whose previous *in vitro* morphogenetic pathways were not clearly defined using conventional methods. TCL technology focuses on the size and origin of the explant, which, when appropriately chosen, serves as a fine-scale developmental block for regeneration and transformation. This review highlights the fundamentals of TCLs, and their application in ornamental plant micropropagation and transformation.

Keywords: Arabidopsis, chimerism, chrysanthemum, lily, plant growth regulators, polyamines, somatic embryogenesis, thin cell layer, tobacco, transformation

Abbreviations: **AA**, aminoglycoside antibiotic; **BA**, 6-benzyladenine; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **lTCL**, longitudinal TCL; **Kin**, kinetin; **NAA**, α-naphthalene acetic acid; **OG**, oligosaccharide; **PA**, polyamine; **PGR**, plant growth regulator; **PLB**, protocorm-like body; **SAM**, shoot apical meristem; **TCL**, thin cell layer; **TSL**, threshold survival level; **tTCL**, transverse TCL

CONTENTS

INTRODUCTION

One of the fields of study that has fascinated plant physiologists and developmental biologists are the mechanisms governing plant growth and development, which is also one of the most fundamental and applied areas of modern plant

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Review

Table 1 Species induced to flower *in vitro* using TCL explants.

Plant	PGR	Other conditions	Explant source	% Flower[1]	Var[2]	Reference
Begonia rex	BA/Z	Epidermis	Leaf vein	100	No	Chlyah and Tran Thanh Van 1975
Cichorium intybus	NAA/BA/IAA	-	Flower stalk	100	No	Li *et al*. 1996
Cymbidium sp.	BA/Kin	Light:dark (1:1)	SAM	7	No	Tran Thanh Van 1974b
Dianthus caryophyllus	NAA/TDZ/BA	Light	Petal/sepal	100	No	Sankhla *et al*. 1995
Geum urbanum	IBA/Kin	Oligosaccharins	SAM	100	No	Tran Thanh Van 1974a
Melia azedarach	None	-	Hypocotyl	5	No	Handro and Floh 2001
Nicotiana plumbaginifolia	IBA/Kin	Oligosaccharins	Floral branch	100	No	Hanh and Tran Thanh Van 1980
Nicotiana tabacum	IAA/Kin	Continuous light	Anther	100	No	Bridgen and Veilleux 1988
N. tabacum	None	T-DNA *rolD*	Leaf epidermis	100	Yes	Mauro *et al*. 1996
N. tabacum	BA/NAA	*Myo*-inositol	Pedicels	100	No	Rajeevan and Lang 1987
N. tabacum	IBA/Kin	3% sucrose; light	Flower stalk	100	No	Tran Thanh Van 1973b, 1974a
Odontoglossum sp.	None	Low light intensity	SAM	100	No	Tran Thanh Van 1974a
Odontonia sp.	None	Low light intensity	SAM	100	No	Tran Thanh Van 1974a
Petunia hybrida	IAA/Kin	-	Internode/pedicel	100	No	Mulin and Tran Thanh Van 1989
Pseudocarpus tetragonolobus	IAA/BA	-	Different organs	unspecified	No	Hanh *et al*. 1981
Silene coeli-rosa	-	*Myo*-inositol	0.5 mm shoot tip	50	No	Donnison and Francis 1993
Tourenia fournieri	IAA/Kin	Epidermis only	Stem epidermis	unspecified	No	Chlyah 1974

[1] Represents the maximum percentage recorded
[2] Variability observed in either anthesis, or total flower morphology

research. Higher plants develop from a single-celled zygote into a multicellular organism through co-ordinated cell divisions, and when this process occurs without patterning, disorganized callus tissue is produced (Teixeira da Silva and Nhut 2003a). Body organization is generated by two distinct processes: the primary organization of the body, represented by the seedling, and including the shoot and root meristems, is laid down by embryonic pattern formation, while the meristems then take over to produce the adult plant during post-embryonic development. Plant organs are composed of ordered collections of various cell types differing in their shape, size, position, function and DNA content. Cell division, separation and morphogenesis are intimately connected, and ontogenesis is determined by the genome and is influenced by external signals. Correct control of the cell-division cycle is required for the elaboration and execution of developmental programmes, while patterning genes determine overall architecture of the plant. Cell division is a critical activity during the growth and development of a plant providing the building blocks for the differentiation of *in vitro* thin cell layers (TCLs) or *in planta* tissues and organs, and contributes to the overall size of the individual.

This review serves to introduce to readers the concept of a TCL, its model systems, and applications in ornamental plant tissue culture and genetic transformation. Moreover TCL technology is a solution to many of the issues currently hindering the efficient progress of ornamental and floricultural crop improvement, since it addresses the issue of plant breeding at the first stage of the problem, regeneration, and using one of the most basic developmental building blocks, cells. Since the regeneration of specific organs may be effectively manipulated through the use of TCLs, in conjunction with specific controlled *in vitro* conditions and exogenously applied plant growth regulators (PGRs), many problems hindering the improvement of *in vitro* plant systems are potentially removed, as has been reviewed by Nhut *et al*. (2006). We will demonstrate, through this short, but comprehensive review, the truth of this claim, and extend that the application and success of this system in floricultural and ornamental crops is widespread. The possibilities of this tool for crop (ornamental and floricultural) improvement are endless, and go tightly hand-in-hand with molecular and genetic engineering tools. Moreover this system provides a means of mass propagation of a species of interest, and has thus profound potential economic spin-offs. The TCL system could in theory provide a simple but efficient micropropagation system for developing countries with limited resources and facilities.

Although beyond the scope of this review, TCL technology has also been effectively used in the micropropagation of vegetable, leguminous, and medicinal plants, including *Amaranthus edulis* (amaranth), *Beta vulgaris* (sugar beet), *Brassica napus* (oilseed rape), *Lupinus* spp. (lupin), *Panax ginseng* (ginseng), and *Phaseolus vulgaris* (common bean) (Nhut *et al*. 2003b); cereals and grasses, including *Digitaria sanguinalis* (large crabgrass), *Oryza sativa* (rice), *Sorghum bicolor* (sorghum), and *Zea mays* (corn) (Nhut *et al*. 2003c); fruits, including *Musa* sp. (banana), *Citrus* spp. (orange, lemon, mandarin), *Poncirus trifoliata* (trifoliate orange), *Cocos nucifera* (coconut palm), *Garcinia mangostana* (mangosteen), *Lycopersicon esculentum* (tomato) (Nhut *et al*. 2003d); woody plants, including *Bambusa* spp. and *Dendrocalamus* spp. (bamboo), *Manihot esculenta* (cassava), *Pinus radiata* (Monterey pine), *Paulownia fortunei* (paulownia), *Populus* spp. (poplar), *Pseudotsuga menziesii* and *Sequoiadendron* spp. (conifers), *Garcinia mangostana* (garcinia/kokum), and *Rosa* spp. (rose) (Nhut *et al*. 2003e, 2003f). Also of great importance to floral developmental scientists is the capacity to induce flowering *in vitro*, although this has thus far had limited success in ornamental plants (Teixeira da Silva and Nhut 2003b; Sudhakaran *et al*. 2006; Taylor *et al*. 2006; **Table 1**).

TCLs: CONCEPT CHECK

The thin cell layer (TCL) system consists of explants of a small size excised from different plant organs (stems, leaves, inflorescences, flower primordia or floral organs, cotyledons, hypo-/epicotyl, apical zone or embryo), either longitudinally (lTCL), or transversally (tTCL). lTCLs contain either only one tissue type, such as a monolayer of epidermal cells, or two tissue types, such as the two-layered TCLs of tobacco, composed of epidermal cells and subepidermal chlorenchyma (Altamura *et al*. 1993), or 4-6 cell layers, i.e., epidermis, subepidermis, cortical parenchyma, and collenchyma, e.g. in tobacco (see below). tTCLs include a small number of cells from different tissue-types: epidermal, cortical, cambium, perivascular and medullar tissue, parenchyma cells (Tran Thanh Van 1980).

TCL systems allow for the isolation of specific cell or tissue layers, which, depending on the genetic state and epigenetic requirements, and in conjunction with strictly controlled growth conditions (light, temperature, pH, PGRs, media additives and others) may lead to the *in vitro* induction of specific morphogenic programs. The capacity of a TCL to enter a program depends upon a number of factors, including correct signal perception and transduction, the capacity of the internal genetic machinery to respond and react to these signals and in the latter case, may depend on the physiological state and origin (tissue and organ) of the TCL, stress factors applied to the TCL, and apoptotic or gene silencing states of the tissue or cells within it. Should

this first phase be achieved then cells within the TCL may redifferentiate into organs exhibiting correct developmental patterns leading to phenotypically normal organ architecture (Teixeira da Silva and Nhut 2003a). TCLs have been used as a model system to analyse the influence of ethylene (Biondi *et al*. 1998) and jasmonates (Biondi *et al*. 2001; Capitani *et al*. 2005) on cell division, and meristemoid and organ formation, and to validate the role of polyamines and polyamine biosynthetic genes as biochemical and molecular markers of differentiation (see below).

Within the TCL system the morphogenic and developmental pathways of specific organs – derived from other specific or non-specific cells, tissues or organs – may be clearly directed and controlled. Moreover, it allows for the study of cytological, physiological, biochemical and molecular changes occurring in a particular morphogenic program. This strict regulation of the morphogenic pathways will allow for, *inter alia*, the controlled production of somatic embryos and their subsequent use as synthetic seeds, or as mass propagation units. It would also enhance the production capacity of secondary metabolites and pharmaceuticals through transgenic organ cultures, such as those produced by *Agrobacterium rhizogenes*, or by other autotrophic bioreactor plant cultures. The efficiency of genetic transformation is clearly enhanced as a result of cell and/or tissue specificity of gene insertion, and the subsequent successful and controlled regeneration of transformed tissue. Moreover, the use of TCLs allows for the potential production of *in vitro* flowers (independent of or in conjunction with photoperiod, vernalization and/or other environmental cues), and can be used as an explant source, or potentially as a new, long-term ornamental propagule, eliminating the problem of post-harvest deterioration.

MODELING DEVELOPMENT USING TCLs

Three plants (*Dendranthema grandiflora*, *Lilium longiflorum*, *Nicotiana tabacum*) have been intensively studied in terms of morphogenesis, organ differentiation and development using TCLs, and consequently have been considered the model plants for TCL systems. However, recently morphogenesis and organogenesis from TCLs were also studied in *Arabidopsis thaliana* and *Antirrhinum majus* (snapdragon), i.e., the eminent model plants in the study of the genetic control of plant development.

Arabidopsis thaliana

A. thaliana is undoubtedly the best model plant for studies on the genetic and biochemical control of *de novo* flower formation and of adventitious rooting (rhizogenesis) because mutants over-producing flowers and roots have been isolated and because of the knowledge that has been acquired on its genome (The Arabidopsis Genome Initiative 2000). Interestingly, the molecular biology, genetic, and morpho-anatomical studies *in planta* may be coupled with *in vitro* culture analyses, because the tissues of *Arabidopsis* are capable of producing organs (Ozawa *et al*. 1998; Falasca *et al*. 2004, and references therein), and embryos (Ikeda-Iwai *et al*. 2002) also through *in vitro* culture.

Zygotic embryogenesis is the first important event in plant development, and somatic embryogenesis is essential for artificial seed production. Indeed, many *Arabidopsis* genes related to both zygotic and somatic embryogenesis have been already isolated (Ikeda-Iwai *et al*. 2002, and references therein). lTCLs are one of the explant types that we tested for obtaining somatic embryos in *Arabidopsis*. In fact, lTCLs, composed of epidermis and two layers of cortical parenchyma, were excised from the hypocotyl of very young seedlings (Columbia ecotype) and cultured on MS medium with 1 μM α-naphthalene acetic acid (NAA) under continuous darkness. The explants were able to produce embryos, however the response was poor because the embryogenic competence was rapidly lost and necrotic events occurred widely. No improvement in embryo formation was obtained by changing the ecotype, and the hormonal and photoperiodic conditions (Altamura, unpublished results).Thus, even if lTCLs seem promising, to date the most efficient way for maintaining high embryogenic competence over long periods in *Arabidopsis* remains the culture of immature zygotic embryos (Ikeda-Iwai *et al*. 2002).

Adventitious rooting *in vitro* provides a suitable

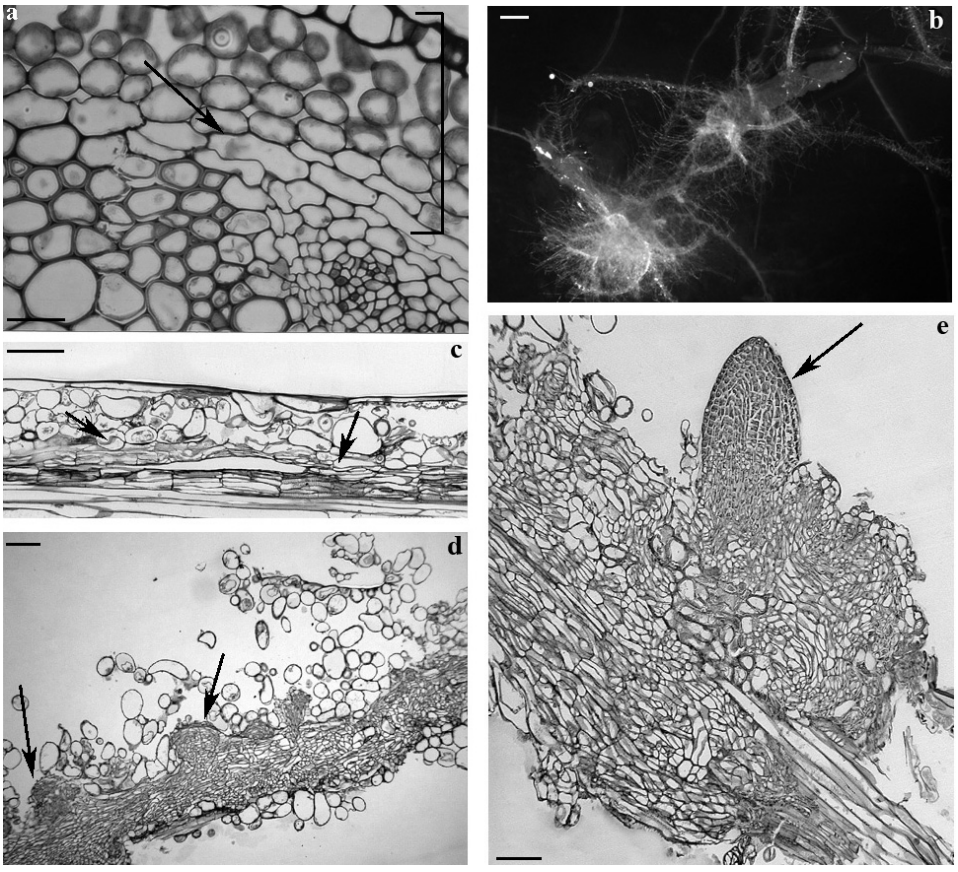

Fig. 1 Rhizogenic response in *Arabidopsis thaliana* (Wassilewskija ecotype) lTCLs cultured in continuous darkness on MS medium ($CaCl_2$ at 0.6 mM) supplemented with 10 μM IBA and 0.1 μM kinetin. (**A**) Detail of a transection of an inflorescence stem internode showing the tissue composition of the explant at day 0 (bracket). The arrow shows the stem endodermis, i.e., the innermost layer of the explant (bar = 10 μm). (**B**) Macroscopic rooting response on two explants at culture end (day 30, bar = 1 mm). (**C**) Formation of endodermal derivatives with meristematic features (arrows), and exfoliation events in the cortex (day 6, bar = 30 μm). (**D**) exfoliated cortex with growing root meristemoids (arrows; day 8, bar= 50 μm). (**E**) elongating root primodium produced by the endodermal derivatives (day 12, bar = 50 μm). (Further details in **Falasca *et al*.** 2004).

system for studying this developmental process under controlled conditions with the aim of identifying the genes and tissues involved, and for transferring the acquired information to root-recalcitrant micro-cuttings of economical value. *Arabidopsis* is useful for these studies because mutants producing an excess of adventitious roots have been isolated (Falasca *et al*. 2004 and references therein), and because genes involved in post-embryonic (i.e., lateral and adventitious) root formation have been isolated (Falasca and Altamura 2003, and references therein). Ozawa *et al*. (1998) induced rhizogenesis from hypocotyl segments of *Arabidopsis* using a two-phase method, i.e. callus induction on a callus inducing medium (CIM), followed by root induction on a root inducing medium (RIM). Unlike these authors, Falasca *et al*. (2004) experimented on lTCLs, consisting of epidermis, cortex, and endodermis (**Fig. 1A**), excised from the stem internodes of *Arabidopsis* (various ecotypes), culturing them with either a mono-phase or a two-phase method. The best results in terms of both rhizogenic explants and number of roots per explant were obtained with a mono-phase method requiring indole-3-butyric acid or IBA (10 μM) and kinetin (0.1 μM) under continuous darkness for 30 days (**Fig. 1B**), and with 0.6 mM $CaCl_2$. Various concentrations of $CaCl_2$ were tested because it is known that exogenous Ca^{2+} is required for various morphogenic/organogenic processes, and may change the hormone-induced organogenic response, e.g. in tobacco lTCLs (Cousson and Tran Thanh Van 1993). The success of the mono-phase method allowed us to study rhizogenesis *ab initio* and *in continuum*, thereby avoiding any alterations in tissue response caused by the stress of medium transfer, and without pre-conditioning the cells to callus induction, as occurs in a CIM/RIM culture. The histological analysis showed that rhizogenesis occurred as a pure programme, i.e. in the absence of competition of other organogenic processes (**Fig. 1B**), and that the stem endodermis (**Fig. 1A**, arrow) was the only tissue involved in proliferation (**Fig. 1C**, arrows) and the formation of root meristemoids (**Fig. 1D**, arrows), from which root primordia developed (**Fig. 1E**, arrow). Furthermore, calcium, supplied at concentrations ranging from 0.3 mM to 3 mM, positively interacts with the hormones, promoting both the early and the late phases of the rhizogenic programme (Falasca *et al*. 2004).

Not only roots and embryos, but also flowers may be obtained through *in vitro* culture of *Arabidopsis* TCLs. However, differently from rhizogenesis, *de novo* flower formation needs to be optimized. It is a goal for developmental biologists for various reasons: 1) a lot of floral organ mutants have been isolated in *Arabidopsis*; 2) genes controlling flowering time, organ identity and senescence have been cloned; 3) techniques for genetic engineering and transformation are available. To know the mechanisms underlying flowering, and to manipulate flower formation and development in this model plant, might open the way to important applications in ornamental plants.

Indeed, Altamura and co-workers obtained flowers and inflorescences from *Arabidopsis* lTCLs (unpublished results). The tissue composition of the explant was the same as that used in rhizogenesis (**Fig. 1A**), as was the physiological stage of the donor plant at the time of excision, i.e. plants with flowers and siliques. *De novo* formation of single flowers and inflorescences was obtained in various ecotypes, and occurred even under continuous darkness, however better results were obtained when explants were exposed to the same photoperiodic requirement of flower induction *in planta*. To date, unlike tobacco (see below), a protocol allowing the realization of a pure flowering programme in *Arabidopsis*, i.e. the formation of only flowers/inflorescences on the explant, has not yet been obtained. In fact, even under the best inductive conditions (NAA, at 1-2 μM, plus kinetin, at 1-2 μM), mixed responses are observed, i.e., flowers associated with vegetative buds on the same sample, and this limits the quantity of *neo*formed flowers and their protrusion from the explant (Altamura, personal communication).

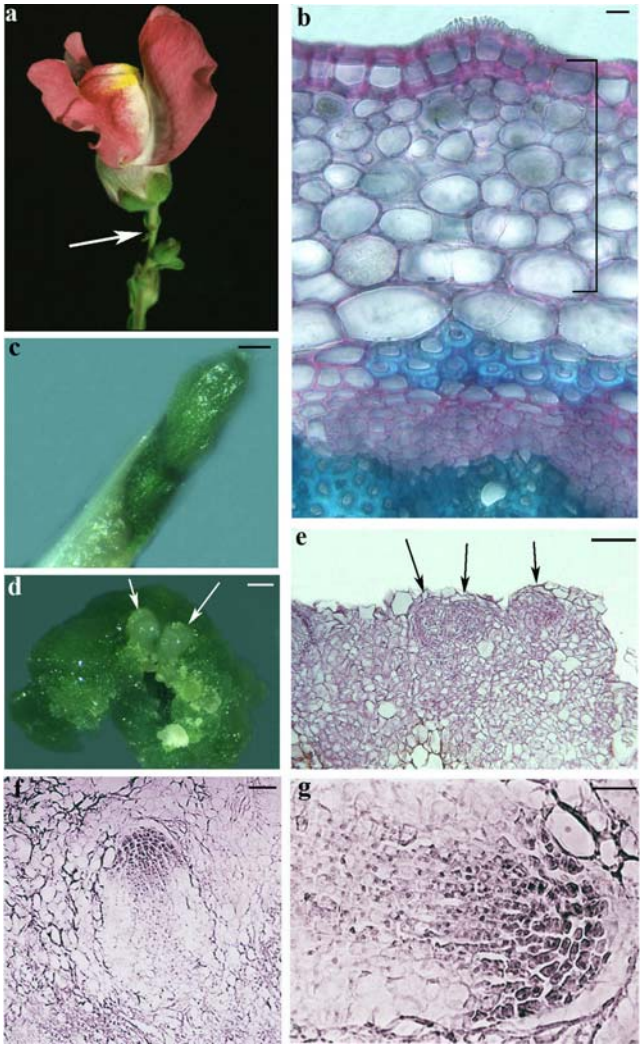

Fig. 2 Organogenic response in *Antirrhinum majus* (snapdragon) lTCLs cultured for 30 days under long-days on MS medium supplemented with 1 μM NAA and 1 μM benzyladenine. (**A**) Snapdragon flower. The arrow shows the excision site of the explants on the floral pedicel. (**B**) Detail of a transection of the pedicel internode showing the tissue composition of the explant at day 0 (bracket, bar = 10 μm). (**C**) Appearance of the macroscopic callus on an explant (day 7, bar = 1 mm). (**D**) An lTCL at culture end (day 30) showing macroscopic callus proliferation and two floral domes (arrows). (**E**) Detail of an explant section showing the morphology of callus at the histological level and the meristemoids (arrows) at its surface (day 22, bar = 100 μm). (**F**) Histological detail of an explant at culture end, showing a root primordium surrounded by callus cells (bar = 100 μm). (**G**) Another root primordium at higher magnification (bar = 10 μm).

Antirrhinum majus (snapdragon)

Antirrhinum majus (**Fig. 2A**), an ornamental species in some countries, is another model plant for the study of the flowering process, and many genes isolated in this species have homology with those of *Arabidopsis*. However, to date it has been more difficult to obtain *de novo* flower formation from snapdragon TCLs than from those of *Arabidopsis* (see above). Indeed, Altamura and co-workers obtained flower neoformation from snapdragon TCLs, but the response was sporadic. In fact, lTCLs, consisting of epidermis and cortical parenchyma (**Fig. 2B**), were excised from floral pedicels (**Fig. 2A**, arrow) by the authors, and cultured under various hormonal and environmental conditions, using both a mono-phase and a two-phase method (see above). Macroscopic callus appeared early in culture on the explants (**Fig. 2C**), the amount increasing exponentially over time, and negatively affecting organogenesis. In fact, the

macroscopic formation of flowers was poor at the end of the culture period (day 30), and the flower developmental process was arrested at the dome stage (**Fig. 2D**, arrows). Histological analysis confirmed that most of the superficial meristemoids (**Fig. 2E**, arrows) were blocked in development, while some roots formed completely within the callus, being, however, unable to protrude from the explant (**Figs. 2F, 2G**) (Altamura and co-workers, unpublished results).

Dendranthema grandiflora (chrysanthemum)

Chrysanthemum, one of the top three global, economically-important ornamental plants will serve as the main model ornamental plant in this review.

PGRs TCLs have been used to study the effect of numerous media additives (carbon source, antibiotics, PGRs, *inter alia*) and conditions on regeneration and morphogenesis in chrysanthemum. The controlled production of roots, shoots or somatic embryos could be achieved when stem internode tTCLs were placed on various media (**Fig. 3**; Teixeira da Silva and Fukai 2003a). More comprehensive reviews on chrysanthemum also highlights these studies in which the use of TCLs (primarily stem tTCLs) as the choice explant for tissue culture and micropropagation, has also been used for the genetic transformation of this species (Teixeira da Silva 2003d; Shinoyama *et al*. 2006). Despite the regeneration of whole plants (primarily adventitious shoots without an intermittent callus phase, and a prerequisite for any transformation

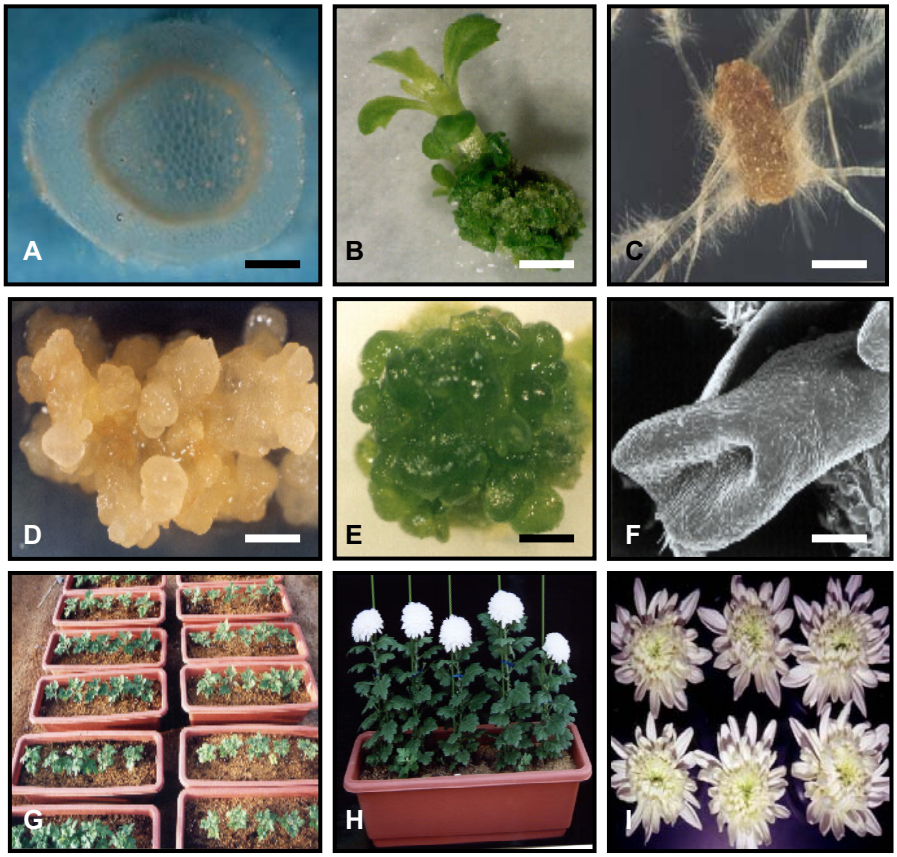

Fig. 3 Chrysanthemum: TCL from *in vitro* organogenesis to *ex vitro*. (**A**) Control spray-type *in vitro Dendranthema grandiflora* Lineker stem tTCL; (**B**) adventitious shoot formation from *in vitro D. grandiflora* Shuhou-no-chikara stem tTCL on MS + 2 mg/l BA and 0.5 mg/l NAA; (**C**) adventitious root formation from *in vitro D. grandiflora* Shuhou-no-chikara stem tTCL on MS + 10% (v/v) coconut water, 4 mg/l 2,4-D, 0.1 mg/l NAA, 1 mg/l IBA or 1 mg/l IAA; (**D**) embryogenic callus induction from *in vitro D. grandiflora* Shuhou-no-chikara stem tTCL on MS + 0.1 mg/l 2,4-D or 1 mg/l IAA in the dark or (**E**) in the light; (**F**) scanning electromicrograph of a single somatic embryo; (**G, H**) greenhouse acclimatization of standard-type *D. grandiflora* Shuhou-no-chikara following treatment with different carbon sources, polyamines or filter paper and subsequent testing of flowering differences (**I**). Bars: 1 cm = 2 mm (**B, C**), 500 µm (**D, E**), 220 µm (**F**).

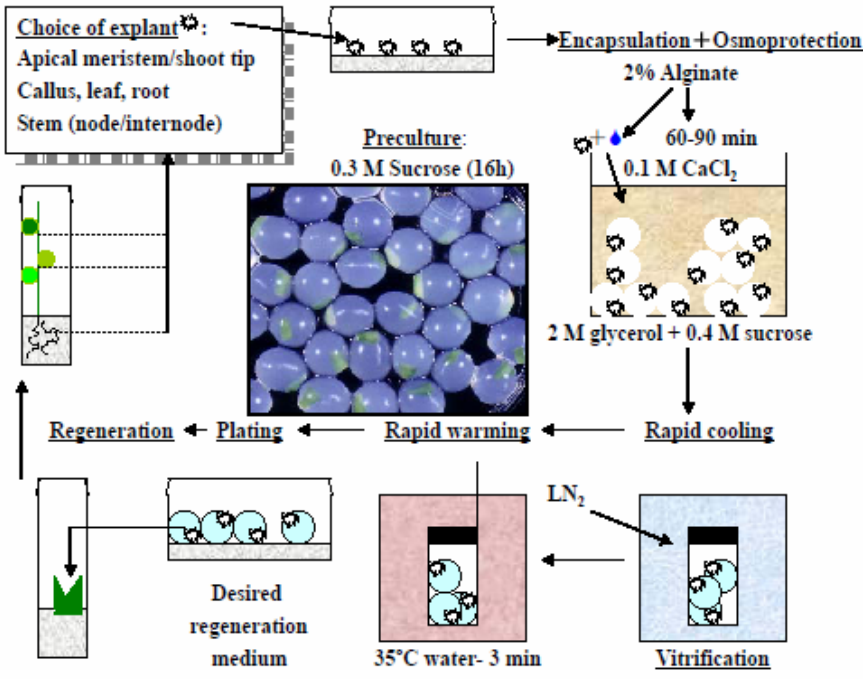

Fig. 4 A generalized scheme for the production of "syn-seed" (centre photo) by a mixture of encapsulation-dehydration and cryopreservation. After choosing a desirable target explant, it is pre-cultured on a sucrose-supplemented medium, encapsulated and osmoprotected using a 2% alginate solution. Following rapid cooling (vitrification) for the desired period of time, rapid warming is conducted and alginate balls are plated onto the desired regeneration medium.

system) from the tissue culture of various explant sources (**Table 1**), few histologically confirmed reports on somatic embryo-genesis in *Dendranthema* exist. A mixed organogenic response (i.e., shoots, roots, callus and embryos forming together) or multiple-organ formation is as a result of the cellular heterogeneity present in the initial explant source but this can be overcome by the use of thin cell layers (TCLs) in conjunction with a single PGR application (Teixeira da Silva 2003c; **Fig. 3**). The *de novo* formation of roots, with or without wounding (Teixeira da Silva 2003a, 2004b; **Fig. 3**) may allow this procedure to be used for cryopreservation, artificial seed production (root "synseeds"; **Fig. 4**), secondary metabolite production (root-specific) and improvement of the genus through genetic engineering. Neither the choice of carbohydrate source or concentration had an effect on somatic embryogenesis, although somatic embryogenic cultures developed better in the dark (Teixeira da Silva 2004a; **Fig. 3**). Numerous studies have been completed on the effect that a number of factors and media additives have on chrysanthemum TCL morphogenesis. To further enhance the medium-dependence of explants, tTCLs, composed of several tissue types, but which are normally too small to separate, as in the case of chrysanthemum were used in the experiments. In most, if not all *in vitro* morphogenetic studies as described below, flow cytometric analyses certified that even though low levels of endopolyploidy (8C cells) could be detected in embryogenic callus cultures, resulting shoot and root cultures, *in vitro* and greenhouse plantlets did not exhibit any endopolyploidy, that is 2C and 4C cells only (**Fig. 5**).

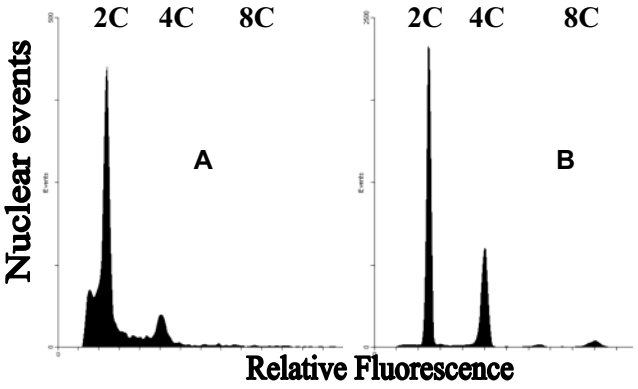

Fig. 5 Analysis of control and TDZ-induced chrysanthemum callus cells derived from tTCL cultures by flow cytometry. Histograms of control *in vitro Dendranthema grandiflora* 'Shuhou-no-chikara', exhibiting 2C and 4C peaks (**A**), and TDZ-induced embryogenic callus cultures exhibiting up to 8C peaks (**B**), i.e. endopolyploidy. Each histogram represents the average of three measurements with a total cell count of ≥5,000 cells, and a CV ≤ 2%. Methodology detailed in **Teixeira da Silva *et al.*** (2003).

Filter paper Despite the wide use of filter paper in tissue culture, only one report truly addressed its direct effect on plant tissue culture and *in vitro* growth and morphogenesis (Teixeira da Silva 2003b). In that study the effect of filter paper on *in vitro* growth and morphogenesis of chrysanthemum tTCLs was examined, focusing on the buffering effect it has on the phytotoxicity of antibiotics; that study further explored the effect that filter paper had on chrysanthemum *in vitro* growth and morphogenesis (callus, shoot, root or somatic embryo formation) when placed on specific morphological programme media. Whatman #1 and Advantec filter paper positively stimulated organogenesis and buffered the phytotoxic activity of aminoglycoside antibiotics (AAs) but Whatman #3 filter paper negatively impacted chrysanthemum organogenesis.

Carbon source Various carbon sources, selective agents for positive selection systems, were tested on chrysanthemum stem explants (Fukai 1986) or stem TCL morphogenesis (Teixeira da Silva 2004a). The rationale behind those experiments lay in the fact that the plant cannot use or metabolise all carbon sources effectively, and thus those could be used as limiting factors to regeneration, growth and development. Sucrose, glucose and fructose were shown to support metabolic growth in 'Lineker' and 'Shuhou-no-chikara' (Teixeira da Silva 2004a). Using this principle it was possible to establish threshold survival levels (TSLs; the level at which a TCL does not differentiate independent of the increase in carbon source) in response to varying concentrations of different carbon sources, since when using a non-antibiotic marker gene, a low level of nutrient medium is utilized, making the TCL highly dependent (more heterotrophic) on the carbon source. Since the shoot regeneration capacity of chrysanthemum is severely hampered by the presence of antibiotics in the selective caulogenic medium (Teixeira da Silva and Fukai 2001), which, despite optimisation of shoot production in the caulogenic program, is disturbed at higher antibiotic concentrations, the search for alternative (positive) selection systems using other carbon sources may benefit the outcome and efficiency of chrysanthemum genetic transformation. TSL levels could be achieved in 64% of the carbon sources tested, enhancing their potential, provided that genes coding for their respective degrading enzymes can be cloned into a vector system.

Polyamines Polyamines (PAs) are regarded as PGRs implicated in the control of cell division cycle, growth, differentiation, and developmental processes such as flowering, and adventitious rooting (Teixeira da Silva 2002a). The involvement of PAs in *in vitro* morphogenesis, in particular *de novo* vegetative bud, floral bud and root formation, has been extensively investigated using several approaches: a) analysis of changes in endogenous PA titres and metabolic pathways (Torrigiani *et al*. 1987; Scaramagli *et al*. 1995); b) treatment with PA biosynthetic inhibitors (Altamura *et al*. 1991b; Torrigiani *et al*. 1993), and c) application of exogenous PAs (Kaur-Sawhney *et al*. 1990). When using putrescine, spermine, spermidine and cadaverine on chrysantheumum tTCLs, Teixeira da Silva (2002a) showed that depending on the choice of PA, either a growth-supporting or growth-inhibiting activity was observed in different *in vitro* morphogenic programmes (callogenic, caulogenic, rhizogenic, somatic embryogenic). Even though all PAs detrimentally affected caulogenesis and somatic embryogenesis, shoots that were harvested from the former were not significantly different from controls, not showing altered morphology and flowering when greenhouse-acclimatized. The use of PAs (spermine, spermidine and putrescine), however, positively stimulated rhizogenesis, while cadaverine reduced the rhizogenic response.

Aminoglycoside antibiotics Aminoglycoside antibiotics (AAs) are still the most common selective agents in genetic transformation experiments both in chrysan-themum and almost every other plant species, the most common being the use of kanamycin A, gentamycin or G-418 as a selective agent for transgenic plants harbouring the *nptII* gene (Teixeira da Silva 2002b). Most AAs had a negative effect on *in vitro* growth and morphogenesis (shoot and root formation) of chrysanthemum TCLs. The effect of the AA concentration on plant morphogenesis and explant survival depended on the size of the explant, the choice of explant source, the timing of infection by *A. tumefaciens* and selection pressure in genetic transformation. In separate experiments on the effect of other antibiotics on TSL values, a gradient of phytotoxicity was shown: bialaphos® > chloramphenicol > rifampicin > streptomycin > minomycin > ampicillin > penicillin G = penicillin V (Teixeira da Silva *et al*. 2003). Other studies (Teixeira da Silva and Fukai 2001, 2003b) showed the importance that *Agrobacterium* selective agent (carbenicillin, cefotaxime or vancomycin) has on maximizing *Dendranthema* SRC, while minimizing phytotoxicity and explant mortality. In the case of cefotaxime, shoot regeneration capacity increased (but not

significantly) when added at 250 mg/l.

Nicotiana tabacum (tobacco)

Tobacco is one of the most well studied plants, and since the inception of the term and concept of a TCL (Tran Thanh Van 1973), it has become the model system on which all other TCL studies have been based. Tobacco TCLs are generally longitudinal strips of tissue (lTCLs) excised from the stem or floral branches, 5-6 cell layers thick, and comprising the epidermis, subepidermal chlorenchyma and cortical parenchyma. Organs arise from the sub-epidermal layer (Altamura *et al*. 1991a). Four morphogenic programs, direct flower formation, direct root formation, direct bud formation, and callus without organogenesis, can be induced (Tran Thanh Van and Trinh 1986). This is made possible by varying the concentration of carbohydrates and/or PGRs, and light conditions. The biological activity of cell wall derived oligosaccharides (OGs) was fully demonstrated by showing that they can modify the morphogenic processes in tobacco TCLs (Eberhard *et al*. 1989; Bellincampi *et al*. 1996). Indeed, a shift from floral to vegetative bud differentiation was possible by the addition of OGs to the medium, suggesting that OGs released by PGR-treatment of TCLs (Tran Thanh Van *et al*. 1985) or by pH (Cousson *et al*. 1989) can act as signaling molecules. Other factors controlling morphogenesis in TCLs could be carried out by controlling light and sugar concentration, and ionic composition of the culture medium and pH (Cousson and Tran Thanh Van 1992; Tran Thanh Van *et al*. 1985). The flower program can only be induced on TCLs excised from floral branches and not from the base of the stem (Tran Thanh Van 1973). There is a more comprehensive review on tobacco tissue culture and TCL applications (Nhut *et al*. 2003a) while the effectiveness of TCLs for tobacco regeneration and transformation using four gene introduction methods was demonstrated by Teixeira da Silva (2005).

Lilium longiflorum (Easter lily)

Lilies are fast becoming one of the most important bulbous crop species globally. The traditional asexual propagation of *Lilium* spp. by bulb scales as well as the lack of efficient micropropagation systems for species within the *Lilium* genus prompted extensive studies of TCL as a tool and solution for these shortcomings (reviewed in Nhut *et al*. 2001a). Since TCLs have been used extensively to study lily differentiation, and with the successful manipulation of all morphogenic programs, it has been considered as a model system. In an attempt to test the effect of tTCL explant source (receptacle (**Fig. 6A**), stem node and internode, pseudo-bulblet, leaf) together with different factors such as sucrose concentration, explant position, activated charcoal (AC) and PGRs on the mass propagation of *Lilium* was studied (Nhut *et al*. 2001b). For all the studies on bulblet formation using TCL methods, a MS/2 medium supplemented with 1-2.7 µM NAA or 10 µM indole-3-butyric acid (IBA) and sucrose at concentrations from 20-30 g/l was used for the rooting of shoots, bulblets and pseudo-bulblets. Plantlets obtained in the light developed well on this medium and were subsequently transferred to the greenhouse, with a 90-100% survival rate. When young leaf explant tTCLs (0.3 mm) were excised and cultured on MS medium supplemented with 3% sucrose and 2 µM BA combined with 6 µM NAA (Nhut 1998), pseudo-bulblets formed, mainly on the adaxial surface. Shoots regenerated from pseudo-bulblet tTCLs using forchlofenuron (CPPU), while a maximum of 15 bulblets can be obtained from one pseudo-bulblet tTCL. At low sucrose concentrations (2-4%) shoots were obtained, but at high concentrations (6-9%) bulblets formed (Bui *et al*. 1999a; Nhut 2001c; Nhut *et al*. 2002b). When receptacle tTCLs were used, buds appeared within 3 weeks of culture, but not in the ovary or flower stalks (Nhut *et al*. 2001a).

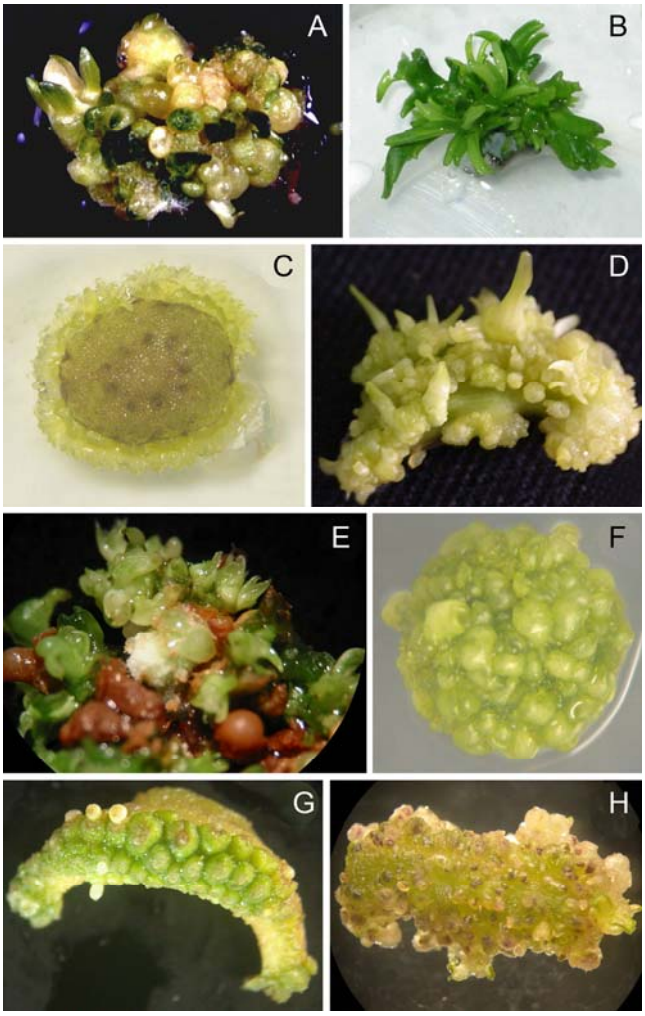

Fig. 6 Application of TCL culture in several ornamental and allied species. Shoot regeneration from (**A**) receptacle tTCL of *Lilium longiflorum*; (**B**) tea young stem tTCL; (**C**) *Begonia* petiole tTCL; (**D**) *Gladiolus* flower stalk lTCL; (**E**) Passion fruit young stem tTCL; (**F**) *Citrus* young stem tTCL; (**G**) *Gerbera* petiole tTCL; (**H**) *Gloxinia* young stem lTCL.

These buds continued to develop into bulblets. Stem node (2-3 mm) tTCL sections from plantlets derived from shoot tips of dormant bulbs formed pseudo-bulblets, which developed into the flowering stage without dormancy being observed (Nhut *et al*. 2001b). tTCL square epidermal layers from young stems could form an average of 4 bulblets was formed per tTCL after 4 weeks culture (Nhut *et al*. 2001a). Shoots did not form in activated charcoal (AC)-free medium, indicating that AC has the same effect as a cytokinin-like hormone on the development of tTCL explants. In other studies (Nhut *et al*. 2001d, 2002a), similarly prepared transverse young stem sections of *L. longiflorum* were shown to form different organs (bulblets, roots, shoots, plantlets, PLBs, somatic embryos) when explants were exposed to different PGRs. Somatic embryogenesis has been achieved in tTCLs of *in vitro L. longiflorum* pseudo-bulblet explants (Nhut *et al*. 2001a).

APPLIED TCL SYSTEMS

Numerous plants are used as ornamentals for decoration, cut flowers, landscaping and gardening. Below are some ornamentals which, using conventional micropropagation systems have resulted in multiple morphogenic programs, but following the use of the TCL system, have resulted in individual morphogenic programs.

Amaranthus edulis (amaranth)

Amaranthus are popular for dry flower production, bedding

and indoor plants. As a crop, they have a rich lysine content, about 18% higher than cereals. Shoots and embryo-like structures (ELSs) were obtained when 0.2-0.4 mm tTCLs excised from apical and subapical zones of *Amaranthus* seedlings formed directly on the epidermal cells of after one week on MS with 3 µM TDZ (Bui *et al*. 1998a).

Begonia rex (begonia)

Over 2,000 species of *Begonia* are already classified with more varieties increasing every year by traditional breeding methods. The major target of breeding is alteration of plant morphology, leaf and/or flower colour. *Begonia* is one of the most popular ornamental plants in the world and is used as garden plants, potted plants, hanging baskets, and greenhouse flowers. One to six epidermal cell layers and subjacent collenchyma cells excised from the main vein of leaves were cultured on a mineral solution containing 1% sucrose and 10 g/l agar, to which 1 µM BA was added for bud formation, or 0.5 µM NAA for root formation, or 0.1 µM NAA for unicellular hair formation. This new morphogenetic program, expressed after 6 days of culture, represents one of the simplest types of differentiation (Chlyah and Tran Thanh Van 1975). TDZ induced a similarly high frequency of shoot regeneration in petiole tTCLs (Nhut *et al*. 2005; **Fig. 6C**).

Gentiana spp. (gentian)

Gentians (*Gentiana* spp.) are herbaceous perennial plants with high ornamental value used as cut and potted flowers, and in landscaping. Gentians, relatively recalcitrant species for shoot regeneration, could be mass propagated when 0.3-0.5 mm tTCLs, excised from floral stalk receptacles, were cultured on 50 µM TDZ and 1 µM NAA (Hosokawa *et al*. 1996). In TDZ-less medium the percentage of buds per tTCL is low. Many buds developed after 2-4 weeks directly on the surface of receptacle tTCLs.

Gladiolus spp. (gladiola)

The genus *Gladiolus* from the family *Iridaceae* comprises about 180 species, and numerous cultivars and hybrids that are of ornamental value as cut flowers. For the mass propagation of *Gladiolus* within a short time interval, *Gladiolus in vitro* cormel tTCLs were aseptically cultured from corms on medium containing 10 µM BA (Stefaniak 1994). After one month cormels developed on the surface of originally cultured cormels, near the axillary buds. *In vitro* cormel tTCL explants (0.3-0.5 mm) were excised and cultured on medium containing different concentrations of TDZ (1-10 µM). After two weeks, direct bud primordia without an intermediate callus phase were observed on the surface of tTCL explants at an optimal concentration of 1-3 µM TDZ. More than 50 buds per tTCL could be recovered after 3 weeks culture. Similar regeneration was obtained when flower stalk lTCLs were used (**Fig. 6D**).

Helianthus annuus (sunflower)

Sunflower is a popular ornamental and cut flower, with many cultivars used in gardening and landscaping. Different hypocotyl *Helianthus annuus* tTCLs were compared for their embryogenic and callogenic capacities: a) 2 cm long segments of hypocotyls, b) hypocotyls without epidermis, c) monolayer of epidermis, d) subepidermal layer, e) epidermis plus parenchyma layers, cultured on medium with 1 mg/l NAA, 1 mg/l BA and 20 ml/l coconut water (Pélissier 1990). The epidermal monolayer, the subepidermal layers and the hypocotyls without epidermis were not embryogenic. Only the tTCLs comprising the epidermis plus parenchyma layer and the hypocotyls segments were embryonic. The primary somatic embryos that differentiated on *Helianthus* TCLs gave rise to secondary embryos, which developed into normal fertile plants.

Heliconia spp. (heliconia)

Several species in this genus are floral crops because of their showy and brightly hued terminal inflorescences. Goh *et al*. (1995) used TCLs to efficiently direct organogenesis. Stem tTCLs from the shoot apex (0.8-1mm) of *H. psittacorum* L. 'Choconiana' were cultured *in vitro* on MS with 80 µM 2,4-D, forming callus and PLBs, which developed and grew into plantlets after two 6-week subcultures on basal MS. The TCL system was used for both mass propagation and germplasm conservation of *Heliconia* species.

Iris pallida (iris)

Many Irises are ornamental plants such as *I. hollandica* or *I. pallida*. Furthemore, *I. pallida* is also a source of γ-irone. Thin sections (0.3-0.5 mm) made across a mature shoot (comprising 5-6 leaves) and from the base toward the apex were cultured. Somatic embryogenesis occurred on young leaf base tTCLs (Tran Thanh Van and Bui 1999). Protocols have been extensively utilized using thin sections for the plant regeneration of Irises (Gozu *et al*. 1993).

Orchids

Orchids are one of the most attractive groups of cut flower and ornamental potted plants. Many commercial firms use *in vitro* systems for the rapid mass plant propagation of various orchids, although only few reports exist in the literature. To mass produce a monopodial orchid hybrid *Aranda* 'Deborah' Laskshmanan *et al*. (1995) used 0.6-0.7 mm thick tTCLs from a single shoot tip (6-7 mm). After 45 days of culture, neoformation of protocorm-like bodies (PBLs) occurred on the same culture medium, 13.6 PBLs per TCL and 2.7 PBLs per shoot tip. The addition of 2.75 µM NAA to the same medium further increased PLB production (19.2 PBLs per TCL). The advantage of the tTCL system is to produce a high frequency of shoot regeneration and to reduce the time interval required, with potentially more than 80,000 plantlets produced from a single tTCL in a year as compared to the 11,000 plantlets produced by the conventional shoot tip method. Among monocot orchid species such as *Phaleanopsis*, young leaf lamina TCL explant (4 mm^2) or floral stalks can be induced to form protocorms directly along the wounded edges of the lamina and on the surface of the TCL (Tran Thanh Van

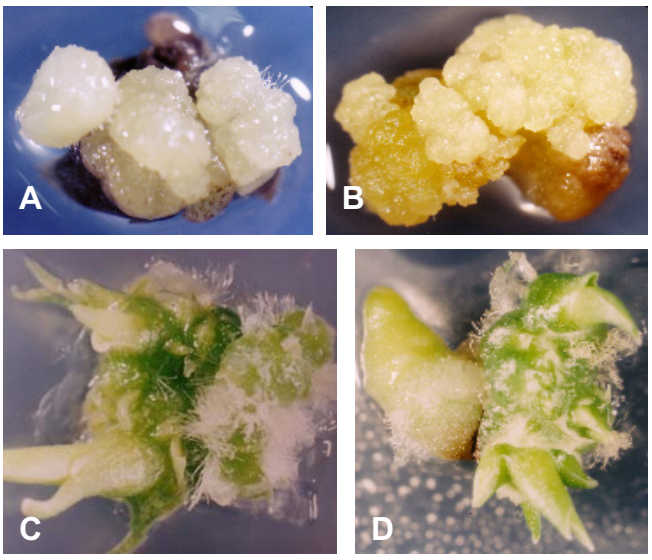

Fig. 7 Organogenesis and somatic embryogenesis in *Cymbidium* from PLB lTCLs and tTCLs. *Cymbidium* Twilight Moon "Day Light" *in vitro* embryogenic callus in the dark (**A**) or in the light (**B**), PLB (**C**) and shoot (**D**) induction from PLB lTCLs and tTCLs.

1974). The use of CPPU (10 µM) with a low sucrose concentration (1%) caused the highest percentage of explant (95%) shoot growth and rooting in *Rhynchostylis gigantean* (Bui *et al*. 1999b). In *Cymbidium* Twilight Moon "Day Light", a hybrid, the use of PLB epidermal lTCLs results in the controlled development of shoots, secondary PLBs, or somatic embryogenic callus (Teixeira da Silva *et al*. 2005, Teixeira da Silva and Tanaka 2006; **Fig. 7**).

Pelargonium x hortorum (geranium hybrid)

Geraniums are popular garden plants used specifically for their decorative or scented foliage while the bushy flowering geraniums have showy flowers suitable for gardens, windowboxes or hanging baskets. Gill *et al*. (1992) showed that in tTCL hypocotyls explants (1 x 10 mm) of 1-week-old geranium hybrid seedlings, somatic embryos could form in response to TDZ or a combination of IAA and BA, but the number of embryos was much less in the latter than with 1-1.5 µM TDZ. The development of somatic embryos was rapid and the number of embryos was about 8-fold higher than in the culture of whole hypocotyl explants. In tTCLs globular or early heart-shaped somatic embryos were formed within one week of culture, whereas in the whole hypocotyl explants they were visible only after 2 weeks. Hypocotyls from a 1-week-old plant can be the source of at least 5 and as many as 10 tTCLs, each of which can develop into about 40-60 embryos, giving a total of 400-600 embryos per hypocotyl, as compared to approximately 50 embryos which can form directly from a whole hypocotyl of similar size and age. tTCLs of 1-week-old seedlings produce a higher number of somatic embryos than those obtained from older seedlings, and regenerated somatic embryos develop into complete plantlets within 6 to 8 weeks of culture initiation.

Petunia hybrida (petunia)

Petunia is one of the world's favourite gardening and potting annuals responsible for rapid and large economic turnovers. It is also an important model plant in understanding the genetics of flower colour. Mulin and Tran Thanh Van (1989) showed that *in vitro* shoots and flowers are formed from thin epidermal cells excised from the first five internodes of basal flowering branches in *Petunia hybrida*. Explants (1 x 10 mm²) consisted of 3-6 layers of subepidermal and epidermal cells and when placed on basal MS with 1 µM each of IAA or Kinetin, vegetative buds formed after 2 weeks and developed into vegetative shoots in all genotypes tested. In contrast, *in vitro* floral buds were obtained when all stages of flowering (floral buds to faded flowers) were present on basal flowering branches of the mother plant. Other morphogenic programs were observed when medium PGRs were modified, such as the substitution of 1 µM Kinetin by 10 µM BAP, resulting in vegetative bud formation, or when 1 µM IAA was replaced by 10 µM IBA or when Kinetin was used at 0.1 µM instead of 1 µM, root formation was obtained. A combination of 100 µM 2,4-D and 0.1 µM Kinetin induced callus.

Rosa spp. (rose)

Rose is one of the four major cut flower species commercially explored worldwide. lTCLs were excised longitudinally from dormant bud floral stalks and cultured on a full- or half-strength MS medium, supplemented with 0.05-5 mg/l 2,4-D or NAA usually in combination with BAP, zeatin or kinetin. Preincubation at a high (100 µM) 2,4-D concentration increased the frequency of both organogenic and embryogenic callus from *Rosa hybrida* 'Baccara' leaf explants (Hsia and Korban 1996). Sucrose at 2-3% was used as the sole carbon source in most cases. However, replacement of sucrose by galactose or fructose increased somatic embryogenesis from leaf explants of some *R. hybrida* cultivars. Breaking of bud dormancy in roses is important for rapid multiplication of roses using grafting. lTCLs cultured on medium with 10 µM BAP and 3 µM GA_3 resulted in more than 7 buds per lTCL after 4 weeks culture.

Saintpaulia ionantha (African violet)

Direct bud organogenesis (vegetative bud, callus, roots, somatic embryo, polyembryonic-like structure) was achieved in this commercially important micropropagated ornamental perennial when the auxin/cytokinin (NAA:BA or TDZ) ratio in the TCL (derived from leaf petioles to the central nerve (leaf vein) and lamina) culture medium was strictly controlled. An average of 100-200 shoots per tTCL explant were obtained from 0.3-0.5 mm petiole or 3x3 mm lamina sections, respectively, within 4 weeks culture. Over 70,000 plants were produced from a single leaf within 3-4 months (Ohki 1994).

Others

Several new experiments have shown that regeneration is possible from tea young stem tTCLs (**Fig. 6A**), passion fruit (**Fig. 6E**), *Citrus* sp. (**Fig. 6F**) and *Gloxinia* (**Fig. 6H**) young stem tTCLs, and *Gerbera* petiole tTCLs (**Fig. 6G**).

TCLs IN GENETIC ENGINEERING AND TRANSFORMATION

The ability to transform economically important cut flower varieties would allow the use of molecular genetic techniques to modify characteristics such as flower colour, shape, height and growth morphology, longevity, horticultural traits, insect and disease resistance, and resistance to environmental stresses. Plant transformation, a core technology in the genetic engineering of plants, is normally composed of three processes: 1) the introduction of genes into plant cells; 2) selection of transgenic cells and 3) regeneration of transgenic plants. However the limiting factor has often been the third step. Without successful regeneration, in a controlled and defined manner, leading to the formation of organs or plants that are genetically and physiologically normal, there will be no success of stable transgene expression. Often initial explants utilized in the regeneration procedure are too large, and the capacity of both transformed and untransformed cells (following the gene introduction method) to regenerate is the same, especially if the selection level is low, or if the regenerating shoots arise from the surface of cells not in contact with the selection medium, or from within the explant, where the selective agent has not had time to diffuse through the explant, and exert its selective pressure. This results in chimerism and the formation of escapes. The utilization of TCLs eliminates the presence of untransformed cells and subsequent chimerism, and exposes all cells on the thin layer to the selective medium. Only cells harbouring the selector gene within their genome proliferate on the selective medium. Success of transformation varies widely between ornamental species, and has been achieved primarily by *Agrobacterium*-mediated gene transfer, and to a lesser extent by direct gene transfer (particle bombardment, electroporation, electrophoresis, silicon fibres, magnetite particles, or protoplast manipulation). The reports on the use of a TCL system as an initial explant for gene transfer are few, but those that exist demonstrate the effectiveness of introducing a gene into an explant with defined cellular structure and with a controlled regeneration program, allowing for the formation of non-chimeric transgenic plants.

Successful transformation of *Dendranthema grandiflora* was obtained when either conventional stem explants, stem tTCLs or leaf lTCLs were used as initial explant sources, using both *Agrobacterium*-mediated transformation, and biolistics (Teixeira da Silva and Fukai 2002a, 2002b, 2004;

Shinoyama *et al*. 2006). The choice of gene introduction method was found to influence the efficiency of gene transfer into stem tTCLs (Teixeira da Silva and Fukai 2003c). Sonication-assisted *Agrobacterium* transformation (SAAT), a method that involves subjecting plant tissue to brief periods of ultrasound in the presence of *Agrobacterium*, usually produces small and uniform fissures and channels throughout the tissue allowing the *Agrobacterium* easier access to internal tissues, especially tissue, such as meristematic tissue, which is buried under several other cell layers. The technique has been quite restricted in its application, and has been used on soybean, cowpea, white spruce, wheat and maize (Trick and Finer 1997). Transient transgene expression and subsequent stable β-glucuronidase transgene expression levels increased in chrysanthemum when SAAT was used on stem tTCLs (Teixeira da Silva and Fukai 2002b; **Fig. 8**). This level differed depending on the plasmid construct and *Agrobacterium* strain. Regeneration of *D. grandiflora* 'Lineker' and 'Shuhou-no-chikara', standard and spray-type chrysanthemum stem tTCLs, following cocultivation with *A. tumefaciens* was severely reduced even when tTCLs were placed on optimized regeneration media (Teixeira da Silva and Fukai 2002a). Both *Agrobacterium*-mediated transformation and shoot regeneration are triggered by wounding of the tTCL (Teixeira da Silva 2004b), and the degree and timing of explants are expected to be important factors in gene transfer and regeneration. Studies of chrysanthemum transformation by biolistics are few (Teixeira da Silva and Fukai 2002a, 2002b; Shinoyama *et al*. 2006). The transformation efficiency in *D. grandiflora* was attributed to the regeneration procedure, the choice of plasmid or gene introduction method while the use of acetosyringone, a phenolic compound and an inducer of T-DNA transfer – independent of the explant – enhanced the transformation efficiency of chrysanthemum and increased transgene expression (Teixeira da Silva and Fukai 2002a), while wounding of tissue (as induced by particle bombardment or SAAT) has been shown to induce shoot formation in chrysanthemum (Teixeira da Silva 2004b). Interestingly, mechanical wounding of TCL tissue does not result in the same level of morphogenesis, possibly due to excessive cell damage resulting from an ultra-small explant size: over 5 minutes sonication in 'Lineker' and 'Shuhou-no-chikara' caused tTCL mortality, but increased GUS transient transgene expression and blue-staining areas (Teixeira da Silva and Fukai 2002a). In a bid to induce virus/viroid resistance in chrysanthemum, *Pac1*, *2,5-A* and *RNaseL* genes were introduced via biolistics or *A. tumefaciens* into either conventional stem explants, or stem tTCLs, confirmed by PCR and Western analyses (**Fig. 8**).

Transformation efficiency values for 'Lineker' and 'Shuhou-no-chikara' fell into the range of 2-27% (GUS positive) and 0-21% (PCR positive) for bombardment related treatments, and in the range of 7-25% (GUS positive) and 0-16% (PCR positive) for *Agro*infection related treatments (Teixeira da Silva and Fukai 2002b). In 'Lineker' and 'Shuhou-no-chikara' an increased pre-culture period positively affected explant survival in both cultivars both with particle bombardment and *Agro*infection (Teixeira da Silva and Fukai 2002a), but also an increase in escape (non-transgenic plant) formation. *In vitro*-derived leaf explants had higher transient GUS transgene expression levels than greenhouse-derived leaves, independent of the *D. grandiflora* cultivar and plasmid type, whether *Agro*infection, *Agro*listics or SAAT were used, but the effect was not tested for biolistics (Teixeira da Silva and Fukai 2002b). In this study an increased GUS transgene expression in older (basal) leaves than in younger (apical) leaves, independent of the cultivar, and the treatment; moreover, GUS expression is primarily found in the veins and in the midrib, consistent with the fact that despite CaMV-35S being a constitutive promoter, that it has a stronger transgene expression in the vascular tissues (Teixeira da Silva and Fukai 2002b; **Fig. 8**). GUS transgene expression chimerism, the presence of both transformed and untransformed cells within the same plant, may be one of the primary reasons to explain the spatial expression of GUS transgene expression observed in 'Lineker' and 'Shuhou-no-chikara' transformants (Teixeira da Silva and Fukai 2002b). For stable transformation, it is necessary for the T-DNA to be incorporated into the host DNA, and T-DNA that is not integrated is gradually lost and inactivated: this phenomenon is evident in the GUS transgene expression in the intron-containing plasmids (pSKGN1, pKT2 and pKT3; see Teixeira da Silva 2003d for plasmid details) where such a gradual loss can be seen by a decrease in the number of GUS focal points up to 72 h and a total loss of GUS focal points by a maximum of 1 week following a gene introduction method (Teixeira da Silva and Fukai 2002a). The amount of *Agro*infection of explants was reduced to 2% after 2 weeks of culture on selective MS

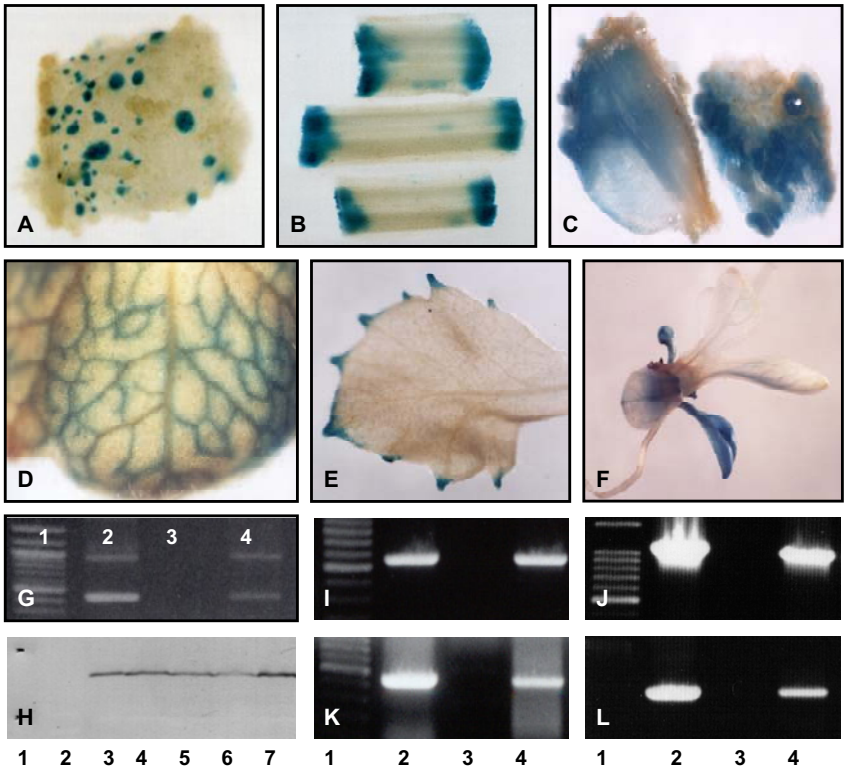

Fig. 8 Genetic transformation following the use of tTCLs as initial explants. Transient GUS expression in *in vitro* stem tTCL 72 h (**A**) after particle bombardment. Infection results in weak transient transgene expression (**B**) localized at cut surfaces only. The use of AGL0 (pKT3) results in stronger GUS transient expression (**C**). Predominant GUS expression in venation – tobacco (**D**) and shoot tips – chrysanthemum (**E**) of *in vitro* plantlet leaves. Chrysanthemum transformant (**F**) grown on kanamycin-supplemented medium. PCR (**G**) of GUS transient and stable gene expression. Lanes L1: size marker; L2: purified plasmid, *GUS* = 954 bp, upper band, *npt*II = 438 bp, lower band; L3: negative control, *in vitro* 'Lineker'. Stable transgene expression (Lane 4) after *Agrobacterium* infection. Western blot analysis (**H**) of Pac1 protein expression after AGL0 *Agro*infection of chrysanthemums. L1: size marker; L2: non-transformed 'Shuhou-no-chikara'; L3: positive Pac1 transformant 'Regan'; L4-7: Pac1 positive 'Shuhou-no-chikara' transformants, L6, low-expression line, L7, high-expression line. PCR analysis of LBA4404 (pKT61) *Agro*infection trials showing: L1 (size marker), L2 (positive control purified pKT61 plasmid), L3 (negative control 'Shuhou-no-chikara'), L4 (+ve transformant line #91 'Shuhou-no-chikara') for 2,5-A (**I**), *npt*II (**J**), *pac1* (**K**) and *RNAase L* (**L**). Details in **Teixeira da Silva and Fukai** (2002a).

medium, indicating that the GUS-positive and PCR-positive blue-staining areas were as a result of stable transformation and transgene integration, and not as a result of *Agro*infection. The tendency was shown to be similar in both conventional stem explants as in stem tTCLs, even though the latter suffered larger losses, possibly as a result of the incapacity of the plant cellular growth rate being unable to compete with that of the Agrobacterial cells.

Transgenic tobacco plants derived from lTCLs transformed with the *rol*B gene have more pronounced rhizogenesis and flowering, and appear to be involved in the promotion of meristem formation (Altamura *et al*. 1994). The effectiveness of transformation also depends on cell competence for both regeneration and transformation (Creemers-Molenaar *et al*. 1994). Tobacco '*Samsun*' lTCLs excised from floral pedicels were cultured to induce vegetative buds to study the cell competence for regeneration and transformation by biolistics (Tran Thanh Van 1980). Ten days pre-culture was the optimal period for obtaining genetic transformants.

Gladiolus tTCLs (**Fig. 6D**) at pre-mature bud stage (15 days on medium with 3 µM TDZ) were transformed using biolistic transformation. Thus the preculture period necessary for induced cell competent for plant regeneration from TCL explants is an important factor for transgenic plant formation. Moreover TCL explants have an increased surface when infected or exposed to *Agrobacterium* or biolistic transformation.

TCLs have been used as an explant source for study of transformation, with transgenic plants obtained for *Brassica napus* (Charest *et al*. 1988), *Nicotiana plumbaginifolia* (Trinh *et al*. 1987), and *Digitaria sanguinalis* (Bui *et al*. 1998b). In *B. napus* ssp. *olifera* 'Westar' tTCLs a high frequency of transformation (40-50%) was obtained when tTCLs were *Agro*infected for 30 s and co-cultivated for 2 days before the addition of an antibiotic (Charest *et al*. 1988). In *N. plumbaginifolia* tTCLs, transformants were obtained on a 100 mg/l kanamycin selective medium after co-cultivation with *A. tumefaciens* for 2 days (Trinh *et al*. 1987). Transgenic plants with the *bar* gene were confirmed by Southern analysis, revealing 3 basta-resistant lines when *D. sanguinalis* tTCLs were biolistically transformed (Bui *et al*. 1998b).

CONCLUDING REMARKS

All patterns of morphogenesis displayed by a plant (callus, root, shoot, flower, somatic embryo) can be induced either separately or in combination, each with a well-defined reversible/irreversible phase. Plant tissue culture and molecular biotechnology are at an important crossroad where one or the other cannot solely be utilized to achieve the successful analysis of *in vitro* or *in planta* physiological and genetic systems. Both have powerful techniques that permit the understanding of mechanisms that control processes such as transgene expression, *in vitro* flowering and morphogenesis. TCL systems have been used extensively as a tool that allows for the understanding of these processes, and opens the way for new research that may further elucidate certain physiological and genetic pathways and processes, which, in plant tissue culture and molecular biotechnology, still remain a paradigm. The TCL system is a simplified system that requires only a small amount of plant material and medium volume (circ. 15 µl per TCL), and provides a good system for the study of fundamental and applied aspects of regeneration and transformation. The TCL system has been effectively utilized to study organogenesis and embryogenesis in ornamental and floricultural species, and promises to be extended to the micropropagation of others.

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