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REVIEW

Insights into the multifaceted application of microscopic techniques in plant tissue culture systems

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Abstract
Main conclusion **Microscopic techniques remain an integral tool which has allowed for the better understanding and manipulation of in vitro plant culture systems. The recent advancements will inevitably help to unlock the long-standing mysteries of fundamental biological mechanisms of plant cells.**

Beyond the classical applications in micropropagation aimed at the conservation of endangered and elite commercial genotypes, plant cell, tissue and organ cultures have become a platform for elucidating a myriad of fundamental physiological and developmental processes. In conjunction with microscopic techniques, in vitro culture technology has been at the centre of important breakthroughs in plant growth and development. Applications of microscopy and plant tissue culture have included elucidation of growth and development processes, detection of in vitro-induced physiological disorders as well as subcellular localization using fluorescent protein probes. Light and electron microscopy have been widely used in confirming the bipolarity of somatic embryos during somatic embryogenesis. The technique highlights basic anatomical, structural and histological evidence for in vitro-induced physiological disorders during plant growth and development. In this review, we discuss some significant biological insights in plant growth and development, breakthroughs and limitations of various microscopic applications and the exciting possibilities offered by emergent in vivo live imaging and fluorescent protein engineering technologies.

Keywords Fluorescent proteins · Histology · Organogenesis · Physiological disorders · Somatic embryogenesis · Subcellular localization

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Introduction

The concept and discovery of microscopy date back approximately four centuries. Basically, microscopy involves the use of microscopes to enlarge samples or objects which originally are beyond the resolution of the human eye (Shur and Price 2012; Thomasson and Macnaughtan 2013). As an indication of the great importance and value of microscopy, it remains a popular and vital tool with a wide range of applications in basic and applied sciences (Tranfield and Walker 2013; Zumbusch et al. 2013; El-Bakry and Sheehan 2014; Whited and Park 2014) as well as in the medical and material engineering fields (Torrealba and Carrasco 2004; Shur and Price 2012; De Boer et al. 2013; Juszczyk et al. 2013). These aforementioned publications also provide excellent reviews highlighting the trends and current updates related to the specifics and significance of microscopy in these various fields. In addition, details on the theory and practice of specific microscopy-based technologies and specimen preparation protocols are well documented (Chalfie and Kain 2005; Kuo 2007; Chandler and Roberson 2009; Murphy and Davidson 2013).

In plant sciences, microscopy is used in an attempt to resolve and understand various aspects of growth and developmental processes including structural and functional properties. It also provides insights on interactions of cells and subcellular components in plants (Chandler and

Roberson 2009; Domozych 2012). Plant science as a field encompasses diverse aspects of plant growth, development and ecology amongst others. With one of the main focus geared at efficient propagation and general plant improvement in terms of quality and quantity, plant biotechnology remains one of the fundamental fields in plant sciences (Vasil 2008). To a certain extent, plant biotechnology was established based on the principles of cellular totipotency and genetic transformation. Inevitably the use of basic in vitro plant culture techniques is essential and vital for the success of several plant biotechnology endeavours. Particularly from a conservation perspective, the value and benefit of in vitro tissue culture systems (micropropagation) are well documented (George 1993; Ramachandra Rao and Ravishankar 2002; Pence 2010). Besides the potential in ensuring food security via mass propagation of different staple crops and fruits (Mondal et al. 2004; Dobránszki and Teixeira da Silva 2010), many plant species with ornamental, horticulture and medicinal values are easily regenerated (Rout et al. 2000, 2006; Teixeira da Silva 2003; Moyo et al. 2011), see Fig. 1.

Recent technological advances are expanding the capabilities of microscopy which are being used to understand and explain commonly observed morphological appearances of in vitro regenerants. Thus, coupled with complementary biochemical, histological and molecular approaches, the increasing diverse microscopy-based technologies can expedite the better understanding of in vitro plant culture systems. Furthermore, physiological disorders in regenerants are often better elucidated with the use of microscopic systems (Chakrabarty et al. 2006; Jausoro et al. 2010a; Bairu et al. 2011). Notwithstanding these aforementioned applications and benefits, a comprehensive review detailing the contribution of microscopy to the understanding of in vitro plant culture systems is still lacking. Thus, this review covers a brief overview on the basic microscopic principles and technological advances in the field as well as summarizing the present applications and gains from the use of microscopy in in vitro plant culture systems. In addition to identifying the current knowledge gap, a critical appraisal of microscopy application in plant cell, tissue and organ culture systems was discussed. Even though the current review is not fully exhaustive of all the available literature, as much as possible, we provide representative and specific references to ascertain the overall objectives of the subject matter.

Recent advances and general overview on microscopic techniques

In recent times, novel and giant technological strides in the form of introduction of laser-based, vibrational, electron and X-ray systems coupled with the rapid evolution of digital image capture and analysis technologies have revolutionized the capabilities and applications of microscopy (Torrealba and Carrasco 2004; Roberts et al. 2007; Domozych 2012; Picas et al. 2012). As postulated by Domozych (2012), these developments have allowed for the visualization of cell dynamics with unprecedented resolution, contrast and experimental versatility. Based on the evidence of its increasing application (Jahn et al. 2012; Picas et al. 2012; Thomasson and Macnaughtan 2013; Zumbusch et al. 2013; El-Bakry and Sheehan 2014; Whited and Park 2014), there is no doubt that microscopy is more valuable than ever before and will remain relevant in all areas of plant science research. In the near future, modern microscopy will ultimately achieve the goal of resolving the three-dimensional (3-D) structural and functional features of cellular life (four-dimensional imaging or 4-DI) (Domozych 2012).

Despite the potential and advances associated with microscopic techniques for biological research, some inherent limitations still exist. The two major 'reality checks' are that (1) light microscopy and confocal laser scanning microscopy (CLSM) used to image dynamic events in live cells are inherently limited in resolution and (2) electron microscopy which possesses better resolution cannot be used to view live cells (Domozych 2012). Although scanning probe microscopy exists as a different technique, optical (light) and electron microscopy are the most commonly used in in vitro culture systems (Tables 1, 2, 3). The conventional light microscope techniques are bright-field, polarized, and fluorescence light microscopy while electron microscopy includes the scanning electron microscopy and transmission electron microscopy (TEM) (Chandler and Roberson 2009; Domozych 2012). In addition to other basic differences such as sample preparation, electron microscopy has a much higher resolution of $\approx$0.1–5 nm than light microscopy with a resolution of 0.2 mm (El-Bakry and Sheehan 2014). Thus, the task to be performed and specific objective generally influence the choice of microscopy technique at any given time.

Practical application of microscopic techniques in in vitro plant culture systems

As a well-established system for rapid proliferation of clonal plantlets (Fig. 1) for the floricultural and ornamental industries, micropropagation allows for year-round and continuous culture (Caponetti et al. 2005; Pence 2010; Ruffoni and Savona 2013). For plant species with medicinal value, their mass propagation is often aimed at ensuring their conservation (Canter et al. 2005). Furthermore, researchers have in recent times unravelled the potential of plant tissue culture as a tool to elucidate

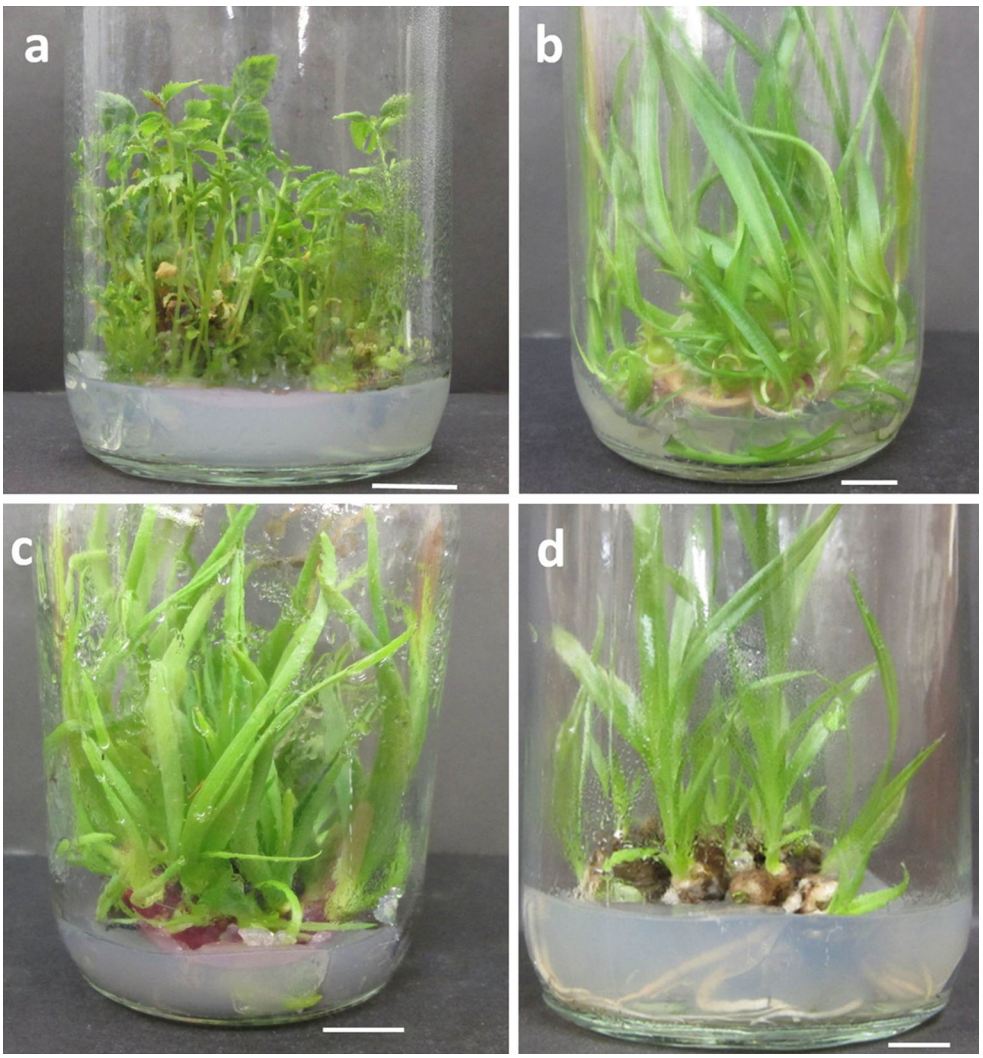

Fig. 1 Morphological appearance of typical high shoot proliferation of clonal plantlets with diverse economical value obtained via organogenesis during micropropagation. **a** *Amelanchier alnifolia* (nutritional). **b** *Merwilla plumbea* (medicinal and ornamental). **c** *Aloe arborescens* (medicinal and ornamental). **d** *Hypoxis hemerocallidea* (medicinal and ornamental). *Scale bar* 10 mm

metabolic pathways and to enhance the production of therapeutic phytochemicals (Ramachandra Rao and Ravishankar 2002; Verpoorte and Memelink 2002; Karuppusamy 2009). In vitro cell culture systems are also valuable avenues for transformation and transgenic studies. The following subsections highlight the basic and practical application of microscopy in different in vitro systems.

Elucidation of growth and development patterns

Even though the frequency of application may differ, the four basic methods for micropropagation are axillary shoot proliferation, node culture, *de novo* formation of adventitious shoots through organogenesis and somatic embryogenesis (Kane 2005). Details of these procedures are outside the scope of this review but have been well documented (Zimmerman 1993; Kane 2005; Rout et al. 2006). There is no doubt that much of the available evidence and theories of in vitro developmental processes were achieved via histological approaches using different microscopic techniques (Trigiano et al. 2005). Although tremendous advances have been recorded in recent times (Motte et al. 2014), more stringent studies are required to fully understand the overall intricate events involved in in vitro plant growth and development. With the ability to generate microscopic structures and characteristics of cells through initiation, assemblage and arrangement phases, researchers have gained in-depth knowledge which allows for manipulation of plant growth using in vitro culture techniques. Both light and electron microscopic techniques have become integral components in studying plant species (Table 1). Plant growth and development processes are

Table 1 The role of microscopic techniques in elucidation of growth, developmental pattern and physiological responses in plant cell, tissue and organ cultures

Microscopic technique	Plant species	Objective(s) of study	Observation(s)	References
Somatic embryogenesis				
Light microscopy	*Aralia elata* Seem.	Repetitive plant regeneration system via primary and secondary somatic embryogenesis	Multi-cellular origin of secondary somatic embryos	Dai et al. (2011)
Light microscopy	*Brassica oleracea* var. *capitata*; *Brassica oleracea* var. *botrytis*	Repetitive somatic embryogenesis and subsequent plant regeneration in two *Brassica oleracea* varieties	Somatic embryos developed from the subepidermal cell layers characterized by prominent nuclei, dense cytoplasm and intensive cell division	Pavlović et al. (2013)
Light microscopy	*Capsicum annuum* L.	Evaluation of embryo and callus origin	Gametophytic origin of embryos and sporophytic origin of calli	Parra-Vega et al. (2013)
Light microscopy	*Cocos nucifera* L.	Histological changes during somatic embryo development	Somatic embryos developed root and caulinar meristems	Sáenz et al. (2006)
Light microscopy	*Crocus heuffelianus* Herbert	Somatic embryogenesis and regeneration from shoot primordia	Confirmation of all stages of somatic embryo development; embryos were bipolar	Demeter et al. (2010)
Light microscopy	*Passiflora cincinnata* Mast.	Somatic embryogenesis from mature zygotic embryos	Histodifferentiation of secondary embryos on the surface of the protodermal layer of primary embryos	da Silva et al. (2009)
Light microscopy	*Picea glehnii* (F. Schmidt) Masters	Effect of polyamines on development of embryonal-suspensor masses and formation of somatic embryos	Starch grains distributed in most cells of the somatic embryos	Nakagawa et al. (2011)
Light microscopy	*Pulsatilla koreana* Nakai	Somatic embryogenesis and shoot organogenesis system	Various developmental stages of somatic embryos observed	Lin et al. (2011)
SEM	*Leucojum aestivum* L.	Influence of ethylene on somatic embryogenesis	Ethylene enhanced the development of globular embryos by nearly 25 %	Ptak et al. (2010)
TEM	*Ceratonia siliqua* L.	Somatic embryogenesis and plant regeneration	Ultrastructural analysis showed that the cells of the globular embryos had a dense cytoplasm	Canhoto et al. (2006)

Table 1 continued

Microscopic technique	Plant species	Objective(s) of study	Observation(s)	References
Organogenesis				
Light microscopy	*Bixa orellana* L.	Organogenic potential of root explants	Adventitious buds originated from cell proliferation within the pericycle opposite the poles of the primary xylem	da Cruz et al. (2014)
Light microscopy	*Daucus carota* L. subsp. *sativus* Hoffm.	Plant regeneration from leaf and hypocotyl-derived protoplasts	Protoplasts enlarged and changed shape from spherical to oval indicating a reconstruction of the cell wall	Grzebelus et al. (2012)
Light microscopy	*Oncidium flexuosum* Sims	Direct regeneration of protocorm-like bodies (PLBs)	PLBs developed from division of epidermal and subepidermal cells	Mayer et al. (2010)
Light microscopy	*Passiflora foetida* L.	In vitro plant regeneration of *P. foetida* via organogenesis using mature zygotic embryos	Globular structures of highly dividing cells similar to meristemoids observed	Rosa and Dornelas (2012)
Light microscopy	*Watsonia lepida* N.E.Br.	Anatomical examination of hypocotyl subsections	Amongst the examined hypocotyl subsections, cells of subsection C1 were developmentally plastic and able to respond to external cues	Ascough et al. (2009)
SEM	*Lycaste* hybrid	Indirect induction of PLBs and shoot proliferation	PLBs with leaf primordia and apical meristem	Huang and Chung (2011)
SEM	*Sutherlandia frutescens* L.	Indirect shoot organogenesis and plant regeneration of *Sutherlandia frutescens*	Nodular callus after 2 weeks of culture	Dewir et al. (2010)
SEM	*Tectona grandis* L.	Determine whether different concentrations of benzyladenine (BA) affect teak plant development	Shoots on either cytokinin-free medium, 2.22 or 4.44 μM BA had elliptical stomata; whereas stomata of shoots grown on 6.66 μM BA were ring-shaped, raised and open	Quiala et al. (2012)
TEM	*Passiflora edulis* Sims	Characterize the anatomical and ultrastructural aspects of direct and indirect organogenesis	Meristemoids that originated either directly or indirectly from calli were ultrastructurally similar	Rocha et al. (2012)

Table 1 continued

Microscopic technique	Plant species	Objective(s) of study	Observation(s)	References
Other developmental patterns				
Light microscopy	*Petunia grandiflora* Juss. 'Prime Time'	Effect of flavonoid 3′,5′-hydroxylase (F3′5′H) delphinidin accumulation in petals	Changes in flower colour pigmentation visualized	Qi et al. (2013)
SEM	*Thymus caespititius* Brot.	Comparison of essential oil production in shoot cultures and field-grown plants	Similar glandular trichomes density for in vitro shoots and field-grown plants	Mendes et al. (2013)
SEM	*Pandanus amaryllifolius* Roxb.	Developmental pattern of leaf lower epidermis papillae	Observation of different stages of papillar development	Wakte et al. (2009)
TEM	*Beta vulgaris* L.	Establish specific patterns of extracellular proteins and identify distinct protein markers of corresponding phenotypes	Number of nuclei in habituated non-organogenic (HNO) > tumour (T) cell lines; HNO and T cells had enlarged, lobed nuclei with high number of nucleoli	Pavoković et al. (2012)
TEM	*Cocos nucifera* L.	Establish whether lauric acid improves growth and development of zygotic coconut embryos	Lauric acid led to large circular oil bodies; large number of plastids, mitochondria, vacuoles, microbodies and extensive endoplasmic reticulum	López-Villalobos et al. (2011)
TEM	*Scopolia parviflora* (Dunn) Nakai	Morphological characterization of transgenic hairy roots (for production of alkaloids)	Presence of large spherical-shaped idioblast cells in transgenic hairy roots	Kang et al. (2011)
TEM	*Triticum aestivum* L.	Effect of polyamines on androgenesis in anthers or embryo-like structures	Chloroplasts (spermine-treated) exhibited slow development and appeared to be smaller in size compared to putrescine and spermidine treatments	Redha and Suleman (2011)
TEM	*Cymbidium Solana Beach* × *C.rincon*	Role of putrescine in rapid shoot regeneration in the callus of orchids pre-treated with mannitol and cadmium chloride	Putrescine-altered $CdCl_2$- and mannitol-treated callus had numerous oil droplets, rough endoplasmic reticulum and dictyosomes	Guha and Rao (2010)
TEM	*Cucumis sativus* L.	Ultrastructural comparison of Cd-tolerant and -sensitive cells	Tolerant cells adjusted to presence of Cd, but without any specific ultrastructural modifications related to increased tolerance	Gzyl et al. (2009)

Table 1 continued

Microscopic technique	Plant species	Objective(s) of study	Observation(s)	References
TEM	*Nicotiana tabacum* L.	Effect of long-term cryopreservation on expression of plant-made vaccines	Organelles with double membranes had intact membranes in both the control and cryopreserved cells	Van Eck and Keen (2009)

BA Benzyladenine, *PLBs* protocorm-like bodies, *SEM* scanning electron microscopy, *TEM* transmission electron microscopy

characteristically dynamic whereas histological techniques only present a narrow momentary glimpse of the process (Trigiano et al. 2005). Notwithstanding, by piecing together a series of the static microscopic observations, plant biologists are able to elucidate the underlying anatomical features involved in plant development.

Somatic embryogenesis

The induction of somatic embryos under in vitro culture conditions in ontogenetic steps is similar to those observed in zygotic embryogenesis and has long fascinated plant biologists (Zimmerman 1993). Somatic embryogenesis (SE) provides a model system for studying the genetic basis of early differentiation events and cellular totipotency of somatic cells (Zimmerman 1993; Fehér et al. 2003; Kurczyńska et al. 2007). Furthermore, SE has become a widely used technique in genetic transformation and mass propagation of elite genotypes (Table 1). Light and electron microscopic studies have reported on the cellular origin of somatic embryos during primary (Blazquez et al. 2009; Capelo et al. 2010; Lin et al. 2011; Parra-Vega et al. 2013) and repetitive/secondary embryogenesis (Dai et al. 2011; Pavlović et al. 2013; Raju et al. 2013). Upon exposure to SE induction medium, the initial events signifying cytological changes in the formation of somatic embryos were evident in 4–6 days (Canhoto et al. 1996; Kurczyńska et al. 2007). Induction of somatic embryos was characterized by formation of embryonic-like centres from single or multi-cells of proto- and subprotodermal origin (Kurczyńska et al. 2007), accumulation of starch grains and differentiation of mitochondria (Canhoto et al. 1996). The unicellular origin of direct somatic embryos has been observed to be the main morphogenic pathway (Rugkhla and Jones 1998; Kurczyńska et al. 2007). Thus, as early as 6 days into the culture period, SE induction can be confirmed, especially for somatic embryo transformation studies. Furthermore, microscopic applications coupled with molecular techniques provides an invaluable OMICS/morphology interface for exploring plant growth and development. During the early stages of embryogenesis, *LEAFY COTYLEDON* (*LEC*) genes play a key role in somatic embryo development (Stone et al. 2001). In *Arabidopsis thaliana*, strong promoter activity of *LEC* was detected at the globular somatic embryo stage after a 12-day culture period (Kurczyńska et al. 2007). In addition, microscopic techniques have been highly valuable in characterizing the non-bipolarity of protocorm-like bodies (Mayer et al. 2010; Huang and Chung 2011), globular embryo-like structures (Woo and Wetzstein 2008; Sharifi et al. 2010) and nodular meristemoids (Moyo et al. 2009; Rosa and Dornelas 2012), which would have been otherwise mistaken for somatic embryos (Fig. 2). Thus,

Table 2 The role of microscopic techniques in detection of in vitro-induced physiological disorders in plant cell, tissue and organ cultures

Microscopic technique	Plant species	Objective(s) of study	Observation(s)	References
CLSM	*Malus* spp cv.'M9 Emla'	Evaluate structural differences between hyperhydric and normal in vitro leaf structure	Compared with healthy leaves, hyperhydric leaves showed abnormal, often discontinuous development of the epidermis and cuticle. Stomata were malformed. The leaf lamina appeared thickened and was characterized by poor differentiation between the palisade and spongy mesophyll tissue	Chakrabarty et al. (2006)
Light microscopy	*Dianthus caryophyllus* L.	Measure epidermal and stomatal cell number and to view surface wax topology and leaf imprints	Hyperhydric plants had fewer epidermis cell and stomata per leaf area compared to the normal ones	Correll and Weathers (2001)
Light microscopy	*Vanilla planifolia* Jacks. ex Andrews	Observation of morphologic changes that occur while progressing towards hyperhydricity	Degradation of the endodermal cells was observed in hyperhydric shoots, whereas the leaf sections showed higher degradation of vascular bundles, loss of firmness of the palisade parenchyma with abnormal enlargement, and more intercellular space	Sreedhar et al. (2009)
Light microscopy	*Jatropha curcas* L.	Effect of browning on callus morphology	Non-browning callus had even, tightly arranged cells and browning callus had a disordered cell pattern	He et al. (2009)
Light microscopy	*Sclerocarya birrea* (A. Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro	Influence of microculture on leaf and root structure	Structural differences in thickness of epidermis and endodermis observed between photoautotrophic and photomixotrophic plants	Moyo et al. (2012)
SEM	*Aloe polyphylla* Schönland ex Pillans	Evaluate structural differences between hyperhydric and normal in vitro leaf structure	Frequency of the stomata was higher and well developed in normal shoots. Hyperhydric leaves had larger and abnormal stomata	Ivanova and Van Staden (2010)
SEM	*Cotoneaster wilsonii* Nakai	Evaluate structural differences between hyperhydric and normal in vitro leaf structure	Hyperhydric leaves showed abnormal stomata which had deformed guard cells	Sivanesan et al. (2011)
SEM	*Solanum melongena* L.	Evaluate structural differences between hyperhydric and normal in vitro plants	Hyperhydric leaves showed abnormal stomata, slightly larger than those of its normal ones and with guard and subsidiary cells drastically deformed	Picoli et al. (2001)
TEM	*Allium sativum* L.	Evaluate structural differences between hyperhydric and normal in vitro cells	Hyperhydric cells had abnormal and poorly developed organelles	Wu et al. (2009)
TEM	*Annona glabra* L.	Role of cytokinins in the differentiation of the photosynthetic apparatus in in vitro-derived plants	Benzyladenine (BA) and kinetin-derived plantlets had chloroplasts with well-developed grana margin system and greater accumulation of starch grains; thidiazuron caused formation of abnormal chloroplasts rich in large, globular, electron dense structures	de Oliveira et al. (2008)

Table 2 continued

Microscopic technique	Plant species	Objective(s) of study	Observation(s)	References
TEM	*Capsicum annuum* L.	Evaluate structural differences and starch accumulation between hyperhydric and normal in vitro cells	Palisade chloroplasts of normal plants showed well-developed thylakoids organized into many grana. Starch accumulation in chloroplasts of hyperhydric plants was significantly higher than in those of non-hyperhydric plants	Fontes et al. (1999)
TEM	*Handroanthus impetiginosus* (Mart. Ex DC) Mattos	Evaluate structural differences between hyperhydric and normal in vitro	Disorganized cortex, epidermal holes, epidermal discontinuity, collapsed cells observed in hyperhydric shoots	Jausoro et al. (2010b)
TEM	*Uniola paniculata* L.	Photosynthetic and carbohydrate status of an easy-to-acclimatize (EK 16-3) and a difficult-to-acclimatize (EK 11-1) genotypes	Chloroplasts in EK11-1 plantlets exhibited larger numbers of plastoglobuli than EK 16-3	Valero-Aracama et al. (2006)

BA Benzyladenine, *CLSM* confocal laser scanning microscopy, *SEM* scanning electron microscopy, *TEM* transmission electron microscopy

microscopy techniques have been invaluable in confirming and ascertaining the bipolar identity of somatic embryos in SE or lack thereof in embryo-like structures during organogenesis.

Organogenesis

Organogenesis refers to *de novo* organ formation involving the processes of dedifferentiation and redifferentiation of plant cells. It is widely proven that the ratio of auxin to cytokinin in plant tissues has the ability to shift the cell physiological state. In particular, the distribution and unique movement of auxins in a polar direction from cell to cell is thought to have a major influence on the organogenic fate of plant tissues (Muday and DeLong 2001; Del Bianco et al. 2013; Motte et al. 2014). However, the underlying mechanisms involved in this process remain to be fully elucidated (Motte et al. 2014). Innovative approaches using reporter genes fused to specific promoters, such as peptidyl-prolyl cis/trans isomerase (PIN1) and microscopy techniques have attempted to decipher the physiological and molecular mechanisms controlling the process of organogenesis (Vieten et al. 2007). When used in conjunction with plant tissue culture model systems, the use of visual markers such as β-glucuronidase (GUS), luciferase (LUC), β-galactosidase (LacZ) and green fluorescent protein (GFP) could be useful in exploring the molecular mechanisms controlling plant growth and development. In plant transformation studies, GFP allows for non-destructive direct observation of gene expression events and the successful recovery of transgenic plants (Hraška et al. 2006). Therefore, microscopy coupled with plant tissue culture offers a platform for molecular/morphology assessments in plant growth and development studies.

Furthermore, regardless of the plant cell, tissue and organ culture technique, establishing the specific origin of structures such as adventitious shoots, roots and somatic embryos remains one of the unsolved mysteries in plant biology (Trigiano et al. 2005). Numerous microscopy studies have provided significant insights into the basic structural features of in vitro plant growth (Table 1). For example, using light microscopy, the regeneration rate of hypocotyl subsections (C1–C4) in *Watsonia lepida* showed that cell division was highest in C2 while in vitro regeneration was significantly lower than in subsection C1 (Ascough et al. 2009). The authors reported that subsection C1 contained the apical meristem which possibly had meristematic cells that are developmentally plastic and responsive to external cues. Using optical microscopy da Cruz et al. (2014) showed that cell proliferation within the pericycle led to adventitious bud formation in *Bixa Orellana* root explants. Rocha et al. (2012) characterized the anatomical events and ultrastructural aspects involved in

Table 3 The role of microscopic techniques in subcellular localization, fluorescent protein tagging and transgene expression in plant cell, tissue and organ cultures

Microscopic technique	Plant species	Objective(s) of study	Probe	Observation(s)	References
CLSM	*Arabidopsis thaliana* (L.) Heynh	Identify molecular factors responsible for cytokinin-induced higher cell division rate in *Arabidopsis amp1* mutant	–	Higher mitotic index (2.5-fold) in *amp1* mutant compared to wild type	Nogué et al. (2000)
CLSM	*Coffea canephora* Pierre ex A.Froehner	Effect of calcium ionophore A23187 on somatic embryogenesis	–	Subcellular localization of calcium ions in cytoplasm	Ramakrishna et al. (2011)
CLSM	*Coffea canephora* Pierre ex A.Froehner	Effect of indoleamines and calcium on somatic embryogenesis	–	Localization of serotonin in vascular tissues of stems, roots, and somatic embryos	Ramakrishna et al. (2012)
CLSM	*Gossypium hirsutum* L.	Evaluate the effect of over-expression of WUSCHEL (*WUS*) gene on somatic embryogenesis and/or organogenesis	GFP	Localization of WUS–GFP in embryogenic tissues	Bouchabké-Coussa et al. (2013)
CLSM	*Hordeum vulgare* L.	Transformation procedure for barley by *Agrobacterium* infection of in vitro-cultured ovules	GFP	Transient GFP expression in cells within the ovule including GFP-expressing cells in the micropylar	Holme et al. (2006)
CLSM	*Lindernia brevidens* Skan	*Agrobacterium tumefaciens*-based transformation protocol for the desiccation-tolerant species	eGFP	Localization of eGFP expression observed specifically in the guard cells of desiccated leaves	Smith-Espinoza et al. (2007)
CLSM	*Nicotiana tabacum* L. ecotype 'Little Havana'	Investigating subcellular localization of *Arabidopsis* ICK proteins and domains responsible for this localization	GFP	All GFP–ICK fusion proteins exclusively localized in the nucleus	Bird et al. (2007)
Light microscopy	*Araucaria angustifolia* (Bert.) O. Kuntze	Expression of a Somatic Embryogenesis Receptor-Like Kinase (SERK) gene family member	–	*In situ* hybridization showed that AaSERK1 transcripts accumulate in groups of cells at the periphery of embryogenic calli	Steiner et al. (2012)
Light microscopy	*Passiflora morifolia* L.	Effect of auxin, cytokinin and sucrose on the regulation of cell cycle machinery	–	Spatial expression of *PmCYCD1* (D-type cyclin gene) observed using in situ hybridization	Rosa et al. (2013)
TEM	*Arabidopsis thaliana* (L.) Heynh	Spatial and temporal distribution of lipid transfer protein 1 (LTP1) epitopes during induction of somatic embryogenesis	GFP	Immunogold localization of LTP1 epitopes in the outer periclinal, anticlinal, cell walls and cytoplasm of protoderm	Potocka et al. (2012)

GFP green fluorescent protein, *ICK* inhibitor/interactor of cyclin-dependent kinase, *CLSM* confocal laser scanning microscopy, *TEM* transmission electron microscopy

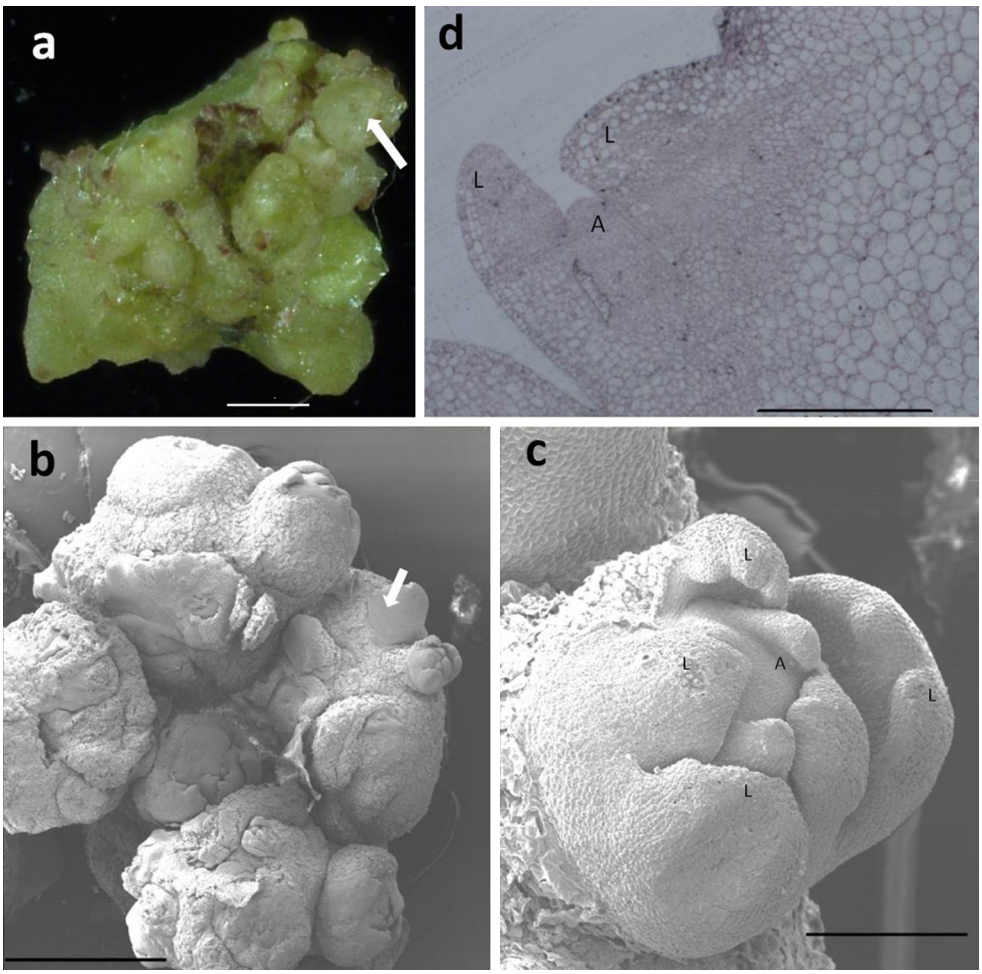

Fig. 2 Induction of asynchronous nodular meristemoids on *Sclerocarya birrea* leaf explants under a 16-h photoperiod. **a** A stereomicrograph showing somatic embryo-like globular structures (*arrowhead*; *bar* 1.0 mm). **b** Scanning electron micrograph of nodular meristemoids at different stages of development (*solid arrow* emerging shoot bud; *bar* 1.5 mm). **c** Shoot bud with characteristic shoot apical meristem (A) and developing leaf primordia (L) (*bar* 430 μm) **d** Longitudinal section of a nodular meristemoid showing its connection to the explant tissues (*bar* 200 μm). Modified from Moyo et al. (2009)

Passiflora edulis direct and indirect in vitro organogenesis. The study showed that irrespective of the organogenic process, *P. edulis* meristemoids had similar ultrastructural characteristics. These and other similar findings provide increased knowledge and critical insights that allow a better understanding of in vitro organogenic processes.

Other cellular developmental patterns

Furthermore, light and electron microscopy have contributed immensely in evaluating the effects of various physiological factors on cellular developmental processes (Table 1). Nakagawa et al. (2011) demonstrated that the distribution of starch grains during SE was induced by exogenous application of polyamines. In addition, spermine-treated *Triticum aestivum* plants exhibited smaller chloroplasts compared to putrescine and spermidine-treated ones, suggesting that the response was dependent on the type of polyamine (Redha and Suleman 2011). In particular, TEM provides ultrastructural details of cellular developments when plants are exposed to different physiological stimuli. Using TEM, López-Villalobos et al. (2011) showed that lauric acid induced the production of large oil bodies and a high number of organelles in *Cocos nucifera* zygotic embryos. The scope for elucidating the ultrastructural developmental patterns arising from physiological stimuli remains limitless.

Detection of in vitro-induced physiological and anatomical disorders

Despite the benefits of micropropagation, the process is often besieged by a number of in vitro-induced challenges which may be anatomical, physiological and biochemical

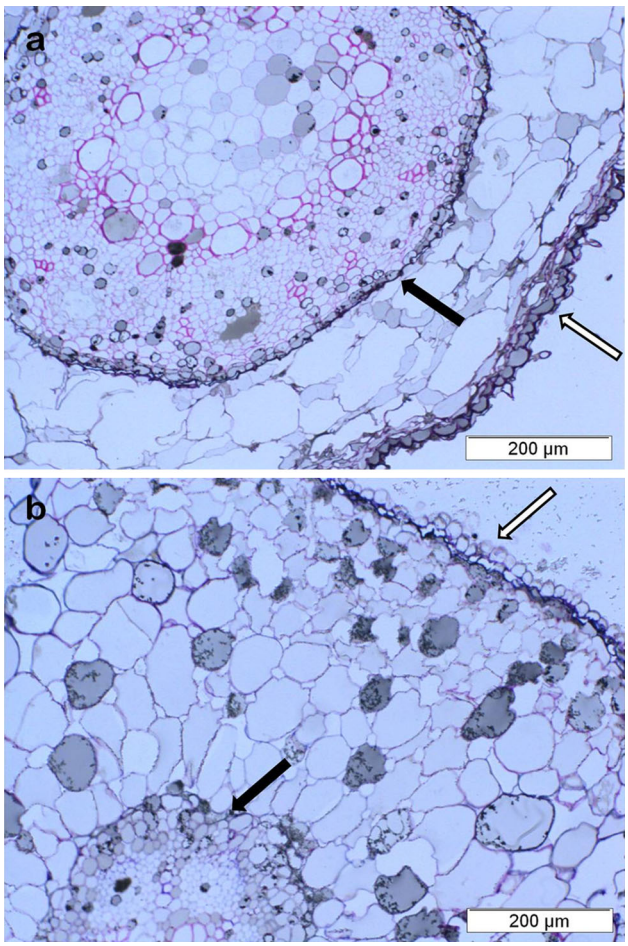

Fig. 3 Effect of the controlled plant tissue culture environment on growth and development. The root structure of **a** ex vitro and **b** in vitro grown *Sclerocarya birrea* plants showing differences in the epidermis (*white arrow*) and endodermis (*solid arrow*). Modified from Moyo et al. (2012)

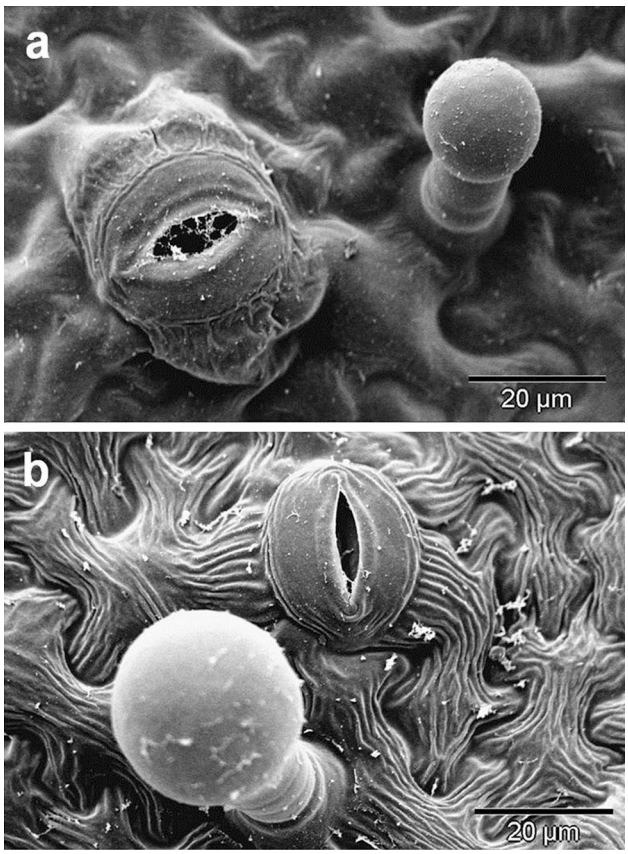

Fig. 4 Scanning electron micrographs showing the effect of photoperiod on stomata and glandular trichome formation on *Pelargonium sidoides* leaf surfaces under in vitro conditions. **a** Plants growing under 16-h photoperiod after 5 weeks in culture. **b** Plants growing under 12-h photoperiod after a 5-week culture period. Modified from Moyo et al. (2014)

in nature (Kaeppler et al. 2000; Hazarika 2006; Bairu et al. 2011; Neelakandan and Wang 2012; Ruffoni and Savona 2013). Several studies have demonstrated the effects of the controlled, largely artificial environment in plant tissue culture systems on the anatomy of in vitro plants (Figs. 3, 4). Researchers have continuously reviewed the subject matter (Bairu and Kane 2011) and suggested means of tackling the recurrent problems such as shoot-tip necrosis (Bairu et al. 2009), hyperhydricity (Ziv 1991; Rojas-Martínez et al. 2010), fasciation (Iliev and Kitin 2011), epigenetic (Kaeppler et al. 2000; Smulders and de Klerk 2011) and somaclonal variations (Larkin and Scowcroft 1981; Bairu et al. 2011). Most of these physiological disorders are not only limited to the period of in vitro growth but become more apparent upon acclimatization of the regenerants (Kozai 1991; Hazarika 2006; Pospíšilová et al. 2007). As a result, the success of plant tissue culture especially on a large scale depends on how these challenges can be alleviated or possibly eradicated (Kozai et al. 1997; Hazarika 2006; Bairu and Kane 2011). A better understanding of these multifaceted problems begins with the availability of appropriate identification tools. In view of the substantial evidence (Table 2), there is no doubt that the application of microscopy remains critical in understanding these challenges. In addition, other approaches such as biochemical and molecular tools provide complementary evidence for overall elucidation of the problems. The importance of microscopy is possibly attributed to the fact that the majority of physiological disorders are often manifested in the anatomy of the tissue-cultured regenerants.

Both light and electron microscopic techniques have demonstrated vital significance in the attempt to elucidate the anatomical and histological basis for in vitro-induced physiological disorders in several plant species (Table 2). Amongst these in vitro-induced challenges, detection of hyperhydricity has received considerable success with the use of different microscopic techniques (Table 2). Considering that hyperhydricity affects several organelles in

the cells of regenerated plants, it becomes necessary to examine the structure for detection of possible aberrations. With the use of light microscope, parameters such as surface wax topology and leaf imprints are recorded while the epidermal and stomatal cell count can be easily achieved (Correll and Weathers 2001). Variations in these aforementioned parameters afford for direct evidence on the possible underlying metabolic processes which are responsible for the incidence of hyperhydricity in regenerated plants. Evidence from scanning electron microscopy revealed that thickening of the stem and retardation of elongation are the first changes observed in hyperhydric carnation plantlets (Werker and Leshem 1987). Examining the ultrastructural differences between the hyperhydric and normal leaves of carnation plantlets, Olmos and Hellin (1998) observed large vacuolated mesophyll cells (showing hypertrophy of cells and large intercellular spaces), lack of cuticular wax and the presence of abundant plastoglobuli on chloroplasts in hyperhydric leaves. The authors also noted differences in the morphology of guard cells with X-ray microanalysis revealing high levels of K^+ on abnormal plants. Furthermore, stomatal density was significantly greater in normal leaves while the crystalline structure of the epicuticular wax was absent in hyperhydric leaves. An irregular assortment of organelles and unorganized spongy mesophyll were also observed in hyperhydric leaves.

With studies involving the use of TEM for hyperhydricity, critical examination of the ultrastructure of plant cells and tissues remains the main objective. In such instances, organelles such as the chloroplast and mitochondria are often the main focus of researchers. In *Allium sativum*, hyperhydric cells had swollen mitochondria and slender chloroplasts (Wu et al. 2009). The authors also observed that the vacuole displaced the organelle to the cell wall edge and the intergranal thylakoids appeared compressed. While the structure of mitochondria and peroxisomes did not change in hyperhydric *Capsicum annuum* plants, the number of peroxisomes was more than in normal plants (Fontes et al. 1999). Furthermore, the chloroplasts in the hyperhydric plants exhibited thylakoid disorganization, low grana number as well as presence of large starch grains and a low accumulation or absence of plastoglobules.

Subcellular localization and characterization

The benchmark discovery of the wild-type green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Shimomura et al. 1962), cloning of the GFP gene (Prasher et al. 1992), and its modification into a functional fluorescent protein (Chalfie et al. 1994) have revolutionized the study of plant cell biology. However, variable outcomes have been reported with the expression of the wild-type *gfp* gene in different plants. The expression of *gfp* in *Arabidopsis* and other plant species was shown to be curtailed by aberrant mRNA splicing in which an 84 nucleotide sequence, recognized as a cryptic intron, codes for a defective protein (Haseloff and Siemering 2005). A modified *gfp* gene without the cryptic intron sequence exhibited improved in vivo expression in a wide range of plant species (Reichel et al. 1996). Further improvements in sensitivity of the marker protein have been achieved through modifications of the GFP mutant cDNA leading to single-amino acid exchanges in the chromophore region. Green fluorescent protein and its derivative fluorophores have emerged as important reporter proteins for monitoring gene expression (Tang et al. 2005; Rosa et al. 2013; Yang et al. 2013), subcellular protein localization (Huai et al. 2009; Lai et al. 2013; Liu et al. 2013), organelle dynamics (Hashimoto et al. 2011; Tewari et al. 2013; Xu et al. 2013) and cell transformation (Holme et al. 2006), both in vivo and real time, as well as in fixed samples (Davidson and Campbell 2009). Furthermore, a combination of plant cell, tissue and organ culture techniques and fluorescent protein tags has provided a powerful tool for unravelling fundamental insights into mechanisms involved in plant morphogenesis. In vitro plant culture provides an ideal environment that can be precisely controlled and modified to achieve specific experimental conditions. Thus, the application of fluorescent probes in plant tissue culture systems has elucidated developmental and molecular mechanisms involved in plant morphogenic processes (Table 3). The most commonly used fluorescent probe application is probably protein tagging for monitoring dynamic cellular events and subcellular protein localization using confocal laser microscopy (Sirerol-Piquer et al. 2012). In addition, dynamic expression patterns of fluorescent probes have revealed interesting spatial and temporal changes in morphogenic events involving plant cell, tissue and organ culture processes such as SE (Ramakrishna et al. 2012; Bouchabké-Coussa et al. 2013) and embryonic cell suspension cultures (Cole et al. 2013).

Beyond the resolution limits of light microscopy, GFP immunogold TEM provides more detailed information on subcellular localization of proteins. The high-resolution property of TEM allows for the detection of immunogold labelled GFP-tagged proteins in the cytoplasm, organelles and plasma membrane (Boevink et al. 1998; Nebenführ et al. 1999; Follet-Gueye et al. 2003). Using this immunocytochemical technique, Potocka et al. (2012) demonstrated spatial and temporal changes in the distribution of lipid transfer protein epitopes during SE. However, the technique has only been sparsely applied in studying morphogenesis in plant cell, tissue and organ cultures (Table 3). Notwithstanding benefits derived from the high resolving power of immunoelectron microscopy,

the technique has inherent drawbacks such as preservation of GFP antigenicity and antibody specificity, arising from denaturization and bleaching of GFP during polymerization; decreased immunogold staining with tissue depth (Sirerol-Piquer et al. 2012) as well as safety concerns associated with the use of uranyl acetate in specimen preparation (Carpentier et al. 2012). In attempts to find alternatives for uranyl acetate, polyphenolic compounds such as tannic acid (Kajikawa et al. 1975) and oolong tea extracts (Sato et al. 2008; Carpentier et al. 2012) have been evaluated for staining ultrathin sections. Other recent protocols using microwave-assisted processing resulted in good preservation of cell antigenicity and high-quality cell ultrastructure for immunocytochemical studies (Carpentier et al. 2012). Polyphenol-containing extracts, for example oolong tea extracts exhibited good counterstaining properties for both ultrathin sections and *in block* staining, making them possible alternatives for the hazardous heavy metal stains such as uranyl acetate and lead citrate. Notwithstanding, specimen fixation with glutaraldehyde and osmium tetroxide (OsO_4), and double electron staining with uranyl acetate and lead salts provide excellent contrast enhancement, hence it has remained standard procedure in most microscopy laboratories (Sato et al. 2008). Thus, until the discovery of suitable alternatives, common stains such as uranyl acetate, uranyl formate, methylamine tungstate and methylamine vanadate will continue to be used but with emphasis on observance of safety regulations.

The search for fluorophores with low phototoxicity and decreased autofluorescence has advanced the boundaries of fluorescent protein engineering. Together with the development of high-resolution imaging techniques, a range of fluorescent protein probes with diverse spectral qualities spanning the orange, red and far-red regions of the electromagnetic spectrum have been developed (Davidson and Campbell 2009). Some studies (Smith-Espinoza et al. 2007; Wu et al. 2011; Sun et al. 2013) used enhanced GFP (eGFP), a variant of the GFP mutant in which exchange of amino acid phenylalanine 64 to leucine (F64L) and serine 65 to threonine (S65T) drastically increased brightness intensity and photostability (Reichel et al. 1996; Zacharias and Tsien 2005). Modifications of GFP have resulted in some variants with better fluorescence characteristics, for example the maturation of eGFP is four times faster than that of the wild type (Ckurshumova et al. 2011).

Conclusions and future perspectives

The discovery and advancement of microscopic technologies have provided plant biologists with a wide array of invaluable techniques to explore cellular structures and dynamics, thereby expanding our knowledge of plant growth and development. When used in conjunction with plant cell, tissue and organ culture methods, microscopic applications have provided critical insights into the dynamics of plant growth and development. In particular, the live imaging capabilities afforded by confocal microscopy and fluorescent protein probes (GFP and its derivatives) have further advanced the boundaries in plant morphogenesis research and expanded the possibilities of what can be achieved in the future with improved resolving power of light microscopy. Development of photostable fluorophores, especially in the red and far-red spectral regions will provide more biological insights through dynamic in vivo live imaging of cellular components. Thus, notwithstanding the limited resolving power of light microscopy, the 'illuminated plant cell' (Mathur 2007) continues to contribute invaluable information on subcellular protein localization, gene expression and transport of molecules, thereby enhancing our understanding of the fundamental mechanisms involved in plant developmental process. Furthermore, immunoelectron microscopy and immunogold labelling have circumvented the drawbacks imposed by the limited resolving power of light microscopy. New advancements and novel innovations in specimen preparation techniques' using high-phenol content plant extracts such as OTE (in place of uranyl acetate) and microwave-assisted processing are likely to expand the utilization of this method. Despite having high resolving power, immunogold labelling using TEM is still limited in its deep-tissue imaging capabilities. In the future, advancements in microscopic technologies have the potential to unlock the fundamental biological mysteries of the plant cell, and thus provide profound insights into plant developmental biology.

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