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In Vitro Embryogenesis in Higher Plants

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In Vitro Embryogenesis in Higher Plants

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Cover image: Somatic embryogenesis in sweet orange (Citrus sinensis (L.) Osbeck). Photo of Maria Antonietta Germanà

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Dedication

To Emanuela,
Maria Luisa,
Antonio and
Gabriele,

the best experiments of my life

Maria Antonietta Germanà

To my beloved children
Matilde and Tommaso

Maurizio Lambardi

Foreword

I was fortunate to start my research career in plant tissue culture in the 1970s when this field of research was expanding rapidly. The next few decades witnessed an exponential growth in knowledge, understanding, and application of many tissue culture protocols to a wide range of plant species. Then followed a period in the 1990s and turn of the century when plant tissue culture research was neglected. Many of the leading researchers of the era such as Toshio Murashige, Pierre Debergh, and Walter Preil retired. Postgraduate students and young researchers now wanted to work in the new field of biotechnology. For a time, plant tissue culture was becoming the "forgotten art" even though it underpinned new biotechnologies such as plant transformation. However, some scientists continued to work on plant tissue culture and applied new molecular genetic techniques, such as gene identification, function, and expression, to an understanding of basic plant pathways such as embryogenesis. It has been encouraging for me, as I now reach retirement, to see the next generation of experienced plant tissue culturists now filling the ranks of the experts who have gone before. Maurizio Lambardi and Maria Antonietta Germanà are two of those scientists who are renowned for their research on plant tissue culture. I have known Maurizio both through his research and his contribution to the International Society for Horticultural Science in his role as Chair of the Commission Molecular Biology and In Vitro Culture. Maurizio is both an accomplished researcher and a genuine person who is passionate about his field of research. Maria Antonietta Germanà is an experienced researcher in gametic and somatic embryogenesis in fruit crops. I recommend them as leaders in their field and ideal authors of this book on embryogenesis.

When I first started working on plant tissue culture in the early 1970s, very little was known about embryogenesis. Why species had a predetermined genetic bias to regenerate from callus by embryogenic or organogenic pathways was a mystery. Of the species that were easy to tissue culture, why was carrot embryogenic and tobacco organogenic? In the 1980s, one of my Ph.D. supervisors advised me not to work on embryogenesis because it appeared to depend on "phases of the moon." The message was that experimental results were inconsistent because of our lack of understanding; thus it was not recommended as a topic for students who were facing a deadline and needed reliable and repeatable results. However, our knowledge of embryogenesis has been greatly expanded in recent years. This book represents a detailed overview of the current status of research on embryogenesis and the advances that have been made by researchers who have worked on biotechnology and in vitro culture. Thus the book contains chapters on "Recent advances on genetic and physiological bases of in vitro somatic embryo formation," "A central role of mitochondria for stress-induced somatic embryogenesis;" "…What can we learn from proteomics?," "Genome-wide approaches and recent insights," and "Microspore embryogenesis." There are chapters on somatic embryogenesis in a range of horticultural species, and an excellent series of protocols for embryogenesis from a range of explants.

I would recommend this book to students, researchers, and those who have an interest in plant tissue culture, and to those who may not realize the importance of knowledge of this "forgotten art."

President of the International Society for Horticultural Science (ISHS)
Leuven, Belgium

Roderick Drew

Preface

Embryogenesis in higher plants, one of the different routes of morphogenesis of the plant kingdom, is a fascinating example of cellular totipotency. In fact, different kinds of plant cells (somatic, gametic, nucellar, and fertilized egg cells) are able to regenerate, in nature or in vitro, an entire organism through the formation of a somatic, gametic, or zygotic embryo, a bipolar structure without vascular connection with the surrounding tissue. In vitro somatic, gametic, and zygotic embryogenesis, apomixis, and secondary embryogenesis are actually valuable tools to support plant breeding, propagation, and conservation, with relevant implications to agriculture, forestry, horticulture, and preservation of plant genetic resources. Advances in plant biotechnology, and particularly in tissue culture, led in time to a better understanding of the physiological and biochemical bases regulating the process of plant embryogenesis, and to the establishment of more and more efficient protocols of in vitro embryo induction, maturation, and conversion to plant. Moreover, the recent molecular, genomic, and proteomic studies have produced additional valuable contributions to the comprehension of the in vitro embryogenic developmental process.

The intent of the book is to present an overview of recent advances, innovative applications, and future prospects of in vitro embryogenesis in higher plants by means of topical reviews and stepwise protocols of selected species. With this goal, the book has been divided into five parts. *Part I* contains reviews on general topics (microspore, zygotic and somatic embryogenesis, in vitro and in vivo asexual embryogenesis, advances on the genetic, physiological, and proteomic knowledge of somatic embryo formation, role of programmed cell death and mitochondria in somatic embryogenesis, and innovation in the use of bioreactors). The remaining part of the book contains stepwise protocols on somatic embryogenesis in selected horticultural plants (*Part II*) and forest trees (*Part III*), on gametic embryogenesis (*Part IV*), and on some pivotal topics (*Part V*), such as the detection of epigenetic modifications during microspore embryogenesis, the in vitro embryogenesis and plant regeneration from isolated zygotes, the synthetic seed production, the induction and maturation of somatic embryos, and the cryostorage of embryogenic cultures. Some useful "Notes," a peculiarity of the series "Methods in Molecular Biology," complete all the stepwise chapters, with additional information directly coming from the authors' valuable daily experience in the tissue culture laboratory.

We are extremely grateful to all the authors for providing such excellent contributions, coming from their remarkable expertise on the different aspects of in vitro plant embryogenesis. It is our hope that this book will be a useful source of information and ideas for plant tissue culturists, cell biologists, embryologists, horticulturists, and operators of commercial nurseries. It is also our hope that it will attract students and young scientists toward the fascinating world of in vitro embryogenesis in higher plants.

Palermo, Italy — ***Maria Antonietta Germanà***
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Part I

Reviews on General Topics

Chapter 1

A Comparison of In Vitro and In Vivo Asexual Embryogenesis

Melanie L. Hand, Sacco de Vries, and Anna M.G. Koltunow

Abstract

In plants, embryogenesis generally occurs through the sexual process of double fertilization, which involves a haploid sperm cell fusing with a haploid egg cell to ultimately give rise to a diploid embryo. Embryogenesis can also occur asexually in the absence of fertilization, both in vitro and in vivo. Somatic or gametic cells are able to differentiate into embryos in vitro following the application of plant growth regulators or stress treatments. Asexual embryogenesis also occurs naturally in some plant species in vivo, from either ovule cells as part of a process defined as apomixis, or from somatic leaf tissue in other species. In both in vitro and in vivo asexual embryogenesis, the embryo precursor cells must attain an embryogenic fate without the act of fertilization. This review compares the processes of in vitro and in vivo asexual embryogenesis including what is known regarding the genetic and epigenetic regulation of each process, and considers how the precursor cells are able to change fate and adopt an embryogenic pathway.

Key words Adventitious embryony, Apomixis, Cell fate, Gametic embryogenesis, *Kalanchoë*, Parthenogenesis, Somatic embryogenesis

1 Introduction

Embryogenesis describes the development of a single cell into an embryo. In plant embryogenesis there is no cell migration, so embryo pattern formation and cell type specification is interrelated with oriented cell division and expansion. Within sexual angiosperm plant species, embryogenesis usually occurs in vivo within floral organs during the events of seed formation. Formation of an embryo can also occur via asexual pathways in seeds, from somatic plant cells in vivo or be induced experimentally from somatic plant explants or gametes in vitro.

This review describes and compares the processes of in vivo and in vitro asexual embryogenesis including what is currently understood regarding the molecular mechanisms underlying each process.

Maria Antonietta Germanà and Maurizio Lambardi (eds.), *In Vitro Embryogenesis in Higher Plants*, Methods in Molecular Biology, vol. 1359, DOI 10.1007/978-1-4939-3061-6_1,

2 Types of Embryogenesis

2.1 Zygotic (Sexual) Embryogenesis

The most prevalent form of embryogenesis in plants occurs following double fertilization in the female gametophyte (embryo sac) found in the ovule of the flower, which gives rise to the embryo and endosperm compartments of the seed (Table 1; Fig. 1a). Haploid male and female gametes form in the anther and ovule, respectively, via meiosis and subsequent mitosis [1, 2]. Double fertilization initiates when the male pollen tube containing two sperm cells enters the ovule. One haploid sperm cell fuses with the meiotically derived haploid egg cell in the female gametophyte to form the single-celled diploid zygote, which then undergoes cell division and pattern forming events to give rise to the diploid embryo [3]. The other haploid sperm cell fuses with the diploid central cell nucleus of the embryo sac, which initiates divisions to form triploid endosperm that provides resources to the developing embryo [4]. Ovule tissues that surround the embryo and endosperm contribute to the seed coat.

Evolutionary speaking, embryogenesis is a much older process than seed formation and initially resulted from the fusion of two homospores into the zygote, gradually evolving in present day heterospory [5, 6]. The zygote formed following fusion of parental gametes is the first cell evident during sexual reproduction with a competence for embryogenesis. In plants, an "embryogenic" state is not only restricted to the zygote and in the following sections, ways of attaining an embryogenic state other than via fertilization will be discussed (Table 1).

2.2 Asexual Embryogenesis in Seeds: Apomixis and Parthenogenesis in Cereals

Apomixis is a term describing a suite of developmental processes resulting in the formation of an asexual seed. Characteristic features of all apomicts include fertilization-independent formation of an egg cell or another somatic ovule cell into an embryo, and the development of functional endosperm in apomicts occurs either with or without fertilization [7, 8]. As a result, plants germinating

Table 1

Characteristics of each type of embryogenesis considered in this review

Type of embryogenesis	Precursor cell	Mode of embryogenesis	Ploidy of embryo	Biological environment
Zygotic	Egg	Sexual	Diploid	In vivo
Parthenogenesis	Egg	Asexual	Diploid	In vivo
Adventitious embryony	Nucellar/integument	Asexual	Diploid	In vivo
Somatic embryogenesis	Somatic cells	Asexual	Diploid	In vitro/in vivo
Gametic embryogenesis	Egg/sperm	Asexual	Haploid	In vitro

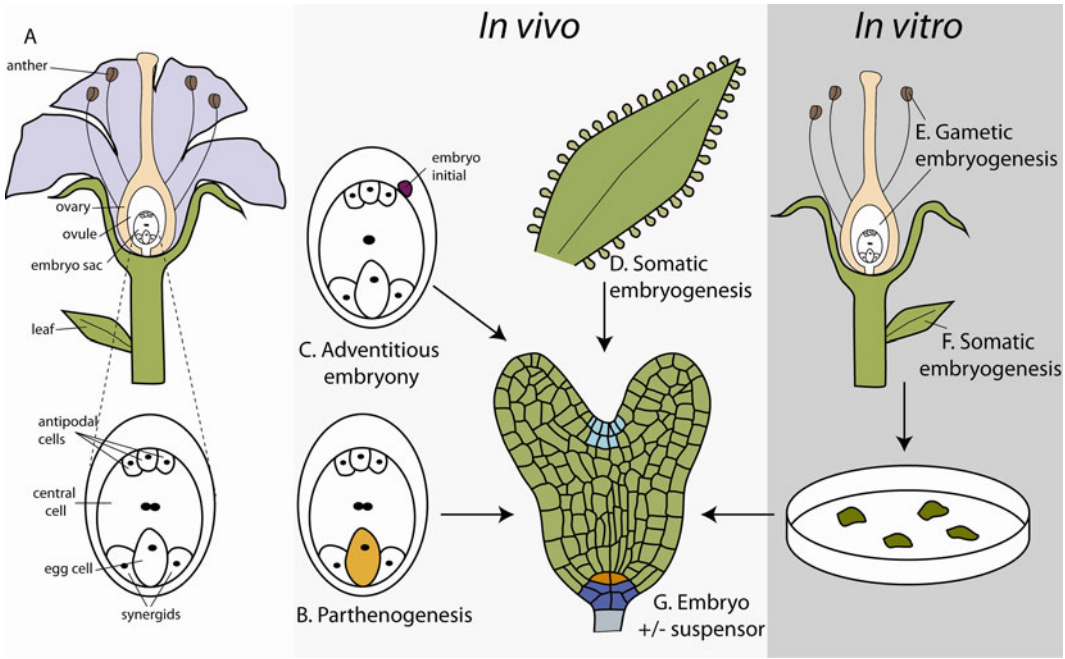

Fig. 1 Asexual embryogenesis occurs in vivo and in vitro from different cell types. (**a**) Floral organs and leaves are some of the source plant tissue for inducing embryogenesis in vitro. Asexual embryos also form in ovules in vivo; (**b**) Parthenogenesis involves the development of a chromosomally reduced or unreduced egg cell (*yellow*) into an embryo without fertilization; (**c**) Nucellar or integument cells (*red*) adjacent to an embryo sac within the ovule develop into embryos through adventitious embryony; (**d**) In vivo somatic embryogenesis is known to occur in species such as *Kalanchoë*, where the embryos develop along leaf margins; (**e**) Gametic embryogenesis involves the experimental induction of embryogenesis from gametic cells such as microspores and ovules; (**f**) Embryogenesis can be induced in somatic cells following experimental treatment; (**g**) Embryos formed via asexual embryogenesis may or may not possess a suspensor. At a heart-shaped stage, the typical plant embryo contains precursor cells for the shoot apical meristem (*blue cells*), and the root apical meristem which consists of a quiescent center (*orange cells*) and columella stem cells (*purple cells*)

from seeds derived via apomixis are genetically identical to the maternal parent.

Apomixis has evolved independently across different angiosperm plant families and genera many times, and has been documented in more than 120 angiosperm genera that belong to approximately 40 families [9]. Apomixis is genetically controlled by dominant loci in studied species and is not prevalent in agronomically important plants [10]. Apomixis mechanisms are generally divided into two categories: gametophytic or sporophytic, based upon the location of the precursor cell which develops into the embryo. In gametophytic apomixis, the embryo develops without fertilization (termed parthenogenesis) from an egg cell found inside an embryo sac that has formed mitotically without prior meiosis, and is thus chromosomally unreduced (Table 1; Fig. 1b).

Two common mechanisms termed diplospory and apospory give rise to such embryo sacs. They are distinguished by whether the starting cell is a megaspore mother cell or another somatic cell in the ovule, respectively (see Hand and Koltunow [7] for further information). Gametophytic apomixis and parthenogenesis are found and studied in species including eudicots *Taraxacum officinale* (dandelion), *Boechera* spp., and *Hieracium* spp. and also in grasses *Pennisetum squamulatum* and *Paspalum simplex* among others [11–14].

During sporophytic apomixis, which is also called adventitious or nucellar embryony, embryos develop without fertilization directly from diploid somatic ovule cells surrounding an embryo sac (Table 1; Fig. 1c). Most commonly, the embryos arise from two different ovule tissues: the nucellus and the inner integument. Nucellar embryony is widespread among *Citrus* species [15, 16]. The embryo initial cells that give rise to the asexual embryos differentiate near the developing embryo sac [17] and they can be specified as early as the 2–4 nuclear stage of embryo sac formation [18, 19]. The embryo initial cells develop and form globular-shaped embryos that can only develop to maturity if the sexually derived embryo sac is fertilized, as the sexual and asexual embryos share the nutritive endosperm. The developing seed therefore consists of one sexual embryo and one or more asexual embryos and is termed polyembryonic. The sexually derived embryo may not develop or survive germination [17].

Asexual embryogenesis is evident within seeds of the "Salmon" system of wheat. In contrast to gametophytic apomixis, a chromosomally reduced embryo sac develops via the usual events of meiosis, spore selection, and mitosis evident in sexually reproducing angiosperms. However, salmon wheat lines are capable of up to 90 % parthenogenesis, whereby the egg is able to initiate embryogenesis without fertilization [20, 21]. Parthenogenesis capability results from translocation of the short arm of wheat chromosome 1B with the short arm of chromosome 1R of rye. This particular translocation results in the loss of two critical loci in wheat: *Suppressor of parthenogenesis* (*Spg*) and *Restorer of fertility* (*Rfv1*), along with the gain of a *Parthenogenesis* (*Ptg*) locus from rye. In addition to this translocation, parthenogenesis is dependent upon organellar DNA from *Aegilops causdata* or *A. kotschyi*, demonstrating the importance of cytoplasmic as well as nuclear factors in asexual embryogenesis in vivo [21]. The existence of fertilization-independent embryo development from different cell types in the ovules of apomicts suggests that multiple cells can acquire an embryogenic state. This contrasts with sexual reproduction where the embryogenic state is suppressed until fertilization and restricted to the egg cell within the female gametophyte. In parthenogenetic cereals the embryogenic state is attained by the egg

in the absence of fertilization whilst embryogenic competency is suppressed in the remaining ovule cell types.

2.3 Somatic Embryogenesis In Vivo from Leaves

Somatic embryogenesis is known to occur in vivo in nature, where embryos develop on the surface of plant tissue (Fig. 1d) [22]. For example, plants of the genus *Kalanchoë* reproduce asexually through the ectopic formation of plantlets along their leaf margins [23]. The plantlets arise following proliferation of cells described as "dormant meristems" that are found in notches along the leaf margin [24, 25]. Some *Kalanchoë* species require stress to induce plantlet formation while others do not and constitutively form asexual plantlets. Because of this form of multiplication, *Kalanchoë* species are known as "mother of thousands." The embryo resulting from somatic embryogenesis is diploid and genetically identical to the somatic precursor cells from which it was formed.

2.4 In Vitro Somatic and Gametic Embryogenesis

It is possible to induce asexual embryogenesis in vitro from gametic cells including male microspores (termed androgenesis), and from egg cells or the associated accessory cells found in the female gametophytes (termed gynogenesis) (Table 1; Fig. 1e). This process requires gametophytic cells to switch to a sporophytic embryo formation pathway. Application of various stress treatments such as cold/heat shock and starvation are applied to the anther, isolated microspores, cultured ovules, ovaries, or flower buds to induce the switch [26–28]. The resulting embryos are haploid, possessing either maternal or paternal chromosomes depending on the gametophytic precursor cell. The production of haploid plants through in vitro gametic embryogenesis is a powerful mechanism to generate homozygous lines much faster than using conventional breeding. Colchicine induced chromosome doubling of haploid embryos during, or just after, embryogenesis results in homozygous doubled-haploid plants which are useful tools in trait discovery and plant breeding applications [29]. Currently, microspore embryogenesis is favored over gynogenesis as a mode of gametic embryogenesis because of its higher efficiency [30].

In vitro somatic embryogenesis can also be induced in vegetative explants or cells following treatment with plant growth regulators (PGR) or stresses such as osmotic shock, dehydration, water stress, and alteration of pH (reviewed in [31]) (Fig. 1f). A few studies have addressed correspondences and differences between zygotic and somatic embryogenesis and suggest that the patterning and specification events are quite similar [32], with the exception of a lack of the suspensor and dormancy in in vitro cultured somatic embryos [33]. Therefore, the most important step in vegetative cells that undergo somatic embryogenesis must be to first gain the "embryogenic" state. Recent work suggests that a release in suppression of the embryogenic state is a plausible mechanism [6, 34].

3 Attaining an Embryogenic State

A prerequisite for embryogenesis in plants is that the precursor cell must attain an embryogenic state which provides the cellular competence for embryo formation. During gametic embryogenesis, and gametophytic apomixis, the developing gametophyte cells respond to induction signals that switch their fate from gametophytic to sporophytic. During zygotic embryogenesis, the zygote has acquired embryogenic competency following fertilization of the egg cell. In somatic embryogenesis in vitro, and adventitious embryony, the embryo precursor cells are somatic sporophytic cells which first must attain the embryogenic state. Changing the developmental fate of a cell is therefore an important component of both in vitro and in vivo asexual embryogenesis.

It has been proposed that somatic embryogenesis consists of two distinct phases which are independent of each other and are controlled by different factors [35]. The initial stage is induction, which involves the somatic cells attaining the embryogenic state usually by the exogenous application of PGR. The following stage is expression, where the newly differentiated embryonic cells develop into an embryo without any further exogenous signals. It is not yet known whether in vivo embryogenesis via adventitious embryony similarly consists of two separate independent phases. However, such a scenario could be envisaged where the sporophytic ovule cells also first acquire embryonic competence by a particular molecular signal, and then develop into an embryo without fertilization via a separate developmental program.

In the process of in vitro somatic embryogenesis, somatic cells attain the embryogenic state following the application of PGR. Auxin is most commonly used [36], although other PGR, including cytokinin and abscisic acid, have proven capable of inducing embryogenesis [37, 38]. Following treatment with PGR, the cells are cultured on a hormone-free medium. Auxin plays major roles in plant growth and morphogenesis including embryo sac development and embryo patterning [39, 40]. In addition to treatment with auxin, the frequency of somatic embryogenesis induction also depends on the species, genotype, tissue, stage of development, and endogenous hormone levels [35, 41]. Therefore although auxin is a universal induction molecule, other factors must be involved in the induction of embryonic competence. The role of cellular stress responses in the induction of somatic embryogenesis is increasingly being recognized. The process of culturing explants for somatic embryogenesis involves wounding, sterilization, and culturing of the explant, all which undoubtedly apply stress to the cells involved. Furthermore, exogenous stresses such as osmotic, heavy metal ion, temperature, and dehydration stresses can enhance

somatic embryogenesis [42–46]. The induction of somatic embryogenesis through the application of auxin or stresses may imply an interaction between auxin and stress signaling. Auxin may therefore activate a stress signaling response, which is involved in inducing embryogenic competence. Many stress-related genes are up-regulated during the early phases of somatic embryogenesis, which supports this theory [47, 48].

Whether somatic cells in vitro and nucellar, integument cells and unreduced egg cells in apomicts in vivo acquire an embryogenic state via the same mechanism is currently unknown. Unlike somatic embryogenesis, embryos formed through parthenogenesis and adventitious embryony in apomicts are subject to the developmental influences of the ovule which may produce alternate cues that induce an embryogenic state. Stress and alterations in ovule pattern formation lead to a deregulation of apomixis in *Hieracium* where embryos form ectopically in different ovule positions [49]. Although no genes have yet been identified that are responsible for inducing adventitious embryony, genes related to stress signaling have been implied in the process of nucellar embryony in *Citrus*. Kumar et al. [50] used suppression subtractive hybridization (SSH) and microarray to detect genes that were differentially expressed during asexual embryo initiation and discovered genes related to stress signaling, including heat shock proteins.

Some similarities exist in the morphology of the embryo precursor cell for in vitro somatic embryogenesis and in vivo adventitious embryony. In *Citrus* species that undergo adventitious embryony, those nucellar cells that ultimately differentiate into embryos are distinguished from surrounding nucellar cells by their large nuclei and dense cytoplasm [51]. These nucellar initial cells also have very thick callosic cell walls and later become thinner walled, rounder, larger, and with a prominent nucleus prior to cell division [17]. Histological observations of embryonic somatic cells cultured in vitro from various species show that these embryonic cells are relatively small and also contain large nuclei and dense cytoplasm when compared to other somatic cells (reviewed in Namasivayam [52]). Large nuclei and dense cytoplasm are also characteristic of cells that are precursors of the female gametophyte, including the aposporous initial cell in aposporous apomictic plants, distinguishing them from surrounding somatic cells [1, 53].

4 Embryo Morphology

Zygotic embryogenesis within angiosperms passes through a series of sequential stages to give rise to the mature differentiated structure. In *Arabidopsis* and some other angiosperms, the first division

of the zygote produces an apical cell that continues to be embryogenic, while the second basal cell is no longer embryogenic and continues to form the multicelled suspensor. Further divisions of the apical cell produce a globular embryo, and differentiation and expansion of the cotyledons leads to heart and torpedo-shaped embryos [54]. Only the suspensor derived hypophyseal suspensor cell continues to form the quiescent center and the columella stem cells of the root meristem (Fig. 1g) [55]. Variation in early cell division patterning exists between different dicotyledonous species, although the typical globular, heart, and torpedo morphological stages still usually occur [54]. Zygotic embryogenesis in monocotyledonous species differs from dicots mostly with respect to planes of symmetry and the position of the shoot apical meristem [56]. Variation in embryo formation also exists between monocot species. The embryo is the only plant structure in which both the root and shoot apical meristem is formed simultaneously. This requires a highly complex series of pattern forming and specification events, including establishment of small populations of stem cells. These cells continue to support the formation and activity of meristems during the remainder of the plant life cycle (for a recent review see [57]). Extensive studies have revealed molecular details of the formation of the major tissue types as well as the meristems themselves during embryogenesis [6].

The processes of asexual embryogenesis, both in vivo and in vitro, often differ from the regular divisions and patterning events that define zygotic embryogenesis. Embryo pattern formation during apomictic embryogenesis (parthenogenesis) can be irregular compared to zygotic embryogenesis in related sexual species. In aposporous *Hieracium*, for example, embryogenesis frequently commences earlier than in sexual plants as once the egg differentiates, it transits rapidly to embryogenesis, and in some cases altered division planes can result in a different embryo appearance. Multiple embryos can also form in aposporous *Hieracium* embryos in either the same or a secondary embryo sac [58]. Although most *Hieracium* parthenogenetic embryos resemble those formed by zygotic embryogenesis in sexual plants, embryos with one or three cotyledons have also been observed. Despite developmental alterations in the primary pattern of embryos formed in aposporous *Hieracium* species, the resulting germinated seedlings eventually exhibit normal plant growth when grown on hormone free media in vitro [58].

In vivo asexual embryogenesis in *Kalanchoë* species proceeds through the typical globular, heart and torpedo stages from meristematic cells along leaf margins [24]. However unlike zygotic embryos, *Kalanchoë* asexual plantlets resemble shoots that then grow adventitious roots from a hypocotyl structure [23]. Once the root system has developed, *Kalanchoë* plantlets detach from the mother plant, fall to the ground and become new plants.

In vitro embryogenesis could also be described as heterogeneous, as multiple developmental pathways are possible which occur at varying frequencies within a single species and even the same culture [33, 59, 60]. Detailed characterization of in vitro embryogenesis pathways has been performed using time-lapse tracking from embryonic cell suspensions [33, 61]. Early development of most microspore derived embryos involves a globular embryo with little cellular organization that undergoes symmetrical division and does not resemble a typical zygotic embryo [54]. Other microspore-derived embryos appear to form via a developmental pathway that involves asymmetric division and consequently more closely resemble zygotic embryos. Recently, microspore embryogenesis systems have been developed that consistently produce such embryos [59, 62]. These systems involve a heat stress period that is either shorter or at a much lower temperature than is usually applied.

Early during zygotic embryogenesis, a region of the embryo differentiates to become a suspensor that functions to connect the embryo to surrounding tissues, thereby positioning the embryo inside the seed [63]. The suspensor also acts to transport nutrients and hormones to the embryo. When microspore embryogenesis more closely mimics zygotic embryogenesis, a recognizable suspensor is always present, which suggests the suspensor plays a role in supporting early patterning events [59, 62, 64]. A suspensor is also formed during in vivo asexual embryogenesis, although throughout *Citrus* nucellar embryony, the suspensor becomes evident at a much later stage of development than in zygotic embryos [15]. In aposporous *Hieracium*, embryos that develop in the micropylar end of the embryo sac always form a suspensor and embryos that develop within secondary chalazal embryo sacs may or may not form a suspensor and often arrest at the globular stage [65]. The development of suspensors in asexual embryogenesis suggests that fertilization is not required for formation of the suspensor.

Unlike asexual embryos formed in apomictic seeds which undergo desiccation and dormancy as part of seed maturation, embryos formed in vitro and in vivo in *Kalanchoë* develop directly into seedlings. Despite not developing within a seed, in vitro somatic embryos also undergo some form of maturation and accumulate late embryogenesis abundant (LEA) proteins, although sometimes treatment with ABA is first required to induce maturation [66]. In vitro somatic embryos also accumulate seed storage proteins, which are recognized as important for the future development of in vitro somatic embryos into plants. Only those embryos that have accumulated enough storage proteins and have acquired desiccation tolerance will develop into normal plants [60]. A comparison between asexual in vivo and somatic in vitro embryogenesis processes was performed by measuring the

accumulation of citrin seed storage proteins in polyembryonic seeds and in vitro cultured embryos in *Citrus*. This study revealed that in vitro embryos accumulate fewer citrins and at a later developmental stage than within the polyembryonic seed, suggesting that despite not being derived from fertilization events, the nucellar embryos are influenced by the seed environment [19].

Formation of endosperm is a crucial component of seed development which does not accompany in vitro embryogenesis. The precursor of the endosperm is the large diploid central cell of the embryo sac. During sexual seed formation, the endosperm will only develop following double fertilization, when one of the two sperm cells fuses with the two central cell nuclei to produce triploid endosperm. Formation of viable seed via apomixis also requires the formation of endosperm. The majority of apomictic species studied require fertilization to develop endosperm, a process which is termed pseudogamy. In some apomictic species, typically members of the Asteraceae, endosperm can develop without fertilization of the central cell. Maternal (m) and paternal (p) genome ratios in the endosperm are typically 2m:1p in sexual species and disturbance in this ratio may lead to seed abortion. Apomicts tend to tolerate variation in endosperm ploidy and maternal and paternal genome ratios which are not easily tolerated in sexually reproducing plants, and have developed various strategies to ensure seed viability [67].

Apomictic *Hieracium* species are able to form endosperm without fertilization. The polar nuclei fuse prior to the development of nuclear and then cellular endosperm, in the absence of fertilization and the resulting endosperm exhibits a 4m:0p genome ratio in aposporous *Hieracium*. The trait of autonomous endosperm (AutE) has recently been separated from fertilization-independent embryogenesis in *Hieracium* through two inter-specific crosses [68]. Two individuals were identified that form reduced embryo sacs containing meiotically derived eggs and central cells through the sexual pathway. However, egg cells within these individuals are unable to commence embryogenesis without fertilization although in the absence of fertilization, the fused polar nuclei undergo proliferation and continue to develop cellular endosperm with a 2m:0p genome ratio. This indicates a paternal genome contribution is neither required for endosperm initiation, nor cellularization in both chromosomally reduced and unreduced embryo sacs. When egg cells from these individuals are fertilized, embryogenesis occurs to completion and viable seed is formed. It is currently unclear if the central cell is also able to be fertilized as this would result in a parental genome ratio of 2m:1p ratio as seen in sexual species [68].

5 Genes Implicated in In Vivo and In Vitro Asexual Embryogenesis

Similarities between asexual embryogenesis in vitro and in vivo raise questions regarding whether these processes are controlled by the same molecular mechanisms. Although no genes responsible for embryogenesis have yet been isolated from apomictic plants, a number of gene candidates have been identified through differential gene expression analysis, genetic mapping and study of sexual mutants with phenotypes that mimic asexual embryogenesis. Attempts to understand in vitro somatic and gametic embryogenesis have also resulted in a range of gene candidates that when expressed ectopically, result in embryo formation.

One of the first genes associated with somatic embryogenesis was *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) when its involvement was demonstrated in carrot cell cultures [69]. *SERK* was identified as a marker for cells transitioning from a somatic to an embryogenic state, due to its transient expression in established suspension cell cultures [69]. *SERK* is a leucine-rich repeat (LRR) receptor-like kinase that is also expressed in developing ovules and embryos *in planta* and may therefore influence somatic embryogenesis through the same mechanisms of the sexual pathway [69, 70]. Overexpression and downregulation of *SERK* increases and decreases the efficiency of somatic embryogenesis, respectively [70, 71]. Interestingly, a *SERK* gene has also been implicated in asexual reproduction within an apomictic grass, *Poa pratensis*. cDNA-AFLPs differentially expressed between apomictic and sexual lines of *P. pratensis* revealed a *SERK* gene that displays differential expression [72]. Apomixis in *P. pratensis* involves development of an embryo sac not from the megaspore mother cell (MMC) which is the typical precursor cell for the sexual pathway, but from a diploid somatic cell positioned nearby the MMC. These somatic precursor cells are found in the nucellar ovule tissue. Within *P. pratensis*, *SERK* is expressed in embryo sac precursor cells: the MMC in sexual plants, and somatic nucellar cells in apomictic plants [72]. The same expression profile was also observed in apomictic and sexual lines of *Paspalum notatum* [73]. *SERK* expression was also examined in apomictic *Hieracium* where it was detected throughout the ovule, and expression was not restricted to the nucellar region or MMC in *Hieracium*. *SERK* expression was also observed in developing *Hieracium* embryos [74]. *SERK* is therefore thought to play an important role in changing developmental fate of cells, both in stages of apomixis and in somatic embryogenesis. *BABYBOOM* (*BBM*) is another gene that has been associated with both in vitro and in vivo asexual embryogenesis. *BBM* is an APETELA2 (AP2) transcription factor that was originally identified following subtractive hybridization of cDNA from *Brassica napus* microspores undergoing

embryogenesis [75]. Ectopic expression of *BBM* in *Arabidopsis* or *B. napus* induces somatic embryos, and constitutive expression of *BBM* genes from other species also results in the emergence of ectopic embryos [75–77]. *BBM* expression was also observed in developing *Arabidopsis* zygotic embryos [75]. These results suggest that *BBM* has a conserved role in the induction and/or maintenance of embryo development. *BBM* genes have also been identified within a genomic region essential for apomixis in the apomictic grass *Pennisetum squamulatum* [78]. The apospory-specific genomic region (ASGR) of *Pennisetum* was identified following marker analysis of a selection of apomictic and sexual plants, which revealed a set of apomixis-specific markers that define the ASGR [79]. Sequencing of BAC clones from within the ASGR revealed putative protein coding regions, including two of which had similarity to *BBM* of rice [78]. The ASGR is thought to contain genetic elements responsible for both the formation of a diploid embryo sac, and the process of parthenogenesis. The *BBM* genes within the ASGR are therefore candidate apomixis genes with strong potential to have a role in the induction or maintenance of asexual embryogenesis in *Pennisetum* apomicts. However, confirmation of a role for *BBM* in parthenogenesis has not yet been reported.

The involvement of common genes in zygotic and asexual embryogenesis implies that despite arising from different activation signals and different tissues, each embryogenesis process converges on a similar developmental pathway. Genes with a known involvement in zygotic embryogenesis have therefore been studied in asexual embryogenesis systems to understand whether such genes are also involved in asexual embryogenesis. The *LEAFY COTYLEDON* (*LEC*) family of transcription factors is crucial for regular embryogenesis and is also implicated in somatic embryogenesis. *Arabidopsis* contains three *LEC* genes: *LEC1*, *LEC2,* and *FUSCA3* (*FUS3*), and each of these genes is expressed exclusively in the embryo [80–82]. Ectopic expression of each of the three *LEC* genes leads to vegetative cells adopting characteristics of maturation-phase embryos, and hence this gene family is associated with the process of somatic embryogenesis [80–82]. The *LEC* genes have been linked to auxin production, as *LEC2* is known to activate the auxin biosynthesis genes *YUCCA2* and *YUCCA4* [83]. *FUS3* expression also increases in response to auxin [84]. This interaction with auxin signaling is thought to be responsible for the ability of *LEC* gene expression to induce embryonic competence.

LEC1 has been studied in *Kalanchoë* species and is implicated in the process of asexual plantlet formation in these species. Compared to *Arabidopsis*, the *LEC1* gene of *Kalanchoë daigremontiana* (*KdLEC1*) is truncated and does not rescue the *Arabidopsis lec1* mutation, suggesting it functions differently to

LEC1 in *Arabidopsis* [23]. A functional full length copy of *LEC1* was created by replacing the deleted nucleotides in *KdLEC1* with the corresponding nucleotides from *Arabidopsis* and transformation of *Kalanchoë daigremontiana* with this synthesized *LEC1-LIKE* gene results in disrupted asexual reproduction and in some instances abortion or absence of plantlet formation [85]. This study strongly supports the involvement of *LEC1* in in vivo asexual embryogenesis in *Kalanchoë* and furthermore suggests that the switch from sexual to asexual propagation in the evolution of *Kalanchoë* was probably activated following truncation of the *KdLEC1* gene [85].

Another gene that appears to be involved in the induction of an embryogenic state is the RWP-RK domain containing (RKD) transcription factor RKD2, which is preferentially expressed in the egg cell of *Arabidopsis* and wheat [86]. Ectopic expression of *RKD2* results in ovule integument cells that become enlarged and densely cytoplasmic with prominent nuclei, suggesting these cells have become pluripotent [87]. Ectopic *RKD2* expression also results in some integument cells adopting an egg cell identity, and a low frequency (ca. 0.1 %) of embryo-like structures also appear outside of the embryo sac [87]. This observation is reminiscent of adventitious embryony and may indicate that RKD2 is involved in the induction of embryogenesis from ovule tissue during adventitious embryony.

Additional genes including *WUSCHEL* and *AGAMOUS-Like 15* (*AGL15*) are known to induce embryo formation from vegetative tissue when ectopically expressed, and have therefore been implicated in somatic embryogenesis [88, 89]. *WUSCHEL* is known to be involved in specifying and maintaining stem cells in the shoot and root meristem [90] while AGL15 is known to accumulate in developing embryos [91], therefore a role in embryogenesis is to be expected for both of these genes. However, with the exception of *SERK*, most of the genes shown to be involved in zygotic and asexual embryogenesis are not specifically expressed in the egg cell or the zygote. Therefore, whilst important for later stages of embryo development, these genes may not be involved in the process of embryo initiation which is possibly the most important aspect of asexual embryogenesis. It has been proposed that the observed ectopic embryo development associated with misexpression of these genes, is a result of cellular stress, rather than a specific initiation signal expressed by the genes [92]. This hypothesis is consistent with embryonic competence being induced by stress factors, as discussed earlier.

To understand the genetic elements responsible for inducing embryonic competence in both in vitro and in vivo asexual embryogenesis, future experiments will likely focus on comparison of gene expression from embryo precursor cells directly before and after the initiation of embryogenesis. Genetic mapping of apomixis loci

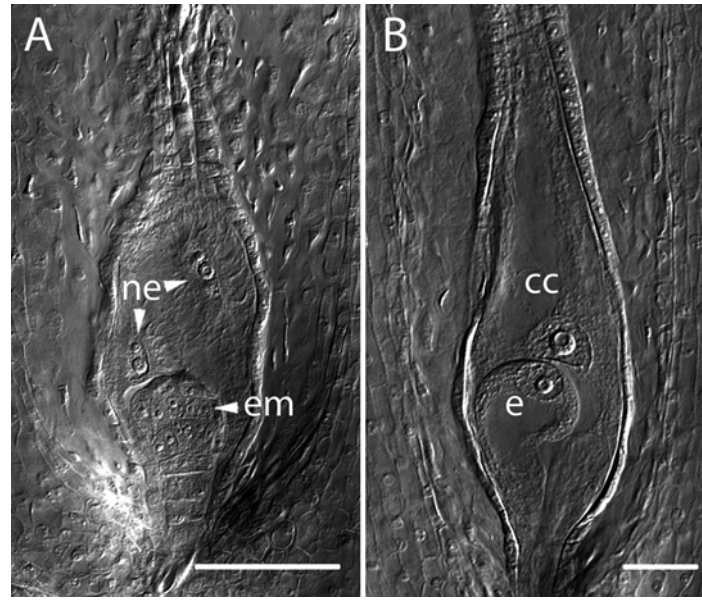

Fig. 2 Cleared ovules from wildtype apomict *Hieracium praealtum* (**a**), and a *H. praealtum lop* mutant (**b**) that has lost the capacity to undergo parthenogenesis and autonomous endosperm development. Within apomictic *H. praealtum* ovules, embryo and endosperm develop from the egg and central cell, respectively, without fertilization (**a**). *H. praealtum lop* deletion mutant m179 (**b**) has lost this capacity and the egg and central cells do not develop further without fertilization. Scale bars = 50 μm. *em* embryo, *e* egg, *cc* central cell, *ne* nuclear endosperm. Ovules were collected at stages 6 (**a**) and 10 (**b**) of capitulum development according to Koltunow et al. [65]

may also reveal which genes are responsible for the initiation of asexual embryogenesis. Genetic analyses of apomicts have shown that gametophytic apomixis is inherited as a dominant trait. In many apomictic species, developmental components of apomixis (meiotic avoidance and parthenogenesis) are controlled by independent loci and further research is underway to isolate the causal sequences that underlie these loci. For example, characterized deletion mutants developed in apomictic *Hieracium praealtum* revealed a genomic region responsible for fertilization-independent embryogenesis and endosperm formation, named *LOSS OF PARTHENOGENESIS* (*LOP*) [93]. Deletion of *LOP* sees the plant become dependent upon fertilization for both embryo and endosperm development (Fig. 2) [13]. Genetic mapping of *LOP* and *AutE* is the focus of current work that may lead to isolation of the causal sequences for both traits.

A genomic locus strongly associated with adventitious embryony in *Citrus* has also been identified [94]. Further characterization of this locus may clarify the mechanism of adventitious embryony and identify the genetic element responsible for

inducing asexual embryogenesis *in planta*. Similarly, the search for genes within the controlling parthenogenesis loci of the salmon wheat system may reveal those genes that are responsible for parthenogenesis in this system. Although controlling genes are currently unknown for the salmon wheat system, it is likely that they have lost those genes required for repressing fertilization independent embryogenesis in sexual plant species.

6 Epigenetic Influence on Asexual Embryogenesis

Various epigenetic marks and pathways have been associated with both sexual and asexual embryogenesis processes, suggesting that the induction and regulation of asexual embryogenesis may involve epigenetic components. For instance, the application of exogenous auxin during somatic embryo induction results in DNA hypermethylation [95], and inhibition of DNA methylation suppresses the formation of embryogenic cells from cultured carrot epidermal cells [96]. Auxin could therefore possibly reprogram gene expression through DNA methylation, leading to the induction of embryogenesis pathways within somatic cells.

Genes within epigenetic pathways have also been implicated in both in vitro and in vivo asexual embryogenesis. One such epigenetic factor implicated in asexual embryogenesis is PICKLE (PKL), a chromatin remodeling protein [97]. *PKL* is responsible for repressing the *LEC* family of transcription factors, as *pkl* mutants display overexpression of *LEC1*, *LEC2* and *FUS3,* and display a phenotype similar to that seen from *LEC1* overexpression [97, 98]. *PKL* activity is therefore acknowledged as an important regulatory mechanism for repressing embryonic identity throughout seedling growth, by suppressing the embryogenic program in somatic cells [99]. For this reason, *PKL* is also a candidate for the induction of asexual embryogenesis. Deregulation of *PKL* in somatic cells or within the egg cell would permit expression of embryogenic genes that are generally only expressed by the developing embryo following fertilization. However to date, *PKL* has not been specifically associated with asexual embryogenesis in any natural apomictic plant.

Strong evidence exists suggesting that epigenetic pathways play a crucial role in asexual embryo and endosperm development during apomixis. Mutants of the Polycomb-Group (PcG) chromatin modeling complex show phenotypes reminiscent of fertilization independent embryogenesis and endosperm formation seen in gametophytic apomixis. In particular, the Polycomb Repressive Complex 2 (PRC2) is known to be involved in the suppression of seed development in the absence of fertilization. The PRC2 is conserved between plants and animals and represses gene expression via trimethylation of histone H3 at lysine 27 (H3K27me3).

Phenotypes of asexual embryo and endosperm development have been observed when core PRC2 genes are mutated in *Arabidopsis*. For instance, the fertilization-independent seed (FIS) PRC2 complex (FIS-PRC2) consists of the genes *MEDEA* (*MEA*), *FIS2*, *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*), and *MULTICOPY SUPPRESSOR OF IRA1* (*MSI1*). Loss of function of any of these genes results in endosperm initiation and proliferation without fertilization. However, the endosperm does not cellularize [100–102]. The role of the FIS-PRC2 complex is therefore considered to inhibit central cell proliferation. In the case of *MSI1* mutants, low levels of parthenogenetic embryo initiation are observed, followed by embryo arrest, so that viable seeds are not formed [103]. The role of some of the FIS-PRC2 genes has been investigated during seed initiation in *Hieracium* spp., one of the few groups of apomicts that develop endosperm without fertilization. Downregulation of *Hieracium* FIE (HFIE), a protein linking multiple PRC2 components inhibiting fertilization-independent endosperm proliferation in *Arabidopsis* does not result in fertilization-independent endosperm proliferation in sexual plants. *HFIE* function is required for completion of both sexual and asexual embryo and endosperm development in examined *Hieracium* species [104]. These results demonstrate that the capacity for embryogenic competence and endosperm formation in apomicts may function via deregulation of other PRC2 complex family members and that additional factors are required to produce viable asexual embryos and endosperm. The identified *Hieracium* AutE plants that form endosperm, but not embryos, without fertilization may help identify and define the roles of genes that regulate the autonomous endosperm mechanism.

The PRC2 complex interacts with other genes implicated in asexual embryogenesis, including the *LEC* gene family. *LEC1*, *LEC2,* and *FUS3* are all overexpressed in *CURLY LEAF* (*CLF*) and *SWINGER* (*SWN*) double mutants, which are PcG gene homologues of the PRC2 gene *MEA* [105]. A *cis* regulatory element has been identified within the *LEC2* promoter which is responsible for recruiting the PRC2 complex [106]. These results suggest that the PcG acts to repress embryonic gene expression by histone methylation. Histone acetylation is another epigenetic mark that in contrast to histone methylation, is generally associated with transcriptional activation. Removal of the acetylation is performed by histone deacetylase (HDAC), which consequently results in transcriptional repression. Interestingly, two histone deacetylase genes (*HDA6* and *HDA19*) are partly responsible for repressing the embryonic program during *Arabidopsis* germination [107]. Another HDAC gene (*HDA7*) in *Arabidopsis* is known to be important for normal embryo development [108]. Inefficient or defective histone deacetylation of key embryonic genes may therefore be a candidate mechanism for inducing asexual embryogenesis.

7 Conclusions

While the pathways involved in developing the embryo itself appear common between the various modes of embryogenesis described here, many differences exist between the initiation processes of asexual embryogenesis in vitro and in vivo. Unlike somatic or gametic embryogenesis, apomixis-associated embryogenesis occurs near maternal reproductive tissue, and develops within a seed structure. Despite these differences, in vitro and in vivo asexual embryogenesis share some common factors: a change in the developmental fate of embryogenic precursor cells; and expression of an embryonic pathway in such cells without fertilization. Identifying molecular mechanisms that underlie these processes within in vitro systems may help to understand pathways that lead to apomixis. While some candidate genes for both in vitro and in vivo asexual embryogenesis have been identified, a role in apomixis has not yet been confirmed for any of these genes. One possibility is that embryogenesis related genes are deregulated by epigenetic factors during asexual embryogenesis. Continued research into asexual embryogenesis will yield important findings related to plant cell fate specification and the molecular regulation of embryogenesis.

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Chapter 2

Somatic Versus Zygotic Embryogenesis: Learning from Seeds

Traud Winkelmann

Abstract

Plant embryogenesis is a fascinating developmental program that is very successfully established in nature in seeds. In case of in vitro somatic embryogenesis this process is subjected to several limitations such as asynchronous differentiation and further development of somatic embryos, malformations and disturbed polarity, precocious germination, lack of maturity, early loss of embryogenic potential, and strong genotypic differences in the regeneration efficiency. Several studies have shown the similarity of somatic and zygotic embryos in terms of morphological, histological, biochemical, and physiological aspects. However, pronounced differences have also been reported and refer to much higher stress levels, less accumulation of storage compounds and a missing distinction of differentiation and germination by a quiescent phase in somatic embryos. Here, an overview on recent literature describing both embryogenesis pathways, comparing somatic and zygotic embryos and analyzing the role of the endosperm is presented. By taking zygotic embryos as the reference and learning from the situation in seeds, somatic embryogenesis can be improved and optimized in order to make use of the enormous potential this regeneration pathway offers for plant propagation and breeding.

Key words Biochemistry, Comparative approach, Maturation, Morphology, Proteome, Storage reserves, Stress response, Transcriptome

1 Introduction

Somatic embryogenesis, a fascinating developmental pathway through which plants can be regenerated from bipolar structures derived from a single or a few somatic cells was first described more than 50 years ago in carrot by Reinert [1] and Steward et al. [2]. This regeneration pathway offers a great potential to be applied in mass propagation, genetic transformation by direct means or via *Agrobacterium tumefaciens* and as a source of protoplasts as well as for long-term storage of germplasm using cryopreservation. Also fundamental studies of early embryogenesis are easier to be performed with somatic than with zygotic embryos. However, up to now the exploitation of this pathway is limited by inherent

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problems that are observed in many different plant species, like asynchronous differentiation and further development of somatic embryos, malformations and disturbed polarity, precocious germination, early loss of embryogenic potential, and strong genotypic differences in the regeneration efficiency. On the other hand, such limitations are not found in zygotic embryos developing within seeds. Thus, this review aims at comparing these two types of embryogenesis by regarding zygotic embryogenesis as a reference as suggested for the first time for wheat by Carman [3]. The identification of the major differences could enable new approaches to optimize somatic embryogenesis. Available literature dealing with comparisons of somatic and zygotic embryos on morphological, histological, biochemical, and also transcriptomic and proteomic level will be summarized, with emphasis on our model plant, the ornamental species *Cyclamen persicum*.

1.1 Zygotic Embryogenesis

The zygote is formed after double fertilization has taken place which is leading to the formation of the embryo and the endosperm. Zygotic embryogenesis is a complex, highly organized process, that has been studied for a long time by histological approaches only [4]. Recently it has been supplemented by molecular genetic studies, mainly based on mutant analyses of *Arabidopsis thaliana* as excellently reviewed in 2013 by Wendrich and Weijers [5] and depicted in Fig. 1. Embryogenesis is divided into (1) embryogenesis *sensu strictu* (morphogenesis of embryo and endosperm) meaning the development of the zygote up to a cotyledonary stage embryo and (2) the subsequent maturation phase that starts with the switch from maternal to filial control [6] and finally (3) the phase of embryo growth and seed filling ending with a desiccation phase [7].

Embryogenesis *sensu strictu* starts with a loss of polarity directly after fertilization of the egg cell which is followed by re-polarization and elongation of the zygote [5]. The important first asymmetric cell division of the zygote results in a more elongated basal cell that gives rise to the suspensor and the hypophysis and a small apical cell that generates the embryo. The suspensor positions the embryo within the embryo sac, conducts nutrients to the developing embryo and is a source of plant hormones that are important for polarity establishment [8]. It is eliminated by programmed cell death between globular and torpedo stage in angiosperms and in late embryogenesis in gymnosperms [9].

Auxin is the predominant plant hormone that has been reported to be involved in polarity and pattern formation. Especially, the PIN (PIN formed proteins) dependent asymmetric auxin efflux regulates these processes in early embryogenesis *(*[10], reviewed in 2010 by De Smet et al., [11]). The role of other plant hormones, among which cytokinins and brassinosteroids were reported to be important in these processes, is not yet clearly resolved [11].

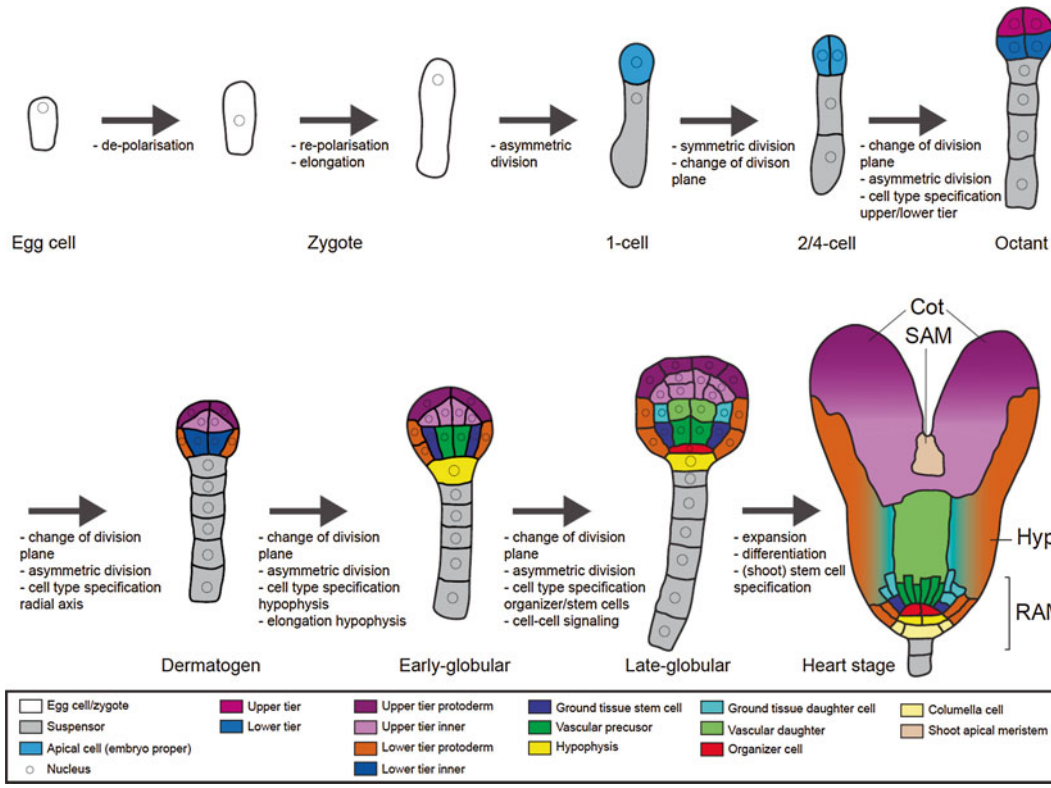

Fig. 1 Morphogenetic processes during *Arabidopsis* embryogenesis. Schematic overview of *Arabidopsis* embryogenesis from the egg cell to the heart stage embryo, highlighting the morphogenetic processes required to progress from one stage to the next. The colors represent cells of (essentially) the same type (*see* color legend), based on marker gene expression and lineage analysis. *Cot* cotyledon, *SAM* shoot apical meristem, *Hyp* hypocotyl, *RAM* root apical meristem (reproduced from [5] with permission from New Phytologist)

Subsequent organized cell division in a symmetric way and only in one direction leads to the formation of the suspensor. In the apical cell division planes change in a strictly regulated way in *A. thaliana* and thereby establish two types of axes, defining upper and lower tiers and radially arranged cell types [5]. Most interestingly, the first cell divisions take place within the space provided by the apical cell. Thus, pattern formation occurs in the globular embryo by which the protoderm cells, vascular and ground tissue are defined. The last stage of embryogenesis *sensu strictu* is the heart stage being characterized by the presence of shoot and root apical meristems as well as early cotyledons. The key genes regulating morphogenesis of the embryo have been identified and encode transcription factors, receptor kinases, proteins involved in plant hormone signaling and micro RNAs pointing to the predominant

transcriptional control, and future research needs to focus on how these regulators hold their function in terms of cell biological implementations [5].

The later phase of seed development (maturation phase) comprises embryo growth, seed filling by deposition of storage reserves and finally desiccation. Mainly seed dormancy has attracted the attention of research in *A. thaliana* and other species (reviewed by Finch-Savage and Leubner-Metzger in 2006, [12]). Seed filling is of importance for many agricultural crops like rape seed or legumes as well (reviewed by Verdier and Thompson in 2008, [13]). At the end of seed development, the zygotic embryo is in a quiescent state which clearly separates embryogenesis from germination.

1.2 Somatic Embryogenesis

The term somatic embryogenesis already points to the pronounced morphological similarity of this vegetative regeneration pathway to zygotic embryogenesis. Somatic embryogenesis generally starts from a single cell or a group of cells of somatic origin and direct somatic embryogenesis is distinguished from indirect somatic embryogenesis in which a callus phase is passed through. The induction of embryogenic cells sometimes refers to all events that reprogram a differentiated cell into an embryogenic cell, but recently was divided into different phases, i.e., dedifferentiation, acquisition of totipotency, and commitment into embryogenic cells [14]. The first important difference compared to zygotic embryogenesis is the need for both, transcriptional and translational reprogramming of a somatic cell. Dedifferentiation of the somatic cells is the prerequisite to gain embryogenic competence and results in genetic reprogramming, loss of fate, and change into meristematic cells [15]. Stress due to wounding, separation from surrounding tissue, in vitro culture conditions, and also auxin are discussed to have a pivotal role in dedifferentiation [15]. Elhiti et al. [14] postulated that cells have to be cytologically separated for dedifferentiation as expression of genes responsible for secondary cell wall formation changed. Moreover, pronounced changes in the network that regulates the response to hormones have to take place. Twenty-five candidate genes being associated with the expression of cellular totipotency were identified by a bioinformatic approach using the CCSB (Center of Cancer Systems Biology) interactome database and *Arabidopsis* as a model for a molecular regulation network [14]. They cover functions in transcription, signal transduction, posttranslational modification, response to plant hormones, DNA repair and DNA methylation, and for the first time protein phosphorylation and salicylic acid signaling. The final step of the induction phase, the commitment into embryogenic cells, involves genes for signal transduction, microtubule organization, DNA methylation, regulation of transcription, apoptosis, and hormone-mediated signaling [14].

The establishment of polarity and a first asymmetric cell division has been observed in early somatic embryogenesis of carrot [16] and alfalfa [17]. By cell tracking experiments it was shown that carrot somatic embryos developed from different single suspension cells either via a symmetric or via an asymmetric first division [18], indicating that an asymmetric division is not decisive for proper somatic embryo development. However, as stated by Feher et al. [15], polarity, in terms of the transcriptional and biochemical status of the cell, is not necessarily expressed at the level of the morphology and symmetry of cell division. Therefore, early polarization is thought to be crucial in somatic embryogenesis as well as in zygotic embryogenesis, but needs to be set up by the cell internally following an external stimulus. The suspensor originating already from the first asymmetric division of the zygote is also formed in somatic embryos of conifers. It is supposed to support polarity and axis establishment in embryos and undergoes programmed cell death also in somatic embryos (reviewed by Smertenko and Bozhkov in 2014, [8]). In contrast, suspensor structures are often not so clearly detectable or completely missing in somatic embryos of plant species other than gymnosperms.

Due to the difficulty of identification of embryogenic cells, the early stages up to the globular embryo, and especially the precise sequence of cell divisions that can be described for *Arabidopsis* zygotic embryogenesis resulting in pattern formation have not often been recorded in somatic embryogenesis systems. Most studies that track the development of somatic embryos start with the globular stage [4]. Further development runs through the typical stages of angiosperm embryogenesis in dicots, namely globular stage, heart stage, torpedo stage, and cotyledonary stage. For a long time, markers for competent cells have been searched for, and most promising are Somatic Embryogenesis Receptor like Kinases (SERKs), that were identified to play a role in zygotic and somatic embryogenesis in *Daucus carota* [19] and *A. thaliana* [20]. They are involved in perception and transduction of extracellular signals and connected to brassinosteroid signaling [21], but their exact function is unknown up to now.

Maturation includes accumulation of storage reserves, growth arrest, and acquisition of desiccation tolerance and is, in case of somatic embryos, induced externally by increasing the osmotic pressure (lowering the osmotic potential) of the culture media (e.g. by addition of polyethylene glycol or increased sugar concentration) and application of abscisic acid (ABA) [22]. Germination requires similar conditions as in the respective zygotic embryos and completes this developmental pathway. Obviously, somatic embryos are completely lacking the effects of the surrounding seed tissues which provide physical (space) constraints and a specific and complex interaction of testa and endosperm supporting embryogenesis in an optimal way. For the induction of embryogenic cells,

external stimuli are mainly coming from the culture media, plant growth regulators, and culture conditions, but thereafter somatic embryogenesis is following an intrinsic autoregulatory developmental program [8]. Most likely, this process can be improved by mimicking conditions found in seeds.

2 Comparison of Somatic and Zygotic Embryos

2.1 Morphological and Histological Comparison

The fact that somatic embryogenesis was named after embryogenesis taking place in seeds clearly indicates a high degree of similarity of somatic and zygotic embryos. Many early studies were devoted to describe morphological aspects involving histological and microscopic investigations. Due to the typical stages both types of embryos pass through, globular, heart, torpedo, and cotyledonary stage, the parallels become obvious. Both kinds of embryos are bipolar structures from the beginning and do not have a vascular connection to maternal tissue which enables the discrimination of somatic embryogenesis and adventitious shoot regeneration.

The first cell division of the zygote is asymmetric while in somatic embryos this is not always the case (see above, [18]). Mathew and Philip [23] described the regeneration of *Ensete superbum* via somatic embryogenesis starting from single cells without the need of strong polarity establishment in these cells. However, all further stages that were compared in this histological approach revealed high similarity of somatic embryos to their zygotic counterparts in terms of structure of the embryonic apex or formation of cotyledons and hypocotyls. In many indirect somatic embryogenesis systems, the so-called proembryogenic masses, being clusters of small, dense cytoplasm rich embryogenic cells, give rise to the differentiating embryos, but their first divisions have not often been observed in detail, since the cell or the cell group from which the embryo originates is difficult to identify. While in gymnosperm somatic embryos the suspensor is a very prominent structure that in late embryogenesis undergoes programmed cell death [8], in many angiosperm systems suspensors are either absent or strongly reduced which might explain the difficulties in root formation reported for some species, especially due to the absence of the hypophysal cell [4].

Maize secondary somatic embryos derived from single primary somatic embryos or somatic embryos developing attached to callus cells, revealed malformations in the shoot meristem formation after direct regeneration of the single somatic embryos, while those that developed next to callus cells perfectly represented zygotic embryo development [24]. The authors discuss a possible role of the neighboring callus cells with similar functions as suspensor cells in the zygotic situation. Interestingly, in our model plant *C. persicum*

[25] embryogenic cultures are mixtures of embryogenic and nonembryogenic cells, and the differentiating somatic embryos are surrounded by a extracellular matrix resembling several cell wall layers (Douglas Steinmacher, Melanie Bartsch, and Traud Winkelmann, unpublished data). One possible explanation, for which further evidence is needed, could be that nonembryogenic cells undergo programmed cell death and thereby enable differentiation. In *Eucalyptus nitens* somatic embryos are only sporadically observed, but then appear on dark brown wounded callus cells [26]. An ultrastructural study not only recorded several analogies in cell and embryo structure when compared to zygotic embryos, it also identified a kind of waxy coat surrounding the somatic embryos which was supposed to originate from phenolic exudates [26]. Somatic embryos of *C. persicum* have three times larger cells than their zygotic counterparts, and their outer surface is more irregular than the smooth protoderm of zygotic embryos [27]. This observation indicates that the physical and chemical constraints of the surrounding tissue, the endosperm, may have an important influence on the cellular organization of zygotic embryos that is lacking in somatic embryogenesis systems (*see* also Subheading 3).

Maturation is a major bottleneck in somatic embryogenesis of several species including *Pinus pinaster* [28] and coffee [29]. Also loblolly pine somatic embryos did not reach full maturity and had lower dry weights than the zygotic ones [30]. Polyethylene glycol (PEG) which is often used in maturation media of conifers had clear effects on the morphology of somatic embryos of *P. pinaster* as numerous and larger vacuoles as well as larger intercellular spaces were induced by this treatment [28]. By the histological comparison of somatic embryos subjected to different maturation treatments (carbohydrates in various concentrations) protein bodies were found to appear earlier in somatic embryos, and to be more abundant in well-developed somatic embryos leading to the suggestion that storage protein accumulation could be regarded as a marker for embryo quality of *Pinus pinaster* [28]. The same authors observed starch accumulating in zygotic embryos in a gradient of higher concentrations at the basal end, whereas in somatic embryos the localization of starch granules strongly depended on the maturation treatment. However, irrespective of the maturation treatment, somatic embryos always contained higher amounts of starch than the zygotic ones again with significant differences between different kinds and concentrations of carbohydrates applied [28].

Another aspect, namely the water status, was studied in *Hevea brasiliensis* embryos [31]. In zygotic embryos the water content decreased sharply from 91 to 53 % within 1 week (14–15 weeks after pollination) and during the remaining maturation phase down to 42 %. In contrast, somatic embryos without maturation treatments had a water content of nearly 80 %, while those that had

been desiccated or cultivated on higher sucrose concentrations plus ABA still contained 71 % water but had much higher germination and conversion rates than the nontreated ones [31]. Also in date palm the zygotic embryos underwent dehydration with a water content of 80 % decreasing to 35 %, whereas somatic embryos had a water content of around 90 % throughout the whole development [32]. Both mentioned species still have high water content in the seed after desiccation. In species with true orthodox seeds and much lower water contents, the drop in water content and thereby the discrepancy between somatic and zygotic embryos can be expected to be even more pronounced.

Somatic embryogenesis is already commercialized in coffee, but its profitability is limited due to losses during conversion into plantlets. Thus, Etienne et al. [29] put special emphasis on studying this phase in the zygotic and somatic system. Differences were found in conversion time which took 22 weeks in somatic and 15 weeks in zygotic embryos, hypocotyl length being shorter in somatic embryos, a more spongy tissue in the somatic embryo axis, earlier differentiation of stomata in somatic embryos and less protein and starch in cotyledonary somatic embryos [29]. The water content of zygotic embryos increased strongly during germination starting from 28 % and reaching 80 % within 4 weeks, whereas the increase in somatic embryos was rather mild (water content from 70 to 85 %). Furthermore, the authors observed asynchronous germination in somatic embryos. It can be concluded that the phase of maturation which includes a growth arrest controlled by plant hormones (mainly ABA) and desiccation is obviously extremely important to allow the development of high quality somatic embryos that will germinate in high rates and in a synchronized way.

2.2 Biochemical Comparison

When screening the literature for studies comparing somatic and zygotic embryos on the biochemical level, mainly analyses of major storage compounds, i.e. storage proteins, carbohydrates, and lipids are found. Depending on the type of seed in a respective species, storage reserves may be found in the embryo itself and here mainly in the cotyledons or in the endosperm. Early studies in *Brassica napus* [33] and cotton [34] have shown that somatic embryos are able to accumulate storage proteins, but in much lower amounts (1/10 of that found in zygotic embryos in *B. napus*) and in earlier stages. In somatic embryos of alfalfa 7S globulin was dominant, while in zygotic embryos 11S globulin and 2S albumin were more abundant [35]. The processing and subcellular localization of 7S and 11S storage proteins in protein bodies was comparable in both embryo types, while 2S albumin in somatic embryos was detected in the cytoplasm, in contrast to zygotic embryos in which 2S albumins were localized in protein bodies [35]. Overall, also in alfalfa lower amounts of storage proteins were determined in somatic embryos, thus supporting the observations in *B. napus* and cotton. Thijssen et al. [36] visualized globulin (storage protein)

2.2.1 Storage Proteins

accumulation by fluorescence labeled antibodies in somatic and zygotic embryos of maize. Starting 10 days after pollination globulins were detected in the scutellum first and later in leaf primordia and roots. Lower amounts of intermediate globulin precursor proteins were found early in development of somatic embryos while mature globulins could be induced by a maturation treatment with ABA [36]. Date palm somatic embryos contained about 20 times lower amounts of total protein than zygotic embryos, a different protein composition, and were lacking glutelin, a storage protein with the typical accumulation and hydrolysis pattern in zygotic embryos [32]. In agreement with these studies are the observations in oil palm embryos in terms of earlier, but 80 times less production of 7S globulins in somatic embryos compared to zygotic ones [37]. A recent follow-up study [38] reported on early mobilization of storage proteins by proteases in somatic embryos, thus providing further evidence that the clear differentiation of the developmental phases of embryogenesis, maturation, and germination is lacking in somatic embryos. Instead there is an overlap of all three programs, since globulin synthesis still occurred during germination of somatic embryos and cystein proteases were active in all phases of somatic embryogenesis [38]. In order to gain insights into glutamine metabolism, a nitrogen compound that is important for embryogenesis, Perez-Rodriguez et al. [39] found cytosolic glutamine synthase 1a (GS1a) to be absent in zygotic, but present in somatic embryos of *P. pinaster* and *Pinus sylvestris* indicating the onset of precocious germination in late stages of somatic embryogenesis, since this gene is a marker for chloroplast differentiation. GS1b expression was detected in procambial tissues of both types of embryos with the level of expression correlating to the quality of somatic embryos [39]. Arginase expression in somatic embryos indicated that storage protein breakdown obviously started before germination [39]. Possibilities to improve storage protein accumulation by ABA treatment were shown for example for cocoa somatic embryos [40] or by increasing sucrose concentrations in maturation media for *Pinus strobus* [41] and cyclamen [42].

2.2.2 Carbohydrates

Cotyledonary white sprucesomatic embryos accumulated more starch, but less proteins and lipids than zygotic embryos in the same stage. This points to the fact that the conversion of starch into the energy rich storage compounds lipids and proteins did not take place in somatic embryos to the same extent [43]. According to this study, adjustment of in vitro culture conditions might be an option to improve this conversion during embryo maturation. Carbohydrates have important functions during plant development and growth as energy sources but also for osmotic adjustment, protein protection, and signaling molecules, and they have been analyzed in comparative approaches during somatic and zygotic embryogenesis. During maturation of cocoa zygotic embryos (*Theobroma cacao*) storage proteins and starch

accumulate, dehydration takes place and monosaccharides and sucrose decrease, while two oligosaccharides, raffinose and stachyose, increase [40]. In contrast, somatic embryos accumulated less protein and starch as detected in histological studies and they had higher levels of sucrose, xylose, and rhamnose [40]. A shift in carbohydrate composition was observed in Norway spruce for both, somatic and zygotic embryos, during later developmental stages with decreasing total carbohydrates and a higher sucrose:hexose ratio within time. However, only mature zygotic embryos contained raffinose and stachyose which play a role in desiccation tolerance [44]. After a maturation treatment with 3.75 % PEG 4000 the sucrose:hexose ratio in Norway spruce somatic embryos raised significantly from 0.88 to 6 which resembled more the ratio of 9.7 found in zygotic embryos, all in the early cotyledonary stage [45]. While in somatic embryos invertase and sucrose synthase were found in high activity during the proliferation and early maturation phase, invertase activity was low in developing zygotic embryos and sucrose synthase was first observed in the cell layer surrounding early zygotic embryos and later inside the embryos. From this the authors conclude that sucrose synthase plays an important role in the transition of the embryo from a metabolic sink to a storage sink [45]. The sucrose distribution within the embryo which is among other factors controlled by epidermal sucrose transporters was suggested to trigger starch accumulation during the maturation phase of *Vicia faba* zygotic embryos [46].

In the fruit tree *Acca sellowiana* that is native to South Brazil, total soluble carbohydrates per gram fresh mass were found to be about twice as high in zygotic compared to somatic embryos in the globular, heart, and torpedo stage, although the principal composition was the same. Especially for sucrose, fructose, myo-inositol, and raffinose (in the later stages of embryogenesis) zygotic embryos showed higher contents, even though somatic embryos were cultured in sucrose containing media. On the other hand starch contents of torpedo and cotyledonary stage somatic embryos exceeded those of their zygotic counterparts [47]. Also in pea changes in soluble sugar composition during maturation of zygotic embryos were observed with sucrose, galactinol, raffinose, verbascose, and stachyose being the most prominent in mature seeds. In contrast, pea somatic embryos contained much lower total soluble sugars being composed of fructose, glucose, myo-inositol, sucrose, raffinose, and galactinol, but lacking stachyose and verbascose. Most interestingly, irregular misshaped somatic embryos differed in their carbohydrate profiles from normal ones [48]. Taken together, these analyses on carbohydrates point to the fact that somatic embryos often contained lower total amounts of soluble sugars, in later stages show different monosaccharide:sucrose ratios and a lack or smaller amounts of raffinose and its derivatives that are considered to be important for desiccation tolerance. Thus, maturation obviously is the major bottleneck for somatic embryogenesis in several species.

2.2.3 Lipids

Comparative lipid analyses in both types of embryos are hardly found in literature, except one report for *Prunus avium* [49]: the lipid profiles of somatic embryos resemble those of zygotic embryos with neutral glycerolipids and phosphatidylcholine being the major lipid classes. However, contents of these two classes of lipids in somatic embryos were comparable to those of immature zygotic embryos, which was in line with the observation that somatic embryos did not develop further, until they received a cold treatment that resulted in increased lipid levels.

2.2.4 Polyamines

Polyamines (among which the commonly occurring spermidine, spermine, and putrescine) are assumed to play a role in embryogenesis [50] and they were quantified in somatic and zygotic embryos of Norway spruce [51]. If mature somatic embryos are contrasted to zygotic ones, the latter contained less spermidine, but more putrescine resulting in a much lower spermidine:putrescine ratio. This ratio as well as the higher absolute polyamine contents of somatic embryos may be connected to the lower germination ability of somatic embryos. However this assumption requires physiological explanations [51].

2.2.5 Plant Hormones

In a comparison of plant hormone contents in somatic and zygotic larch embryos, 100 times higher concentrations of ABA were found in somatic embryos that were cultivated on medium containing the nonphysiological ABA concentration of 60 μM. During maturation the ABA content increased in somatic embryos while it declined in zygotic ones [52]. Among the cytokinins, only for isopentenyladenine differences were detected with much higher levels in zygotic embryos, whereas IAA contents were similar in both embryo types [52]. The set of enzymes detoxifying reactive oxygen species differed between zygotic and somatic embryos of horse chestnut [53]: catalases and superoxide dismutases showed different courses of expression and different isoforms, especially in the maturation phase that resembled more the germination phase in case of somatic embryos. These authors concluded that somatic embryos seem to be exposed to higher stress levels than their zygotic counterparts.

2.3 Comparison of Transcriptomes

While an increasing number of studies on gene expression during embryogenesis of either the somatic (e.g. soybean, [54]) or the zygotic type (e.g. loblolly pine, [55]), are available, only very few reports deal with transcriptomic comparisons of somatic and zygotic embryos. In *C. persicum,* Hoenemann et al. [27] compared zygotic and somatic embryos and also embryogenic and nonembryogenic cell lines using a cDNA microarray with 1216 transcripts. They observed an upregulation of oxidative stress response genes in somatic embryos, as for glutathione S-transferases, catalase, and superoxide dismutase. These genes were upregulated not only in early stages of somatic embryogenesis but also 3 weeks

after induction, pointing at lingered stress and/or the induction of secondary somatic embryos. The importance of pectin-mediated cell adhesion as a prerequisite for embryogenicity was proposed by these authors based on the higher abundance of several genes encoding pectin-modifying enzymes in embryogenic than in non-embryogenic cells. Moreover, a cationic peroxidase that prevents cell expansion was suggested to be important for early embryogenesis [27]. Thus, the early cell divisions that do not result in expansion in size in early zygotic embryogenesis could be realized in a similar way in somatic embryos.

Recently, next generation sequencing was applied in cotton to compare the transcriptome of three comparable stages of both somatic and zygotic embryos [56]. Among a total of more than 20,000 unigenes, 4242 were found to be differentially expressed in these six samples. Of the differentially expressed genes a higher number was upregulated in somatic embryos at all stages [56]. Especially, stress response genes including hormone-related genes (mainly ABA and jasmonic acid signaling), kinase genes, transcription factors, and downstream stress responsive genes—e.g. late embryogenesis abundant (LEA) genes, heat shock proteins—were found at higher expression levels in somatic embryos. Moreover, cotton somatic embryos were found to be metabolically more active than their zygotic counterparts as indicated by gene expression data, the number of mitochondria, bigger vacuoles, and more lipid droplets [56]. Stress on the one hand can be considered as an important trigger of embryo development which also occurs in the zygotic system during maturation to prepare the embryo for desiccation stress. On the other hand, if cells experience too much stress as it might be the case under in vitro conditions, this might disturb the developmental program or even lead to cell death.

2.4 Comparison of Proteomes

The proteome reflects the total set of proteins that is present in a defined tissue in a specific developmental stage under defined conditions and thus provides direct evidence of the biochemical and physiological status of these cells. A possible disadvantage of proteomic studies is that proteins of very low abundance such as important transcription factors may be not detected. Although the number of proteins that can be detected is limited if gel-based proteomics is used, the comparison of two proteomes can be visualized very well using 2D-SDS-PAGE (two-dimensional isoelectric focussing/sodium dodecylsulfate polyacrylamide gel electrophoresis). In our own comparative studies we used a gel-based proteomic comparison of somatic and zygotic embryos of *C. persicum*, the work-flow of which is depicted in Fig. 2 [57]. The first and essential step is to select the biological material that will allow a meaningful proteomic comparison; in our studies, the selection of comparable stages was based on embryo morphology [42, 58, 59]. Spots of interest being either more abundant or even specific for

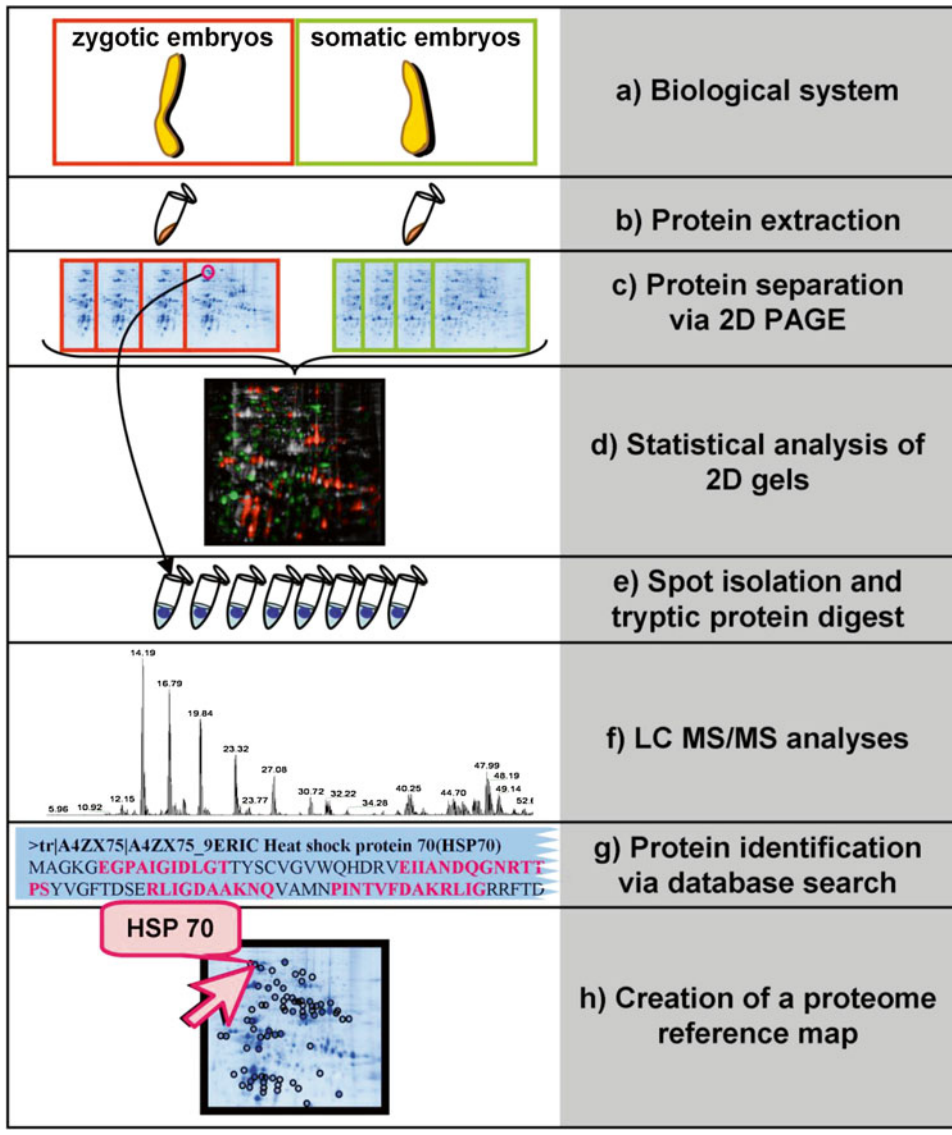

Fig. 2 Workflow of a gel-based proteomic approach combined with mass spectrometry. The biological system represents one or more samples to be analyzed via a gel based proteomic approach. In the example given in this diagram, proteomes of zygotic and somatic embryos of *Cyclamen persicum* are analyzed and compared (**a**). Therefore, total proteins are extracted from each tissue (**b**) and separated via IEF-SDS PAGE (**c**). To perform statistical analyses with gels of different tissues, at least a set of three replicates for each tissue is required. Spots that differ significantly in abundance are labeled (*green* and *red*) in an overlay image of all gels analyzed (**d**). Protein of interest (e.g., differentially abundant proteins) are isolated from 2D gels and subsequently a tryptic protein digest is performed (**e**). The resulting peptides are separated via liquid chromatography (LC) before tandem mass spectrometry analyses (**f**). Protein identification is performed based on resulting peptide sequences (*pink*) via a database search matching to known sequences (**g**). Finally, a digital proteome reference map can be designed indicating all identified proteins (**h**). Using a gel-free shotgun approach, the steps (**c**) and (**d**) are replaced by digestion of a complex protein sample which is then further analyzed (reproduced from [57] with permission from author and Leibniz Universität Hannover)

one sample can then be eluted from the gel and subjected to mass spectrometry in order to identify the protein or proteins within this spot by comparison to databases. Finally, the obtained data can be combined to an interactive reference map which in our case was made publicly accessible and allows filtering spots by their abundance, metabolic function, or tissue specificity [60]. This technique was already applied in the '90s. Comparing somatic and zygotic embryos of *D. carota* torpedo shaped somatic embryos had a clearly distinct protein pattern from zygotic embryos and lacked the maturation specific proteins, namely two globulin-type storage proteins and a LEA protein [61]. In the gymnosperm species Norway spruce (*Picea abies*), similar protein patterns of zygotic and somatic embryos, the latter cultivated on maturation medium containing 90 mM sucrose and 7.6 μM ABA, were reported and both types were dominated by storage proteins [62].

Our model to study somatic embryogenesis is the ornamental plant *C. persicum*. In a pilot study, the proteomes of cyclamen somatic embryos grown in differentiation medium with 30 and 60 g/L sucrose were compared to zygotic embryos and endosperm [42]. When somatic embryos were differentiated in medium containing 60 g/L sucrose, 74 % of the protein spots were found in comparable abundance as in the zygotic embryos' proteome, while 11 % and 15 % were found in higher abundance in zygotic and somatic embryos, respectively. Enzymes of the carbohydrate metabolism, as well as heat shock proteins and a glutathione-S-transferase, were more abundant in somatic embryos. Thus, again evidence was presented for differences in stress response of both types of embryos. Furthermore, first insights into cyclamen seed storage protein accumulation and the synthesis of the storage carbohydrates xyloglucans were gained [42]. A follow-up study made use of the advances achieved in protein extraction, resolution, evaluation, more sensitive mass spectrometrical analyses and, most important, sequence information available in the data bases leading to higher identification rates even for this nonmodel organism [58]. In both embryo types glycolytic enzymes were identified as a high percentage of the identified proteins. In somatic embryos four protein spots showed six- to more than tenfold increased abundance, and the identified proteins within these spots were involved in oxidative stress defense: osmotin-like protein and antioxidant 1, peroxiredoxin type 2, and catalase. This finding is a clear indication that somatic embryos are much more stressed than zygotic ones [58]. The occurrence of truncated forms of enolases in zygotic embryos in relatively high amounts that disappear during germination suggested a new role of parts of this glycolytic enzyme as storage proteins [58]. We followed the original idea of taking the proteome of zygotic embryos as a reference for the optimized development of high quality somatic embryos: we could show that in somatic embryos a change of the proteome towards

the zygotic status was induced after the application of a maturation treatment with ABA [59]. After ABA treatment, the proposed new storage proteins ("small" enolases) appeared in the proteome of somatic embryos, thus resembling more the proteome of zygotic embryos (Fig. 3). Sghaier-Hammami et al. [64] found the total

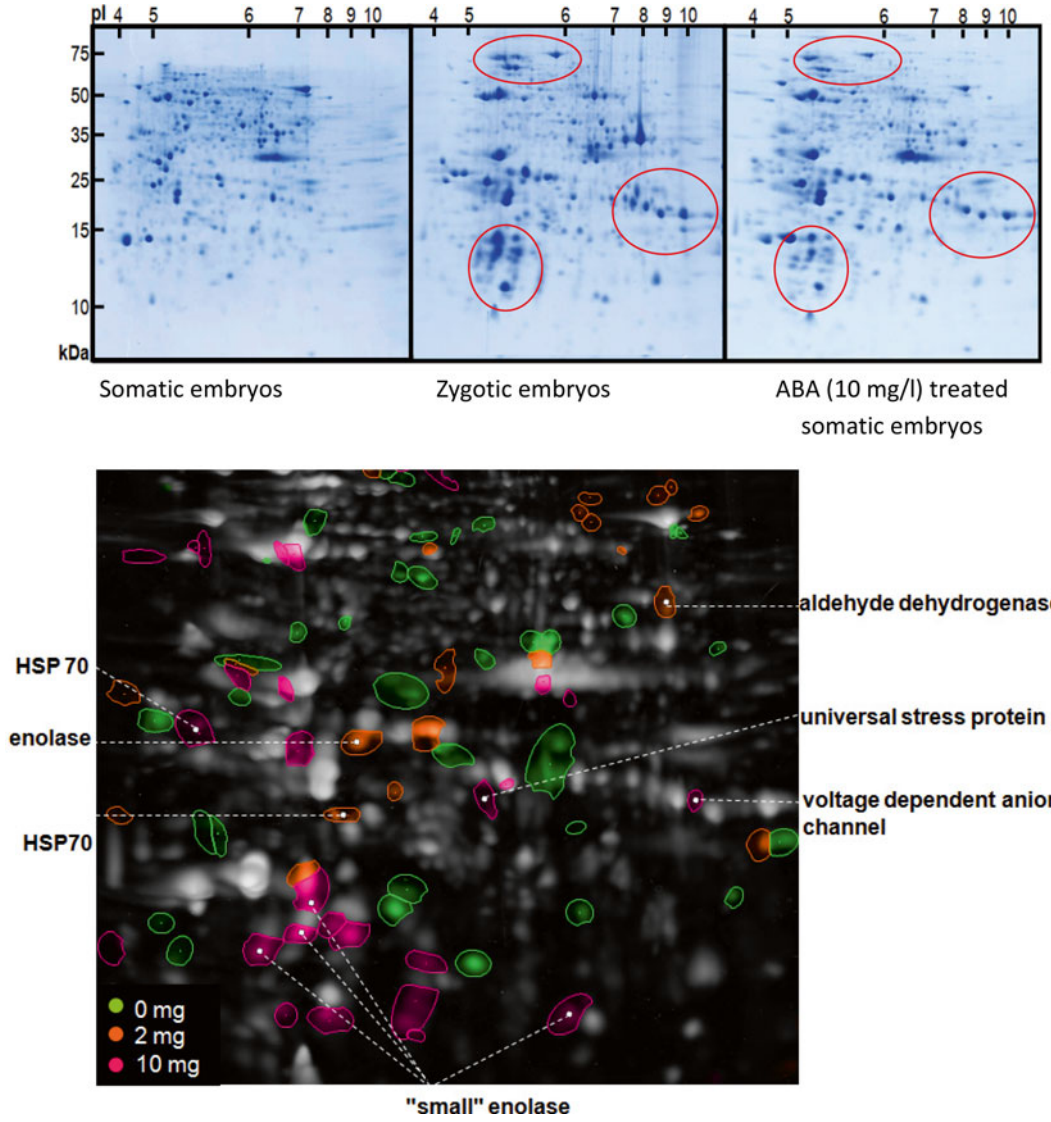

Fig. 3 *Upper Part:* Comparison of protein gels of torpedo-shaped somatic embryos, zygotic embryos, and somatic embryos treated with 10 mg/L ABA for 28 days (taken from different studies [57, 63], *encircled* are parts of the gels which show high similarity in zygotic and ABA-treated embryos). *Lower Part:* Alterations in protein abundance of 56 days old somatic embryos after cultivation on medium containing 0, 2, and 10 mg/L ABA for 28 days. *Green labeled spots* are at least 1.5 times higher abundant in controls, *orange labeled spots* are at least 1.5 times more abundant in the 2 mg/L ABA treatment, and *pink labeled spots* are at least 1.5 times more abundant in the 10 mg/L ABA treatment (compared to control) (lower part of the figure reproduced from [63] with permission from the author and Leibniz Universität Hannover)

protein content as well as the number of spots to be higher in zygotic than in somatic embryos of date palm in a comparative 2-DE proteomic approach. Sixty percent of the protein spots differed in their abundance between the two embryo types, and out of 63 spots of differential abundance that were eluted from the gels, 23 were identified. Most of the proteins of higher abundance in somatic embryos were involved in the glycolysis pathway, citrate cycle, and ATP synthesis pointing to a higher energy demand, while in zygotic embryos a high abundance of storage proteins and stress-related proteins of the heat shock family indicated maturation and preparation of dehydration [64].

Also in cocoa, enzymes of the carbohydrate and energy metabolism were very prominent in torpedo stage somatic and zygotic embryos [65]. Interestingly, somatic embryos had a more active oxidative/respiration pathway while in zygotic embryos anaerobic fermentation might be the more important energy pathway. Again stress-induced proteins such as peroxidases, pathogenesis-related proteins, and glutathione S-transferase were more abundant in somatic embryos [65].

3 Role of the Endosperm

Somatic embryos lack an endosperm, which is not only a tissue that nourishes the developing embryo and the germinating seedling, but insulates the embryo from mechanical pressure and has important signaling function for embryo development, maturation and growth arrest, and finally germination timing [66]. Thus, for optimization of somatic embryogenesis a detailed look into the endosperm during seed development seems reasonable.

In order to develop optimal culture media for somatic embryo development in wheat, Carman et al. [67] analyzed minerals and primary metabolites of the endosperm during seed development. Maltose concentrations in the extracted kernel fluid increased between 6 and 18 days after pollination indicating that this product of starch hydrolysis is the major carbon source for the developing embryo. For the development of improved tissue culture media, the addition of free amino acids, the adjustment of phosphate and sulfur which were detected in relatively high concentrations in the kernel fluids probably because of their presence in phosphorylated sugars and amino acids, respectively, and the addition of maltose and short chain fructans were suggested [67]. Likewise in white spruce, somatic and zygotic embryos and the megagametophyte which is the haploid nourishing tissue of gymnosperms were analyzed with respect to their mineral contents [68]. The female gametophytes and zygotic embryos contained more phosphorus, potassium, magnesium, and zinc on a dry-weight basis than somatic embryos, whereas the female megagametophyte stood out due to

its high calcium content when compared to the embryo tissues [68]. However, if this information is going to be integrated into optimization of culture media, more data sets will be necessary for the mineral contents in different developmental phases, and also the forms in which the minerals are found in the respective tissue. Arabinogalactan proteins were identified in conditioned culture media of embryogenic cells by Kreuger and van Holst [69] and found to be essential for somatic embryo development [70]. Most interestingly, an endochitinase gene (EP3) which is involved in the generation of arabinogalactan proteins was expressed in carrot seeds by cells in the integuments and the protein localized in the endosperm and also in nonembryogenic cells of embryogenic cultures [70]. Also the formation of arabinogalactan proteins in the developing carrot seed was shown to be developmentally regulated [71]. In a review Matthys-Rochon [72] came to the conclusion that nonembryogenic cells within embryogenic cultures might take over some functions of the endosperm by secretion of signal molecules that control embryo development.

For *C. persicum* the proteomic analysis of the endosperm during seed development revealed a general shift from high molecular weight proteins to low molecular weight proteins and the accumulation of storage proteins (including "small" enolases) from 7 weeks after pollination when the endosperm is still liquid [73]. Furthermore proteins involved in synthesis of other storage compounds, namely lipids and xyloglucans were identified in the endosperm. Obviously, stress response including reactive oxygen species detoxification and ABA signaling also play a role in endosperm and embryo development [73].

4 Conclusions and Outlook

It can be concluded from the aforementioned literature that:

1. Somatic embryos are more exposed to stress than their zygotic counterparts,
2. Somatic embryos accumulate less storage compounds,
3. Somatic embryos do not undergo a proper maturation phase that would include a growth arrest but instead germinate precociously.

The role of stress which is on the one hand an important trigger of embryogenesis and, on the other hand, induces severe changes in the cellular metabolism; here especially the role of reactive oxygen species deserves further investigations. Obviously, particularly somatic embryogenesis is a process that only is successfully realized if the cells experience the right stress level at the right developmental time frame. Also programmed cell death which has

an impact in zygotic and somatic embryogenesis should be taken into consideration in coming research projects. The importance of the maturation phase for accumulation of storage reserves, and also for the clear distinction of differentiation and germination, has been noticed in many systems. Nevertheless, input is needed particularly to improve this phase of somatic embryogenesis in the future. At physical culture conditions, attention is not often paid, except at the oxygen concentration, for example in wheat embryogenesis [3, 74]. Here it has been shown that installing reduced O_2 levels, mimicking the situation found in seeds, improved growth and development of somatic embryos. However, the O_2 levels changed not only with time of development and spatially but also during the day due to photosynthesis [74]. Our own studies in cyclamen revealed hypoxic conditions in seeds at the position where the embryo is found about 5–6 weeks after pollination in unpublished measurements according to [75]. Thus, in vitro cultured somatic embryos which grow at ambient oxygen concentrations may establish too high or altered metabolic activity as indicated by some studies cited above (e.g. [64], [65]) and/or oxidation of plant growth regulators such as cytokinins, ABA, and indole acetic acid due to increased activity of oxidases as discussed by Carman and Bishop (2004) [74].

The "omics" tools (transcriptomics, proteomics, metabolomics…) will substantially improve in terms of sensitivity, resolution and identification, and affordable analyses of different genotypes over time and thereby enable us to gain deeper insights into plant embryogenesis and to optimize the in vitro protocols for somatic embryogenesis. Moreover, epigenetic regulation of embryogenesis by methylation/demethylation and histone modifications, post-transcriptional and posttranslational modifications should be studied in detail especially during the early phases. The role of specific micro RNAs as regulators of plant development including embryogenesis has to be elucidated, since Oh et al. [76] found differences in the abundance of five micro RNAs between somatic and zygotic embryos in loblolly pine. Although zygotic embryogenesis is more and more understood because of mutant analyses and molecular genetic studies of embryogenesis-related genes and both kinds of embryogenesis are studied in detail on a transcriptional and proteomic level, many aspects of the fascinating regeneration pathway of plant embryogenesis are still not explained. One interesting aspect for instance is the fact that somatic embryogenesis is highly dependent on the genotype, whereas zygotic embryogenesis is not. Especially for the recalcitrant genotypes improvements would be desirable by learning from seeds.

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Chapter 3

Recent Advances on Genetic and Physiological Bases of In Vitro Somatic Embryo Formation

Maria Maddalena Altamura, Federica Della Rovere, Laura Fattorini, Simone D'Angeli, and Giuseppina Falasca

Abstract

Somatic embryogenesis involves a broad repertoire of genes, and complex expression patterns controlled by a concerted gene regulatory network. The present work describes this regulatory network focusing on the main aspects involved, with the aim of providing a deeper insight into understanding the total reprogramming of cells into a new organism through a somatic way. To the aim, the chromatin remodeling necessary to totipotent stem cell establishment is described, as the activity of numerous transcription factors necessary to cellular totipotency reprogramming. The eliciting effects of various plant growth regulators on the induction of somatic embryogenesis is also described and put in relation with the activity of specific transcription factors. The role of programmed cell death in the process, and the related function of specific hemoglobins as anti-stress and anti-death compounds is also described. The tools for biotechnology coming from this information is highlighted in the concluding remarks.

Key words Auxins, Chromatin remodeling, Hemoglobins, Osmotin, Programmed cell death, Receptor-kinases, Stem cells, Stress signaling, Somatic embryos, Transcription factors

1 The Concept of Stem Cell in Somatic Embryogenesis[1]

Most plant cells express developmental plasticity allowing their reprogramming. The developmental plasticity is linked to the stem cell condition, because the stem cells have the unique characteristics

[1] Genetics nomenclature adopted in the chapter:

- Wild-type gene names are uppercase italic, e.g., *EMBRYOMAKER*.
- Wild-type gene symbols consist of three uppercase letters in italics, e.g., *EMK*, and may be preceded by two letters in italics, the first one in uppercase, showing the species to which the gene belongs, e.g., *AtEMK* means *Arabidopsis thaliana EMBRYOMAKER*.
- Mutant gene names are lowercase italic, e.g., *embryomaker*.
- Mutant gene symbols are designed by three lowercase letters in italics corresponding to the gene locus name, e.g., *emk*.
- Different genes with the same symbol are distinguished by different numbers, e.g., *LEC1* and *LEC2*.
- Different alleles of the same gene are distinguished with a number following a hyphen, e.g., *Hb1-2*.

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to both self-renew and develop into precursors that can form different cell types and tissues. During zygotic embryo formation the number of stem cells reduces progressively, and the stem cells become limited to the opposite poles of the mature embryo, forming niches in the shoot and root meristems. The zygote, and its early derivatives, are totipotent stem cells, whereas the niches in the root and shoot meristems of the mature embryo are formed by pluripotent stem cells. Pluripotent stem cells are also present out of the apical meristems, e.g. in the procambium, cribro-vascular cambium and phellogen, and in the meristemoids, as those originating stomata and hairs ([1] and references therein). Plant somatic embryogenesis (SE) is the process through which differentiated cells, single or in small groups, reverse their developmental program during in vitro culture, and rarely *in planta*, giving rise to embryos which follow a developmental pattern similar to the one of zygotic embryogenesis [2]. The cells that initiate the somatic embryo, and their early derivatives (trans-amplifying cells), have been recently included in the plant stem cell concept [3]. Moreover, stem cell niches are maintained in the apical meristems of the somatic-embryo-derived plants, and are pluripotent as in zygotic embryo-derived plants [1]. However, the mechanisms underlying the initiation of somatic embryos are still poorly understood [4, 5]. Deciphering the molecular determinants of SE can contribute to revealing the genetic program underlying the phenomenon of stem cell totipotency and pluripotency, and somatic embryo formation and maturation. The study of regulatory molecules and associated gene networks during SE is essential for understanding embryogenic competence and plant regeneration, which are necessary for crop improvement and the establishment of new protocols, e.g., aimed to the production of synthetic seeds and the maintenance of elite germoplasm.

2 Chromatin Remodeling as a Prerequisite for Totipotent Stem Cell Establishment in SE

In animals, chromatin remodeling is an important tool of stem cell conversions [6]. In plants, chromatin structure is continuously remodeled during development, whereas a chromatin-dependent gene silencing is a common mechanism for maintaining the

– Proteins are written in non-italic uppercase, with the same full descriptive name, and the symbol of the corresponding gene, e.g., EMBRYOMAKER, and EMK.
– The symbol of either a gene or a protein known by multiple names is given by the known symbols separated by a slash, e.g., *BAK1/SERK3* and BAK1/SERK3, respectively.

differentiated cellular state. Thus, as in animals, a role of chromatin-structure changes in pluripotency of plant stem cells has been proposed [7–9]. The stem cells show a more dynamic chromatin state than the differentiated cells because structural chromatin proteins, like histones, are exchanged more rapidly in stem cells than in differentiated cells, and this favors rapid changes in the gene expression program [10].

It has been hypothesized that chromatin restructuring plays two major roles during early stages of SE. An unfolding of the super-coiled chromatin structure is required for the dedifferentiation of the somatic cells, both when they produce embryos directly (direct SE), and when they are engaged into callus formation before embryogenesis (indirect SE). This early step is necessary to allow the expression of genes which had been inactivated by heterochromatization during the cell differentiation process, but which are specifically required for the embryogenic pathway [7]. A chromatin remodeling is also required at the end of embryogenic process to repress the embryo-specific genes, thus reactivating the differentiation process, at least in specific cellular districts of the somatic embryos. Formation of somatic embryos by somatic embryo cells (i.e., secondary SE) occurs when the repression of the embryo-specific gene pathway and the chromatin re-folding are delayed/altered, not allowing the transition from the embryo to the seedling. In plants, as well as in animals, two major epigenetic pathways play important roles in the regulation of cell-fate decisions by modifying chromatin, namely DNA methylation and histone methylation. In animals, there is increasing evidence for a complex regulatory interplay between the two pathways, with a direct mechanistic link based on physical interactions of histone-modifying proteins and DNA methyltransferases ([11], and references therein).

Polycomb group (PcG) proteins are present in animals and plants. They maintain the inactive state of the target genes by establishing repressive histone modifications, e.g. by methylation. During *Arabidopsis* life cycle, distinct variants of the POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) are involved in the regulation of the developmental processes, such as gametophyte and seed development and embryo-to-seedling transition [11]. The plant variants of PRC2 catalyze the Histone H3 Lysine 27 Trimethylation (H3K27me3) [12]. It is known that H3K27me3 is a repressive mark that plays a crucial role in the dynamic regulation of gene expression in plant development [13], acting as a major silencing mechanism. In *Arabidopsis*, *prc2* mutants with substantially reduced levels of H3K27me3 exhibit extensive derepression of the embryonic traits ([14], and references therein). The ATP-dependent chromatin remodeler PICKLE (PKL) belongs to a protein family that can participate in multiple remodeling pathways

and can either repress or activate gene expression depending on the other factors it associates with. In *Arabidopsis*, PKL promotes the epigenetic mark H3K27me3 facilitating repression of specific genes. In fact, the phenotype of *pickle* (*pkl*) mutant is characterized by the postembryonic expression of embryo-specific markers, e.g. the *LEAFY COTYLEDON* (*LEC*) genes (see below), and by the spontaneous regeneration of somatic embryos in the roots [15]. Thus, the loss of PKL generates a window of opportunity throughout which the embryo transcriptional program has the potential to become reestablished [14], maintaining the embryo-related gene expression.

In contrast with the effects of histone methylation, DNA (hyper)methylation, for example caused by exogenous auxin, which is, in general, necessary to induce SE, is positively related to SE ([16], and references therein) (Fig. 1).

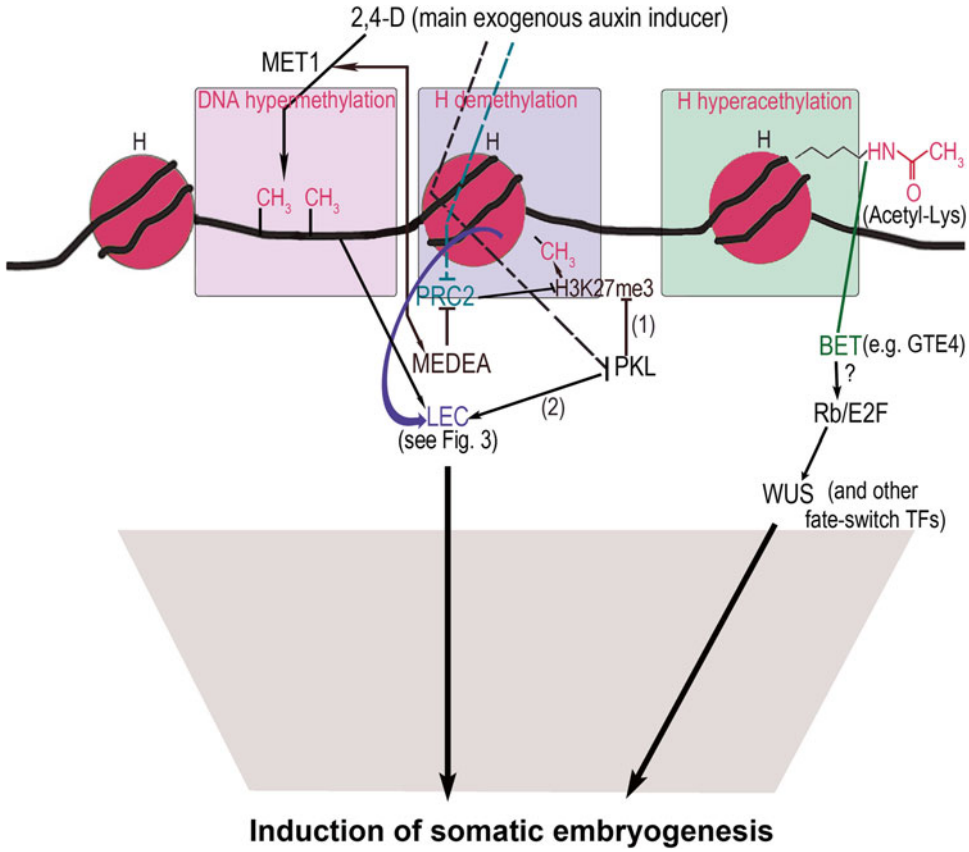

Fig. 1 Model of chromatin remodeling involved in SE-induction. 2,4-D-activated DNA hypermethylation, histone (H) demethylation and hyperacetylation events, and early-activated/repressed proteins are shown (see the text for further explanation). Numbers (1) and (2) consequences of *PKL* knock-out

In *Arabidopsis*, DNA methylation is mediated by at least three classes of methyltransferases. The METHYLTRANSFERASE1 (MET1) is one of these classes. Zygotic embryos with loss-of-function mutations in *MET1* develop improperly, displaying altered cell divisions, reduced viability, mis-expression of genes specifying embryo cell identity, and altered auxin hormone gradients [17]. In carrot, a *MET1* gene is expressed transiently after the application of the synthetic auxin 2,4-D, and before the formation of the SE-cell-clumps, and 5-azacytidine, an inhibitor of DNA methylation, suppresses the embryogenic clump formation [18]. All together, these results highlight a role of MET1 in both zygotic and somatic embryogenesis. Very recently, it has been observed that during seed development *Arabidopsis* MET1 interacts with MEDEA, one of the core components of the FERTILIZATION INDEPENDENT SEED (FIS)-PRC2 complex, with MEDEA involved in *PRC2* repression ([12], Fig. 1). The interaction between MET1 and MEDEA (Fig. 1) demonstrates, for the first time in plants, that a concerted action of the epigenetic pathways of DNA methylation and histone methylation regulates the switching of developmental changes [11], and sustains that a concerted action of DNA methylation and histone demethylation might be essential for SE induction (Fig. 1). The modifications of chromatin also include the acetylation of histone tails for relaxing the packing of the DNA, thus facilitating the access to DNA of many regulatory proteins. Thus, the hyperacetylation of histones is associated with active gene expression, while hypoacetylation correlates with gene repression ([19], and references therein). In animals, a dynamic reprogramming of both histone acetylation and methylation has been demonstrated, e.g. in cloned mouse embryos [20], whereas full evidence of this interplay is still lacking for plant zygotic/somatic embryogenesis.

Proteins containing bromodomains have the role of deciphering the histone acetylation codes. There are bromodomain proteins that also contain an Extra Terminal domain that is a protein-protein interaction motif [21]. BROMODOMAIN and EXTRA TERMINAL DOMAIN proteins (BET proteins) bind to acetylated lysines of histone tails and control gene transcription ([22], and references therein) (Fig. 1). In a variety of organisms the BET proteins contribute to the transmission of the transcriptional memory from one generation of cells to the next ([23], and references therein). In *Arabidopsis*, the BET bromodomain factor GENERAL TRANSCRIPTION FACTOR GROUP E4 (GTE4) is involved in the activation and maintenance of cell division in the meristems. The loss of GTE4 negatively affects both zygotic embryogenesis by altering meristem organization in the mature embryo, e.g., at the root pole (Fig. 2a, b), and post-embryonic growth by altering the stem cell niche formation in the root apical meristems [22, 23]. The RETINOBLASTOMA (Rb)-E2

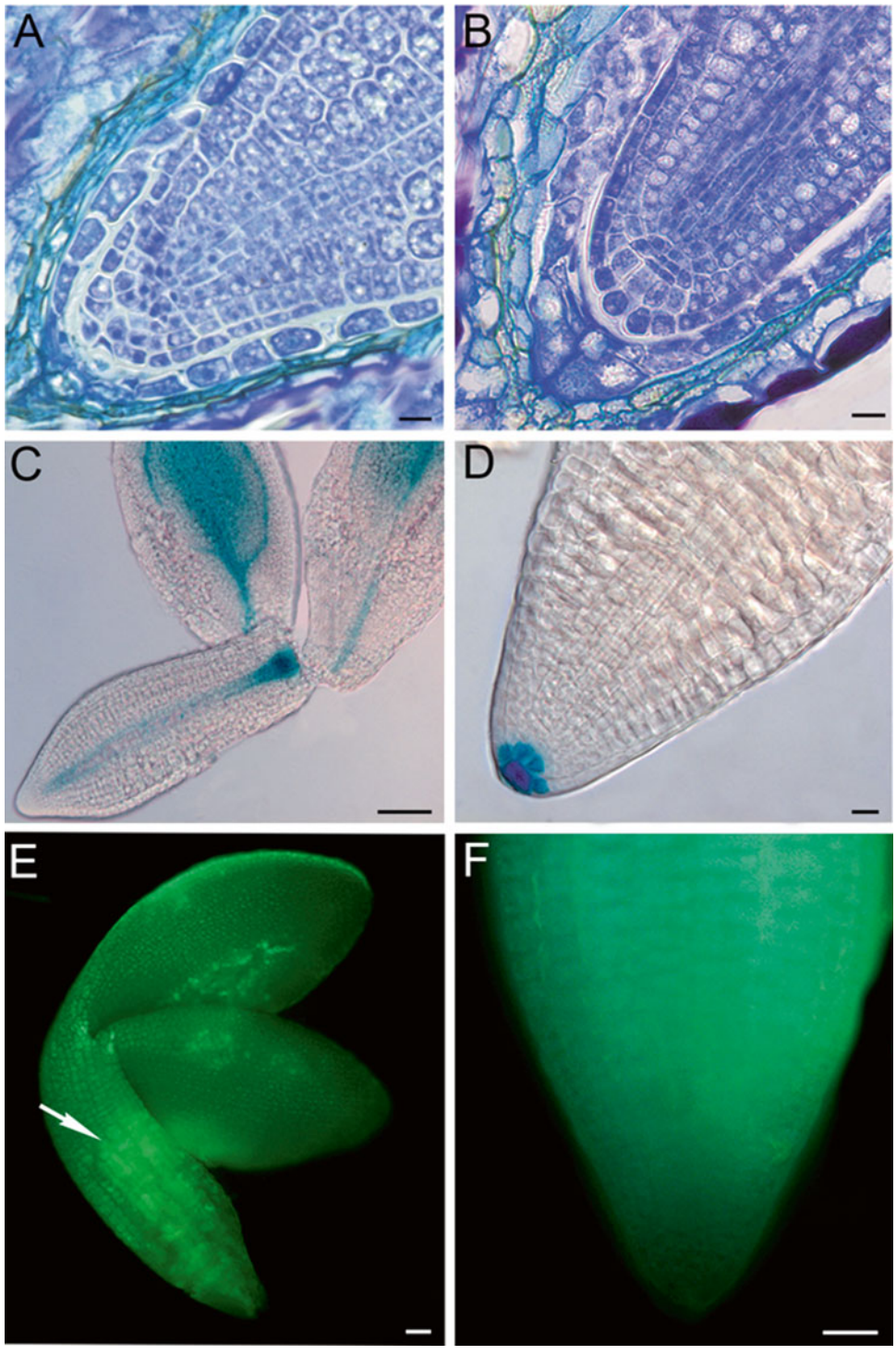

Fig. 2 *Arabidopsis* zygotic embryos (**a**–**b**). Altered root pole meristem in a mature *gte4* embryo (**a**), in comparison with the well-organized meristem of the wild-type (**b**). (**c**–**d**) Auxin transport and localization in the mature embryo. The expression pattern of *PIN1* auxin-efflux carrier in a *PIN1::GUS* embryo [i.e., a transgenic embryo expressing the *uidA* gene coding for a β-GLUCURONIDASE (GUS) under the control of *PIN1* promoter] (**c**), and

FACTOR (E2F) pathway is considered an essential link between chromatin restructuring, dedifferentiation, and fate switch. In tobacco protoplasts, the Rb/E2F-target genes *RNA RIBOSOMAL CLUSTER 2* (*RNR2*), i.e., the small subunit of ribonucleotide reductase, and *PROLIFERATING CELL NUCLEAR ANTIGEN* (*PCNA*) are condensed and silent in differentiated leaf cells, but become de-condensed as cells acquire competence for fate-switch, and turn transcriptionally active during progression into S phase, concomitantly with Rb phosphorylation [24]. Moreover, Rb has been shown to bind to a transcription factor (TF) that functions in the *Arabidopsis* root stem cell niche, and a relationship with WUSCHEL (WUS)/CLAVATA (CLV) has been also shown ([19], and references therein). Roles for Rb proteins in human embryogenesis are widely known [25]. GTE4 might be a good candidate in the control of histone-acetylation during plant SE, because, as the Bromodomain containing 2 (BRD2/RING3) protein in animals, its activity is related to the Rb-E2F pathway [22] (Fig. 1). Among the possible factors causing chromatin modifications there are also small RNAs, such as the small-interfering RNAs (siRNAs) and the microRNAs (miRNAs). The small RNAs not only function at the posttranscriptional level by guiding sequence-specific transcript degradation and/or translational repression ([26], and references therein), but can also play a role in targeting DNA methylation through RNA-directed DNA methylation [27, 28]. These events lead to chromatin modifications eventually resulting in transcriptional silencing and heterochromatin formation [29]. ARGONAUTE (AGO) effectors of RNA silencing bind small RNA molecules and mediate mRNA cleavage, translational repression, or DNA methylation [30]. The possible regulation of SE by small RNAs has been investigated using various systems. In rice callus, a unique set of miRNAs, only expressed or differentially expressed in embryogenic cells, was identified [31, 32]. Moreover, miRNA expression during SE was also characterized in orange [33], longan [34], and cotton, where four trans-acting small interfering siRNAs (tas3-siRNAs) were also identified [35]. On the other hand, the expression of *AGO* genes during SE is well known, and in both gymnosperms and angiosperms, e.g., spruce [36], carrot [37], *Cichorium intybus* [38], *Auracaria angustifolia* [39]. Nonetheless, the roles of miRNAs and siRNAs, and related effectors, in the induction of plant SE remain to be understood.

Fig. 2 (continued) the strictly apical localization of auxin in the root pole of a *DR5::GUS* embryo [i.e., a transgenic embryo expressing the *GUS*-encoding reporter gene under the control of the synthetic auxin-responsive promoter *DR5*] (**d**), are shown. (**e**–**f**) Calcium distribution in the embryo monitored by the CTC-Ca^{2+} epifluorescence signal (*yellow-green color*). The apical-basal distribution along the hypocotyl (**e**, *arrow*), and the strong signal-reduction at the root apex (**f**), are shown. Bars = 10 μm (**a**, **b**, **d**–**f**) and 50 μm (**c**)

3 Transcription Factor Activity Involved in Cellular Totipotency Reprogramming

The developmental cell switching to SE induction involves activation of various signal cascades and differential gene expression. The inductive role of Plant Growth Regulators (PGRs), auxin in particular, has been well established, and will be summarized below. However there is increasing evidence for a role of numerous Transcription Factors (TFs) in accordance with human somatic cells in which a specific combination of TFs re-programs the differentiated cells into embryonic stem cells [40]. In plants, TFs involved in SE induction have been reported. For example, an extensive modulation of the TF-transcriptome has been recently described during SE induction by in vitro culture in *Arabidopsis* suggesting directions for further research on functional genomics of SE [41]. In this model plant, it has been demonstrated that the embryo-induction stage is associated with a robust change of the TF-transcriptome by a drastic upregulation of transcripts related with plant development, PGRs and stress responses. By contrast, the advanced embryo stages are associated with the stabilization of the transcript levels of the majority of the TFs [41]. TFs are known to play fundamental roles in the control of plant cell totipotency, which is essential for SE (see above), and the list of TFs affecting SE induction includes *BABY BOOM* (*BBM*) [42], *WUS* [43], some *WUSCHEL RELATED HOMEOBOX* genes (*WOX*) [44], *AGAMOUS-LIKE15* (*AGL15*) [45], *LEAFY COTYLEDON* (*LEC*) [46], genes encoding MYELOBLASTOSIS (MYB) TFs, i.e., *AtMYB115* and *AtMYB118* [47], and *EMBRYOMAKER* [48].

BBM was isolated from microspore embryo cultures of *Brassica napus* [42]. It belongs to a family of genes [*APETALA2* (*AP2*)/*ETHYLENE RESPONSE FACTOR* (*ERF*)] known to enhance regeneration in vitro, and to be involved into meristem cell fate and organ development. Interestingly, in *Arabidopsis*, BBM induces somatic embryo formation from seedlings in the absence of exogenously applied PGRs ([49], and references therein). *Arabidopsis EMBRYOMAKER* (*AtEMK*) is another AP2-domain TF. It is homologous to *BnGemb-18* of *Brassica napus* which is specifically expressed in microspore embryogenesis, as *BBM* ([48], and references therein). *AtEMK* ectopic expression results into embryo-like structures from cotyledons *in planta*, enhancing somatic embryogenesis in in vitro culture, and a role for this gene in conferring embryonic identity to cells has been proposed [48].

Another initiator of ectopic embryogenesis is *AGL15*. This gene was identified from *Arabidopsis* and soybean as a MADS domain-containing TF specifically expressed in the embryonic cells [50, 51]. The MADS name derives from the initials of the founding members of the gene family, i.e., *MINICHROMOSOME*

MAINTENANCE1 (*MCM1*), *AGAMOUS* (*AG*), *DEFICIENS* (*DEF*), and *SERUM RESPONSE FACTOR* (*SRF*). In plants, e.g. *Arabidopsis* and rice, MADS-domain proteins are central players of many developmental processes including flowering time, floral organogenesis, fruit and seed development ([52], and references therein). About embryogenesis, Harding and coworkers [45] have demonstrated that in *Arabidopsis* the ectopic expression of *AGL15* enhances somatic embryo formation both from zygotic embryos removed from the seed at the green cotyledon stage and cultured on a germination medium without exogenous PGRs, and from the shoot apical meristem of seedlings growing in a liquid medium in the presence of 2,4-D. It is also known that AGL15 enhances SE reducing gibberellic acid (GA) levels by inducing a GA2-oxidase which inactivates GA [45, 53, 54]. Moreover, it is a component of SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1) protein complex [55], as detailed in a following paragraph.

MYB proteins are TFs with a specific DNA-binding domain comprising up to three imperfect tandem repeats (R1, R2, R3), that fold into a helix-turn-helix motif. In vertebrates, the *MYB* gene family is small and includes *c-MYB*, *A-MYB*, and *B-MYB*; the products of these genes are involved in the control of cell proliferation, differentiation, and apoptosis [56]. In plants, the *MYB* family is much more extensive: at least 198 *MYB* genes have been identified in *Arabidopsis*, and, similarly to the *MADS*-domain genes, are involved in a wide range of developmental processes, including cell cycle progression, cell differentiation, lateral organ polarity, flower and seed development. Plant MYB proteins are classified according to the number of MYB repeats, and those with R2R3 repeats constitute the largest group ([57], and references therein). AtMYB118 and the closely related AtMYB115 encode R2R3-type MYB TFs. In *Arabidopsis* their overexpression efficiently induces SE from root explants, resulting in elevated expression levels of *Arabidopsis LEAFY COTYLEDON 1* (*LEC1*) (see below), suggesting that they may act as positive regulators of vegetative-to-embryonic transition in a *WUS*-independent manner [47], possibly acting upstream to *LEC1*.

The *WUS* gene, encoding a homeodomain protein, is critical for stem cell fate determination in the shoot apical meristem of higher plants. WUS activity results in signaling to the overlaying stem cells, inducing *CLAVATA3* (*CLV3*). CLV3 acts as the ligand for the CLAVATA1/CLAVATA2 (CLV1/CLV2) receptor complex that limits the expression areas of *WUS* in the shoot apical meristem, with this negative CLV3/WUS feedback loop ensuring the shoot apical meristem homeostasis by regulating the number of stem cells in the central zone [58, 59]. During zygotic embryogenesis in *Arabidopsis WUS* is expressed before the stem cell establishment in the embryonic shoot [60]. In the same plant,

WUS-induced overexpression causes increased SE without any external PGR, suggesting the involvement of this TF in the SE process by promoting the vegetative-to-embryonic transition [43]. Moreover, *WUS* induction occurs earlier than that of *CLV3*, marking the initial cell clumps of SE. These clumps exhibit the same cytological features of the pre-embryogenic aggregates formed in *Cyclamen persicum* SE-callus, and which express other stem cell markers which are not TFs (i.e., CpSERK1 and SERK2, as described below) [3]. The induction of *WUS* expression in the embryogenic callus of *Arabidopsis* requires the removal of auxin from the medium; however, the cell status in the embryonic callus is regulated by auxin in a concentration-dependent manner, with the levels of this exogenously applied PGR essential for determining *WUS* expression pattern. Moreover, the auxin gradients activate the polar auxin transporter PIN-FORMED 1 (PIN1) in the embryogenic callus, and the suppression of both *WUS* and *PIN1* show that both genes are necessary for somatic embryo induction [3] (*see* the following paragraph).

The *WUS-RELATED HOMEOBOX* (*WOX*) gene family is a class of TFs involved in the early phases of *Arabidopsis*zygotic embryogenesis and in lateral and adventitious organogenesis [44, 61]. In particular, WOX5 is a stem cell marker in the root apical meristem and is very early expressed in in vitro adventitious root induction ([61], and references therein). Moreover, in *Arabidopsis* WOX2, WOX8, and WOX9 are important cell fate regulators of zygotic pro-embryos, acting as embryo-identity genes [44, 62]. During the SE-process *WOX5* and *WUS* are expressed before the embryo-identity genes, e.g. *WOX2* [63]. An extensive study on the expression of *WOX* gene family in *Vitis vinifera* SE shows that the *WOX* genes play important roles in coordinating the gene transcription involved in the early phases of the process. *VvWOX2* and *VvWOX9* are the principal *WOX* genes expressed, and the low aptitude to SE shown by specific grape cultivars correlates with a very low expression of these genes [64].

ArabidopsisLEC genes, *LEC1*, *LEC2*, and *FUSCA3* (*FUS3*), were identified originally as loss-of-function mutations resulting in defects in both embryo identity and seed maturation processes [65], and are all essential for SE induction in *Arabidopsis* [46]. *LEC1* encodes a protein with sequence similarity to the *HEME-ACTIVATED PROTEINS 3* (*HAP3*) subunit of the CCAAT binding factor [66]. *LEC1-LIKE* (*LIL*) gene is also required for somatic embryo development and is active in numerous plants. For example, in cocoa, *TcLIL* mRNA levels are detected in young somatic embryos and undetected in nonembryogenic explants and mature somatic embryos, suggesting that also *LEC-like* genes may be important in coordinating primary events leading to embryonic competence ([49], and references therein). LEC2- and FUS3-proteins share greatest similarity with the B3 domain, a DNA-

binding motif unique to plant TFs, acting primarily in developing seeds ([46], and references therein). FUS3 activates transcription of maturation-specific genes containing RY domains [67], and the ectopic expression of *LEC2* causes accumulation of lipids and seed storage proteins in transgenic seedlings [68]. In addition to its versatile regulatory functions in zygotic embryogenesis and seed development ([69], and references therein), *LEC2*, the same as *LEC1*, is sufficient to induce embryo development in vegetative cells when expressed ectopically ([49, 68], and references therein). Moreover, it directly induces genes involved in maturation processes before formation of somatic embryos [68, 70, 71]. The role of LEC1 in maintaining embryonic characteristics in vegetative organs requires auxin and sugar ([72], and references therein), and the capacity of SE in *lec1lec2* double mutants is very low even in the presence of auxin, suggesting that in *Arabidopsis* the formation of somatic embryos by auxin needs the function of *LEC* genes [46]. By the use of an inducible chimeric fusion construct it has been recently shown that ectopic expression of these *LECs* confers embryonic characteristics also to tobacco [72]. PKL, the chromatin-remodeling factor described before, seems to be the master regulator of *LEC* genes because, in the *pkl* mutant, roots express the ability to form somatic embryos through a derepression of the *LEC* genes [73] (Fig. 1). Interestingly *LEC2* overexpression leads to spontaneous embryo formation *in planta*, but impairs SE in vitro under auxin treatment, suggesting an auxin-mediated mechanism of action [70]. In line with this hypothesis, it has been shown that LEC2 controls the *INDOLE-3-ACETIC ACID INDICIBLE30* (*IAA30*), an auxin signaling gene, the *PIN1* and *PIN2* auxin efflux carrier genes, the auxin biosynthesis *ϒUCCA2* (*ϒUC2*) and *ϒUCCA4* (*ϒUC4*) genes, the *AUXIN RESPONSE FACTOR* (*ARF*) genes *ARF5*, *ARF8*, and *ARF10*, and *SERK1*, the auxin-induced marker-gene of SE in a lot of plants (*see* the next paragraph) [70–72]. Again in accordance, *LEC2* is upregulated in a SE culture induced on an auxin-containing medium, and the gene overexpression compensates for the auxin treatment as somatic embryos are formed in explants cultured under auxin-free conditions [74]. Very recently, it has been shown that de novo auxin production via the tryptophan-dependent indole-3-pyruvate (IPA)-YUC auxin biosynthesis pathway is implicated in SE induction, with LEC2 playing a key role in this mechanism [69]. The possibility that LEC2 may also promote SE due to a repression of GA levels via a positive control on *AGL15*, as well as of other GA-related factors, has been also proposed [69, 71, 72]. Moreover, most genes involved in ethylene signaling pathway are downregulated by *LEC2* during SE in transgenic tobacco, suggesting a LEC2-mediated negative role of this PGR, at least in this species [72]. Controversial results about ethylene-control of SE are discussed in the following paragraph.

4 The Eliciting Effects of PGRs on SE Induction and the Crosstalk with TFs

Auxin, but also other PGRs, for example cytokinins, are involved in the specification and maintenance of stem cells in the zygotic embryo and in the meristems, both *in planta* [75–78], and in in vitro culture [61]. Usually, auxin is required to induce SE in in vitro culture, in particular the synthetic auxin 2,4-D ([49], and references therein). Moreover, auxin gradients are needed to trigger the formation of stem cells in the zygotic and somatic embryos [3]. A significant amount of literature on auxin biosynthesis, metabolism, and transport, in somatic embryos shows that auxin plays important roles, both in the induction of embryo formation in culture, and in the subsequent elaboration of the proper morphogenetic events during embryo development ([49], and references therein). However, there are also species in which a cytokinin applied alone induces SE, e.g., in sunflower [79], and species in which a cytokinin must be combined with an auxin to induce the process, for example, in *Medicago truncatula*, *Vitis vinifera*, *Cyclamen persicum* [1, 80, 81]. The SE-inductive role of abscisic acid (ABA) in carrot seedlings has been also reported [82, 83]. The external PGR supply has been supposed to cause local variations in the internal auxin concentration of explants, possibly triggering de novo synthesis/relocation of endogenous auxin forms, which contribute to somatic embryo induction. Moreover, 2,4-D might act as an auxin either directly or modifying intracellular IAA metabolism, and/or it may act as an inducer of stress-related genes [7, 84, 85]. For example, in the induction of SE in wheat leaf explants, genes characteristic of a response to oxidative burst are upregulated during the first hours of 2,4-D treatment [86]. Moreover, as described above, 2,4-D might cause a DNA hypermethylation, as in *Cucurbita pepo* [38], and induce *MET1* gene expression leading to genome reprogramming and acquisition of SE competence.

In *Arabidopsis*, transcriptional regulatory networks, controlling stem cell population and maintenance, have been demonstrated *in planta* in the shoot and root apices, and in the procambium. Moreover, homologous TFs have been found to be involved ([9, 87], and references therein), with activity beginning during zygotic embryo formation. The relationship between specific TF-networks and PGR synthesis, transport, activity, catabolism is also elucidating for SE.

By the action of the ubiquitin protein ligase SCF^{TIR1}, formed by the SKP, CULLIN, F-BOX CONTAINING COMPLEX (or SCF) and the TRANSPORT INHIBITOR RESPONSE 1 (TIR1), the endogenous auxin is known to promote the breakdown of certain auxin/IAA (AUX/IAA) repressor proteins which, when active, block the ARFs by forming inactive dimers. The AUX/IAA

inactivation allows ARFs to bind to the auxin-response elements present on the auxin-responding genes, causing their activation. Because AUX/IAA genes themselves are rapidly induced by auxin, a negative-feedback loop is established with the AUX/IAA, newly synthesized by auxin, restoring ARF-repression [88]. The expression of *IAA9* and *IAA8*, two *AUX/IAA* transcriptional regulators, has been observed in the SE of *Cyclamen persicum* and *Gossypium hirsutum* [89, 90]. Recently in *Arabidopsis* it has been shown that about 70 % of the *AUX/IAA* members display modulated expression during SE, and the corresponding mutants are impaired in somatic embryo formation [41]. Taken together, it seems evident that members of the ARF and AUX/IAA transcription regulator/signaling families act in concert to modulate expression of auxin-responsive genes that are essential for SE. Interestingly, in *Arabidopsis* and soybean, *ARF5/MONOPTEROS* (*MP*) is upregulated during SE-induction [41, 85]. In addition, during SE in transgenic tobacco, *MP* is activated by LEC2 [72], and Fig. 3.

MP is a key gene in zygotic embryo patterning, affecting polar auxin transport through activation of *PIN1* auxin carrier [91]. Embryo formation is impaired in vitro when auxin transport is inhibited and *MP* repressed ([41], and references therein). This highlights the importance of a correct polar auxin-transport for

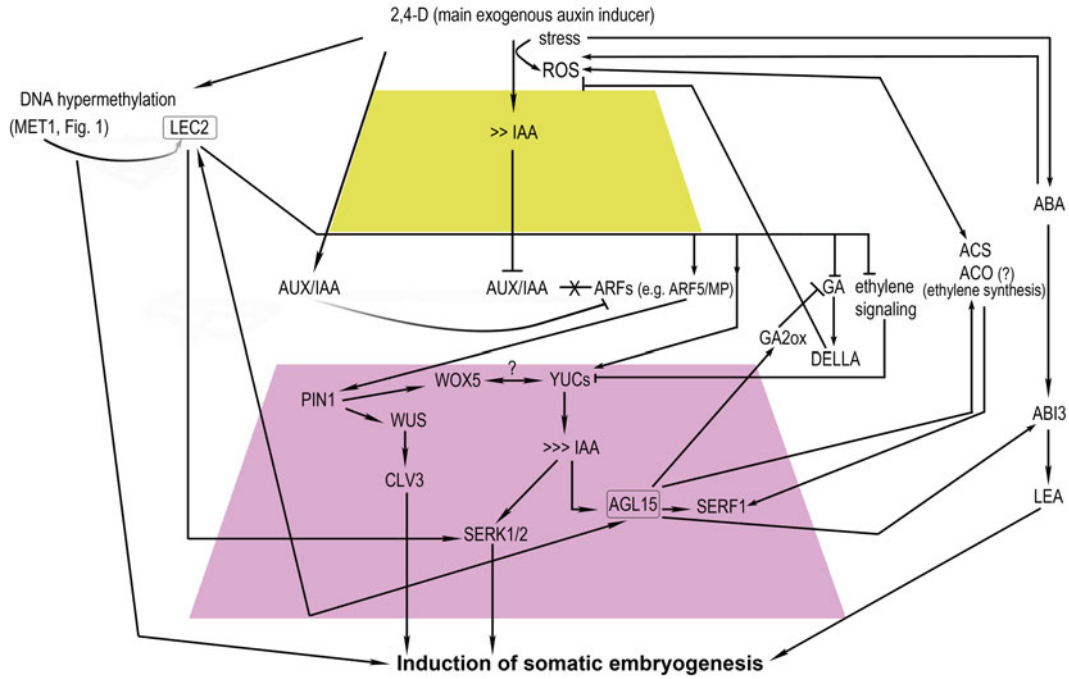

Fig. 3 Model of the cross talk among PGRs, stress, and TFs in SE-induction. Other proteins, exhibiting a pivotal role in the process, are also shown. Early and further increases in endogenous IAA are shown by different colors (details in the text)

proper SE formation, as well as for zygotic embryo formation (Fig. 2). In addition, the early establishment of auxin gradient and PIN1-mediated polar auxin transport are essential, at least in *Arabidopsis*, for the induction of *WUS* in the callus before the morphological identification of the somatic embryonic cells [92]. *CLV3* transcripts appear later than those of *WUS*, and are localized in the stem cells of the somatic proembryo [3]. Like *WUS* role in early defining the shoot apical stem cell niche during SE, the *WUS-RELATED HOMEOBOX* TF *WOX5* is also early activated by the auxin present in the medium, early defining the root apical stem cell niche, e.g. in grapevine, *Arabidopsis* and *Medicago truncatula* SE [64, 93, 94]. As detailed in the paragraph about Programmed Cell Death (PCD), PCD takes part to somatic embryo development. Polar auxin transport is essential for apical-basal patterning and related stem niche positioning during early embryogenesis, and disturbed transport causes aberrant embryo development, as well as altered PCD, e.g. in Scots pine [95].

It is known that auxin regulates stem cell positioning and maintenance during plant developmental processes via an auxin gradient resulting from a local auxin biosynthesis, coupled with polar auxin transport ([61], and references therein). A family of *YUC* genes encoding flavin mono-oxygenases, key enzymes in auxin biosynthesis, is also required for the establishment of the basal part of the zygotic embryo and for embryogenic organ initiation. Multiple mutations of *YUCs* impair local auxin distribution, resulting into severe developmental defects which resemble those caused by multiple mutations in *PIN* genes [96, 97] and indicating that auxin biosynthesis and transport are both required for zygotic embryogenesis. This seems also the case for SE, because together with the essential role of the auxin transport discussed before, also YUC-mediated auxin biosynthesis has been demonstrated to occur during SE [4, 69]. Moreover, the above-described LEC2 TF exhibits a positive role in controlling the YUC-mediated auxin biosynthesis, associated with SE induction in *Arabidopsis* [69]. Moreover, LEC2 downregulates genes involved in ethylene signaling pathway in SE of transgenic tobacco [72]. Interestingly ethylene disturbs SE initiation in *Arabidopsis* through inhibiting *YUC* gene expression [4]. Roles for ethylene in SE are not well understood, because SE-promotive/inhibiting results have been obtained in different species and culture systems. Ethylene is considered a stress hormone, and "stress" is a major factor in inducing SE, as discussed in a following paragraph. Stress response can take different forms depending on the species, the immature/mature features of the explant tissues, and the environmental parameters used for the culture. As discussed below, wounding and 2,4-D application are also stress-inducers, and ethylene synthesis is rapidly induced in response to various stresses, including wounding

and auxin application [16]. In *Medicago truncatula*, SOMATIC EMBRYO-RELATED FACTOR1 (MtSERF1) has been demonstrated to be essential for SE [98]. *MtSERF1* encodes one of the *ERF* subfamily B-3 members of the AP2/ERF TF family. Its transcript accumulation depends on ethylene, but also on auxin and cytokinin, i.e. the SE-inductive PGRs in this plant [98]. Its ortholog in *Arabidopsis* (*At5g61590*) is the direct upregulated target of AtAGL15, both positively involved in the promotion of SE in *Arabidopsis* [99]. In addition, the ortholog of AtAGL15 in soybean (i.e. GmAGL15) upregulates genes involved in ethylene biosynthesis, including a *1-AMINOCYCLOPROPANE-1-CARBOXYLIC-ACID* (*ACC*) *SYNTHASE* (*ACS*) and an *ACC OXIDASE* (*ACO*) which generates ethylene from ACC, and in ethylene response, including the TFs which are the orthologs of *AtERF1* and *MtSERF1* (i.e., *GmSERF1/SERF2*). This upregulation results into increased ethylene production and SE in soybean [99]. Likewise, in *Pinus sylvestris* an increased content of endogenous ethylene appears to be required for SE [100]. Interestingly, none of the *ACS* or *ACO* genes upregulated by GmAGL15 in soybean appear to be upregulated in response to AtAGL15 accumulation in *Arabidopsis*, and two putative *ACO* genes are repressed, hypothesizing differences between AGL15 regulation in the SE of different species [99], and perhaps in ethylene levels and effects on SE. In accordance, ethylene biosynthesis has been reported to decrease during SE in *Arabidopsis*, with excessive ethylene reducing *YUC* expression and disrupting local auxin distribution [4]. Ethylene is known to affect auxin transport and regulate the asymmetric distribution of auxin in various plant tissues [101]. Once synthesized, ethylene is perceived by a family of receptors. *CONSTITUTIVE TRIPLE RESPONSE 1* (*CTR1*) is a receptor-interaction protein kinase whose expression negatively regulates ethylene response ([102], and references therein). In *Arabidopsis*, mutation at *CTR1* causes costitutive ethylene signaling, and inhibition of SE initiation, but also downregulation of most *YUC* genes, highlighting the possibility of a negative effect of ethylene signaling on SE through inhibition of *YUC* expression [4]. Moreover, the endogenous levels of GA are negatively related to SE potential. The *lec* mutants show increased GA levels and reduced SE [103], and the exogenously supplied GA_3 decreases tissue capacity for SE induction [104]. In support of the inhibitory effect, several genes important for the negative regulation of GA responses display a SE-specific upregulation, including genes coding proteins containing the conserved amino-acid sequence *Asp-Glu-Leu-Leu-Ala*, named *DELLA*-domain [105]. Similarly the GA addition reduces SE in both nontransgenic and transgenic (i.e., *35Spro:GmAGL15*) soybean, and genes encoding DELLA proteins are upregulated at some stages of SE induction [99]. Ethylene and auxin are known to impact their biosynthesis

reciprocally, and ethylene is known to act cooperatively/antagonistically with GA depending on the context [99], and a central role for AGL15 seems to exist at least during SE (Fig. 3).

In carrot, the application of ABA to seedlings efficiently induces SE [82] and plays an important role in the induction of secondary SE [106]. By the use of different approaches to reduce cellular ABA levels in *Nicotiana plumbaginifolia*, it was demonstrated that the ABA deficiency disturbs morphogenesis at the preglobular somatic embryo stage, but the effect is reverted by exogenous ABA application [107]. ABA is known to induce the expression of *LATE-EMBRYOGENESIS-ABUNDANT* (*LEA*) genes in late-stage zygotic embryos. The expression of some carrot *LEA* genes is also observed during SE after treatment with ABA, and occurs via a *C-ABSCISIC ACID–INSENSITIVE3* (*ABI3*)-mediated signal transduction [108]. In *Arabidopsis*, the genes coding for the EMBRYOGENIC CELL PROTEIN 31 and 63 (*AtECP31* and *AtECP63*) represent the homologous to the carrot *LEA* genes, and are similarly induced by ABA during SE and equally involve an *ABI3* gene expression [109]. In carrot, the endogenous levels of ABA increase in response to stress treatments, and they are particularly high during the induction of SE in comparison to further embryo developmental stages, suggesting the importance of an early stress-induced accumulation of ABA for SE-induction [83]. Interestingly, LEC1 upregulates the expression of *ABI3* and *FUS3* [110, 111], and all the three genes are expressed during early microsporeembryogenesis in *Brassica napus* [112]. It seems that ABI3 and FUS3 positively regulate each other through a feedback loop, and the GA-ABA ratio seems to determine the developmental mode, with low GA-ABA ratio promoting the embryo mode of development [112, 113]. AGL15 directly controls the genes encoding these TFs, in both *Arabidopsis* and soybean [99, 114]. Thus, the roles of AGL15 are multiple, in particular having in mind that it responds to auxin levels, but it is also capable to repress its own expression and to activate *LEC2* [114, 115] (Fig. 3).

Independently of the presence/absence of cytokinin in the SE inductive medium, the involvement of cytokinin-related TFs in SE may be expected due to the known crosstalk between auxin and cytokinin in the control of the respective synthesis, transport, and signaling during morphogenesis in vitro (e.g. adventitious rhizogenesis, [61]). In the auxin-induced embryogenic cultures of *Arabidopsis* numerous cytokinin-response associated TFs are affected, including key cytokinin regulatory genes, i.e. *CYTOKININ RESPONSE FACTORS* (*CRFs*) and *Arabidopsis RESPONSE REGULATORs* (*AtARRs*) [41]. CRFs mediate the transcriptional responses to cytokinin involved in the regulation of embryo and leaf development, and function together with type-B ARRs [116]. One of these *ARRs*, i.e. *ARR10*, is upregulated in the SE of *Arabidopsis*

[41], similarly to its homolog in *Medicago truncatula*, i.e. *MtRR1* [117]. ARR10 has been proposed to play a general role in cytokinin signal transduction throughout the life cycle of *Arabidopsis*, working redundantly with other typeB-ARRs [118]. However the upregulation of specific TFs may also inhibit SE. It is known that the embryogenic competence of the callus induced from alfalfa petioles is inhibited when kinetin is replaced by thidiazuron (TDZ) ([5], and references therein). This inhibitory effect of TDZ is associated with the upregulation of a HD-Zip II TF, named MEDICAGO SATIVA HOMEOBOX 1 (MSHB1) [5].

Taken together, the regulatory network of TFs for cellular reprogramming leading to SE is complex, and still far to be fully elucidated. The mechanisms by which this regulatory network communicate with PGRs to coordinate SE is still widely unknown, however, a model summarizing the relationships between the main TFs and PGRs during the reprogramming of vegetative cells for SE induction is proposed in Fig. 3.

5 Somatic-Receptor-Kinases Involvement in the Regulatory Network for Stem Cell Induction and Maintenance During SE

In addition to PGRs and TFs, ligand-receptor-like kinase signaling pathways have been revealed as crucial regulators in stem cell specification, and an intercellular leucine-rich repeat receptor-mediated pathway has been proposed for the maintenance of plant stem cells ([119, 120] and references therein). *SERK* genes form a subgroup among the genes coding for membrane-located LEUCINE-RICH REPEAT-RECEPTOR-LIKE KINASE proteins (LRR-RLKs), which play important roles in plant signaling pathways ([1], and references therein). Auxin, combined or not combined with cytokinin, upregulates *SERK* genes, depending on the species ([121], and references therein). Some *SERKs* are positively related to zygotic embryogenesis, e.g., in carrot [122], *Arabidopsis* [123], cacao [124], *Medicago truncatula* [98], wheat [125], as well as to apomixis [126, 127]. The positive involvement of some *SERKs* in the induction of SE has been reported for a lot of dicots and monocots, e.g., carrot [122], *Dactylis glomerata* [128], *Arabidopsis* [123], *Medicago truncatula* [80], *Ocotea catharinensis* [129], sunflower [79], cacao [124], rice [130], *Citrus unshiu* [131], grapevine [81], potato [132], wheat [125], coconut [133], banana [134], maize [121], and *Cyclamen persicum* ([1], and references therein). Interestingly, numerous studies suggest a role in development, at least for specific SERKs, broader than in SE and zygotic embryogenesis. For example, in sunflower, a *SERK* gene is expressed in both SE and shoot organogenesis [79]. In *Medicago truncatula*, *MtSERK1* is expressed in somatic and zygotic embryogenesis, but also in rhizogenesis in vitro, and in all types of primary

meristems *in planta* [80, 135, 136]. In addition, *AtSERK1* and *AtSERK2* redundantly control microsporogenesis in *Arabidopsis* [137]. All together these results suggest an involvement of specific SERKs in stem cell formation and maintenance *in planta* and in vitro. The in vitro culture of *Cyclamen persicum* immature ovules has provided useful outcomes for demonstrating SERK(s) involvement in stem cell formation/maintenance, because lines forming either organs or embryos, as well as callus lines recalcitrant to organ/embryo formation, are available for the same cultivar and PGR condition (Fig. 4a–c). Using this system, Savona and coworkers [1] isolated two *SERK* genes, *CpSERK1* and *CpSERK2*, from the embryogenic callus. The expression of both genes was high in the embryogenic callus, moderate in the organogenic callus, and null in the callus showing neither SE nor organogenesis. The expression of both genes has been shown to start in the stem cell clumps from which the pre-embryogenic aggregates (PEAs,

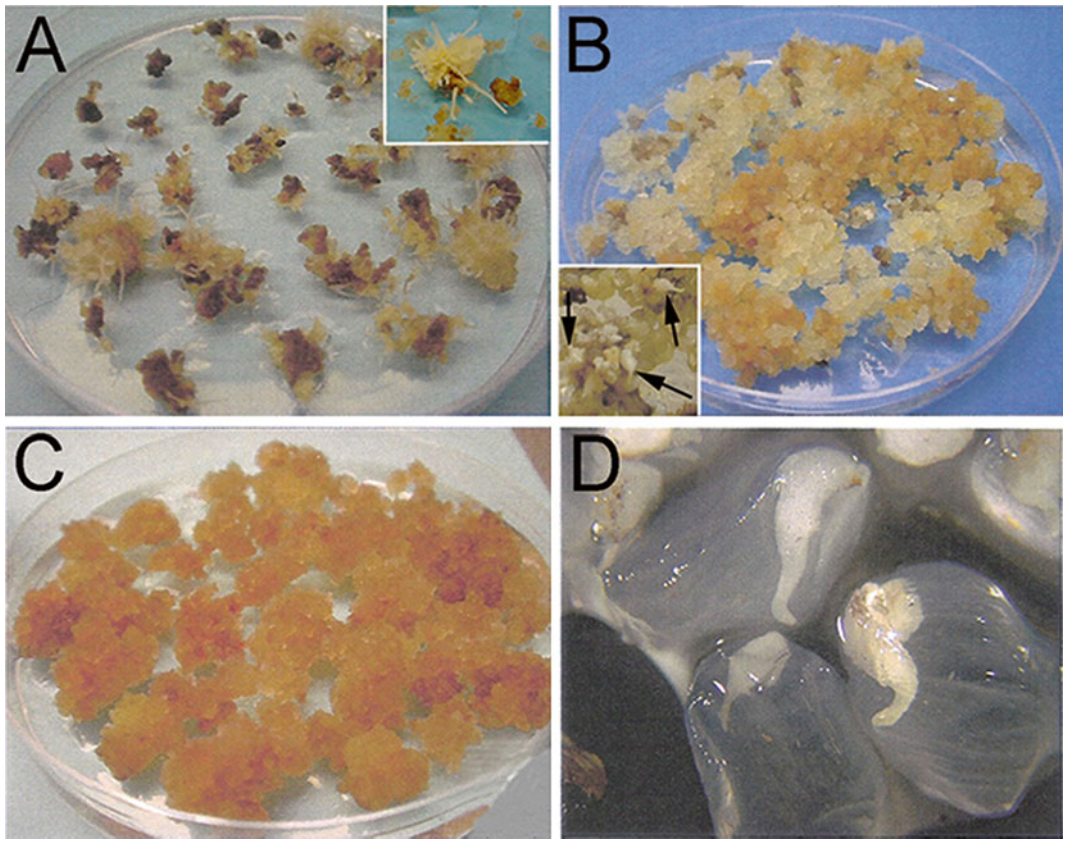

Fig. 4 *Cyclamen persicum* callus lines obtained under the same hormonal conditions but forming either shoots and roots (magnified in the *inset*) (**a**), or only somatic embryos (magnified in the *inset*, *arrows*) (**b**), or only callus (**c**) [see also the text, and [1] for further details]. (**d**) Encapsulated seeds of cyclamen (courtesy of B. Ruffoni and M. Savona)

Fig. 5c) and the organ meristemoids, respectively, originate. Expression continues in the pro-embryogenic masses (PEMs, Fig. 5d, m), but progressively declines (Fig. 5d). A similar expression pattern occurs in the organ meristemoids. In mature somatic embryos developing from the PEMs (Fig. 5g), and in the shoot and root primordia developing from the meristemoids (Fig. 5e, f), *CpSERK1* and *CpSERK2* are expressed with patterns similar to those of the zygotic embryos (Fig. 5b) and the primary meristems *in planta* (Fig. 5a). Thus, *CpSERK1* and *CpSERK2*, being expressed in the stem cells, may be regarded as markers of pluripotency. Moreover, their relation with the embryogenic potential is of interest, because their high expression maintains the trans-amplifying derivatives of the original stem cells (PEAs) in a pluripotent condition over time, and this leads to the totipotency necessary for somatic embryo formation [1]. Consequently, even if not peculiar of SE, the two genes may be used as markers of SE in cyclamen, allowing the screening of the calli before the macroscopic expression of their fate, i.e., embryogenesis, organogenesis, recalcitrance, with this early screening important for a large-scale production of synthetic seeds (Fig. 4d). The feature of *CpSERK1/SERK2* of pluripotency/totipotency markers is in accordance with their lack of expression during PCD occurring in the xylogenic nodules of the organogenic calli [1]. Similarly, AtSERK1 and AtSERK2 do not seem to be involved in PCD in *Arabidopsis* [138, 139]. As discussed in a following paragraph, PCD has an important role in SE, but uncoupled with stem cells.

In *Arabidopsis* and other species, *SERK* genes form a gene family ([1], and references therein). SERKs tend to function in pairs of redundant proteins evolutionarily organized in clades related to either AtSERK1/2 or AtSERK3/4/5 [140, 141]. CpSERK1 and CpSERK2 are tightly evolutionarily related, and relatively close to AtSERK1/2, and more distant to the other three AtSERKs [1]. In accordance, *CpSERK1/2* and *AtSERK1/2* share a common localization *in planta* ([1], and references therein), and *CpSERK1* and *CpSERK2* are expressed in *Arabidopsis*, e.g. in the root apex (Fig. 5h, k). In addition, a pro(promoter)*AtSERK1-AtSERK1-YELLOW*-FLUORESCENT-PROTEIN (YFP) construct [142], introduced into cyclamen embryogenic calli, expresses correctly the fusion protein and localizes exactly as *CpSERK1/2* (Fig. 5j, c, in comparison). All together these data sustain a role for SERK1/SERK2 complex in SE control in different species.

It is interesting to note that SERK1 and AGL15 are associated in complexes that include components of the brassinosteroid (BR) signaling pathway, i.e., BR-INSENSITIVE 1 (BRI1), and its coreceptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1)/SERK3 [55, 140]. Evidence has been presented that BAK1-LIKE 1 (BKK1)/SERK4 also participates in BR signaling [138]. However, the involvement of the latter complex, as well as of BR

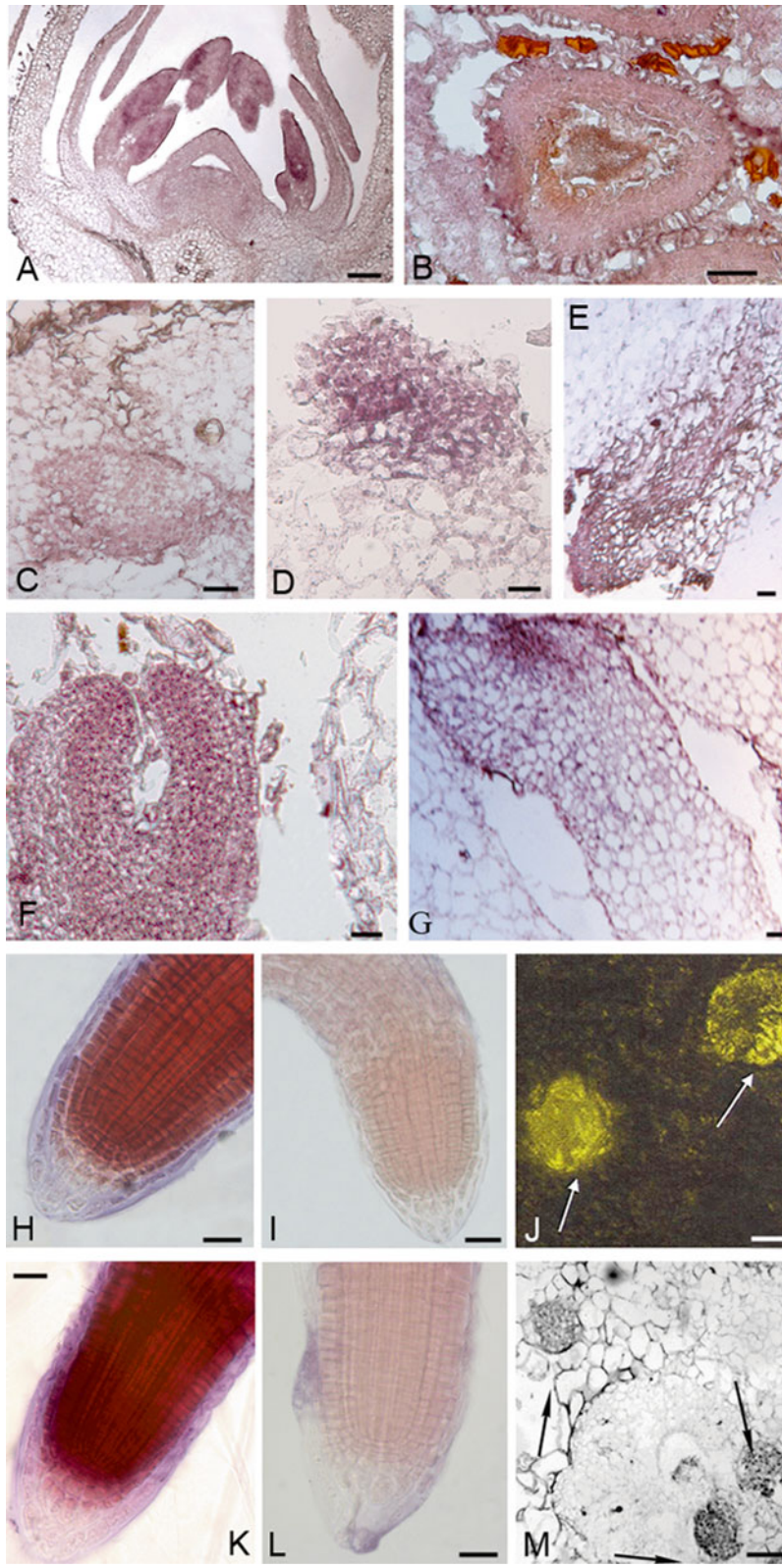

Fig. 5 Investigating *SERK1/2* genes in *Cyclamen persicum* and *Arabidopsis*. (**a**–**g**) *CpSERK1/2* RNA in situ hybridizations on cyclamen sections. (**h**, **i**, **k**, **l**) expression of *CpSERK1/2* in *Arabidopsis*, and (**j**, **m**) cyclamen callus with PEMs, with expression of *AtSERK1* (**j**). (**a**) Flower with strong *CpSERK2* expression in stamen

signaling in SE control, remains to be elucidated. It might not be excluded that SERK1 and AGL15, and perhaps LEC2, interact in controlling SE independently on the BR pathway. In fact, it is known that in *Arabidopsis AGL15*-overexpressing tissues show enhanced SE (see above), and have increased expression of *SERK1* and reduced GA levels by the AGL15-induced GA2-oxidase ([45, 53], and Fig. 3). Also in *Medicago truncatula* SE induction, *MtSERK1* reveals a binding recognition site for AGL15 and upregulation of *GA2-oxidase* [98]. Moreover, upregulation of *SERK1* also occurs in LEC2 transgenic tobacco in which SE is promoted [72], and a relationship between AGL15 and LEC2 in the positive control of SE through a GA-lowering mechanism has been proposed, as discussed above.

6 The Multifacet Significance of Stress as SE-Inducer

In vitro culture experiments have widely shown that the differentiated fate of plant cells, which depends on positional information and developmental signals *in planta*, can be altered under the in vitro culture conditions, with the changes in cellular environment perceived as, and generating, significant stress effects. When the stress level exceeds cellular tolerance, cells die, whereas when the level is lower, it enhances metabolism and induces adaptation, including gene expression reprogramming, cellular reorganization, and developmental switch. Somatic embryogenesis may be considered among the adaptation responses to stress, because various stresses are useful to induce this process (Fig. 3). For example, SE in carrot can be stimulated by the application of heavy metal ions (Cd^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}), high osmotic pressure (sucrose, NaCl) and high temperature in the absence of exogenous PGRs ([83], and references therein). Heavy metals also induce SE in *Arabidopsis* and wheat [143, 144], and high osmotic pressure or dehydration are SE-inducers in *Arabidopsis*, wheat, and cotton [143–145]. Of course, also the excision of the explant (i.e., wounding) must be

Fig. 5 (continued) primordia and procambia. (**b**) Immature seed containing an embryo at torpedo-stage showing *CpSERK1* expression. (**c**) Large PEA showing uniform *CpSERK1* expression. (**d**) *CpSERK2* expression only in a part of a PEM. (**e**) Adventitious root apex with *CpSERK1* expression in the apical meristem and procambium. (**f**) Meristematic shoot apex with strong *CpSERK2* expression. (**g**) Mature somatic embryo with *CpSERK1* expression in the shoot pole. (**h**, **k**) Whole mounts RNA hybridizations of *Arabidopsis* primary root apices with *CpSERK1* (**h**) and *CpSERK2* (**k**). (**i**, **l**) Sense-probe controls. (**j**, **m**) Cyclamen embryogenic calli transformed with *proAtSERK1-AtSERK1-YFP* construct during the induction phase. (**j**) Yellow fluorescence signal under confocal microscopy (*arrows*) localizing *AtSERK1* expression in the PEMs (courtesy of M. Savona). (**m**) Histological control section of PEMs. See text and [1] for further details. Bars = 10 μm (**b–g**), 20 μm (**h**, **i**, **k**, **l**), 50 μm (**a**, **m**), 1 mm (**j**)

considered as a "stressor" by a very early upregulation of the endogenous levels of auxin, as in potato adventitious shooting [146], and of ethylene [147], but also by acting as a source of Reactive Oxygen Species (ROS), as occurs in *Medicago truncatula* SE [136]. Moreover, ROS may induce ethylene biosynthesis [148].

It is widely known that 2-Dichlorophenoxyacetic acid (2,4-D) may be considered as a "stressor" for the explant ([16], and references therein). For example, in soybean and potato cotyledons SE induced by 2,4-D is associated with upregulation of oxidative stress and defense genes [85, 132]. In agreement with a role of ROS as stress-inducers (Fig. 3), SE is enhanced by increased levels of ROS, e.g., in wheat and alfalfa [149, 150], and the application of antioxidants to the inductive medium reduces SE in *Eucalyptus globulus* [151].

ABA serves as a critical messenger for stress responses, and is considered one of the SE-inductive PGRs (Fig. 3). ABA increases ROS levels in maize embryos, supporting roles of ROS in ABA-signaling through a mechanism that still requires investigation [16].

Also the micronutrient boron triggers stress-mediated pathways during SE, as recently reviewed [152]. Boron-stress has a direct impact on levels of ABA, e.g. increasing them as reported in carrot SE [153]. However, after ROS-signaling, a mechanism to protect the embryogenic-potential cells against the harmful effects of ROS is activated. GLUTATHIONE-S-TRANSFERASES (GSTs) seem involved in this protective activity, considering the accumulating evidence that the redox status and the glutathione content of the cells are interrelated in plant developmental processes. In accordance, members of the *GST* gene family are upregulated during auxin-induced SE of soybean [85], and *GST* transcripts accumulate in the somatic embryos of numerous plants ([49], and references therein).

GA has a negative effect on SE, and, in accordance, several genes important for the negative regulation of GA show SE-specific upregulation in *Arabidopsis*, including *DELLA* genes [41] (Fig. 3). The stimulation of *DELLA* genes may be put in relation with the stress response, because DELLA accumulation has been reported to elevate the expression of genes encoding ROS detoxification enzymes, reducing ROS levels [154]. Moreover, results on SE in transgenic lettuce support the hypothesis of an involvement of *SERK* genes in stress-perception [155].

Ca^{2+} ions are key regulators of many plant developmental processes, including sexual reproduction of gymnosperms and angiosperms [156–158]. In plant cells the free ionized form of calcium is located in the cytosol, and frequently acts as second messenger in signaling, whereas the loosely bond calcium, which is in dynamic equilibrium with free calcium, is present in middle lamellae and cell walls [157]. Exogenous treatments with calcium salts have demonstrated that calcium ions are active during organogenesis in vitro,

e.g., in vegetative bud formation from tobacco leaf and pith explants, flower and root formation from tobacco pith explants, and root formation from *Arabidopsis*thin cell layers [159–161]. Depending on the culture system and the exogenous concentration, calcium ions affect organogenesis independently/dependently of the exogenous hormone(s) [159, 161]. A positive Ca^{2+}/auxin interaction has been demonstrated in the pollenandrogenesis of *Solanum carolinense* [162]. In the SE of carrot the process coincides with significant variations in calcium ion distribution and levels. In particular, a positive interrelation with the inductive auxin 2,4-D seems to exist because the first SE stages are characterized by a strong and uniform Ca^{2+} presence in the cells. Calcium-presence, monitored by a fluorescent dye, becomes lower in PEMs, but again rises in the somatic embryos from the globular to the torpedo stage. At the latter stage, an apical-basal gradient of calcium-distribution appears along the longitudinal axis of the somatic embryo [163]. The role of PIN efflux-carriers in the establishment of the auxin gradient, necessary to the apical-basal axis of the *Arabidopsis* zygotic embryo, is well known ([96], Fig. 2c). Our unpublished results show that calcium distribution parallels this gradient (Fig. 2e, f), strengthening the possible link between auxin and calcium in axial patterning in both zygotic and somatic embryos [163]. Ca^{2+} ions are also known to regulate the transcript abundance of early auxin-inducible *AUX/IAA* genes [164]. Moreover, in SE-induction in wheat [165, 166], Ca^{2+} ions seem to have a different role, i.e., to be involved in the induction of lipid-transfer proteins [86], which are also active compounds in SE, as described in the following paragraph. Taken together, these evidences show that there is a connection among auxin, calcium ions, and SE induction, but this connection also involves SERKs, because, as in wheat, *SERK1* and *SERK2* expression is auxin- and calcium-dependent [125]. It is important to note that calcium is also important after SE-induction. In fact, an increase in calcium ion supply in the medium at the time of the transfer to the auxin-free differentiation medium, and an increased uptake by the somatic embryos, highly enhances the number of embryos reaching maturity, e.g., in carrot and sandalwood ([7], and references therein). The levels of cytosolic calcium are well known to change transiently in response to various stresses and to auxin, as well [167], and there are evidences that this also occurs during SE-induction, e.g., by an effect on the transduction of the auxin signal ([86], and references therein). Thus, in addition to the long-lasting changes described above, the very rapid changes affecting the free-calcium-pool are also of interest. For example, blocking calcium-signaling in sandalwood cells, SE frequency decreases [168]. In this species, Ca^{2+} signaling is associated with the activity of two Ca^{2+}-DEPENDENT PROTEIN KINASEs (CDPKs), and the expression of a *CDPK* gene also increases during early phases

of 2,4-D induction of SE in cultured alfalfa cells [169]. In *Arabidopsis*, two CDPKs have been demonstrated to activate a stress and an ABA-inducible promoter, suggesting connection of CDPKs to ABA-signaling pathways, with a link with calcium, because elevation of calcium ions is sufficient to trigger ABA-responsive gene expression [170].

Nitric oxide (NO) is being recognized as a critical factor in growth, development and stress response in plants [171], and there is emerging evidence that it may act as a stressor for SE-induction. NO might exhibit its inductive role on SE affecting the availability of Ca^{2+} within the cells via protein kinases [172]. Alternatively it might increase auxin production, repressing the basic HELIX-LOOP-HELIX protein 6 (bHLH006/MYC2), a repressor of auxin biosynthesis, as observed in *Arabidopsis* SE [173]. Interestingly, NO and calcium ions are also related to PCD, which is essential to successful embryogenesis, as discussed in the following paragraph.

7 Programmed Cell Death vs. Cell Survival in Somatic Embryogenesis, Two Faces of the Same Coin

In the zygotic embryogenesis of angiosperms and gymnosperms, the suspensor is a terminally differentiated structure committed to programmed cell death (PCD) and elimination. The suspensor cells must die at a certain stage of embryo-proper development. This usually occurs at the end of embryo-heart-stage, in the angiosperms, and at the end of the early embryogeny phase in the gymnosperms ([174], and references therein). The suspensor is not always formed by angiosperm somatic embryos, but, when present such as in carrot [175] and *Vitis rupestris* (Fig. 6a), its cells die via PCD similarly to what occurs in zygotic embryogenesis. Suspensor death by PCD also occurs in gymnosperm somatic embryos, as described for Norway spruce (*Picea abies*) and silver fir (*Abies alba*) [176, 177]. It is important to highlight that the proper timing of PCD in the suspensor cells is determinant to correct zygotic and somatic embryogenesis. In *Arabidopsis*, mutants with altered

Fig. 6 (continued) mature somatic embryo. (**b**) Barrier formed by cells with degenerating nuclei (*arrow*) and cutinized cell wall around a PEA of *Vitis rupestris*. (**c**) PEM flanked by callus cells in PCD (magnified in the *inset*), and (**d**) mature somatic embryos of *Vitis rupestris*. (**e**, **f**) Strong Ca^{2+} signal in the anther-wall tissues and microspores of kiwifruit male-sterile anthers (**e**), and weaker Ca^{2+} signal in male-fertile ones (**f**). (**g**) Nuclear fragments showing OSMOTIN immunolocalization in a degenerating epidermal cell of an olive tree twig, also showing a thick cuticle. (**h**) PCD nuclei of late-vacuolated microspores of kiwifruit male-sterile anthers monitored by OSMOTIN immunolocalization. (**i**, **j**) Kiwifruit male-sterile anthers shortly before dehiscence showing OSMOTIN-positive nuclear fragments in the middle-layer (**i**, *arrows*), and in the endothecium (**j**, *arrow*). See text and [158, 181, 187] for further details. Bars = 10 μm (**a**–**c**, **e**, **f**, **i**, **j**), 40 μm (**d**, **g**)

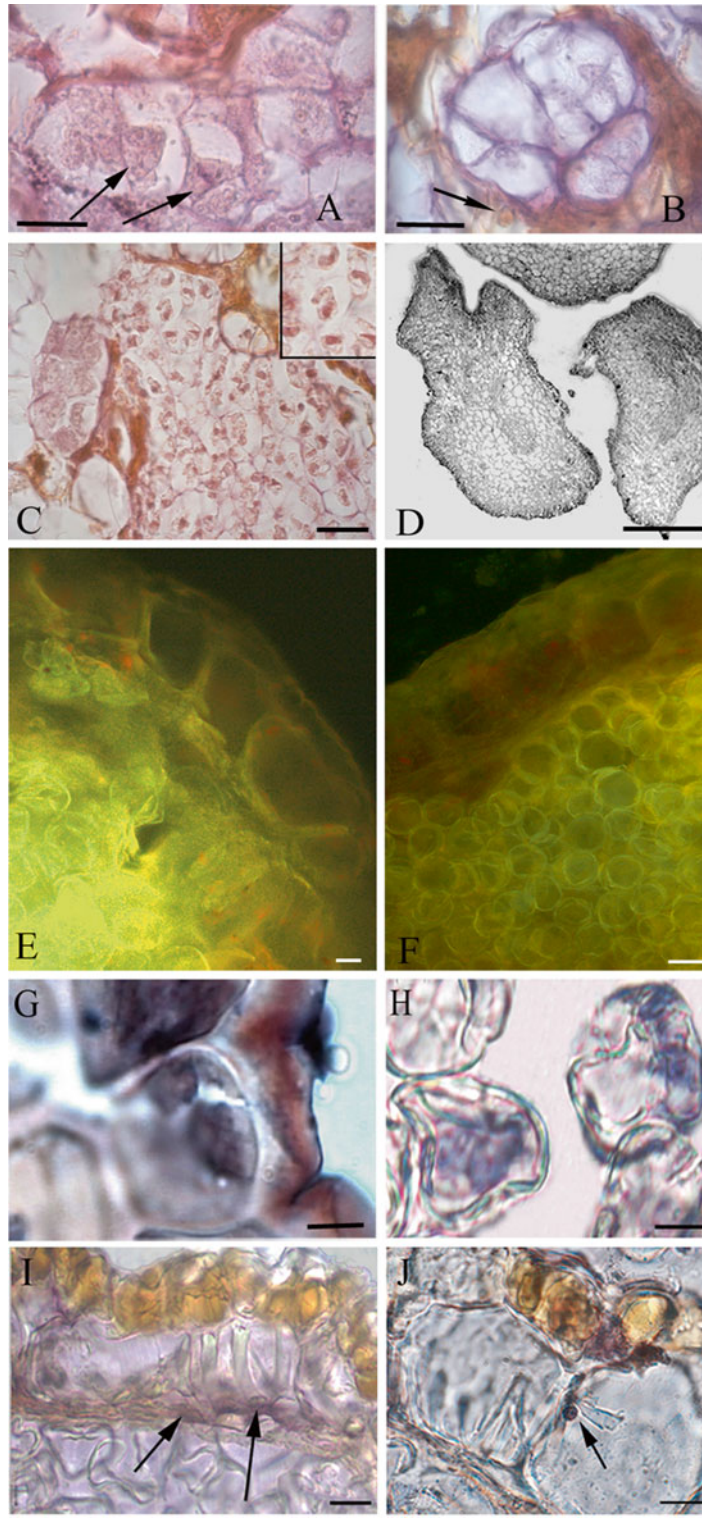

Fig. 6 Somatic embryogenesis from the sporophytic tissues of *Vitis rupestris* anthers (**a**–**d**), CTC-Ca^{2+} signal in kiwifruit male-sterile and male-fertile anthers (**e**, **f**), and PCD monitored by OSMOTIN immunolocalization in different cell types (**g**–**j**). (**a**) Degenerating multilobed nuclei in the suspensor cells (*arrows*) of a *Vitis rupestris*

regulation of PCD in the suspensor, such as *twin* and *raspberry*, exhibit altered embryo development. In both mutants the suspensor proliferates, and this results either into multiple embryo formation or failure of embryo transition from globular to heart stage [178, 179]. Moreover, in *Picea abies*, the prolonged longevity of suspensors delays the onset of histogenesis in the somatic embryos, finally resulting into disintegration of their tissues [176].

Research in gymnosperms (i.e., *Picea abies* and *Abies alba*) shows that there is another, and earlier, wave of PCD which is specific of SE and essential for its success [174, 177]. This first wave of PCD occurs in the PEMs. In *Picea abies*, time lapse-tracking analysis has shown that each PEM may either multiply in the presence of the inductive PGRs (auxin and cytokinin are necessary for SE-induction in this plant), giving rise to new PEMs, or transdifferentiate to embryo upon withdrawal of the PGRs. The latter pathway is only executed when massive PCD occurs in the PEM, establishing a positive correlation between cells in PCD in the PEM and frequency of somatic embryos [174]. This concept is strengthened by the results of the experiments with cell-lines composed of PCD-deficient PEMs, which are unable to form embryos regardless of treatment [180]. The presence of a PCD wave at the PEM stage of angiosperm-SE still needs investigation. However, in cyclamen SE, the progressive loss of stem trans-amplifying condition in the derivatives by the PEM couples with *CpSERK1/2* loss of expression (Fig. 5d). Because the expression of these genes is not compatible with PCD occurrence (see above), it is possible that in the angiosperms PCD occurs as a late event in the PEM cells, and only in those not engaged into somatic embryo formation and no more expressing *SERK1/2* genes. In a lot of cases of indirect SE in angiosperms, e.g., in SE from *Vitis rupestris* anther tissues [181], PEAs become separated from the callus cells by a barrier of cells with a cutinized cell wall (Fig. 6b). Events of nuclear fragmentation leading to PCD widely occur around the encased PEMs (Fig. 6c, and inset) and in the barrier cells (Altamura, unpublished results). It is plausible that this out-of-PEM PCD wave reduces the embryo-inductive potentialities in the PEMs, giving the already existing embryogenic cells the chance to develop further, i.e., to become a mature embryo without competition with further forming embryonic structures. All together, in both gymnosperms and angiosperms, PCD seems necessary to obtain a correct SE up to the mature embryo stage (Fig. 6d), and a PCD-signal needs to be cell-to-cell communicated.

Zinc is a potent regulator of PCD in animals and is crucial for correct SE patterning, as demonstrated in *Picea abies* SE ([182], and references therein). In this plant, high zinc accumulation in the somatic embryo couples with a strong decrease of the ion in the suspensor. In accordance, exposure of early embryos to a zinc chelating agent leads to embryonic lethality, and exogenous zinc

supplementation suppresses suspensor terminal differentiation and elimination, causing inhibition of embryo maturation [182]. However, Zn^{2+} can also have a pro-apoptotic effect on mammalian cells [183]. In accordance, in plants, when applied at high level, it may exhibit a positive function in the PCD process. This is caused by an increase in nitric oxide (NO) in the cells, which induces an NO-mediated PCD ([171], and references therein). All together these results show that the exact level of free intracellular zinc mediates PCD-survival decisions in embryogenesis, however other ions seem to be also involved.

In the previous paragraph the importance of calcium in the control of somatic and zygotic embryogenesis has been highlighted (Fig. 2e, f); however, the role(s) of calcium in these processes also include a relationship with PCD. In fact, transient changes in cytosolic free calcium and long-lasting changes in cytosolic and cell wall/membrane-associated Ca^{2+} affect development also by inducing PCD [184–186]. Male sterility is known to occur by PCD, and studies on microsporogenesis and microgametogenesis in numerous dioecious plants have demonstrated that calcium ion distribution and content are related to this sterility ([158], and references therein). The sporophytic tissues of the anther degenerate by PCD in both male-fertile and male-sterile plants, but PCD is delayed in kiwifruit male-sterile genotypes, with a calcium signal in the tapetum, middle-layer and exine of the microspores that is higher than in the male-fertile anthers (Fig. 6e, f). A prolonged secretion of calcium by the anther tissues seems to induce the inability of the microspores to transit to microgametogenesis, causing, instead, their PCD [158].

OSMOTIN is a pathogenesis-related type-5 protein involved in abiotic/biotic defense responses ([187], and references therein). The protein is also positively involved in PCD-induction [188], e.g., in the stem epidermal cells during cork formation (Fig. 6g), in degenerating sterile microspores (Fig. 6h, [158]), and in the endosperm of olive tree seed [189]. In accordance with the results by the use of other PCD markers, OSMOTIN immunolocalization in kiwifruit demonstrates the existence of a delay in PCD in the sporophytic tissues of the male-sterile anthers (Fig. 6i, j). Kiwifruit anther tissues are able to dedifferentiate in the presence of IAA and produce somatic embryos, but SE only occurs from anthers of male-fertile genotypes [190]. Taken together, it seems that the excess of calcium and the altered timing of PCD [158] are positively related with the SE-inability of kiwifruit male-sterile genotypes [190]. In accordance, it is known that Ca^{2+} concentration can change the hormone-induced organogenic response. For example, tobaccothin cell layers were induced to produce flowers by a specific combination of auxin and cytokinin and a specific concentration of $CaCl_2$, but formed vegetative buds instead of flowers when $CaCl_2$ concentration was increased fourfold [191].

In addition, NO has been demonstrated to influence the Ca^{2+} availability within the cells ([171], and references therein), and NO and PCD have been shown to be involved in the stress-induced microsporeembryogenesis of barley [192]. A role of OSMOTIN as a stress-acclimating protein involved in both blocking $[Ca^{2+}]_{cyt}$ transients, and in PCD has been demonstrated in the vegetative organs (Fig. 6g, [187]). *Osmotin*, and *osmotin*-like mRNAs have been also found in seeds, e.g., those of *Benincasa hispida* [193], tobacco [194] and olive tree [189], and the gene has been over-expressed in tea and olive tree somatic embryos [195, 196]. Also ABA, ethylene and wounding activate the *osmotin* gene ([187], and references therein). The transcriptionally active form *NAC* [name from the first letters of *NAM* (*No Apical Meristem*), *ATAF* (*Arabidopsis Transcription Activation Factor*), and *CUC* (*Cup-shaped Cotyledon*) genes] of AtNTL6 (*Arabidopsis thaliana* NAC with TRANSMEMBRANE MOTIF 1-LIKE) protein causes the expression of *Pathogenesis-Related-5* (*PR-5*) genes [197], and LEC2 (Fig. 3) induces the expression of *NAC* TFs ([72], and references therein). In *Olea europaea*, the *NAC* domain of the homologous gene (*OeNTL6*) induces *osmotin* transcription in both seed coat and embryo, but the protein is absent in the embryo, because of a downregulation after transcription. Concomitantly, cuticular lipids are produced in the seed coat and extruded towards the endosperm to enhance its cutinisation, suggesting a further role for OSMOTIN as lipid-transfer protein [189]. In accordance, *osmotin* over-expression induces accumulation of oil bodies in tea somatic embryos [195]. It has been previously mentioned that there is a phase during PEM growth characterized by the formation of a cutinized barrier around the PEMs, with PCD events occurring in the barrier cells and in the callus around (Fig. 6b). A role of OSMOTIN as a lipid-transfer protein during PEM encasing, and in inducing PCD around, is possible because genes involved in encoding lipid-transfer proteins elicit PCD, e.g., in the anther of *Hordeum vulgare* [198], and because an OSMOTIN-like protein accumulates and is secreted in the embryogenic cultures of *Cichorium* [199].

8 Hemoglobins Function as Anti-stress and Anti-PCD Compounds in SE and Their Repression is required in the Inductive Phase

The existence of plant hemoglobins, distinct from leghemoglobin, has been demonstrated in over 50 species ([200], and references therein). A relationship between NO, hemoglobins and PCD is well known in mammalians, but it is also appearing in plants, e.g., in chicory and *Arabidopsis* SE [173, 201]. The main role of specific hemoglobins is to reduce NO levels as a result of NO scavenging, resulting in reduction of NO toxicity and cell survival [171]. Plant

hemoglobins have been classified into three groups. Class 2 hemoglobins are upregulated by cold, cytokinin and ABA ([171], and references therein). For example, the promoter of a rice hemoglobin gene is activated by ARR1 TF [202], a type-B cytokinin-responsive regulator. As discussed before, stress-induced compounds, e.g., ROS (Fig. 3) and NO, are important for triggering SE, and PCD occurrence is determinant at PEM and suspensor stages. Based on this premise, compounds causing NO-detoxification and acting as anti-PCD might have a negative effect at specific time-points of the process. In accordance, the suppression of the type-2 hemoglobin identified in *Arabidopsis* (GLB2/AHB2, NON-SYMBIONTIC HEMOGLOBIN-2) enhances SE in this species by increasing levels of NO within the embryogenic cells. This increase causes a repression of the IAA-biosynthesis-repressor *MYC2*. Relieving the inhibition of IAA synthesis, the hormone increases in the cells promoting *WUS* and *SERK1* expression, and embryogenic competence. Moreover, the repression of *GLB2* increases the expression of *PIN1* [173], which is also essential to SE-induction (Fig. 3). Similarly the suppression of *Hemoglobin 1* (*Hb1-2*) gene in maize results in stimulating somatic embryo formation [171]. Taken together, SE is induced by compounds, such as NO, which are known to induce PCD, but PCD does not occur in the inductive phase because the activity of auxin, and not of anti-PCD compounds, such as hemoglobins. Thus, a working hypothesis in which SE-induction involves auxin and NO activities and suppression of hemoglobins and PCD may be suggested. However, the activity of hemoglobins might become essential later in the SE process, e.g., to scavenge NO and repress PCD, at the times when this becomes necessary.

9 Concluding Remarks

The broad repertoire of genes and complex expression patterns in SE show that multiple cellular pathways are controlled by a concerted gene regulatory network. SE is an ideal model system for investigating developmental flexibility and stem cell formation, maintenance and polarization, in particular because there is a strict similarity in the genetic control of zygotic and somatic embryogenesis in both gymnosperms and angiosperms. The first message coming from the actors described in the work is that pluripotent stem cells need to be defined before the realization of the totipotent condition. The totipotent cells result from trans-amplification and maintenance over time of the original pluripotent stem cell commitment. The switch from trans-amplification to trans-differentiation (i.e., the construction of the somatic embryo) is still obscure, but seems to share aspects with animal metamorphosis. The lack of the switch stabilizes the cells in the embryogenic fate,

leading to secondary embryogenesis. As in animals, death (PCD) and life (embryo-proper) must be coordinated processes. Differently from animals, a feedback loop exists in plants between pluripotent and totipotent stem cells, because niches of no more totipotent, but again pluripotent, stem cells must be present in the mature somatic and zygotic embryos to allow the polarized growth of the seedling. The second message is about the importance of the epigenome in the control of SE. Unraveling the interplay between DNA methylation, histone modifications, and small RNA activities in the establishment of the epigenetic program leading to SE will contribute to understand the behavior of plant cells in vitro and the molecular basis of cell totipotency. A third message is about the lipidome importance in SE, and its emerging functions in cellular communication, trafficking control, and PCD.

The tools for biotechnology coming from these messages are evident, because to maintain cells in a trans-amplification state will improve the massive production of plant stem cells, e.g., for innovative cosmetics industry. By contrast, to stimulate trans-differentiation will provide a tool for improving large-scale production of mature somatic embryos, which is the essential prerequisite for massive artificial seed production.

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Chapter 4

Do Mitochondria Play a Central Role in Stress-Induced Somatic Embryogenesis?

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Abstract

This review highlights a four-step rational for the hypothesis that mitochondria play an upstream central role for stress-induced somatic embryogenesis (SE): (1) Initiation of SE is linked to programmed cell death (PCD) (2) Mitochondria are crucially connected to cell death (3) SE is challenged by stress *per se* (4) Mitochondria are centrally linked to plant stress response and its management. Additionally the review provides a rough perspective for the use of mitochondrial-derived functional marker (FM) candidates to improve SE efficiency. It is proposed to apply SE systems as phenotyping tool for identifying superior genotypes with high general plasticity under severe plant stress conditions.

Key words Somatic embryogenesis, Mitochondria, PCD, Severe stress, Cell reprogramming, Phenotype plasticity, Phenotyping tool, Genotype selection

1 Introduction

It is state-of-the-art understanding that mitochondria play an upstream role in stress response management and cell network integration. This view is shared among scientists from various research fields who study organisms from diverse kingdoms, including plants and animals [1–4]. Mitochondria are seen central for stress perception and orchestrating the effects of external signaling by connecting them to growth and development control mechanisms. They are movable organelles that are able to sense where their action is required and seem to act as tuners [2, 3]. The numbers per cell, size, and shape of mitochondria are highly flexible and related to development and cell function. SE is an example of inducible developmental plasticity. It is based on molecular and metabolic cell reprogramming that covers typically phases of dedifferentiation and de novo-differentiation. SE can occur naturally during agamospermy along a species-dependent developmental plan and it can be induced artificially under in vitro culture conditions ([5, 6] *and other chapters in this book*).

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Changing environmental conditions is commonly referred to as being "stressful" to organisms. This definition of stress encloses regular daily and seasonal, as well as unpredictable, events. These events can have positive, negative, or even neutral effects. In cell biology and organism physiology, stress at various levels is judged by its effect. However, exploring stress responses is a never ending story, since diversity is high and evolution is an ongoing process. Nevertheless, despite this dynamics, in biotechnology and plant breeding the view is forced to be more focused, which helps to acknowledge that functional biology can only be understood in case-by-case studies that respect individuality. This understanding is getting even more important in view of the increasing awareness of the ubiquity of endophytes and the subsequent fact that organisms typically exist as complex holobionts [7, 8]. Thus, the term "individuality" must refer also to organisms that need to coordinate various living units within the same body along developmental and environmental constraints. It includes not only coordinating the diversity of cells with potentially diverse genetic, epigenetic, and metabolic identities, but encloses also endophytes, whenever present. Consequently, stress effects on organism's performance should be assessed by upstream and superimposed measures, because this is what will count for validating final stress responses. In this context, it was proposed that plant stress responses are optimization strategies dictated by thermodynamic demands, in order to reach harmony with the environment [9–11], and carbon balance is seen as the master integrator for plant stress responses [11–13]. The effect of stress depends on timing, severity, and novelty of stress events. Whether a stress will be harmful, neutral, or a driving force for tolerance or resistance depends on the capacity and actual engagement of the cell/tissue/organism to respond and on the level that the organism has achieved for "optimality" in a trait-specific, as well as in a wider sense related to the general capacity for phenotype plasticity [14].

Nevertheless, it is possible to identify also typical stress response patterns. In explants or cultivated plantlets exposed to successful in vitro culture conditions for SE, the process starts typically in distinct plant cells or group of cells. Afterwards, these cells directly develop embryos or indirectly proliferate and form pro-embryogenic cell structures and/or masses (PEMs) from which somatic embryos can form. Original cells and PEMs are networking with their neighbors via plant common internal communication pathways, contributing to the stimulation of surrounding cells to regain totipotency in order to run into the same developmental direction [6, 15]. Developing embryogenic cells reproduce and propagate the multicellular organism via a bipolar structure originating a new plantlet. These processes run similar to zygotic embryogenesis [15]. Nevertheless, the new plantlets may show genetic differences to each other through differential genetic

identities in the original cells, or due to stress-induced genetic changes (somaclonal variation). These changes can be expected to result from the multiple interactions among the original cell, the developmental stage, and the stress [16–21]. The developmental program for SE is heritable, and consecutively occurs in a repetitive manner. Thus, it can be used for mass propagation. However, SE performance can happen with varying efficiency depending on the characteristics of individual genotypes. Low efficiency can be due to lower strength in response and/or postponed responses. Both can make a relevant difference for the efficiency of a SE system. The molecular biological reasoning for differential efficiency during both induction and initiation is still quite obscure. However, genetic differences for efficiency in SE systems are supposed to be mainly seen during the initiation phase [6, 22].

2 Initiation of SE Is Linked to Programmed Cell Death (PCD)

It is now accepted that the death of cells from the suspensor and the final exclusion of the suspensor itself are prerequisite to the process of SE during bipolar patterning [15]. Thus, successful SE seems to depend on the dying of neighboring cells that initially helped to feed the later core cells of the embryo proper. This observation is more obvious in gymnosperms, where suspensor structures during early and late embryogenesis are more strongly pronounced. However, McCabe et al. [23] have reported also for carrot (*Daucus carota*) that suspensor cells die during initiation of embryo formation via programmed cell death (PCD). Bozhkov et al. [24] found a two wave rhythm of PCD in *Picea abies*. While one wave of PCD occurs during maturation as vacuolated PCD and is linked to gradual degradation of the suspensor [25], a first wave of PCD happened already during proliferation. This first period is connected to the transition of PEMs to somatic embryos. Smertenko and Bozhkov [15] reviewed the life and death processes during apical-basal patterning for angiosperms and gymnosperms both in comparison to zygotic embryogenesis. The authors stress that the balance between survival and embryo development and PCD together with the elimination of the suspensor are critical for SE efficiency. Petrussa et al. [26] found that in *Abies alba* the rate of PCD was substantial during proliferation as well as during the maturation stage, although much higher during proliferation.

3 Mitochondria Are Crucially Connected to Cell Death

PCD and necrotic cell death events form part of stress management strategies for organism survival and are both related to mitochondrial functionality. However, while necrosis is based on

mitochondrial dysfunction not involved in SE observed in plants, vacuolated PCD is connected to SE, but mitochondrial involvement is more sophisticated [27]. Smertenko and Bozhkov [15] reported that mitochondria remained intact at the final stages of PCD during SE, although with altered biochemical activities. The role of mitochondria in plant PCD was described by Vianello et al. [28] and Reape et al. [29]. It was reported that mitochondrial electrical potential and ATP levels dropped down during PCD process [30, 31]. However, how the balance between survival and death is established and maintained in proliferating embryogenic cells and during the maturation phase of SE remains unclear. Smertenko and Bozhkov [15] underlined that the same groups of protein can play a role in proliferation and cell death, depending on their molecular environment. In maturating cells in *A. alba*, Petrussa et al. [26] observed that mitochondrial activities changed when compared to cells during the proliferation phase. The authors found higher activity of the mitochondrial alternative oxidase enzyme (AOX) in maturing cells than in proliferating cells, which were characterized by a higher amount of dying cells. This led them to suggest a correlation between mitochondrial activities and the manifestation of PCD during the formation of somatic embryos. The alternative respiration pathway (AR) seemed to act in the *A. alba* SE system as anti-apoptotic factor via reactive oxygen species (ROS) capturing. The activities of external NADH dehydrogenases, AOX, and the free-fatty acid circuit system were higher in mitochondria from maturing tissues. The alternative cyanide-resistant pathway seemed to be activated and functional only in maturing tissue reaching about 50 % of total O_2 uptake. It was demonstrated a fivefold increase in this pathway compared to proliferating cells [26]. In contrast, the mitochondrial K+ATP channel activity was decreased, which seemed to reduce the destructive release of cytochrome c from mitochondria. Overall, it is supposed that mitochondria play a crucial role in the manifestation of the two waves of PCDs during SE in conifers. A protective role of AOX in PCD had been indicated earlier by the group of Vanlerberghe [32, 33].

4 SE Is Challenged by Stress Per Se

It is now commonly accepted that stress induces the in vitro induction of SE ([34]; reviewed by [5, 6]). Moreover, SE is the most pronounced example for stress-related phenotype plasticity reactions [6]. It is well known also that plant growth regulators (PGRs) are involved in wounding, plant development, and growth processes, likewise that they are integrated in the process of external environmental signal transmission towards the interior of organismic life, and that they interfere with gene regulatory networking.

This is reviewed in Zavattieri et al. [5], Yang and Zhang [35], Zeng et al. [36], Osakabe et al. [37] and Fehér [6]. Several reports show the generation of ROS or the involvement of oxidative stress (OS) responsive genes upon SE induction conditions [38–40]. Following a transgenic approach, Zheng and Perry [41] demonstrated that SE could be more rapid and prolific by differential regulation of genes involved in stress response.

Appropriate abiotic stress *stimuli* for in vitro SE have been empirically explored over long time, among which osmotic shock, dehydration, water stress, heavy metal ions, pH changes, heat and cool treatments, hypoxia, ultraviolet radiation, and mechanical or chemical treatments, including also antibiotics (*reviewed in* [5]). Several PGRs have been applied and a diversity of combinations have been optimized not only to induce SE, but also to promote embryo differentiation when SE is indirect and embryos are developed from a previously generated callogenic mass. However, SE in carrot was also induced in PGR-free medium by different chemicals, such as sucrose, sodium salt, or CdCl [5]. SE can be regulated by cell wall components, diverse extracellular proteins, arabinogalactan proteins (AGPs), oligosaccharins, and through the perception and transduction of extracellular signals by receptor kinases, Ca^{2+} and its effectors, as well as by diverse transcription factors (reviewed in [15]). Several studies show that changes in chromatin organization and in epigenomic marks (DNA methylation, histone posttranslational modifications, micro RNAs) accompanies SE induction and somatic embryo development and growth [6, 15]. These observations are not surprising, since they confirm the role of global genome organization during normal and adaptive development and its involvement in stress responses, also seen in other in vitro culture systems or stress treatments [16, 17, 19]. Nevertheless, future studies should more strongly consider differences in cell identity in the first responsive cells, marked not only by differential transcript patterns [42, 6] but also by genetic and/or epigenetic factors. This is justified by the current knowledge on DNA variability at single cell and tissue levels due to single nucleotide polymorphisms (SNPs), insertion/deletions (InDels), copy number variation (CNV), and/or DNA methylation [43–45].

Stress provided by changing environmental conditions can promote both induction of dedifferentiation (e.g., [46]) and the realization of induced SE programs by somatic embryo development (e.g., [6]). However, due to the high diversity of inducers, SE cannot be defined as a specific response to a unique stress or stress composition. On the contrary, it must be recognized that stress per se plays critical role as an embryonic *stimulus* [5, 6, 47]. The so-called stress-induced morphogenic response (SIMR) depends on the stress-management capacity of the plant, or of a cell and tissue at a given developmental stage. Fehér [6] pointed to the large variation observed in SE between genotypes. He

highlighted also the fact that main differences among various embryonic pathways will be found in the phase of the initiated stress response.

5 Mitochondria Are Centrally Linked to Plant Stress Response and Its Management

Under stress mitochondria play multiple roles. They regulate cell homeostasis through controlling cell redox states and adapt the supply of energy and metabolic compounds to target cell locations, integrating stress responses with plant growth and development both in photosynthetic and nonphotosynthetic cells [4, 48]. How mitochondria can take over this upstream role superimposed to all types of adaptive metabolic and morphologic cell processes related to growth and development is currently in the focus of ambitious research efforts and was excellently reviewed for plants by Ng et al. [4]. Crucial is the central role of mitochondria in stress perception and transmission to cell functioning through anterograde and retrograde signaling pathway networks, including dual location strategies that integrate cell nucleus, cell organelles, and endoplasmic reticulum (ER) [2, 4, 49, 50]. Recently, Wallace and Fan [51] and Wallace [52] highlighted in the context of human diseases the critical role of mitochondria (via bioenergetics) also for epigenetic cell regulation.

A role of mitochondria for stress responses was confirmed for diverse types of environmental stress *stimuli* that also account as *stimuli* for SE. This includes importantly osmotic stress [53, 54], salinity [54], water stress [48], and temperature [55]. As reported above some of those factors have been successful to induce SE without any PGR application (*reviewed in* [5]). Sugar and hormone signaling pathways interplay for the modulation of developmental transition [56] and it was reported that mitochondrial invertase functions in developmental energy-demanding processes [12]. It has been shown that Glucose-TOR (Target-Of-Rapamycin) signaling reprograms the transcriptome and activates meristems in the control of developmental transition and growth [13]. TOR complexes constitute an ancestral signaling network, which is conserved throughout eukaryotic evolution to control the fundamental process of cell growth. As a central controller of cell growth, TOR plays a key role in development and aging, and has been implicated in stress-induced disorders. This master metabolic regulator was shown to be involved also in mitochondrial shaping, which is impressively linked to mitochondrial functioning [57]. It is well known that the number of mitochondria is adaptive, depending on environmental signaling that interacts with plant development. For example, in root cells the number of mitochondria is plastic and correlates to induced root exudation and plant growth

performance. Mitochondria are highly dynamic with respect to their biogenesis, frequent fusion and fission events, and size and shape restructuring, which is related to consecutive functioning. This dynamic seems to be regulated by tissue specificity, developmental and internal, as well as external, *stimuli* [57–60]. Vice versa, mitochondria can influence morphogenesis as reported for cancer [61]. In plants, significance of mitochondria for cell fate decisions that enclose dedifferentiation and de novo differentiation is also recognized [62, 63]. In this context, the AR is increasingly getting into the focus of research on stress acclimation and adaptation [64–66]. Most studies on AR focus on AOX, an inner mitochondrial membrane protein that functions as terminal oxidase generating water from ubiquinol [67]. The enzyme is encoded by a nuclear gene family, which in higher plants is composed by 1–6 gene members distributed in two subfamilies (*AOX1* and *AOX2*) [68, 69]. AOX employs activity in the mitochondria at the cutting edge of stress signal perception, cell signaling, and maintenance of homeostasis. Several authors highlighted the involvement of also other components of the mitochondrial energy-dissipating systems in stress responses, such as uncoupling proteins (UCPs) and external NADH dehydrogenases [14, 70, 71] or other antioxidant molecules. From those, glutathione [72], superoxide dismutase and catalase [73] have also been suggested as being involved in SE.

For AOX, many studies confirm a central role for cell redox homeostasis [74, 75], a link between AOX and responses to osmotic stress [64], salinity [54, 76, 77], temperature [70, 78–80], drought [65, 81], pH changes [82], nutrient limitation [83, 84], ozone, metal toxicity, as well as to low oxygen and high irradiance (*reviewed in* [64, 70]). Several reports are available also in reference to biotic stress showing a contribution of AOX in resistance against insects, virus, fungi, and pathogenic bacteria (*reviewed in* [64, 66]). AOX was proposed as "master regulator" for stress responses [85], and it is known for its involvement in the regulation of seed germination [86], plant growth [83] and development [87], as well in fruit development [88] and ripening [89]. The involvement of AOX in SE was firstly reported by Frederico et al. [22]. These authors demonstrated early differential expression of AOX gene members during SE initiation ("realization phase"). Application of SHAM (salicylhydroxamic acid) inhibited AOX activities and completely suppressed embryo development. Recently, a role for AOX in early events of dedifferentiation was also indicated during the lag-phase of callus growth induction in explants from carrot tap-root secondary phloem ([90], Campos et al., *personal communication*). SHAM application can obviously reveal discriminatory inhibiting effects on callus growth and developmental morphogenesis. During auxin-stimulated adventitious rooting in microshoots from olive, SHAM application suppressed the rooting process as expected, while simultaneously occurring

callus growth in the same region was not influenced [91–93]. Fehér [6] emphasized that calli correspond not necessarily to a dedifferentiation state, but can also be the result of disturbed differentiation of adult stem cells under unphysiological conditions ([6], *and references therein*). These results hypothesize a role for AOX in dedifferentiation, but not a role in "misdifferentiation."

6 Perspective View on Future Experimentation

There is no doubt that mitochondria and mitochondrial proteins play a relevant role in plant stress responses. SE is a clear demonstration of the capability of plants to respond upon severe stress by strong morphogenic plasticity in order to enable survival. Further, SE is an example for SIMR, i.e., the stress *per se* is the *stimulus*. Consequently, efficient biomarker and DNA marker for SE related to applications in biotechnology or plant breeding can be supposed to come from the mitochondrial machinery linked to cell networking. This is a promising and wide field for future research. Since long time it is known that induction and initiation of SE depend on multiple interaction of [genotype × development × explant × environment]. Recalcitrant species are well recognized (*see in this book*), but genotype-specific responses are also known in non-recalcitrant species. Even within easy-to-induce species, such as *D. carota*, differential responsiveness to *stimuli* and conditions are found at subspecies and variety level. Based on the insight that SE is a response upon stress, SE was proposed as a screening tool to study stress-inducible plant plasticity as a trait per se [14, 94, 95]. This was also the underlying idea of initiating about 10 years ago research on carrot SE as one of several experimental in vitro and in vivo plant systems from diverse species, subspecies, and cultivars that show clearly defined developmental plasticity upon stress. In these systems the role of AOX on stress performance and its appropriateness as functional marker (FM) for stress behavior was studied [22, 90, 91, 94, 96–98], Campos et al. (*personal communication*). Findings from this integrated research across species and systems is expected to contribute to a better understanding of stress behavior and phenotype plasticity as well as to advance FM development for stress responses, including also the identification of markers for the efficiency of the SE process.

The idea of Frederico et al. [22] of using SE as a screening tool for stress behavior was already taken by the breeding community. Afuape et al. [99] applied SE in cassava as a system to study stress responsiveness of the heterologous *AtAOX1a* gene in search for a linkage to post-harvest stress and its use in molecular breeding. Similarly, a primary culture test system for carrot root explant growth induction was used to confirm the significantly higher

responsiveness of a hybrid to yield-determining cytokinin activity compared to growth of the according parental inbred lines [100]. The system could also discriminate carrot cultivars and plant-specific response to temperature and reveals now *AOX* involvement in dedifferentiation and growth maintenance ([90], Campos et al., *personal communication*). Recently, bioenergetics and mitochondrial respiration are in the focus of plant breeding research on abiotic and biotic stress tolerance and FM development. SE systems might develop as important species-specific deep phenotyping tool, in order to screen for superior genotypes that can cope with severe stress conditions.

Recognizing the ubiquity of endophytes in organismic life challenges not only our fundamental understanding of functional biology but will also drive innovation in conventional and FM-assisted plant breeding [8]. Future research needs to consider the significance of plants as holobionts that should also be explored to understand the origins of variable competence for SE. Studying the meaning of endophytes in SE systems will be a fascinating area of research for the coming generations of scientists. Mitochondria are one of the most prominent examples of invasion of organisms with mutually beneficial effects and shared coordination of the whole organism structure and function. Improving our understanding of mitochondrial dynamics (variable number per cell, mobility and, plastic sizes and shapes) related to plant morphogenesis will certainly contribute to improve SE efficiency.

Finally, linking bioenergetics and thus the importance of mitochondria for epigenome regulation may become highly instrumental for application in biotechnology and breeding. This is a research area which can be excellently studied on SE as an experimental system.

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Chapter 5

Dying with Style: Death Decision in Plant Embryogenesis

Shuanglong Huang, Mohamed M. Mira, and Claudio Stasolla

Abstract

Embryogenesis is a fascinating event during the plant life cycle encompassing several steps whereby the zygote develops into a fully developed embryo which, in angiosperms, is composed of an axis separating the apical meristems, and two cotyledons. Recapitulation of embryogenesis can also occur in vitro through somatic embryogenesis, where somatic cells are induced to form embryos, and androgenesis, in which embryos originate from immature male gametophytes. Besides cell division and differentiation, embryo patterning in vivo and in vitro requires the dismantling and selective elimination of cells and tissues via programmed cell death (PCD). While the manifestation of the death program has long been acknowledged in vivo, especially in relation to the elimination of the suspensor during the late phases of embryo development, PCD during in vitro embryogenesis has only been described in more recent years. Independent studies using the gymnosperm Norway spruce and the angiosperm maize have shown that the death program is crucial for the proper formation and further development of immature somatic embryos. This chapter summarizes the recent advances in the field of PCD during embryogenesis and proposes novel regulatory mechanisms activating the death program in plants.

Key words Androgenesis, Embryogenesis, Hemoglobins, Programmed cell death, Somatic embryogenesis

1 Programmed Cell Death in Plant Growth and Development

The term programmed cell death (PCD) encompasses several distinct pathways unique to eukaryotes [1] which lead to the selective dismantling and elimination of cells, tissues, and/or organs. This active process, which together with cell division and differentiation contributes to the "shaping" of the organism, is controlled by endogenous factors and relies on energy-dependent events [2]. Manifestation of PCD in plants is developmentally and environmentally regulated and observable throughout the life cycle. The most dramatic examples of development-regulated PCD are apparent during xylogenesis, the maturation and death of xylem cells required for the formation of the vascular system, reproduction, involving the elimination of specific embryogenic cells or the selective killing of female primordia, and senescence, where PCD

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ensures the removal of old tissue and the turnover of macromolecules [3]. Activation of PCD is also triggered by some abiotic and biotic stresses. While during flooding conditions selective removal of cortical cells maintains a continuous supply of oxygen to the under-water organs through the formation of aerenchyma, during plant-pathogen interaction programmed elimination of cells limits pathogen growth and reduces the infection sites [3].

In animal systems, the mechanisms regulating PCD have been well investigated and, based on morphological, biochemical and molecular characteristics, three types of PCD are distinguished: apoptosis, autophagy, and necrosis [1]. In plants, despite the early recognition of PCD, knowledge on the biochemical and molecular events underlying PCD is scarce and classification of the death pathways is solely based on morphological criteria [4]. According to van Doorn [5], PCD in plant cells can be categorized as necrosis and vacuolar cell death. While the former is generally caused by the rupture of the plasma membrane and the shrinkage of the cytoplasmic components, as often observed under abiotic stresses, the latter is characterized by the clearance of the cytoplasm triggered by the rupture of the tonoplast and the release of vacuolar hydrolytic enzymes. Vacuolar cell death is very common during development where it is involved in organ formation. It must be kept in mind that the categorization of cell death into these two pathways, i.e., necrosis and vacuolar cell death, is somehow simplistic as some atypical examples of cell death do not follow in either category [5].

2 Ultrastructural and Cytological Characteristics of Necrotic and Vacuolar Cell Death in Plants

Considered for a long time an "unprogrammed" event, necrosis has been recently included as an integral pathway of PCD [5] characterized by two early hallmarks: the increase in cellular volume and the rupture of the cytoplasm leading to the release of the intracellular content [1]. Although poorly characterized in plants, necrosis in animals is also accompanied by increases in cytosolic Ca^{2+} and changes in mitochondrial and lysosomal function leading to the accumulation of reactive oxygen species (ROS) [6]. As summarized by van Doorn [5], necrosis is typical of the hypersensitive response and cells challenged with necrotrophic pathogens.

Unlike necrosis, vacuolar cell death is better characterized and manifested by the rupture of the tonoplast and the release of the hydrolytic enzymes. Plant cells are equipped with two major types of vacuoles: storage vacuoles which accumulate preferentially proteins, and lytic vacuoles enriched with several hydrolytic enzymes including aspartate and cysteine proteases and nucleases [5]. Manifestation of vacuolar cell death can be non-disruptive, if the tonoplast fuses with the plasma membrane and releases the

hydrolytic enzymes in the apoplast, or disruptive, if the collapse of the tonoplast discharges the hydrolytic enzymes within the cytoplasm [7]. This second series of events has been shown to occur during lysogenous aerenchyma formation through three temporally distinct steps. The first step is characterized by the swelling of the lytic vacuoles which occupy most of the symplast. During the second step, the tonoplast invaginates and through processes analogous to autophagy of animal cells engulfs and degrades cytoplasmic regions [8]. Microscopy studies revealed shrinkage of the plasma membrane and the formation of granular bodies within the lytic vacuoles and around the organelles engulfed by the tonoplasts [9]. The third and final step is characterized by the lysis of the tonoplast and the release of the hydrolytic enzymes which clear cytoplasmic components starting with the endoplasmic reticulum and terminating with the nucleus and mitochondria [5]. Deviations from this sequence, such as the early disruption of the cell wall prior to the rupture of the vacuole [10], are observed and confirm the simplistic classification of the proposed death pathways.

The most characteristic cytological events of PCD are visible in the nucleus and compromise the ability of the DNA to transcribe and replicate; these include the degradation of DNA, the condensation of chromatin, and nuclear fragmentation [11]. Degradation of DNA is executed by nucleases and occurs in two distinct phases: the initial cleavage of the DNA at the interloop sites of the chromatin producing DNA fragments of about 50–300 kbp, followed by cleavage at the internucleosomal sites which generate 200 bp DNA fragments [12]. These events occur in conjunction with the condensation of chromatin which requires de-polymerization of F-actin [13], and the fragmentation of the nucleus which is very typical of animal apoptosis [14]. Although nuclear fragmentation is generally one of the last events of PCD, it was reported as the first sign of PCD during aerenchyma formation in oxygen-deprived plants [15].

3 Execution of PCD During Plant Embryogenesis

Embryogenesis is an important event during the plant's life cycle. The zygote, originating from a single fertilization event in gymnosperms and a double-fertilization event in angiosperms, undergoes a precise pattern of cell divisions culminating in the formation of a fully developed embryo. The subsequent imposition of a maturation period, in which the seed experiences water stress, is required for the termination of the developmental program and the initiation of germination [16]. Recapitulation of embryogenesis can also be achieved in culture through judicious manipulations of media and culture environment. Two methods routinely used to generate in vitro embryos are somatic and gametophytic

embryogenesis. While the former method is employed to generate embryos from somatic cells, i.e., cells other than gametes, the latter uses male or female gametophytes as explants. The utilization of male gametophytes to produce embryos (microsporeembryogenesis, sometimes referred as androgenesis) exploits the ability to reroute the developmental fate of immature pollen, i.e., microspores, from a gametophytic to an embryogenic pathway [17]. Both somatic and gametophytic embryogenic systems are used as model systems to investigate biochemical and molecular events governing embryo development.

Execution of PCD is an integral component of embryonic development both in vivo and in vitro as it shapes the body of the embryo through the elimination of specific cells and organs. Experimental interference with the death program compromises the formation of the embryos [18].

3.1 Role of PCD During In Vivo Plant Embryogenesis

Manifestation of PCD is apparent during different phases of in vivo embryogenesis. It participates in the dismantling of the suspensor, the removal of supernumerary embryos produced by polyembryonic seeds, and degradation of nucellus, endosperm, and aleurone layer. This chapter only deals with the first two events as they are intimately related to the formation of embryos. Detailed descriptions of the last events are available [19].

3.1.1 Elimination of the Suspensor

Formation of the suspensor is concomitant to that of the embryo proper. In angiosperms, the first asymmetric division of the zygote originates an apical cell and a sub-apical cell. While the apical cell gives rise to the embryo proper (which will progress though a globular, heart, cotyledon, and torpedo stage of development), transverse divisions of the subapical cells generate the suspensor [20]. Besides its passive function in anchoring the embryo to the seed, the suspensor plays two key roles. It transfers nutrients to the embryo proper and it participates in the establishment of the polar-basal embryonic axis by modulating the flow of auxin [21]. The suspensor is short-lived and once its functions are no longer needed, generally at the cotyledon stage of development, it is dismantled through the execution of the death program [22]. In all cases examined, PCD is required for the elimination of the suspensor regardless of its shape and morphology which differ remarkably among species. While in orchids the suspensor consists of a single cell, the *Arabidopsis* suspensor is composed of about seven cells while runner bean suspensors have more than 200 cells [23]. Variations in the number of suspensor cells are also observed within the same family [24]. Profound differences in suspensor morphology are also apparent. In angiosperms, the suspensor is generally composed by a file of single cells characterized by two regions: the neck including suspensor cells adjacent to the embryo proper and the knob comprising suspensor cells in close proximity to the seed

integuments [25]. More complex morphological arrangements are observed in gymnosperms, such as in *Picea abies*, where the suspensor consists of defined tiers of cells with the upper tier "embryonal tube cells" produced by the asymmetric division of the embryo proper [22]. Independent evidence suggests that elimination of the suspensor by PCD progresses basipetally, starting from the top suspensor cells adjacent to the embryo proper and terminating to the bottom portion of the suspensor. Using *Phaseolus coccineus* as a model system, Lombardi et al. [25] showed the basipetal spreading of DNA fragmentation, a hallmark of PCD, from the neck region (top) to the knob region (bottom) of the suspensor. This "death" pattern was also observed in maize [26] and in spruce [22]. Contrasting reports describing an acropetal movement of PCD in suspensor cells exist, but they are solely based on ultrastructural evidences and are not substantiated by PCD marker analyses [27]. As reviewed by Bozhkov et al. [22] two scenarios have been proposed to account for the progressive development of PCD. The first involves the presence of a "cell-death" signal produced by the embryo proper which is released basipetally towards the suspensor cells, while the second would require the depletion of an "anti-death factor." The generation and analyses of suspensor mutants might resolve the nature of the PCD progression. An intriguing question arising from the progressive spreading of PCD is whether the suspensor cells are committed to die only after they are fully differentiated. *Arabidopsis* suspensor cells are targeted by PCD only after the suspensor is fully formed, thus suggesting that death occurs in terminally differentiated cells. This notion is also substantiated by analyses of *tween* mutant embryos. In these mutants, suspensors cells can re-differentiate into embryogenic cells and this ability is retained only up to the globular stage, after which cells become fully differentiated and committed to die [28]. In spruce, however, the death program is initiated in newly formed suspensor cells which are not terminally differentiated. Suspensor cells are added from asymmetric divisions of the embryo proper and they start dying soon after they are formed [22].

Elimination of the suspensor cells is a slow process and cells are not subjected to rapid disruption. This is possibly required for the proper differentiation of the embryo, given the function of the suspensor in transporting nutrients [22]. Time-course analyses of zygotic and somatic embryogenesis in gymnosperms [29, 30] suggest that the death and removal of a suspensor cell occurs in a period of at least 5 days [22]. While the information above argues strongly for the involvement of PCD in the elimination of the suspensor, it provides a rather simplistic picture on the timing and progression of PCD, as variations in both are apparent. Different patterns of PCD timing and progression in suspensor cells exist and, in the case of the *Leguminosae* family, have been categorized [31].

3.1.2 Monozygotic Polyembryony: Survival of a Single Dominant Embryo

Generation of multiple embryos from one zygote is referred to as monozygotic polyembryony, a common event in animal reproduction [32]. This process, the genetic bases of which are unknown, requires the physical splitting of cells after a few rounds of mitotic divisions leading to the formation of two or more genetically identical embryos. Although several plant species develop supernumerary embryos, monozygotic polyembryony is particularly common in gymnosperms where several embryos are produced in one seed, but only a "dominant" one continues to complete the developmental process. The remaining "subordinate" embryos are eliminated [33]. Based on the growth rate of the supernumerary embryos, Filonova et al. [34] divided the development of pine seeds into three distinct phases. The first phase is characterized by the formation of multiple embryos from the same zygote. The embryos share the same growth rate with no dominance. Acquisition of "dominant" characteristics of one embryo, which overgrows the subordinate embryos, demarks the second phase. In the third phases, the subordinate embryos are eliminated by PCD, while the dominant embryo completes the maturation process. Manifestation of PCD in the subordinate embryos follows a specific pattern and is part of two distinct death programs [22]. By using terminal deoxynucleotydil transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) to detect DNA degradation in the subordinate embryos, Filonova et al. [34] showed that PCD is initiated in the basal part of the embryo proper and progresses acropetally reaching the apical cells in approximately 4 weeks. As reviewed by Bozhkov et al. [22], this pattern can be established by the presence of a "death-inducing signal" moving acropetally, or by a survival signal which accumulates preferentially in the apical region of the embryo. The nature and origin of the signal are unknown, but the signal might be produced by the megagametophyte where PCD precedes the autodestruction of the embryonic cells [34]. This idea is also resonated by Young and Gallie [35] who proposed that death in the megagametophyte precludes the transport of nutrient (and a putative survival signal) to the supernumerary embryos. Orchestration of the death program in megagametophytic and embryonic cells is in fact required for normal seed development [22]. As in any other developmental process governed by PCD, the response to the death program can be quite flexible and environmentally influenced, as demonstrated by the presence of more than one dominant embryo able to develop to maturity [36].

3.2 Role of PCD During In Vitro Plant Embryogenesis

As outlined above, recapitulation of the embryogenic process can also occur in vitro through somatic and microspore-derived embryogenesis. In both processes removal of cells through PCD is an integral component of embryo development, and recent studies have emerged on the molecular components governing the death program.

3.2.1 PCD During Somatic Embryogenesis

While descriptions of hormone- and density-induced death programs have been shown in several somatic embryogenic systems [37–39], it was von Arnold's group to first prove the requirement of PCD for proper spruceembryo development in vitro [30, 34]. The spruce system is suitable for these studies as its developmental pathway has been well characterized [30]. In this system the proembryogenic masses (PEMs), generated from cultured zygotic embryos, are maintained with the plant growth regulators auxin and cytokinin (PGRs), and consist of three defined cellular aggregates (PEM I-III). Proembryogenic masses I (PEM I) are composed of clusters of highly cytoplasmic cells subtended by a single suspensor-like cell. Addition of other suspensor cells to PEM I forms PEM II. As more cells are added to the PEM II the cluster grows in size and differentiates into PEM III. In the presence of PGRs, the three PEMs co-exist without forming embryos. Removal of PGRs from the culture medium stimulates the transdifferentiation of PEM III into somatic embryos through processes involving PCD [30]. The massive execution of the death program in the PEM III re-shapes the cell cluster and allows the formation of somatic embryos. Independent evidence suggests that PCD is obligatory for proper and successful embryogenesis. Besides the positive correlation observed between the extent of PCD in the PEM III and the number of somatic embryos produced [30], inhibition of PCD through manipulations of the culture medium compromises the differentiation of PEM III into somatic embryos [18]. Consistent with the requirement of the death program, lines composed by PCD-deficient PEMs are not able to form embryos [40], possibly because of their inability to reprogram their transcriptional machinery [41]. These results are analogous to *Drosophila* studies showing that blockage of PCD by mutagenesis results in prenatal death [42].

Ablation of the PEMs III by the death program is followed by a second wave of PCD which removes the suspensor of the somatic embryos during the late phases of development. This second wave follows a basipetal gradient, starting from the suspensor cells adjacent to the embryo proper and proceeding towards the basal region of the suspensor [43].

3.2.2 PCD During Microspore-Derived Embryogenesis

Microspore-derived embryogenesis relies on the ability of immature microspores to redirect their normal gametophytic developmental pathway toward a sporophytic route. This cell reprogramming can be triggered in culture by imposition of diverse stress treatments including heat shock, starvation, cold conditions, and ethanol and gamma irradiation [44]. According to Touraev et al. [17] the embryogenic process involves two steps: the formation of multicellular structures (MCS) within the exine wall of the isolated microspores, and the differentiation of MCSs into embryo-like structures (ELS). Formation of MCSs can occur through

different pathways. In the first pathway, the microspore nucleus divides asymmetrically forming a generative and a vegetative cell. Divisions of the vegetative cells give rise to MCSs [45]. In the second pathway, common to rapeseed, potato and tobacco, MCSs are formed directly from symmetric divisions of the microspore nucleus. The first pathway is characterized by the simultaneous divisions of the vegetative and generative cells, both contributing to the formation of MCSs [46].

Manifestation of PCD is an integral component of microspore-derived embryogenesis, especially during the early phases, as most of the anther tissue harboring the microspores undergoes massive death. Degeneration of cells by PCD is first apparent in the tapetum of the anthers at the pre-meiosis stage [47]. As reviewed by Varnier et al. [48], this first death wave, which contributes to the total elimination of the tapetal cell layer, has a temporary effect on the microspores soon after meiosis, as some death "information" might migrate from the dying tapetum to the microspores. Signs of microspore degradation are often observed [49] and this might compromise their redirection towards the embryogenic pathway. Therefore, the ability to control and manipulate the course of the PCD process in the microspores is crucial for ensuring a high recovery of embryos. Does the stress pretreatment, which redirects the developmental fate of the microspores towards the embryogenic pathway, interfere with the death program? Wang et al. [50] showed that while inducing death in the tapetal cells, the stress pretreatment does not accelerate death in the microspores. Furthermore, at a metabolic level components of the PCD machinery, including the bax inhibitor Bi1, are induced during the stress pretreatments [51]. Based on the above results, Varnier et al. [47] suggested that the arrest of the death pathway in the microspores is a necessary prerequisite for redirecting their fate towards embryogenesis. Once the redirection step has occurred the microspores undergo a symmetric division and no evidence of PCD is apparent, as revealed by transcriptome and proteomic studies [52, 53]. Contrasting observations were reported in barley, where microspores subjected to stress pre-treatment exhibited increasing levels of cell death [54]. Discrepancies in results are possibly due to different systems and stress conditions utilized.

The second wave of PCD during microspore-derived embryogenesis occurs during the differentiation of MCSs into ELSs. The development of mannitol-stressed barley microspores into haploid embryos is characterized by formation of MCSs composed by two distinct cell domains derived from proliferation of the vegetative cell and generative cell, respectively. These two domains have different fates; the generative domain is eliminated by PCD, while the vegetative domain develops into ELS [55]. According to the authors, the elimination of the generative domain marks the site of exine rupture from where the globular embryos will emerge.

Collectively, these studies suggest that PCD plays an integral and important role during (1) the redirection of the microspores from a gametophytic to an embryogenic pathway, and (2) the early morphogenetic events associated to embryo development. The capacity to experimentally manipulate the death program during both processes would provide valuable insights into the requirement of PCD for microspore-derived embryogenesis.

4 Regulation of PCD During Embryogenesis

Regulation of the death program in plants is complex and relies on the participation of many components, some of which participate in unrelated responses. A premise to any investigation on plant PCD should be that the death program in plants is mediated by factors fulfilling similar functions to regulators of animal PCD. The following section outlines the role of some proteins and signal molecules in modulating the cell survival/death decision during embryogenesis.

4.1 Bax-Inhibitor-1

Animal apoptosis is mainly orchestrated by the Bcl-2 related proteins, which include pro-survival and pro-apoptotic members. While pro-apoptotic members, such as those of the Bax subfamily, trigger death events through the release of cytochrome C from the mitochondria, pro-survival components, such as Bax-inhibitor 1 (BI-1) abrogate these events [56]. Initially characterized in humans for its ability to repress the yeast death pathway activated by the over-expression of the mouse Bax gene [57], BI-1 has been isolated in many species of yeasts, plants and animals where it is expressed under stress conditions and in senescent tissues [58, 59]. The pro-survival nature of this protein was also defined in plants through transformation studies. Cell death induced by pathogens, fungal elicitors, temperature stress and hydrogen peroxide was repressed in *Arabidopsis* plants ectopically expressing *BI-1* [60]. In the same line, a down-regulation of BI-1 accelerated death in carbon starved tobacco cells [61]. Although the role of BI-1 has not been investigated during embryogenesis, Maraschin et al. [51] showed a transcriptional activation of the barley BI-1 following stress treatments which induce embryogenesis possibly through the suppression the PCD pathway. Localization studies together with analyses of structural and functional domains suggest BI-1 proteins reside in the ER membranes where they have a protective role against the ER-stress induced PCD, a condition where basic ER functions are compromised [62]. This cytoprotective role of BI-1 is mediated by its ability to modulate Ca^{2+} homeostasis and response in the ER by interacting with several calcium-binding proteins [63].

As indicated above, the pro-survival role of BI-1 proteins is to counteract the pro-death effect of other factors, including kiss of death, a small amino acid peptide which triggers PCD [64]. Using two mutant *kod* alleles and *KOD* over-expressing lines, the authors demonstrated the involvement of KOD in the elimination of the *Arabidopsis* suspensor during embryogenesis and its participation in early PCD events including the depolarization of mitochondrial membrane [64]. The ability to trigger the suicide program by the sole expression of *KOD* makes this gene a suitable tool to target and dismantle cells by PCD.

4.2 Metacaspases

Apoptosis in animal cells is triggered by the activation of caspases, proteolytic enzymes able to cleave proteins at specific amino acid residues. Expressed as inactive pro-enzymes, i.e., pro-caspases, caspases are activated at the onset of the death program where they initiate an irreversible proteolytic cascade of events involving the induction of other caspases and culminating to rapid cell death [65]. Based on their position and function along this cascade, caspases are broadly divided into initiators (caspase 2, 8, 9, and 10), executioners (caspase 3, 6, and 7) and inflammatory (caspase 1, 4, and 5) [66, 67].

While direct homologues of caspases are not found in plants, proteins with similar functions have been identified as metacaspases, characterized by caspase-like secondary structures and catalytic domains [68]. Involvement of metacaspases in PCD has been demonstrated in yeast, where the survival/death fate is dependent upon metacaspase expression [69], and more recently in plant embryos [70]. In this latter study, it was showed that a spruce metacaspase (mcII-Pa) is expressed during spruce somatic embryogenesis in tissues committed to PCD, i.e. suspensor of immature embryos and procambium of late embryos, and that RNAi-mediated suppression of *mcII-Pa* prevents the differentiation of somatic embryos from PEMs III by repressing the death program. Besides establishing metacaspases, and mcII-Pa specifically, as executioners of PCD in plant embryogenesis, this work emphasized the relevance of the death program for the formation of embryos in culture. The cellular function exercised by mcII-Pa requires its cysteine-dependent arginine-specific proteolytic activity and its ability to migrate from the cytoplasm into the nucleus to induce the fragmentation of DNA and the disassembly of the nuclear envelope in cells committed to die [71].

4.3 Nitric Oxide

Nitric oxide (NO) is a signal molecule fundamental for a broad range of plant developmental and environmental responses, including hormone signaling, cell cycle mechanisms, and biotic and abiotic stress responses [72]. Over the past years its role as modulator of PCD has emerged and NO participation during the embryogenic process has received increasing attention. In animals, the

pro-apoptotic role of NO occurs through several mechanisms. Besides inducing two caspase activators: p53 and CD95 [73], NO influences the death program by modulating protein nitrosation/nitrosylation and the level of cellular cGMP [74]. During plant pathogen interaction, protein nitrosylation via reaction with NO regulates the activity of many stress-related enzymes, including metacaspase 9 [75]. Nitric oxide also influences the pool of cGMP by binding to the ferrous heme group of the guanylate cyclase-coupled receptor converting GTP to cGMP, an effector of apoptosis [76]. In plants, administration of NO increases PCD through an elevation of cGMP which opens Ca^{2+} channels through intermediates including cyclicADP-ribose [77]. A spike in cellular Ca^{2+} increases mitochondria permeability and triggers the death program [78]. Of note, applications of 8-Br-cGMP, a cGMP analog, suppress caspase activity and PCD [79]. While these regulatory mechanisms have not been demonstrated during embryogenesis, the NO-mediated activation of caspase activity and PCD has been recently shown to occur during the early phases of microspore-derived embryogenesis [54].

As suggested above, NO homeostasis is crucial for cell death/survival decision and plant hemoglobins (Hbs) are active NO scavengers [74]. Plant HBs have been classified into three classes depending on their structural and chemical characteristics, but all of them react with NO producing nitrate and oxidizing ferrous hemoglobin to methemoglobin [72]. Studies in animal systems have shown the ability of Hbs to influence the death program by modulating NO, a function that we have shown to be retained during plant embryogenesis [80, 81]. Suppression of two *Hbs* (*ZmHb1* and *ZmHb2*) in maize embryogenic tissue induces PCD by increasing NO levels in cells in which Hbs are repressed. This increase of NO produces opposite outcomes on embryo yield depending on the expression patterns of the two *Hbs*. While suppression of *ZmHb1*, which is expressed in both suspensor cells and embryo proper, triggers massive death resulting in embryo abortion, suppression of *ZmHb2*, which is expressed solely in a few cells anchoring the embryos to the embryogenic tissue, eliminates these "anchor" cells releasing the embryos in the culture medium, encouraging their growth, and increasing total embryo production [81]. The induction of PCD in *Hb*-suppressing cells fits a model in which repression of *Hbs* causes localized NO maxima which increase intracellular Zn^{2+} levels, by favoring its release from metallothioneins through the destruction of the zinc-sulphur clusters [81, 82]. Changes in cellular Zn^{2+} homeostasis influence the death/survival decision in a system-dependent fashion. While in some embryogenic systems the PCD program is induced by depletion of Zn^{2+} level [83], in others, including maize, an elevation in Zn^{2+} level triggers cell suicide through the MAPK cascade which activates NADPH oxidase and induces production of reactive

oxygen species [81]. Based on these observations, the authors identified Hbs as potential regulators of in vitro embryogenesis by elevating NO levels and promoting the suicide program.

5 Conclusions

Removal of unwanted plant cells by PCD is an important factor for embryonic and post-embryonic development. During in vivo embryogenesis activation of the death program is required for the elimination of the suspensor, once the function of this organ is not needed, and for the selective elimination of supernumerary embryos in polyembryonic seeds. Recent advances on the role of PCD during in vitro embryogenesis have evidenced the death program as an obligatory event for the early phases of embryo formation. While the ability to alter the embryonic death program with molecular and pharmacological approaches has been pivotal in the identification of some executors of the death pathway, more information is required to identify the early inductive signals triggering death. Specific attention should be addressed on the initial steps of the death commitment, the reversibility of the commitment process, and most importantly the identification of cues (positional?) responsible for selective death occurring within the cultured tissue. Answers to these questions will open new avenues for targeted applications and manipulations of PCD to enhance embryo quality and yield.

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Chapter 6

Somatic Embryogenesis in Broad-Leaf Woody Plants: What We Can Learn from Proteomics

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Abstract

Proteomic approaches have been used to understand several regulatory aspects of plant development. Somatic embryogenesis is one of those developmental pathways that have beneficiated from the integration of proteomics data to the understanding of the molecular mechanisms that control embryogenic competence acquisition, somatic embryo development and conversion into viable plants. Nevertheless, most of the results obtained are based on the traditional model systems, very often not easily compared with the somatic embryogenesis systems of economical relevant woody species. The aim of this work is to summarize some of the applications of proteomics in the understanding of particular aspects of the somatic embryogenesis process in broad-leaf woody plants (model and non-model systems).

Key words Angiosperms, 2D electrophoresis, Embryogenic competence, Embryo maturation, Heat-shock proteins, Mass spectrometry analysis, Metabolism-related proteins, Stress-related proteins, Zygotic embryo

1 Introduction

Proteomics studies the total proteins expressed in any given system, whether by abundance, activity, structure, state of posttranslational or other modification, or how these proteins interact with each other in networks or complexes [1]. In recent years proteome studies have been employed to generate reference maps of the most abundant soluble proteins of plant organs, at defined developmental stages, for several purposes such as genetic studies comparing the proteomes of different plant genotypes, physiological studies analyzing the influences of exogenous signals on a particular plant organ, and developmental studies investigating proteome changes during development [2, 3]. Technical advances provide now a proteomic dissection of individual cell types, thus greatly increasing the information revealed by proteome analyses [2].

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Proteomics has been successfully applied to the systematic analysis of protein expression during somatic embryo formation and development in various plant species [3]. Following the pioneering studies in carrot [4, 5], somatic embryogenesis (SE) has been considered not only as an efficient system for in vitro clonal propagation, but also as an outstanding model system quite appropriate to better understand totipotency in higher plants, as well as embryo development, considering the difficulties that have been encountered to analyze the early stages of zygotic embryogenesis during development of the embryo inside the ovular tissues [6]. Nevertheless, most knowledge on the general principles underlining the SE regulatory pathways has been focused on traditional model organisms. With the recent increase in the number of genome-sequencing projects, the definition of model organism has broadened [7]. For example, the whole sequence genome of *Populus trichocarpa* was published in 2006 [8]. In addition, several other tree species have been sequenced, including conifers [9–11], *Eucalyptus* [12], and Fagaceae [13]. Moreover, the genomic data for fruit trees such as citrus or apple also became available [14, 15]. Considering that there are approximately 300,000 botanically described species of plants and that model plants represent only a handful of species and families, even the arrival of these new model plants cannot reflect the biodiversity of the plant kingdom and all the economic or agricultural interests [16]. Some features and processes are unique and cannot be approached via a model plant. Woody plants for example, are perennials with a quite long life cycle and special features to be analyzed, including in what concerns their SE systems. Proteomic approaches have a great potential to study non-model species, because protein sequences have the advantage of being more conserved, making the high-throughput identification of non-model gene products quite effective by comparison to orthologous proteins [17]. However, it is important to recognize that there is a possible discrepancy between the messenger (transcript) and its final effector (mature protein). As most biological functions in a cell are executed by proteins rather than by mRNA, transcript expression profiling does not always provide pertinent information for the description of a biological system. Several post-transcriptional and posttranslational control mechanisms such as the translation rate, the half-lives of mRNAs and proteins, protein modifications and intercellular protein trafficking, have an important influence on the phenotype [18].

The main goal of this work is not to give a full review of all the proteomic studies carried out on broad-leaf woody plant species, but to summarize some of the applications of proteomics to understand different aspects of the SE process in these plants (model and non-model systems). First, a general perspective of the most common methodologies followed in the proteomics workflow is given, followed by a short review of what we could learn in the last years

from the proteomic approaches to embryogenic competence induction and somatic embryomaturation and germination in woody plants.

2 Proteomics Workflow

The most common proteomic workflow (Fig. 1) consists of protein extraction, protein (peptide) separation and quantification, protein identification, and data integration [19, 20]. Several approaches have been developed to address proteomic investigations, either trough top-down or bottom-up strategies, applying "gel-based" or "gel-free" procedures. These procedures differ in the way proteins are isolated (extracted), separated, and detected, and consequently, each of them covers a typical subset of proteins [19]. The "gel-based" approaches based on two-dimensional polyacrylamide gel electrophoresis (2DE-PAGE) are the most common referred proteome analysis of the plant SE process. The classical 2DE protocol separates denatured proteins according to two independent properties: isoelectric point (p*I*), by isoelectric focusing (IEF), and molecular weight (MW). One of the most challenging steps of the process is usually protein extraction from plant samples, due to the relatively low protein content and high level of contaminants [21]. The cell wall and the vacuole are associated with numerous substances responsible for irreproducible results such as proteolytic breakdown, streaking and charge heterogeneity. Most common interfering substances are phenolic compounds, proteolytic and oxidative enzymes, terpenes, pigments, organic acids, ionic species and carbohydrates. The majority of the plant protocols introduce a precipitation step to concentrate the proteins and to separate them from the interfering compounds. The most commonly used method for extraction of plant proteins is the trichloroacetic acid (TCA)/acetone precipitation method [22]. Apart from the optimization of the extraction protocol, also protein solubilization is a critical factor. Proteins are solubilized in the presence of high concentrations of chaotropes, a reductant and a neutral detergent. The use of a detergent in conjunction with chaotropes is of paramount importance and is decisive for the subset of proteins that can be analyzed [20, 21]. Proteins of several samples can be labeled prior to an electrophoretic separation with spectrally distinct fluorescent dyes, and mixed together to run on the same 2D gel. This 2D difference gel electrophoresis (DIGE) approach allows to simultaneously comparing the proteomic profiles of different samples that migrate under identical conditions [20]. After separation through 2DE, data are generated through image analysis software that detects and quantifies the protein abundances and matches the proteins across the different gels. Though the matching quality is dependent on the software

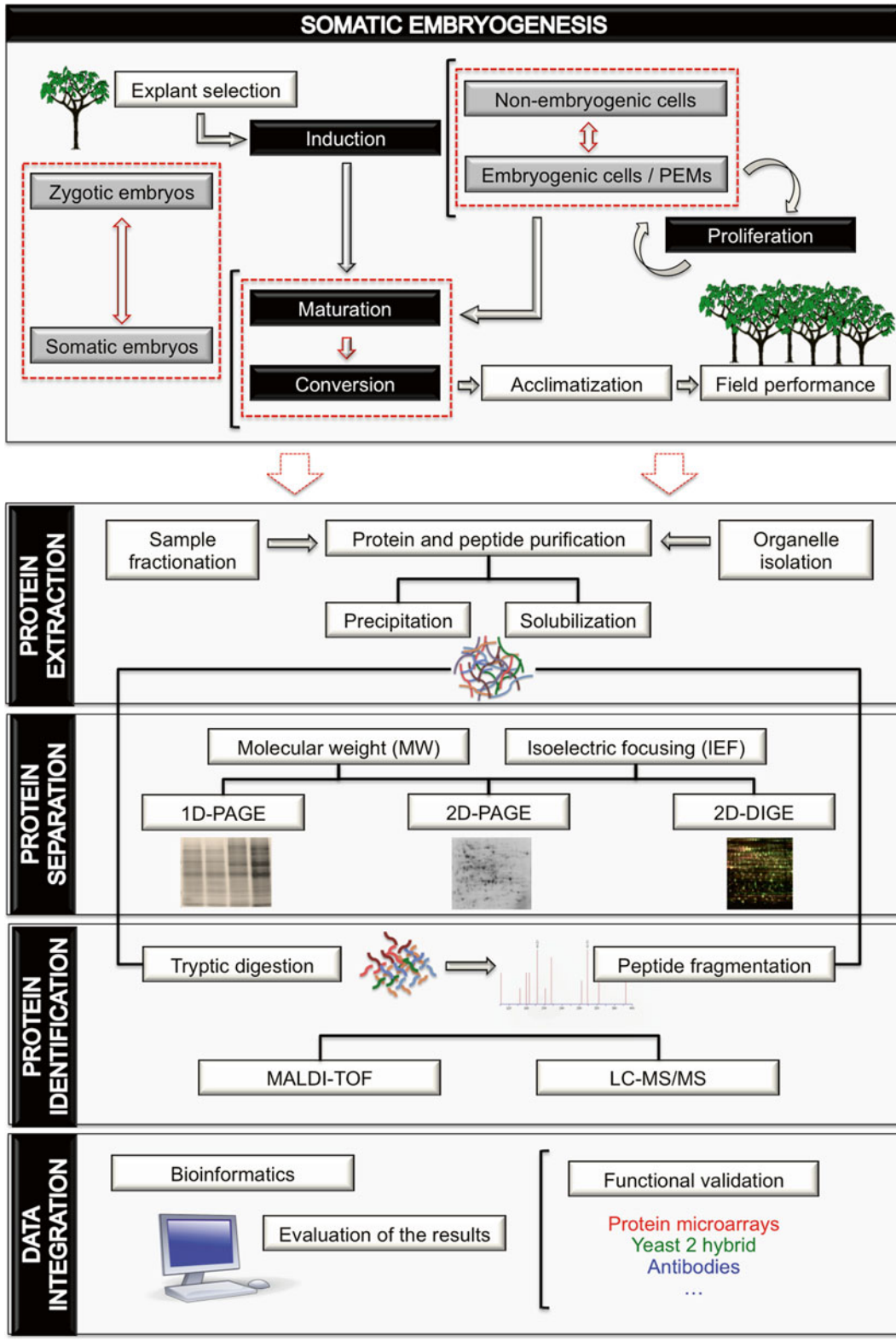

Fig. 1 Workflow for the proteomic analysis of somatic embryogenesis in woody plants

algorithm, it is above all determined by the quality and reproducibility of the gels. The standard approach for the identification of 2DE-separated proteins involves an enzymatic digestion of the protein in the spot of interest and extraction of the peptides followed by mass spectral (MS) analysis. The traditional way of analysis involves peptide mass fingerprint (PMF) analysis, typically performed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS, since it provides a simple profile by producing a single peak per peptide [19]. However, PMF data have very little resolution power to identify proteins from species with a fragmentary genome and protein repository (non-model organism). Hence, the chance of finding significant and conserved peptides decreases and PMF fails or results in false positive hits [23]. For that reason, tandem mass spectrometry (MS/MS) has been often used to generate sequence specific information and the information content of such spectra is thus much higher than for PMF. Unfortunately, separation of peptides prior to MS/MS is expensive and time consuming, and MALDI-TOF is often preferred because of easiness of using, speed and ability to include MALDI-TOF spotting in automated digestion protocols on liquid handling systems [19].

An emerging method gaining popularity combines one-dimension (1D) gel separations with reversed-phase (RP) liquid chromatography. Here proteins are first separated by size on standard polyacrylamide gels or by isoelectric point on IPG strips, normally used for the first dimensional separation in 2D-PAGE. After separation, the lane of the gel or the strip containing the proteins is extracted, divided into slices and treated similarly to spots excised from 2D gels. The peptides are then separated on an integrated and reusable RP column coupled to a standard HPLC pump. The RP eluent is then analyzed by MS/MS [24]. Although the platform based on 2-DE is still the most commonly used [25], the use of "gel-free" approaches offers several advantages, since 2-DE is difficult to automate. Most of the protocols use a bottom-up strategy where proteins are first digested with a proteolytic enzyme and the obtained complex peptide mixture is then separated via reversed-phase (RP) chromatography coupled to a tandem mass spectrometer [24]. The whole dataset of acquired tandem mass spectra is subsequently used to search protein databases and to link the individual peptides to the original proteins. However, this concept is only successful when identifying proteins in relatively simple mixtures. In general, such peptide centered bottom-up approaches have the disadvantage that both qualitative and quantitative information on protein isoforms and differential posttranslational modifications are lost [20].

To summarize, an optimized workflow for a non-model organism comprises (1) the investment in a powerful protein extraction method capable to minimize the effects of interfering compounds,

(2) the combination of different complementary protein fractionation, separation and quantification techniques to maximize the resolution and to cover the proteome as good as possible, and (3) the usage of different complementary MS techniques and error tolerant database searches [19].

3 Proteomics Approaches to Somatic Embryogenesis Analysis in Broad-Leaf Woody Plants

Proteomic studies have shown to be powerful tools for monitoring the physiological status of plant organs under specific developmental conditions [3]. SE is one form of non-zygotic embryogenesis by which somatic cells, under suitable induction conditions, undergo a complete genome shift and embark into a new developmental pathway ending in the formation of asexual embryos morphologically identical to their zygotic counterparts [6, 26, 27]. During this unique developmental process, cells have to dedifferentiate, activate cell division, and reprogram their physiology, metabolism and gene expression patterns [28]. Thus, SE can be considered the clearest demonstration of totipotency, showing that somatic cells contain the essential genetic blueprint to complete plant development, and that embryogenesis is not exclusive of the zygote formation and can proceed in absence of fertilization [26]. Since the first observations of somatic embryo formation in carrotcell suspension cultures [4, 5], the potential for SE has been shown to be characteristic of a wide range of tissue culture systems from both gymnosperms and angiosperms plants [28–30].

In recent years, there has been a growing interest in proteomic approaches to better understand SE. Since proteins directly influence cellular biochemistry and provide a more accurate analysis of cellular changes during growth and development [31], the identification of proteins associated with somatic embryo development may provide insights onto SE. Thus, several proteomic approaches were applied to study somatic embryogenesis of several broad-leaf woody plant species such as cork oak (*Quercus suber*) [32], Valencia sweet orange (*Citrus sinensis*) [33], grape wine (*Vitis vinifera*) [34], cacao tree (*Theobroma cacao*) [35], feijoa (*Acca sellowiana*) [36], and tamarillo (*Cyphomandra betacea*) [37]. These reports included studies on protein expression changes during SE and comparative studies between embryogenic and non-embryogenic cells as well as between zygotic and somatic embryogenesis.

3.1 Embryogenic Competence Acquisition

SE induction involves differentiated somatic cells acquiring embryogenic competence and proliferating as embryogenic cells [28]. This switching of somatic cells into embryogenic cells involves a series of events associated with the molecular recognition of internal signals and external stimuli [38, 39]. In recent

years, an increasing number of works have indicated that the stress-response of cultured tissues plays a major role in somatic embryo induction [39, 40], and that plants respond to abiotic stresses by altering the expression of many of their genes. This altered expression is a major mechanism of adaptation and survival during the stress periods [41]. Actually, proteomics helps the investigation of changes in proteome profiles emphasizing the participation of stress-related proteins in all developmental processes [3].

One important line of investigation to analyze embryogenic competence acquisition by woody plants is by the comparison of responsive and nonresponsive explants during the SE induction process [37, 42–44]. In the late 1990s, following the pioneering works of De Vries and collaborators in carrot [45], the detection of embryogenesis-related proteins from total protein extracts was reported for several woody species, such as *Betula pendula* [41], *Camelia japonica* [46], and *Cupressus sempervirens* [47]. In *Betula pendula*, the changes in protein patterns and the expression of "embryo-specific" proteins during embryogenesis were observed when comparing two cell lines, one potentially embryogenic, under the right inductive conditions, and one which never has shown any embryogenic capacity. In the following years, the improvements in high-resolution 2-DE and mass spectrometry contributed to the large-scale profiling and identification of the proteins associated to embryogenic competence acquisition. SE systems in which embryogenic (EC) and non-embryogenic (NEC) cell lines can be induced from the same cultured explant, like the ones of wine grape (*Vitis vinifera*) [34, 43] and tamarillo (*Cyphomandra betacea*) [37], have been explored to obtain more information on important regulatory proteins. Proteins, exclusively or predominantly expressed in EC, included iron-deficiency-responsive proteins, acidic ascorbate peroxidases and isoflavone reductase-like proteins [43] and metabolism-related proteins, such as enolases and threonine synthases, and also heat-shock proteins (HSP) and ribosomal proteins [37]. Ascorbate peroxidases, catalases, calcineurin B-like proteins, 1,3-*b*-glucanases, cyclin-dependent kinases A1 [43] and pathogenesis-related (PR) proteins were found mainly in NEC [37, 43]. The examination of differentially expressed proteins between ECs and NECs suggests that the embryogenic status of EC cells could be related to a better ability to regulate the effects of stress conditions, namely through the controlling of oxidative stress by regulation of the reactive oxygen species (ROS) scavenging system [34], and by the action of HSP [34, 37]. A hypothesis is that the expression of totipotency in cultured somatic cells is part of a general stress adaptative process that implies a fine regulation of auxin and stress signaling resulting in the restart of cell division and embryogenic competence acquisition. The observation that embryogenic tissues of different origins and obtained with the use of different auxins display similar protein profiles suggests a general behavior of cellular metabolism that

can give important insights about the mechanisms triggering and controlling somatic embryo formation [37]. Also for cork oak [32], the role of ROS in the proliferative stages during SE and the upregulation of proteins involved in cell division were reported. The comparison between somatic embryo cells type (SE-type) and pro-embryogenic masses type (PEM-type) of avocado (*Persea americana*) have confirmed the observations previously made in other systems [44]. In this work, the identification of high levels of HSP, glutathione *S*-transferases (GST), and superoxide dismutases (SOD) proteins in SE-type cells suggested that the generation of a significant amount of stress and ROS are prerequisites to induce somatic embryogenesis, and SE lines seems to be more efficient to cope with the necessary ROS and stress and, hence, have a higher regeneration capacity.

In order to develop into somatic embryos, somatic cells must regain their cell division activity. Hence, the division associated proteins, such as proliferating cell nuclear antigen in grape wine [34] and putative citrus DRT102 in Valencia sweet orange [33] are activated during embryogenesis. Besides, cytoskeletal proteins, such as tubulins associated to cell division, are also differentially regulated [33]. During the last decades, proteomic studies also described several extracellular proteins as markers for SE, which could offer the possibility of determining embryogenic potential of plant cells in culture [38–49]. Arabinogalactan proteins, nonspecific lipid transfer proteins and germin/germin-like proteins are important groups of extracellular proteins that help triggering embryogenic potential in plant cells [50]. More recently, results obtained with EC and NEC suspension cultures of coffee species (*Coffea* sp.) [51] showed that a particular set of proteins is exclusively secreted under embryogenic conditions.

3.2 Somatic Embryo Maturation and Conversion

In several plant regeneration processes through SE, one of the major problems is an effective transition from the proembryogenic masses, forming the embryogenic tissue, toward embryo development, which is often impaired by the formation of abnormal embryos and precocious germination of many others. This situation may be caused by an inadequate maturation of the embryos, an important phase of somatic and zygotic embryo development, following the classic morphogenic phases from globular to cotyledonary embryos [52]. During maturation, embryo cells undergo various physiological changes, which become evident by the deposition of storage materials, repression of germination and acquisition of desiccation tolerance [53, 54]. In cork oak, the activation of diverse ROS detoxification enzymes and the accumulation of reserve products (carbohydrates and proteins mostly) have been reported during the transition phase between proliferation and cotyledonar stages, suggesting the requirement that cell division should be replaced with cell expansion for proper embryo differentiation [32]. Energy requirements reach a maximum at the

cotyledonary stage, suggesting the relevance of primary metabolite production, such as amino acids and fatty acids, whereas fermentation could constitute an alternative source of energy at the early steps of somatic embryo development [32]. Also, for Valencia sweet orange [33] several proteins involved in antioxidative stress response (GST), cell division (tubulins), photosynthesis (ferritins), and cyanide detoxification (rhodanese) exhibited different expression patterns and were likely to be associated with SE. Another species often referred in studies aimed to detect and identify proteins expressed during the different developmental stages of somatic embryos is the myrtaceous feijoa (*Acca sellowiana*) [36, 55]. The results obtained with this SE system indicate a high similarity in the profiles of the assayed somatic embryos, suggesting that only a few specific genes are involved in the different developmental stages, and that gene expression occurs prior to morphological changes. The hypothetical protein similar to l-isoaspartyl-*O*-methyltransferase in torpedo stage, and an osmotin-like protein in the pre-cotyledonar stage of somatic embryos were suggested as embryonic markers for feijoa [55]. The expression of the protein phenylalanine ammonia lyase in all the assayed developmental stages confirmed the synthesis and accumulation of several phenolic compounds observed during the induction of feijoa embryogenic cultures and the development of somatic embryos. The presence of cytosolic glutamine synthetase and NmrA-like proteins revealed the activation of nitrogen metabolism, observed particularly in the later developmental stages in which the accumulation of storage compounds (mostly in the cotyledonary leaves) is enhanced [55]. More recently, the comparison between "off-type" and normal phenotype proteomes of somatic plantlets of feijoa has brought new insights to somatic embryo abnormal development [36]. The presence of HSP was observed only during the formation of normal phenotype somatic plantlets, indicating that these proteins may be involved in the morphogenesis of normally developed plantlets. A vicilin-like storage protein was only found in "off-types" at 20-day conversion, indicating that plantlets may present an abnormality in the mobilization of storage compounds, causing reduced vigor in the development of derived plantlets.

3.3 Somatic Versus Zygotic Embryos

The understanding of seed development is an important approach to overcome the difficulties in somatic embryoconversion and germination. Proteomic analyses have been made on zygotic embryos of several woody species, such as araucaria (*Araucaria angustifolia*) [56], coffee (*Coffeaarabica*) [57], and Masson's pine (*Pinus massoniana*) [58]. These analyses revealed significant requirements for energy production/carbon metabolism during the early stages of zygotic embryo development [35, 59]. Over accumulated proteins in early seed development also indicated a higher control on oxidative stress metabolism during this phase [56]. Besides, early zygotic embryos showed changes in the abundance of proteins involved in

mRNA splicing and signaling [57]. The advanced stages of zygotic embryogenesis were complemented by differential expression of Rubisco, Myb transcription factor, and by changed biosynthetic activity of phosphatidylcholine [57], but also by an active metabolism, leading to carbon assimilation and storage compounds accumulation [56]. Comparison of somatic and zygotic embryos revealed that their proteomes reflected mainly the different environmental conditions, which caused differential expression of proteins involved in metabolic pathways and stress response [59]. Noah and collaborators systematically compared at the proteome level the physiological mechanisms underlying somatic and zygotic embryogenesis in cacao [35]. Many of the identified proteins were involved in genetic information processing, carbohydrate metabolism and stress response. Somatic embryos especially displayed many stress related proteins, few enzymes involved in storage compound synthesis and an exceptional high abundance of endopeptidase inhibitors. Phosphoenolpyruvate carboxylase, which was accumulated more than threefold higher in zygotic embryos, represents a prominent enzyme in the storage compound metabolism in cacao seeds. More information on this topic are reported elsewhere in this book (*see* Chapter 2).

4 Conclusions and Future Perspectives

The results obtained with the proteomic studies over the last decades strongly emphasize the role of stress proteins in somatic embryogenesis, revealing an intricate dynamism, variability, and behavior of several regulatory proteins. Nevertheless, and unlike the classical biological model systems, the full potential of proteomics is far from being fully exploited in woody plant research. Only a low number of woody species has been investigated at the proteomics level and the predominant use of strategies based on 2DE coupled to MS results have so far resulted in a low proteome coverage. Although proteome analyses are still significantly less representative in the literature than those based on genomic approaches, the integration of the expressed protein data, together with transcriptome and even metabolome data, has the potential to provide the most comprehensive and informative clues on somatic embryogenesis in plants. Future research in this field should include new and/or complementary approaches, including more sensitive methods for protein detection and identification. The laser-capture microdissection tool is one of those technical improvements that could overcome the limitations in tissue accessibility, allowing more accurate molecular profiles of isolated embryogenic tissues. Also, "gel-free" approaches, with higher levels of automation and less inherent technical variability, should be applied in order to obtain a better reproducibility. These approaches are becoming more effective with the integration of new data from

several genome-sequencing projects. Furthermore, efforts should be taken in the functional validation of the specific identified proteins, in order to use them as markers for the SE process. The coordination of all this knowledge will give an insight into future studies addressing the optimization of the somatic embryogenesis protocols for mass propagation and conservation strategies in several economical relevant woody species.

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Chapter 7

Advances in Conifer Somatic Embryogenesis Since Year 2000

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Abstract

This review compiles research results published over the last 14 years on conifer somatic embryogenesis (SE). Emphasis is placed on the newest findings that affect the response of seed embryos (typical explants) and shoot primordia (rare explants) to the induction of SE and long-term culture of early somatic embryos. Much research in recent years has focused on maturation of somatic embryos, with respect to both yield and quality, as an important stage for the production of a large number of vigorous somatic seedlings. Attempts to scale up somatic embryo production numbers and handling have resulted in a few bioreactor designs, the utility of which may prove beneficial for an industrial application. A few simplified cryopreservation methods for embryonal masses (EM) were developed as a means to ensure cost-efficient long-term storage of genotypes during clonal field testing. Finally, recent long-term studies on the growth of somatic trees in the field, including seed production yield and comparison of seed parameters produced by somatic versus seed-derived trees, are described.

Key words Cryopreservation, Field tests, Somatic embryos, Somatic trees, Tree improvement

1 Discovery of Somatic Embryogenesis in Conifers and the General Pattern of Somatic Embryo Development

Since its discovery in *Picea abies* and *Larix decidua* [1, 2], somatic embryogenesis (SE) in conifers has been reported in many other species, with a large majority of them belonging to Pinaceae, and only a few to Cupressaceae, Taxaceae, Cephalotaxaceae, and Araucariaceae families. The general differentiation and developmental pattern of conifer somatic embryos is highly similar among most species tested to date and starts with an immature seed embryo (enclosed in a megagametophyte or excised) or with an excised mature embryo that is cultured on a nutrient medium containing plant growth regulators (PGRs) from either both the auxin and cytokinin groups or from cytokinin only. Subsequently, the

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cells of the embryo deviate from their previous pattern of division and differentiation into a mature embryo; instead, they start dividing profusely and differentiate into multiple early somatic embryos also known as embryonal masses (EM). For example, 50 mg fresh mass of white spruce proliferating cell culture may contain, at any given time, approximately 300 single early somatic embryos, 39–75 cleaving early somatic embryos, 60–90 multiple cleaving somatic embryos, small and large cell aggregates, and single small and large cells, the latter with large vacuole(s) (Klimaszewska, unpublished). Typically, the composition of the culture is highly heterogeneous and may change over culture time, a period that may last from several months to several years, during which EM has to be subcultured every 10–21 days (depending on the species) onto fresh medium of the same or slightly modified composition. EM of some species can be cultured on a semisolid medium or in liquid medium. The cultures are amenable to long-term storage through cryopreservation without losing their viability and growth characteristics. In most species, early somatic embryos will not develop further unless the culture conditions are changed. High frequency development and maturation of early somatic embryos in most conifer species of the Pinaceae family take place under remarkably similar conditions, namely in the presence of abscisic acid (ABA), sucrose and/or other sugars, and in a medium that imposes restriction on water availability either by physical means (high concentration of solidifying agents) or by a high molecular weight solute such as polyethylene glycol (PEG, MW 4000–8000) that mimics drought. Often the sugar concentration is also increased to lower the osmotic water potential of the medium. Once the somatic embryos resemble mature seed embryos (usually after 6–12 weeks, depending on the species), they are harvested and desiccated if matured on a medium with PEG for normal germination, or they can be germinated directly if developed on a medium with high gel strength. On a germination medium, the somatic embryos display a rapid radicle and hypocotyl elongation (usually within 1 or 2 days) followed by the growth of a shoot and a root. Once the plantlets reach the desired size, they are transferred into containers with a suitable substrate and acclimatized in a greenhouse or a nursery. Subsequently, they are planted in the field for research purposes, clonal selection or, eventually, for commercial production.

Several reviews describing various aspects of SE research in conifers have been published in both international journals and in books [3–9]. The main focus of the present review is to summarize the progress in SE research in conifers made over the last 14 years and to determine its impact on understanding the basic mechanisms governing the process and on the development of new, more efficient protocols for the production of somatic trees. We also include research results on conifer species for which somatic embryogenesis has been achieved only recently and the results obtained with adult trees.

2 Genetic Control in Somatic Embryogenesis

Perhaps the most basic factor that determines whether SE is initiated from the seed embryos is the genetics of parental trees, providing that suitable culture medium and conditions have been established. Experiments designed to establish the extent of genetic control in SE initiation have been conducted with a few pine species. A large study was undertaken with *Pinus sylvestris* using 49 seed families from diallel crosses among seven elite trees including reciprocals and selfing [10]. Four of the experimental trees were preselected for their propensity for SE based on an earlier study that tested 138 trees. Analysis of the data suggested a stronger maternal than paternal effect on culture initiation; however, specific combining ability (SCA) had no detectable effect. The maternal effect at the initiation stage could be explained by both the genotype and the developmental or physiological stage of the mother tree and the inherited maternal alleles of the zygotic embryo. Similarly, MacKay et al. [11] quantified the genetic control of SE initiation in *P. taeda* using seeds from diallel crosses and factorial matings. Thirty seed families were used in the experiments that tested two different culture treatments and resulted in large differences between treatments in SE initiation frequency among families. The variance due to treatments accounted for 41 % of total phenotypic variance, whereas that due to families accounted for 22 %. Significant variance due to interactions between families and treatments was also found, accounting for 13 % of the phenotypic variance. The latter indicated that different culture media might be better suited for different genotypes. In another study with 20 control-pollinated seed families of *P. radiata*, Hargreaves et al. [12], also challenged the notion that poor results should be attributed to genetic effects only and showed that it was possible to create laboratory conditions that increased the number of responding explants across all families. However, previous work with *P. taeda* showed that many mother trees produce seeds that do not respond to SE initiation, leading to the hypothesis that such trees possess unfavorable alleles at loci expressed in the mother tree, whereas favorable alleles at other loci may be inherited by zygotic embryos [11]. The estimates of large general combining ability (GCA) variance component and narrow sense heritability suggested that targeted breeding could influence SE initiation in *P. taeda*. To test this hypothesis, an experiment was carried out that involved a small number of control reciprocal crosses among trees that ranged from low to high SE initiation capacity when tested with seed from open-pollinated mother trees. By selecting a favorable mother tree for cross-pollination for each pair of parental trees, it was possible to increase SE initiation frequency from 1.5- to 9-fold. Also, some trees had strong additive effects as male parents, but had negative maternal effects; hence, using them in control

crosses as pollen donors might be yet another solution to increase SE initiation. The authors concluded that this knowledge of genetic control in SE initiation can now be easily applied to breeding schemes to capture valued genotypes. Smaller studies with seeds from control crosses of *P. pinaster* [13] and *P. sylvestris* [14] supported the above conclusions.

3 Improvements of Previously Established Protocols for Somatic Embryogenesis

SE biotechnology of conifers has constantly evolved since its discovery in 1985, and incremental improvements are being made according to the time and effort committed to a given species. The literature search revealed that since the year 2000, about 46 journal articles reporting improvements of SE protocols were published for *Pinus taeda*, *Pinus strobus*, *Pinus sylvestris*, *Pinus pinaster*, *Pinus radiata*, *Pinus patula*, *Pseudotsuga menziesii*, *Abies nordmanniana*, *Picea abies*, *Picea glauca*, *Picea mariana*, and *Larix* hybrids. In addition, the publication of approximately 40 articles on species for which SE is described for the first time is a clear indicator of the importance of this technology for conifer clonal propagation. For species of economic importance that are grown in forest plantations, a lot of research has been carried out by companies and patented, for example for *Pinus taeda* (Arbogen, Weyerhauser, WA, USA), *P. radiata* (Forest Genetics Ltd and Arborgen, NZ), *P. abies*, *P. pinaster*, and *P. radiata* (FCBA, France), *Pseudotsuga menziesii* (Weyerhauser, WA, USA), *Picea glauca* and *P. abies* (Natural Resources Quebec, QC, Canada and JD Irving Inc., NB, Canada). Among these economically valued plantation species, the largest body of literature exists for *Pinus taeda* (loblolly pine), which reports on the stepwise optimization approach to overcome low efficiencies at each stage of SE. In their recent review, Pullmann and Bucalo [9] have attributed these improvements to medium supplements including specific sugars, vitamins, organic acids, and redox potential modifiers. Other controlled factors, including medium water potential, pH, adsorption of medium components by activated carbon and use of liquid versus semisolid medium, also positively influenced SE. These modifications resulted from the analytical studies of *P. taeda* seed tissue, the seed environment, and gene expression in megagametophytes, zygotic embryos, and somatic embryos. The premise of the study was that duplication of the seed environment in vitro would lead to the design of efficient protocols for SE.

3.1 Initiation of SE and Growth of Early Somatic Embryos

Major improvements were made in the frequency of SE initiation in either open-pollinated or control-pollinated seed sources of several European and North American *Pinus* species, and *Pseudotsuga menziesii*. In *P. strobus*, the number of responding immature seed

embryos increased from the average of 20 % to the average of 53 % across five open-pollinated seed families by reducing the 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA) concentrations from 9.5 to 2.2 μM and from 4.5 to 2.2 μM, respectively. Both concentrations were tested in modified [15, 16] Litvay's medium (MLV). The most striking difference in initiations occurred when the embryos were at the pre-cleavage and early post-cleavage stages, which were also linked to the morphological appearance of the megagametophytes becoming opaque as opposed to being translucent [15]. The same medium modifications were tested with a few control-pollinated seed families of *P. sylvestris* and the result was better on a medium with reduced PGRs, i.e., 24 % initiation versus 9 % [14]. However, contrary to the response of *P. strobus* and *P. sylvestris*, when eight control-pollinated seed families of *P. pinaster* were tested, MLV with reduced concentrations of PGRs decreased the initiation frequency from 93 to 80 %; nevertheless both culture medium variants were very productive [13]. The high response of the latter was also attributed to the selection of embryos that were at the uniform pre-cotyledonary stage of development by excising the embryos from the surrounding megagametophytes. When the embryos were cultured within megagametophytes, the SE response was only slightly reduced, suggesting that in the tested cones the development of embryos was relatively synchronized. This was in contrast to *P. radiata* for which the zygotic embryos had to be excised for the best response [17].

Another medium that is considered suitable for SE initiation in *P. pinaster* and *P. sylvestris* is Gupta and Durzan's medium (DCR [18]). Like other commonly used media [7, 19, 20], it also includes glutamine and casein hydrolysate as well as 2,4-D and BA. Recent research aimed at improving SE initiation in *P. radiata* showed that by making another modification to Litvay's medium (designated as GLITZ) it was possible to achieve considerably higher responses from both 19 open-pollinated and 20 control-pollinated seed families compared with those obtained on Embryo Development Medium 6 (EDM6) [12, 17, 21]. Average initiations were 70 % for both types of seed families when embryos were excised from the megagametophytes at an early stage of development. GLITZ medium contained glutamine (0.5 g/L), casein hydrolysate (1.0 g/L), 2,4-D (4.5 μM), and BA (2.25 μM). Likewise, a 2-year study on SE initiation in *P. nigra* demonstrated higher potential for two out of four tested medium formulations [22]. DCR and MLV media consistently supported approximately 10 % explants producing EM as opposed to Litvay medium (LV [16]) and Quoirin and Lepoivre (QP [23]) media, on which the response was negligible.

Among pine species, SE in *P. taeda* has been the most researched owing to its high commercial value in the USA and

elsewhere, but relatively low responses obtained in earlier work. Not surprisingly, a considerable effort was undertaken to improve the efficiencies of SE and understand the bottlenecks at each stage (*see* ref. 9 and references therein). Various supplements were tested in a unique loblolly pine (LP) medium formulation and found beneficial for SE initiation, such as $AgNO_3$,maltose instead of sucrose, high level of myo-inositol (up to 20 g/L), glutamine, casamino acids, 2-(*N*-morpholino) ethanesulfonic acid (MES, as pH stabilizer), biotin, folic acid, vitamins B_{12} and E, α-ketoglutaric acid, kinetin together with BA, activated charcoal, abscisic acid (ABA), brassinolide as well as D-xylose and D-*chiro*-inositol [24]. The number of initiated SE cultures increased further by adding liquid overlays 14 days after placement of explants on gelled medium. This technique allows replenishment or addition of nutrients and PGRs, or adjustment of pH without disturbing the tissue.

All the medium supplements that were beneficial to *P. taeda* were also tested with immature seed embryos of *P. menziesii*, which differ from those of pine species by the lack of cleavage polyembryony and the need for an embryo to be cultured while partially excised and still attached to the megagametophyte by a suspensor [25]. These tests resulted in the development of an effective medium formulation for initiation of SE in *P. menziesii* that included activated charcoal, ABA, biotin, brassinolide, folic acid, MES, pyruvic acid, D-xylose, and D-*chiro*-inositol in addition to 1 g/L myoinositol, 0.5 g/L casamino acids, 0.45 g/L glutamine and 2,4-D, BA, and kinetin [24, 25]. Tests with seeds from high-value crosses conducted over 2 years gave initiation frequencies of 40 and 57 %, respectively. Based on the above results, a new medium was designed for the culture of mature embryos of *P. abies* that resulted in doubling SE initiation from around 14–30 % when the medium contained 100 mg/L D-xylose. Some other medium additives were asparagine and brassinolide, and the PGRs were α-naphthaleneacetic acid (NAA) and BA [24].

Research on initiation of SE in somatic embryos of *Larix* x *leptoeuropaea* showed that 98 % of cotyledonary somatic embryos matured for 3 weeks produced SE; those matured for 6 weeks produced SE at a frequency of only 2 % [26]. The authors suggested that the loss of ability of somatic embryos to respond to the induction treatment might be caused by the synthesis/accumulation of ethylene, because enrichment of the vessel headspace with ethylene reduced the induction of SE from 3-week-old somatic embryos from 98 to 4 %. Ethylene was also found to influence the development of early somatic embryos as described below.

3.2 Growth of Initiated EM

Once SE is initiated and EM can be identified (after several days to several weeks), the next challenge is to ensure a rapid proliferation of EM upon subculture onto fresh medium to generate the amounts that are needed for various steps, such as cryopreservation

and/or production of mature somatic embryos. The majority of conifer species are usually subcultured onto media of the same composition, but in a few cases, such as *P. pinaster*, it has been shown that medium modifications were required to obtain better growth and/or to maintain the embryogenic potential of the cultures [27, 28]. These modifications included weekly subcultures, substitution of sucrose with maltose and withdrawal of PGRs (2,4-D and BA). To maintain satisfactory growth of *P. radiata* EM, it was necessary to increase the amino acid content in LV medium after initiation [12]. In some species and genotypes, the application of a culture technique that is based on dispersing the cells in a liquid medium, and then collecting the cells on a filter paper, draining the liquid, and placing the filter paper with the cells onto a fresh medium has been the most important for the survival and growth of *P. monticola* and *P. sylvestris* [14, 29].

Embryogenic cultures of *Cryptomeria japonica* (Cupressaceae) were composed of a mixture of EM and callus cells, and when an attempt was made to culture the EM separately; their embryogenic capacity was lost [30]. The culture medium was that of Campbell and Durzan [31], containing 1 μM 2,4-D and 0.6 g/L glutamine; however, when the medium was supplied with 2.46 g/L glutamine, the culture remained embryogenic and simultaneously its dry mass and endogenous level of glutamine increased. The high glutamine treatment might have increased the synthesis of certain macromolecules or metabolites that were essential for SE. The ability of EM to grow in the presence of callus cells was attributed to the high content of endogenous glutamine in the latter that might have supported the growth of EM in mixed culture. Based on the research results of others, the authors concluded that without an adequate supply of glutamine/glutamate, the embryogenic culture of *C. japonica* would lose its embryogenic characteristics. Phytosulfokine, which is a small sulfated peptide, was also found beneficial for *C. japonica* culture growth when included in the medium at 32 nM [32]. This peptide acts as an extracellular ligand at the onset of cell dedifferentiation, proliferation, and redifferentiation and plays a stimulatory role in SE. In particular, phytosulfokine promoted suspensor regeneration from basal cells of somatic embryos of *Larix leptolepis* [33]. The presence of suspensor in somatic embryo development and in the maintenance of the culture embryogenic characteristics was established as critical by Umehara et al. [34] and later by Larsson et al. [35] and Abrahamsson et al. [36] for *Picea abies* and *Pinus sylvestris*, respectively.

3.3 Development and Maturation of Early Somatic Embryos

To promote further development and maturation of early somatic embryos in a majority of conifer species, the proliferating cultures of early somatic embryos are transferred (after a pretreatment or without it) onto a medium with ABA that replaces auxin and/or cytokinin. Most often, the medium water potential is lowered at the

same time by increasing the concentration of sugar(s) and creating permeating osmotic stress, or by including PEG (MW 4000–8000), thus creating a non-permeating osmotic stress, the latter is due to the larger than cellular pores molecule size [37]. An alternative method of affecting somatic embryo development is to increase the gelling agent concentration in the ABA medium, which increases gel strength and reduces water availability to the cells [38].

3.3.1 Abscisic Acid

A study that unequivocally confirmed that ABA was crucial for the normal somatic embryo development and maturation in conifers was carried out with *Larix* x *leptoeuropaea* [39]. This larch hybrid is somewhat unique because it can produce cotyledonary somatic embryos and plantlets on a medium with ABA or without it, hence providing an ideal material for this study. However, the somatic embryos that developed on both media differed in structure, cell types, intracellular secondary metabolites and storage product accumulation, endogenous ABA concentrations, and extracellular mucilage build-up. Clearly, those from ABA medium displayed a coordinated growth and better-shaped somatic embryos with the concomitant accumulation of lipids and storage proteins that were lacking in embryos developed in the absence of ABA. Hence, somatic embryos developed without ABA did not go through maturation. Still, in all conifer species studied to date, a certain number of genotypes in a given species fails to produce mature somatic embryos even in the presence of optimized concentrations of ABA. It has been shown that the ability of embryogenic tissue to utilize ABA from the medium may reflect the capability of embryo maturation in different genotypes of *Picea glauca* x *engelmanni* [40]. The genotypes that produced mature somatic embryos on gelled medium with racemic ABA (equal amounts of (+)-*cis*, *trans-ABA* and (−)-*cis*, *trans*-ABA) were characterized by a greater utilization of exogenous ABA, when grown as cell suspensions, compared with a non-productive genotype. Furthermore, different forms of ABA were metabolized to various levels. For example, only half of racemic ABA was metabolized by the 22nd day of culture; the remainder was exclusively (−)-ABA. The natural ABA ((+)-cis, trans-ABA) was still available at the end of the test, but its amount may be influenced by species, cell density of the initial inoculation, tissue growth rates, and initial ABA concentrations. The natural ABA exerted by far the best bio-effect compared with racemic ABA and the mixture of ABA isomers.

3.3.2 Activated Charcoal

Improvement of somatic embryoquality and yield was achieved by combining ABA with activated charcoal (AC). In *P. abies*, AC introduced into a medium at 0.125 % with 189 μM ABA promoted a zygotic-like appearance of somatic embryos with more elongation and taper in the hypocotyl region as well as formation of a prominent shoot apical region compared with those developed

without AC [41]. These embryos grew faster and were produced at a reduced material and labor cost because the cultures did not require subculturing onto fresh medium. However, the authors cautioned against the types of AC to be utilized as these vary with respect to particle sizes and hence the adsorption properties causing potential deficiencies in the medium components. Alternatively, AC was used with *P. pinaster* by coating the cells with AC and culture on a filter paper placed on the maturation medium [13]. This method of culture resulted in a greater number of mature somatic embryos produced in a shorter time compared with cultures without AC coating. Similarly, *P. sylvestris* aged cultures (24 weeks old) responded favorably when coated with AC, whereas there was no effect on young cultures (8 weeks old) [14].

3.3.3 Carbohydrates, PEG (Medium Water Potential), and Gel Strength (Water Availability)

Changes in water status that occur during conifer zygotic embryo development and maturation are also critical for the progression of the development of early somatic embryos, but the type of compounds used to alter the medium water status must be the "right" type for a given species. For example, when mannitol (a plasmolyzing agent) was tested against PEG (a non-plasmolyzing agent) in cultures of *P. glauca*, the better quality of somatic embryos from the latter was accompanied by the accumulation of higher levels of reduced ascorbate, resulting in a physiological state similar to that of zygotic embryos [42]. Moreover, in the presence of PEG, there was a constant decline in the GSH (reduced)/GSSG (oxidized) ratio of glutathione, suggesting seed-like fluctuations of the ascorbate-glutathione metabolism in somatic embryos. In another study with somatic embryos of *P. glauca* and *P. mariana* it was found that sucrose at 6 % (in the absence of PEG) was highly beneficial when added to the maturation medium for both the number of matured somatic embryos and the accumulation of soluble and insoluble storage proteins [43]. The maturation response could not be matched by osmotic equivalents of glucose and fructose (products of sucrose hydrolysis) in the medium. Moreover, the embryo carbohydrate content was independent from the carbohydrate used in the maturation medium. The same conclusion was later reached for somatic embryos of *P. abies*, where endogenous carbohydrate patterns were stable irrespective of culture conditions, which indicated the carbohydrate status to be a robust feature of normal somatic embryo development [44]. Experiments aimed at the separation of the sucrose osmotic influence from its role as carbon and energy source suggested that sucrose might have an additional regulatory role in the maturation process. In *P. abies*, a medium with 7.5 % PEG and 3 % maltose promoted the development of a large number of somatic embryos, but with low germination frequency in spite of the post-maturation partial desiccation [45]. Conversely, somatic embryos developed on a medium with 3 % sucrose (without additional osmotic agent),

although low in numbers, were able to germinate. A combination of sugar assays, metabolic and proteomic analyses revealed that somatic embryos grown on sucrose medium contained high levels of sucrose, raffinose, and late embryogenesis abundant proteins, all involved in the acquisition of desiccation tolerance (reviewed by Trontin et al., Chapter 8). These embryos also accumulated starch whereas those from PEG and maltose medium had high levels of storage proteins. Therefore the poor germination of *P. abies* somatic embryos grown on PEG and maltose medium was most likely caused by the reduced desiccation tolerance.

Manipulation of water availability to the cells of EM was also achieved by physical means, without affecting water potential of the medium, by increasing the amount of gelling agent that increased medium gel strength and consequently reduced the amount of water available to the cells [16, 46]. By applying this method of water control to *P. strobus* early embryo cultures, high quality mature somatic embryos were produced on medium with 1 % gellan gum that were characterized by a lower water content compared with those from 0.4 % gellan gum medium. Combination of 0.8 or 1 % gellan gum with 6 % sucrose (instead of 3 %) in the maturation medium was even more beneficial because the somatic embryos accumulated higher quantities of storage proteins [47]. An intuitive interpretation of these results is that the developing embryos must have been exposed to the water stress (drought type of conditions) on the media with high gelling agent concentrations, a condition similar to that of a developing zygotic embryo, when the maturation of the embryo is accompanied by desiccation (loss of water). However, in a later study involving cultures of *Larix* x *eurolepis*, an opposite conclusion was reached [48]. The more numerous and higher quality (lower water content) somatic embryos that developed on medium with 0.8 % gellan gum were in fact less stressed than those developed on 0.4 % gellan gum. This conclusion was based on the measurements of physiological parameters and on the two-dimensional (2-D) protein gels that identified 62 proteins that differed between the two somatic embryo groups from the two treatments. Fifty six proteins were subsequently identified, and among them 6-phosphogluconate dehydrogenase (decarboxylating), actin, enolase, fructose phosphate aldolase, phosphoglucomutase, and superoxide dismutase, which are known to be associated with water stress, were expressed at a higher level in somatic embryos developing on medium with 0.4 % gellan gum. In addition to the increased abundance of heat shock proteins in somatic embryos cultured on the 0.4 % gellan gum medium, the observed increases in expression of pyruvate decarboxylase (which directs carbon metabolism toward glycolysis) and apparent detoxification capacity (indicated by the increased expression of superoxide dismutase) suggested that maturation medium containing 0.4 % gellan gum induced a water stress response in the developing somatic embryos. Contrary to this, somatic embryos developed on

0.8 % gellan gum medium accumulated stress proteins at a much lower level. Further evidence supporting utilization of high gellan gum medium for the maturation of somatic embryos came from a large study of *P. pinaster* where multi-scale integrated analysis was used to follow early molecular and physiological events involved in somatic embryo development [49]. Similarly to *P. strobus*, early somatic embryos of *P. pinaster* do not develop on medium with 0.4 % gellan gum; instead, abundant proliferation of EM occurs, which is not conducive to subsequent embryo development. According to the transcriptomic and proteomic analysis results, these cultures had enhanced glycolysis whereas those from medium with 0.9 % gellan gum had adaptive, ABA-mediated molecular and physiological responses marked by active protein synthesis and overexpression of proteins involved in cell division, embryogenesis and starch synthesis. Concomitantly, synthesis of protective secondary metabolites and regulation of oxidative stress were activated, most likely to adapt to the culture conditions. Furthermore, two genotypes of cotyledonary *P. pinaster* somatic embryos, after 10–14 weeks of culture on maturation medium, were compared with zygotic embryos excised from developing fresh and desiccated seeds with respect to dry mass, water content, sucrose and raffinose contents, raffinose/sucrose ratio, and total proteins ([50]; reviewed by Trontin et al., Chapter 8). The study demonstrated that somatic embryos were the most similar to zygotic embryos in seeds collected from late July to early August, and with respect to total protein content up to October (Northern Hemisphere). The somatic embryos, which typically are harvested after 12 weeks, are not at the same maturity level as their zygotic counterparts at the mature, desiccated stage.

3.3.4 Ethylene

There is evidence suggesting that ethylene, a gaseous plant growth hormone produced by cultured plant cells and tissues, may also affect the development of somatic embryos in *P. glauca* [51]. Due to the volatile nature of ethylene, it is difficult to control it in culture. However, the reduction in endogenous ethylene synthesis was achieved by incorporating α-aminooxyamino acid (AOA), a potent inhibitor of ethylene biosynthesis, into the medium, which proved to be beneficial to somatic embryo development. The mechanism of this stimulation was not elucidated, only suggesting that AOA may have interfered with the metabolism of other compounds, most likely through the availability of S-adenosylmethionine, a common precursor for both ethylene and polyamine biosynthesis. In *P. mariana*, it has been shown that limiting ethylene biosynthesis or its physiological action was beneficial to somatic embryo development in a poor line, but not beneficial in a line that was a good embryo producer [52]. These opposite reactions could stem from different initial ethylene levels in the two cultures, one having a super-optimal and the other an optimal level. Later study with cultures of *P. sylvestris* confirmed that ethylene production

varied among five embryogenic lines, both when cultured on proliferation medium with 2,4-D and on maturation medium, indicating a lack of definite trend [53]. Future experiments should include many more genotypes to realize the full impact of ethylene on SE in *P. sylvestris*. Also, any generalization to other conifer species should be avoided.

3.3.5 Antiauxin and Auxin Transport Inhibitor

Among *Abies* species, *A. nordmanniana* somatic embryos were stimulated to develop on maturation medium (with ABA) after a 4- to 8-week treatment with PCIB, 2-(*p*-chlorophenoxy)2-methylpropionic acid, an auxin antagonist that is believed to reduce the activity of endogenous indole-3-acetic acid (IAA) by competitive binding to auxin receptors [54]. *Abies* species do not require an auxin for initiation or proliferation of early somatic embryos and the subsequent problems pertaining to somatic embryo development have been attributed to the high activity of endogenous auxin, at least in *A. nordmanniana*. Treatment of *P. sylvestris* early somatic embryos with the auxin transport inhibitor 1-*N*-naphtylphthalamic acid (NPA) caused the embryos to form supernumerary suspensor cells at high frequency, which led to abnormal development [36]. Although treatment with PCIB increased the yield of somatic embryos, their morphology was not affected, suggesting that the supernumerary suspensor cells in early somatic embryos were stimulated by disturbed polar auxin transport.

3.3.6 Redox Compounds

P. glauca somatic embryo development and maturation were greatly improved through the manipulation of glutathione redox status in EM cultures. By employing a two-step protocol that first included reduced glutathione (GSH) and then its oxidized form (GSSG) in culture medium, which caused a shift in the total glutathione pool towards its oxidized state, proper somatic embryo development was achieved [42]. However, due to the high cost and labor associated with this protocol, a simpler alternative was developed involving dl-buthionine-[*S* , *R*]-sulfoximine (BSO) [55]. BSO is effective in reducing endogenous GSH levels through the inhibition of its de novo synthesis without affecting glutathione reductase, the GSH-recycling enzyme. These changes are similar to those observed when GSH and GSSG are applied sequentially to impose an oxidized environment. To maximize somatic embryo development, BSO concentration had to be at 0.01 mM, while higher concentrations were inhibitory. Therefore, it appears that certain threshold of cellular GSH must be maintained for embryo development to continue. In *Araucaria angustifolia* cultures of EM, manipulation of the GSH/GSSG ratio of the culture medium proved to be beneficial to somatic embryo development up to the pre-cotyledonary stage, but to achieve a complete development would require further modification of the redox potential of the cultures [56].

3.3.7 Inhibitors of SE

When *Larix laricina* early somatic embryos were cultured in high density suspension in a liquid medium the differentiation of suspensors was inhibited, thus negatively impacting the development of new somatic embryos [57]. It was confirmed that an inhibitory compound was present in the conditioned culture medium, which was subsequently purified and the compound was identified as vanillyl benzyl ether (VBE) [34]. Tests with synthetic VBE in the medium produced similar results. Interestingly, the low density suspension cultures also contained VBE, but at much lower concentrations, which did not prevent differentiation of the suspensors. This finding emphasizes the importance of the presence of suspensors in somatic embryo development of a conifer and increases awareness of the influence of cell density on the embryogenic characteristics of a culture. Another modifier of normal somatic embryo development is an inhibitor of polar auxin transport, 1-*N*-naphtylphthalamic acid (NPA), which was tested in *P. abies* [35]. Polar auxin transport is essential to proper embryo patterning and establishment of root/shoot polarity. During early somatic embryo development, treatment with NPA caused an increase in IAA content, abnormal cell division, and decreased programmed cell death resulting in the aberrant development of embryonal tube cells and suspensors. These embryos had abnormal morphology marked by malformed and fused cotyledons and irregular cell divisions at the site of root meristem.

4 Extending Somatic Embryogenesis Protocols to Numerous Conifer Species

Previously published protocols have been utilized, often with slight modifications and with various degrees of success, to test/achieve SE in numerous other conifer species.

4.1 Pinaceae

Among *Picea* species, results that showed regenerated somatic seedlings were published for *P. morrisonicola* [58], *P. koraiensis* [59], and *P. likiangensis* [60]; however, it is not clear whether any of the somatic trees were established in the field.

In the *Pinus* genus, at least 16 new species were reported to display SE; however, for many of them, initiation and/or maturation efficiencies and plant regeneration were low and needed further research and improvements. Notoriously low initiation and survival of EM has been reported for *P. contorta* [61], *P. monticola* [29], *P. roxburghii* [62, 63], *P. pinaea* [64], *P. banksiana* [65], *P. densiflora* [66, 67], *P. rigida x taeda* [68], *P. kesiya* [69], *P. thunbergii* [70], *P. armandii* [71], and *P. luchuensis* [72]. On the other hand, species such as *P. patula* [73], *P. nigra* [22], *P. bungeana* [74], *P. brutia* [75], *P. oocarpa* [76], and *P. halepensis* [77] responded at frequencies ranging from 9 to 30 %.

Significant progress in SE response and plant regeneration has been achieved for *Larix leptolepis* [78, 79], *L.* x *eurolepis* and *L.* x *marschlinsii* [80]. The reciprocal hybrids between *L. decidua* and *L. leptolepis* are important species in Europe, and the first hybrid variety ('REVE-VERT') was registered in France in 2005. SE modified protocols were subsequently tested as a means for rapid cloning of limited numbers of hybrid seeds and resulted in up to 48 % of initial zygotic embryos producing high numbers of vigorous somatic plants (Fig. 1). It is anticipated that SE will influence breeding strategies for these hybrids by offering an additional tool for the production of large quantities of plants for clonal field tests.

SE in *Abies* species has been very challenging, but recent progress made with some species is encouraging. A study with *A. alba* by Krajňáková et al. [81] tested several variables for maturation of somatic embryos, which improved the maturation yield by utilizing a method of spreading the cultures in a layer on Whatman #2 filter paper placed on the surface of a semisolid medium [82] supplemented with 32 μM ABA, maltose, organic N additives, and devoid of PEG. The medium formulation was Murashige and Skoog [83] modified by 50 % reduction in inorganic salt strength. The somatic embryos required desiccation for proper germination

Fig. 1 *Larix* x *eurolepis* "Reve-Vert" somatic seedlings (INRA, France, improved variety) acclimatized at the XYLOBIOTECH nursery (XYLOFOREST platform, www.xyloforest.org) located at the FCBA, Pierroton, France (0.8×)

but still the conversion to plants remained inefficient. In *A. lasiocarpa*, a novel medium was designed based on the elemental analysis of megagametophytes and designated AL, which was free of inorganic nitrogen and in which L-glutamine was supplied at 2 g/L as the sole nitrogen source [84]. AL medium was compared with Schenk and Hildebrand [85], and although initiation of SE (up to 37 %) did not differ significantly between the two media, EM growth after subculture and its survival was better on AL. However, the conversion to plants was very low (approximately 8 % of germinated somatic embryos), which seems to be a norm in this genus. Similarly, in *A. cilicica* and *A. cilicica* x *A. nordmaniana*, relatively high initiations of EM were obtained (63 and 28 %, respectively) but only a third of EM lines developed cotyledonary stage somatic embryos on both maltose or lactose media [86]. In *A. numidica*, both the maturation and germination of somatic embryos were studied. PEG and 6 % maltose in maturation medium were very effective followed by partial desiccation and germination of somatic embryos on medium with activated charcoal and indolbutyric acid (IBA) [87, 88]. In *A. cephalonica*, up to 25 % of the seeds produced EM lines, and somatic embryo development was achieved on medium with PEG and sucrose followed by medium without PEG for up to 12 weeks [89, 90]. However, germination was poor, most likely due to the omission of partial desiccation of somatic embryos before germination, which appears to be a requisite in this genus.

A complete protocol for SE and plant production, including cryopreservation, was reported recently for *Tsuga caroliniana* and *T. canadensis* [91]. Induction frequencies were from 17 to 52 % for immature embryos, respectively. The results confirmed the interplay among the collection date of the cones, medium composition and source tree on the frequency of SE induction, which has been reported in all previous publications. Maturation of somatic embryos was completed by slow drying under permeable plastic film. However, conversion of somatic embryos to plants was very low and requires further research.

4.2 Cupressaceae

Cryptomeria japonica SE was successful with up to 17 % of immature seed explants tested over 3 years, and the presence of PGRs was not required in the initiation medium [92]. The embryogenic characteristics of the cultures could be improved by increased glutamine concentration in the medium [30]. Somatic embryos developed better on a medium that in addition to ABA, PEG, charcoal and maltose, also contained 32 nM phytosulfokine [32].

Two species of *Chamaecyparis* produced plants through SE, namely *C. pisifera* [93] and *C. obtusa* [94, 95]. Initiation of SE occurred on both a medium with PGRs (2,4-D and BA) and on PGR-free medium at the frequency up to 33 and 48 %, respectively. Development of somatic embryos was promoted by PEG,

AC, ABA, and maltose in a medium gelled with 0.3 or 0.5 % gellan gum. Sixty-eight percent of *C. obtusa* EM lines produced cotyledonary somatic embryos, 91 % of which germinated and the plants were subsequently transferred to a greenhouse [95]. In both species, somatic embryo germination was very high and conversion to plants was not problematic. Field tests of *C. pisifera* somatic trees are underway [93].

Another species that was recently studied for its propensity to undergo SE in the same family was *Juniperus communis* [96]. This work confirmed that similarly to *Cryptomeria* and *Chamaecyparis*, the presence of an auxin and/or a cytokinin was not necessary for SE to be initiated and was even inhibitory in *J. communis*, with a reduction from 50 to 25 %. Proliferation of EM was rapid on PGR-free medium as well, but the maturation of somatic embryos was stimulated by 60 μM ABA after brief culture on ABA-free medium with a lower concentration of N and Ca. The development of somatic embryos was highly asynchronous and the culture produced a continuous supply of early and mature somatic embryos. The latter germinated after partial desiccation and converted to plants at a low frequency. A major impediment to plant production was the growth of new EM at the basal part of the somatic plant, which could not be controlled by the application of the gibberellin ($GA_{4/7}$) in the medium.

4.3 Taxaceae

In *Taxus wallichiana*, SE was achieved indirectly from calli that grew on zygotic embryos in culture [97]. Initially, the explants were cultured in the presence of BA and NAA, and after 8 weeks they produced compact yellow calli. Subsequently, when transferred onto a medium with 2,4-D, NAA, and BA, the calli changed in morphology, and two out of four displayed embryogenic characteristics. Somatic embryo development was achieved on medium with ABA and charcoal after 12 weeks; however, the conversion rate to plants was only 10 %.

4.4 Cephalotaxaceae

A complete SE protocol was developed for *Torreya taxifolia* [98]. High initiation of SE (60–100 %) from six seed families was accomplished on medium with various additives, including PGRs (2,4-D, BA, kinetin, and ABA) and maltose. The EMs were cryopreserved using the standard protocol. Somatic embryos developed on a medium with ABA, activated charcoal, maltose, biotin, brassinolide, MES, folic acid, and pyruvic acid. Two genotypes of clonal mature somatic embryos germinated at 64 and 95 %, respectively, and after 2 months the somatic seedlings were planted in a substrate. The species is under threat of extinction and SE will assist in the present and future conservation of this ancient plant.

4.5 Araucariaceae

Despite extensive research to develop SE protocols for *Araucaria angustifolia*, only pre-cotyledonary somatic embryos were obtained in culture [99, 100].

5 Genetic Stability of Cultured Embryonal Mass and Somatic Seedlings

Embryogenic cell lines from ten half-sib seed families of *P. sylvestris* were analyzed using four nuclear single sequence repeat (SSR) markers, also known as microsatellites or short tandem repeats (STR) [101]. The aim was to determine whether the genetic stability of the lines changes during in vitro culture. The results indicated that mutations occurred in the cell lines and that their frequency was dependent on the seed family. Interestingly, mutations were detected in cell lines cultured on both a medium with PGRs and on a medium without PGRs, undermining the present notion that 2,4-D is the main culprit causing genetic instability. The authors also found a considerable mutation rate in zygotic embryos, albeit at a much lower rate compared with cultured EM, which led them to conclude that the in vitro culture stress triggered the mutations in the microsatellite regions in *P. sylvestris*. Whether the instability in the studied microsatellite loci reflect alteration in functional genes remains to be investigated. In a similar study with *P. pinaster*, 17 EM lines from six seed families were analyzed using seven nuclear SSR markers after 6, 14 and 22 months of culture as well as regenerated somatic seedlings [102]. The SSR pattern at the time of line establishment was used as a reference for the cultures of increasing age. Genetic variation was detected in cultures of all ages and in 5 out of 52 somatic seedlings. Some somatic seedlings displayed plagiotropism and loss of apical shoot dominance, but no correlation was found between genetic instability at the analyzed loci and the abnormal phenotype. Nevertheless, there is a risk of genetic mutations during the cell proliferation stage in vitro, which may lead to the regeneration of mutant plants with different mutations occurring among somatic plants regenerated even from the same EM line. The latter could be caused by the presence of a mixture of cells that accumulated different mutations in a given cell line and, hence, the regenerated plants were not clonal. There is some speculation that the loss of embryo development capacity in aged EM lines might be attributed to the accumulation of mutations, perhaps together with epigenetic changes, during prolonged in vitro culture, as described below.

6 Aging of Embryogenic Cultures and its Influence on the Ability to Produce Mature Somatic Embryos

The age of embryogenic cultures maintained on semisolid medium can negatively influence their ability to produce mature somatic embryos in some conifer species. However, aging was not counterproductive in cultures of *Picea* and *Larix* hybrids. In *L.* x *eurolepis*, a line was still productive after 9 years of subculturing [80]. On the contrary, the somatic embryo regeneration ability of cultures of

A. lasiocarpa [84] and *C. japonica* [92] decreased overtime whereas in *Larix leptolepis*, embryogenic cultures became non-embryogenic [103]. The aging and associated changes appear critical in *Pinus* sp. In *P. pinaster*, maturation yield decreased rapidly within 6 months of culture [28, 104]. Reduction in the quantity of somatic embryos or cessation of somatic embryo development was accompanied by substantial modifications to the cellular organization/composition of the culture during proliferation [27]. Total culture time also affected the quality of cotyledonary somatic embryos, with progressive reduction of size and germination rate [28]. However, the embryogenic culture's ability to regenerate cotyledonary somatic embryos could be prolonged by modifying the culture medium composition and subculture frequency [27].

In *Larix* sp., the effect of aging on embryogenic ability has been studied at the molecular level (reviewed by Trontin et al., Chapter 8). Differential expression of various microRNAs (four major miRNA families: miR171, miR159, miR169, miR172) has been detected in embryogenic and in non-embryogenic cultures of *Larix kaempferi* [105]. In particular, miR171 and miR159 were found downregulated and upregulated in non-embryogenic cultures, respectively. Subsequently, the authors identified a MYB transcription factor (*LaMYB33* from *L. kaempferi*) as a target gene for miR159 and *Larix SCARECROW-LIKE 6* homolog (*LaSCL6*) was targeted by miR171 [106]. Post-transcriptional regulation of *LaMYB33* and *LaSCL6* by miRNAs may participate in the maintenance of embryogenic potential as part of the epigenetic complex of regulation of gene expression [103, 106].

To circumvent the recurrent problem of aging, the embryogenic cultures of most conifer species are routinely cryopreserved shortly after initiation (*see* Ozudogru and Lambardi, Chapter 32). Full embryogenic competence of old embryogenic lines could be restored in *P. pinaster* by inducing new cultures from cotyledonary somatic embryos (secondary SE; [104]).

7 Desiccation and Cryopreservation

The ability to cryopreserve EM has been a critical success feature of the development of SE to the level we utilize this technology in the afforestation programs today. The maturation competences of SE cultures under conditions of continuous subculture are highly variable both within and between species. Some species of *Picea* and *Larix* are seemingly unaffected by long periods of minimal or erratic subculture, but many *Pinus* species show a sharp decline in both quantitative and qualitative mature embryo production [28, 80, 107]. These differences, to some degree, influence the urgency to cryopreserve cultures. However, the cost savings associated with not subculturing and the importance of retention of genetic fidelity

and maturation competence make cryopreservation as critical as the other stages of the SE process.

Considering the progress made in cryopreservation over the past decade, key developments include a better understanding of the morphology of cell lines, the interaction of cryoprotectant treatments with these and the development of protocols that facilitate storage of immature and mature somatic embryos [108, 109]. It is also clear that relatively simple pretreatments and freezing protocols are proving as effective as earlier more complex methodologies. Embryogenic cell lines on proliferation media are highly heterogeneous, consisting of a range of cell types in a state of constant differentiation and dedifferentiation. This variability in cell types has been thought to differentially influence the responsiveness of cellular components to osmotic treatment, colligate cryoprotection and controlled cooling [107]. Recent results with a number of species indicate that many of the factors that contribute to successful cryopreservation still remain elusive [108]. The increasing body of work using more differentiated tissues may lead to this being the preferential material for long-term storage of conifers.

7.1 Cryotolerance of Embryonal Masses

Cryotolerance of *P. abies* was studied in association with growth rate, anatomical features and polyamines (putrescine, spermidine and spermine) in five embryogenic cultures [108]. The authors found that the ability to produce normal mature embryos was the only characteristic shown to have a positive correlation with cryotolerance. Of the two lines showing a high percentage of cryotolerance, one was a highly productive line, in terms of maturation ability, the other one had a negligible ability to produce mature embryos. Anatomically, the contrast between the embryo initials for these two lines prior to cryopreservation was striking, with the poor-embryo-producing line showing highly dedifferentiated initials. The same contrast was seen with total polyamine contents, with the two cell lines with the highest contents giving opposite results with regard to cryotolerance at 94 and 0 %. These observations indicate that the factors that confer cryotolerance in EM are yet to be fully elucidated.

New species to show successful recovery from liquid nitrogen storage include *P. nigra* and *P. omorika* [110–112], respectively. More unusually for conifer embryogenic tissues, the pretreatment stages for *P. omorika* were done on semisolid medium with increasing sucrose concentrations followed by air drying of the EM to 20 % of original fresh weight and subsequent immersion directly into liquid nitrogen. No other cryoprotectant agents were used. After cryostorage, *P. nigra* demonstrated growth rates and ability to produce mature embryos similar to the control material maintained in long-term culture [110]. Another less common pretreatment (maltose) and cryoprotectant formulation was applied to *P. pinaster*

that included PEG 4000 with dimethylsulfoxide (DMSO), resulting in 97 % recovery of the cell lines tested [113].

Vitrification using a modified plant vitrification solution (PVS2), developed primarily for nonembryogenic tissues and shoot apices, has been tested with the aim of developing a simplified cryopreservation procedure for conifer embryogenic tissues. Successful vitrification of tissues would facilitate immediate immersion into liquid nitrogen storage without intervening steps including transient storage at –40 to –80 °C in freezers or programmable cooling incubators. This was successfully achieved with some cell lines of *P. mariana* [114]. An encapsulation/dehydration method was tested with immature somatic embryos of *P. sitchensis* and resulted in the regeneration of EM following immersion in liquid nitrogen [115]. No –40 to –80 °C or programmable freezer are required, but the tissue treatment is labor intensive prior to storage.

A novel method for tissue regrowth was tested with *P. radiata* and resulted in significantly improved post-thaw growth with 60 cell lines stored from 6 months to 4 years prior to thawing [116]. The authors used a vigorous culture (nurse culture) of *P. radiata* to nurse the thawed cells; the nurse culture and thawed cells were separated from each other by a nylon screen. Further simplification of methods was achieved with *P. glaucax engelmannii* and *P. menziesii*. The method eliminated both the use of toxic cryoprotectants and freezing environments. Following culture on ABA medium at 4 °C, the tissue was immersed directly into liquid nitrogen [109]. The method relied on preconditioning of early somatic embryos and these retained the ability to regenerate EM following storage in liquid nitrogen.

Contamination of EM lines can still plague this step of the SE process, with a number of authors reporting significant losses of cell lines upon thawing from liquid nitrogen storage [108, 112]. *Picea omorika* cryopreserved as clumps of tissues rather than as cell suspensions had a decreased frequency of contamination if liquid nitrogen was prevented from entering the vials during freezing [112]. In embryogenic cultures of *P. radiata* cell lines stored for 6 months, none of the 37 genotypes (222 vials) were contaminated and only 5 % of the vials from a further 23 genotypes (138 vials) stored for 4 years were contaminated, despite the fact that all vials had been immersed in liquid nitrogen upon freezing [116]. Interestingly, antibiotic cephotaxime (100 mg/L) was used in the proliferation medium to reduce the risk of bacterial contamination of cultures during the frequent treatments before cryostorage of *P. abies* [108].

7.2 Mature Somatic Embryo Storage

Mature somatic embryo storage could potentially confer a range of advantages over the cryopreservation of embryogenic masses and would be especially useful in the application of this propagation technology. Effective storage would facilitate both the synchrony

of seed orchard and laboratory production with seasonal nursery and planting programs. Added advantages are that with careful pretreatment, no cryoprotectant chemicals or programmable freezing equipment is required. Successful desiccation without cryopreservation may also be an important aspect of improving quality and synchronizing germination in somatic embryos in many species. Continued development of direct sowing and artificial seed technologies may also benefit from more effective desiccation protocols.

The desiccation environment seems to be one of the key elements for successful storage. *Picea mariana* and *P. glauca* somatic embryos were slowly dried at 97 or 88 % relative humidity (RH) to reach a water content of 0.23 H_2O g/L dry weight before achieving a high post liquid nitrogen germination frequency of 93.8 % [117]. Desiccation at a lower RH of 63 % had a significantly negative effect on subsequent germination following cryopreservation. Interestingly, the somatic embryos that managed to survive this treatment showed a 100 % conversion to plantlets whereas the conversion of the somatic embryos from the 97 or 88 % RH treatments ranged from 26.7 to 46.7 %. These authors also tested the stored embryo potential for embryogenic tissue reinduction following thawing. Embryos that had been desiccated at high RH (97 %) and were rehydrated for 12 h at 100 % RH had reinduction rates that were similar to those of the controls [117]. Further work from this team has elucidated some of the mechanisms linked to fast desiccation tolerance in *P. mariana* [118]. Their studies showed that an initial short period of slow desiccation of the embryos increased their subsequent tolerance to a fast desiccation treatment. The mechanisms behind this indicated an increase in sucrose accumulation, occurrence of raffinose, and depletion of starch reserves within the somatic embryos. The occurrence of dehydrins was also investigated in reference to their suspected role in the development of desiccation tolerance. The authors noted a doubling of the dehydrin signal intensity after 48 h of slow desiccation (24–48 kDA), which coincided with the best treatment for subsequent germination of rapidly desiccated embryos.

Picea glauca and *P. glauca*×*engelmannii* complex somatic embryos were gradually dried over salt solutions to the level of dry seed embryos and retained their viability upon rehydration [119]. Desiccated somatic embryos also survived subsequent freezing in liquid nitrogen, without the addition of cryoprotectant or preculture steps. Highest survival (>80 %) after freezing in liquid nitrogen was in embryos pre-dried to Ψ of –15 to –20 MPa, which yielded relative water content (RWC) close to predicted bound (apoplastic) water values. In another study, somatic embryos of *P. glauca* survived a rapid desiccation treatment (2 h of air drying on a laminar flow bench at ambient temperature and humidity) if they were carefully preconditioned on maturation medium [120].

The optimum treatment was leaving embryogenic tissue on maturation medium for 51 days, making it possible for embryos to become cotyledonary before placing the Petri dishes into 5 °C for 8 more weeks of incubation. It should be noted that in *P. glauca*, cotyledonary embryos are fully developed after 51 days and precocious germination was observed in some embryos prior to incubation at 5 °C. In contrast to the results presented for *P. mariana* (and *P. glauca*) [118], shorter periods of incubation were detrimental to the quality of the germinant following rapid desiccation [120]. Elucidation of the mechanisms behind the cold tolerance of *P. glauca* somatic embryos has subsequently shown, with freezing damage tests based on electrolyte leakage, that somatic embryos matured at lower temperatures possessed significantly higher freezing tolerances than somatic embryos matured at 20 °C [121].

8 SE from Vegetative Tissues of Adult Conifers

Vegetative (also known as clonal) propagation of adult trees has a major advantage over propagation through seed because large genetic gains are achieved by capturing a large proportion of tree genetic diversity in a single selection cycle [122]. Hence, vegetative propagation of select superior forest conifers through SE is highly desirable, particularly because it has the potential to deliver a stable supply of superior seedlings for forest plantations. However, in spite of decades of research efforts, efficient propagation of adult conifers by any means is still beyond reach [61, 123, 124]. The first work that raised expectations in this area of research was induction of SE in buds (primordial shoots/needles) of 2- to 3-year-old *P. abies* grown from a somatic embryo [125] and *Ceratozamia* spp. [126], but no results on somatic plant growth have been published. Using four genotypes of somatic trees of *P. glauca*, it was subsequently demonstrated that one genotype produced SE from primordial shoot explants (Fig. 2a–f) consistently from age 2 (in 2002; [127]) to 15 years (in 2015; Klimaszewska, personal communication). The media for each stage of SE were the same as those used for seed embryo SE and a large number of juvenile propagules (somatic seedlings) have been grown in a greenhouse and subsequently planted in the field. These somatic trees derived from donor trees of increasing chronological and ontogenic ages

Fig. 2 (continued) (17.5×). (**c**) Primordial shoot cut longitudinally and showing slightly elongated needle primordia (24×).Fig. 2 (continued) (**d**) Embryonal masses growing from the explant after 33 days (magnification 25×). (**e**) Mature somatic embryos produced from embryonal masses induced from the same donor trees of different ages; 2, 7, and 8 years old (0.5×). (**f**) Somatic seedlings cultured on germination medium for 7 weeks (1.1×). (**g**) Clonal, juvenile G6 trees produced from primordial shoots collected from 7-year-old (on the *left*) and 8-year-old (on the *right*) donor trees (1×) growing in the nursery of NRCan-CFS, Valcartier, QC, Canada

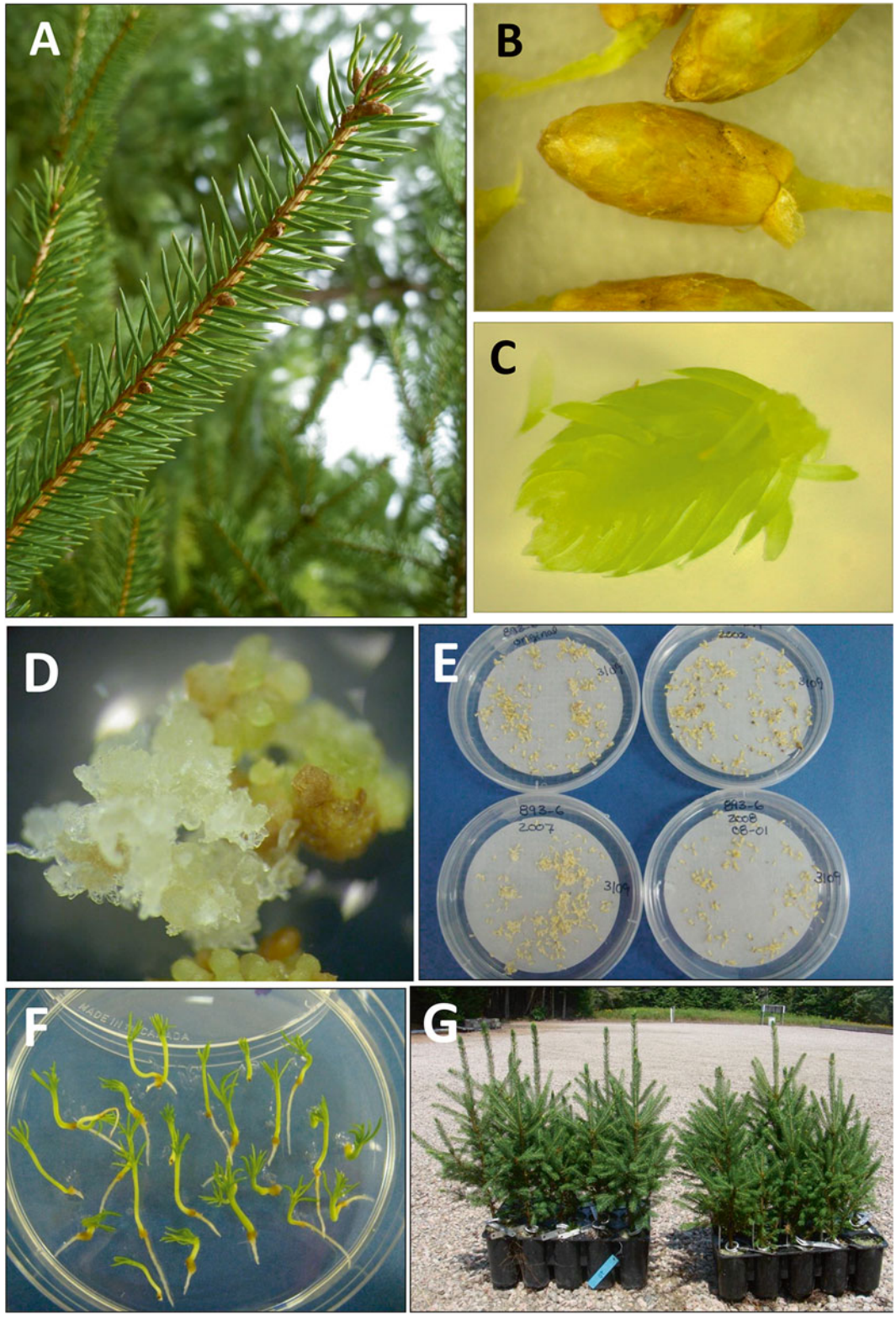

Fig. 2 Induction of somatic embryogenesis (SE) within primordial shoots of somatic *Picea glauca* 13-year-old trees, genotype G6. Vegetative buds were collected on May 6, 2013 from a plantation established in 2003 by NRCan-CFS in Valcartier, QC, Canada. (**a**) A branch with pre-flush buds (2.5×). (**b**) Cleaned and disinfected buds

are being evaluated for their growth rate and morphology, and are expected to provide evidence of true rejuvenation. Simultaneously, the donor trees of responsive and nonresponsive genotypes provided a unique opportunity to examine the molecular aspects underpinning SE within shoot tissues of adult *P. glauca* trees (reviewed by Trontin et al., Chapter 8). A 32,000 oligo-probe microarray was used for transcriptome-wide expression profiling of explants at day 0 and day 7 of culture, which led to the identification of four of the most differentially expressed genes in each of the two genotypes [128]. The absolute quantitative PCR (qPCR) of these genes was expanded to 21 days of SE induction and showed that the expression of all eight genes was maintained throughout the induction period. In contrast to the responsive genotype, explants of the nonresponsive genotype expressed high levels of stress-related genes, such as two extracellular serine protease inhibitors, a cell wall invertase, and a class III apoplastic peroxidase, whereas the former showed temperate expression of these genes. Instead, high expression of dehydrins and the QT-repeat and proline rich proteins that are conifer-specific were identified in the responsive genotype and suggested an adaptive stress response. These results further suggested that the possible causes of the lack of SE induction in an explant may not be necessarily due to an innate lack of SE promoting activity, but that biotic defense activation could potentially be a dominant antagonist. Therefore, future work should focus on determining how and if suppressing biotic defense activation could be used to promote SE induction in nonresponsive explants.

9 Field Growth of SE Trees

Clonal forestry offers significant advantages for forest productivity due to the genetic gain (volume and quality improvements) that can be realized through selection and mass propagation of elite individuals (clones) [129]. Somatic embryogenesis, with its capacity for long-term germplasm cryopreservation and scale-up technologies, is the preferred avenue to accelerate the selection and operational deployment of value-added genotypes, especially through multivarietal forestry [4, 7]. Over the past decade, more information has become available from field performance trials of planting stock derived from SE. As described in an earlier review, the majority of reports was for *Picea* spp. due to their responsiveness to SE relative to other genera [4]. A number of early trials with *Picea* spp. and *P. menziesii* have been established for several decades and while the *Pinus* spp. have been more recalcitrant to SE, some information is available [7].

Further studies looking at the possible long-term effects on field growth of somatic seedlings caused by in vitro conditions

was undertaken with *P. abies* [130]. The somatic plants were assessed for survival and early growth after 4 months in the field. The authors confirmed that prolonged exposure to ABA during the maturation period of somatic embryo formation inhibited early growth. Another treatment of continuous light routinely given to *P. abies* seedlings to improve early growth in the greenhouse had a negative effect on the growth of somatic plants. The authors concluded that direct inwintering of somatic plants after transfer to ex vitro conditions should be avoided. Early greenhouse work studying clonal variation in morphology, growth, physiology, anatomy, and ultrastructure of 6-month-old container-grown *P. glauca* somatic plants found a number of differences when compared with zygotic seedlings of the same families [131]. Height ranges of clones were greater (14.4–31.8 cm) than that of seedlings (15.8–24.3 cm), and root collar diameters were generally greater in clones. Variation within families was larger among somatic clones than among zygotic seedlings for height, needle dry mass and branch density. Light microscopy showed that tannins were more abundant in somatic plants than seedlings; otherwise all needle samples displayed a similar morphology. Of more concern was the incidence of root deformation in somatic plants which had to be transplanted from culture vessels to styroblock containers. Only 52 % of somatic plants had a normal root form, a rate that is comparable with that observed in zygotic seedlings that were not transplanted. Another interesting observation was that plants from specific clones suffered from copper deficiency symptoms in all replications despite fertilizer application. What was clear and encouraging from this study was the early screening potential for selection of superior clones based on both physiological and morphological characteristics [131]. Subsequent work presenting pooled data that compared zygotic seedlings and somatic plants of *P. menziesii* for gas exchange rates, water relations and frost hardiness after 2 years in the field concluded that there were no significant differences between the two stock types [132]. However, no data was presented for individual clone performance. There were only three clones in this trial derived from control crosses versus the seedling controls, which were from bulked open pollinated seed collected from the same orchard. When considering frost hardiness and bud break, no significant differences were found between the two stock types in the latter study. More recent work raised the possibility that there may be some interaction between temperature at the time of somatic embryo maturation and subsequent frost hardiness and bud break especially in the first few years of plant establishment [117]. Based on field performance studies, it appears that clones produced from SE, at least those of *P. menziesii* and *P. glauca*, can be highly acclimated to different climatic conditions [133, 134].

Evaluation of genetic parameters and examination of genotype x environment interactions to characterize the genetic stability of somatic seedlings of *P. glauca* have been done 4 years after establishment of the field tests [134]. In these tests, 52 clones (from 14 control-crossed families) were compared and they are the first of a series of trials established under different ecological site conditions comparing over 1000 somatic clones. Encouragingly, the percentage of somatic seedlings (52 clones) exhibiting normal adaptive characteristics for survival (98–99 %) and bud frost damage and stem form (90–99 %) characteristics were high, and therefore, genetic parameters were not calculated for these characteristics. Strong positive genotypic correlations were found between height, diameter, annual shoot length and volume. The authors felt that the stability of the clonal performance at the two sites reflected the efficiency of clonal selection and was therefore a good reason to promote the selection of generalist clones for future applications in multiclonal forestry [134]. Older somatic plantings (5.5 years) of *P. menziesii* var. *menziesii* have been assessed for survival and performance, clonal genetic parameters such as variances, heritability, and correlations, and for stability of clonal performance across five sites in Washington and Oregon, in the Pacific Northwest, USA [133]. There were 70 clones in the test and the somatic seedlings were grown in the same greenhouse for 1 year prior to planting. All exhibited growth rates and morphology within the normal range exhibited by zygotic seedlings in nurseries. The survival of the somatic seedling clones at 5.5 years ranged from 92 to 99 % and the general conclusion from this study was that the stability of these clones was encouraging for future clonal forestry applications in coastal Douglas fir.

A set of *P. radiata* trials was established in New Zealand and Australia (three in each country) to investigate clonal stability focusing on growth and form traits [135] (Fig. 3). The planting stock was derived from cuttings taken from hedges established from somatic embryos rather than using germinated somatic embryos directly. One reason for this approach was to improve plant quality within clones (height, root mass, and stem diameter, all of which were positively affected). There were 245–280 clones tested at the three New Zealand trials and 44–69 clones at the three Australian sites. In general, clonal stability was good across the New Zealand sites, and although there was only a small number of clones that were common between Australia and New Zealand, clones stable for growth could be identified across both countries. The authors did note that age 5 may still be too young to draw firm conclusions with regard to genotype rankings. Forest Genetics Ltd. planted their first trials of *P. radiata* derived from somatic seedlings in 1999. They have been able to clearly identify outstanding clones, which now form the basis of field-proven material being sold to commercial clients (www.forest-genetics.com).

Fig. 3 Somatic *Pinus radiata* in a field test of the Forest Genetics Ltd., New Zealand

These plants command a premium price relative to seedlings of control pollinated seed lots. Evaluation of somatic seedlings has been also ongoing in France since 1999 with *P. pinaster* for which ca. 3200 clonal trees from more than 200 genotypes were established in eight field tests [7]. Data analysis at age 6, from 24 clones planted in 2004, indicated that somatic seedlings are producing normal trees but usually with a lower initial growth rate than those from seedlings (Trontin, personal communication). However, it has been shown that mean relative increase in height was similar or even higher in specific somatic lines after 6 years, suggesting that normal growth can be recovered later.

Seed production from somatic clonal trees has been studied in *P. mariana* [136]. The authors found that the somatic trees produced both viable pollen and female cones that were able to be crossed to produce equally viable seeds. The authors noted that male strobili were produced about 6 years after planting and 2 years after the early onset of female flower production, which is earlier than what is generally observed in zygotic trees. The authors also noted the incidence of albino germinants (up to 14 % in one particular inbred cross). No direct non-somatic clone controls were used in this research to determine if earlier male and female cone production had an adverse effect on vegetative growth. Results were generally compared with other data available for *P. mariana* and there were no outstanding anomalies (pollen germination, seed mass, and morphophysiological standards for planting stock). The authors concluded that the stock produced

through SE and selected for exceptional performance could be used for subsequent seed production and would enhance gains from multivarietal forestry.

10 Bioreactor/Scale-Up Studies

For commercial application of SE, laboratory-scale protocols must be scaled up and fulfill several criteria such as production of high quantities of uniform somatic embryos at a given time and at a reasonable cost per unit. This can be achieved by utilizing bioreactors, which are amenable to automation and allow continuous monitoring and control of growth conditions (agitation, pH, oxygen, and carbon dioxide), large volumes, and maintenance of a homogeneous culture. In a study with *P. sitchensis*, two EM lines were grown in bioreactors of different configurations (air-lift, bubble, stirred tank, and hanging stirrer bar) and compared with shake flask cultures [137]. The bioreactors were 5, 2, or 1 L in volume. Both lines exhibited larger increases in biomass when grown in bioreactors, but one line proliferated as single early somatic embryos while the other one formed large aggregates of somatic embryos. Samples taken from all cultures were transferred onto maturation medium with 40 μM ABA, 1 μM IBA, 3 % sucrose, and 0.1 % activated charcoal. There were two medium variants in Petri dishes: one semisolid (0.6 % agar) and another semisolid covered with 7 mL of liquid medium for submerged culture. One line produced cotyledonary somatic embryos at the highest number when proliferated in bubble bioreactor on both variants of maturation medium. For the second line, the submerged way of culture was unsuitable. The results suggested that the bioreactor configuration, design, and operating conditions must be adequately chosen to suit the physiological, metabolic, and morphological characteristics of a line. For *P. menziesii*, a large-scale somatic embryo production system was developed by Weyerhauser Co. (WA, USA) [138]. It involved growing the EM in 1 L flasks in liquid medium on a rotary shaker in darkness, followed by culture in perfusion bioreactors on liquid medium soaked pads containing PEG, ABA, $GA_{4/7}$, and charcoal for development and maturation. The development medium was pumped from the reservoir into the bioreactor until it made contact with the lower surface of the pads. The medium was absorbed in the pads by capillary action and, after a few hours, it was pumped out to the reservoir. This was repeated at regular intervals until mature cotyledonary embryos developed. Twenty to fifty cotyledonary somatic embryos were produced from the initial 1 mL of settled EM, but different genotypes showed variations in both the number and quality of mature somatic embryos. The somatic embryos were cold-treated before germination. The authors concluded

that the solution for low-cost mass production of conifers was the combination of liquid culture with bioreactors, automation technology, and manufactured seed delivery system.

11 Conclusions and Future Research

The multidiscipline approach that is taking place to find solutions to some of the problems still facing large-scale production of conifer trees through SE should prove fruitful in the future. SE research has generated knowledge and protocols that can be immediately applied from one species to another and have their utility verified. For example, in *Pinus* species and other conifer genera where initiation of SE is proving problematic, the viability of crosses prior to sampling needs to be considered. Following sampling for embryogenesis, a further collection of cones should be made when the seed is mature to verify that the seed is viable. It has been shown with a range of conifers that embryos often form early in development only to abort prior to becoming cotyledonary, especially in situations where self-fertilization or hybridization may be occurring. In some cases, SE may be a way of rescuing these embryos that could be advantageous, especially with the establishment of novel first generation hybrids. However, for general protocol development, it is better to have known high viability crosses to work with. Another confounding factor with protocol development is the presence of the megagametophyte, which is likely to have a confounding effect with regard to development of an optimal induction medium. The megagametophyte tissue dies as soon as it is dissected away from the seed coat and may start to produce toxic leachates. Examination of the literature does show mixed responses and it is possible that the megagametophyte acts as a buffer against suboptimal media interactions, and that it even provides some nutritive benefits in the short term. It is recommended that both methods of dissection be tested as part of the matrix including media modifications when protocols are being developed or improved for conifer species. Optimization of culture medium should benefit from using software tools for experimental design, computation, and graphic visualization of multifactor interactions that together influence the culture productivity from its onset in vitro to the plants in a greenhouse/nursery as demonstrated for herbaceous species by Halloran et al. [139] and Adelberg et al. [140].

We expect to see increased integration of somatic embryogenesis within current nursery practices. In the laboratory, methods to reduce plant production costs will continue to develop; these include liquid culture and sorting mechanisms for mature somatic embryos. Somatic embryos of a number of conifer species are amenable to organogenesis, and this step will be used to provide

plants for stool bed and subsequent cutting regeneration. Where physiological age constraints limit the life of stool beds, liquid nitrogen storage will ensure a continuous supply of juvenile stock plants. Soft tissue manipulation robotics for medical procedures is improving as well as visual multidimensional graphics, and this may create new opportunities for automation of the SE process.

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Chapter 8

Molecular Aspects of Conifer Zygotic and Somatic Embryo Development: A Review of Genome-Wide Approaches and Recent Insights

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Abstract

Genome-wide profiling (transcriptomics, proteomics, metabolomics) is providing unprecedented opportunities to unravel the complexity of coordinated gene expression during embryo development in trees, especially conifer species harboring "giga-genome." This knowledge should be critical for the efficient delivery of improved varieties through seeds and/or somatic embryos in fluctuating markets and to cope with climate change. We reviewed "omics" as well as targeted gene expression studies during both somatic and zygotic embryo development in conifers and tentatively puzzled over the critical processes and genes involved at the specific developmental and transition stages. Current limitations to the interpretation of these large datasets are going to be lifted through the ongoing development of comprehensive genome resources in conifers. Nevertheless omics already confirmed that master regulators (e.g., transcription and epigenetic factors) play central roles. As in model angiosperms, the molecular regulation from early to late embryogenesis may mainly arise from spatiotemporal modulation of auxin-, gibberellin-, and abscisic acid-mediated responses. Omics also showed the potential for the development of tools to assess the progress of embryo development or to build genotype-independent, predictive models of embryogenesis-specific characteristics.

Key words Developmental regulator, Embryo patterning, Gymnosperm, Metabolome, Proteome, Somatic embryogenesis, Transcriptome, Stress

1 Introduction

Compared with herbaceous angiosperms such as *Arabidopsis thaliana*, elucidation of the molecular events regulating embryo development in trees, and particularly in conifers (the primary source for wood production worldwide), has been hindered by their large physical size, slow growth, long generation time, and very large genome. A number of powerful genetic approaches whose efficiency does not require the availability of large genomic resources or full genome sequence (e.g., embryo defective mutants, T-DNA insertional mutagenesis) are therefore impracticable with conifers

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[1, 2]. The recent implementation of qualitative and quantitative methods for the genome-wide profiling of genes [3] (transcriptomics), proteins [4] (proteomics), and metabolites [5, 6] (metabolomics) provided unprecedented opportunities to unravel the complexity of coordinated gene expression during conifer embryo development. Analysis of the "omic" data now benefits from the extensive cDNA resources and proteome databases established in several spruce and pine species of commercial and ecological interest [7–10]. It is also anticipated that the identification rate of multiple transcripts and proteins in genome-wide data sets will considerably increase as decoding the conifer "giga-genome" is ongoing in *Picea glauca* [11], *Picea abies* [12], *Pinus taeda* [13], and other pine species such as *Pinus radiata* (Scion-led project, New Zealand, Scion Annual Report 2013, p. 15, http://www.scionresearch.com/__data/assets/pdf_file/0017/42443/ScionAnnualReport2013-Highlights.pdf), *Pinus pinaster* and *Pinus sylvestris* (ProCoGen European project 2012–2015, http://www.procogen.eu/). Even if annotation of this enormous genomic resource is still challenging [9, 14], "omic" approaches to elucidate embryo development are rapidly developing. The resulting knowledge might ultimately provide (epi)genomic tools for the efficient production of improved and better adapted varieties through seedlings and/or emblings (somatic embryogenesis) to cope with both market evolution and the changing environment. It is also of particular interest to complement the tedious "trial and error" strategy currently in use, to refine somatic embryogenesis protocols (reviewed by Klimaszewska et al., Chapter 7) and achieve commercial application in conifer species, especially through multivarietal forestry [15, 16].

In this chapter we review the recent advances in transcriptomics (and targeted gene expression studies), proteomics and metabolomics of both somatic (SE) and zygotic embryo (ZE) development in conifers. The review is mostly focused on SE, as somatic embryogenesis has become a model in vitro system in conifers to study the molecular biology of embryo development [2, 17], including epigenetic aspects as background genetic load can be ruled out from clonal material [18]. It is comparatively difficult to sample manageable quantities of embryonal mass (EM) during early zygotic embryogenesis [19, 20]. Our aims were (1) to emphasize the critical processes and genes at specific embryo developmental and transition stages, and (2) to highlight practical application for somatic embryogenesis in conifers.

2 Transcriptomics of Conifer Embryo Development

The development of cDNA or oligonucleotide-based microarray technologies, and more recently, of next generation sequencing RNA methods has provided critical advances for genome-wide

screening of quantitative gene expression in forest trees [3]. Quantitative real-time polymerase chain reaction (qPCR) also provides an accurate tool for analyzing the expression of individual candidate genes selected from microarray and sequencing-based data. The increasing use of absolute quantification may raise the possibility for universal comparison of gene expression [21]. There are still limitations at both technical [22] and data interpretation levels [9]. However, microarrays developed in major conifers were found suitable for transcriptome profiling in other species [9, 23]. Both microarrays and RNA sequencing methods are now increasingly used to obtain either initial insights into embryogenesis-related transcriptomes when public resources are limited, e.g., *Larix kaempferi* [24], or to perform comprehensive transcriptome analyses of somatic [2, 18, 25], and/or zygotic material [19, 26, 27].

2.1 Conifer Transcriptomic Analyses Benefit from Advances in Model Plants

Despite various spatiotemporal differences between gymnosperms and angiosperms embryogenesis [28], it is increasingly debated how to efficiently translate the molecular information gained in *A. thaliana* to domesticated species [29]. The information delivered from the completed genome sequence in *A. thaliana* resulted in a deeper understanding of complex, regulated gene network (300–450 genes) involved in embryo patterning [20, 29–31]. Interestingly, most embryogenesis-related genes identified in *A. thaliana* have homologous sequences with strong congruity in conifers [28] such as in *P. taeda* (83 %) [32] and *L. kaempferi* (78 %) [24]. Differences in molecular regulation of embryogenesis between *A. thaliana* and conifers may therefore mainly arise from variation in gene expression [2, 28], especially temporal differences at the transition between embryo developmental stages [33]. Regulation of gene expression is thought to result partly from epigenetic modifications as a possible adaptive mechanism in long-lived trees [18]. A large transcriptomic study further indicated that ZE transcript profiles are highly correlated between *P. pinaster* and *A. thaliana* [19, 34], with gymnosperm-specific transcripts estimated to be only 3 %. There is thus some evidence that conifers will benefit from angiosperms reference data [20, 35, 36]. Because transcripts abundance is not always predictive of effector molecules underpinning the physiological process involved in embryo development, one more challenge is to integrate transcriptomic with proteomic and metabolomic datasets. This promising systems biology approach to modeling the genetic regulation of plant embryogenesis is ongoing in *A. thaliana* [20], with expected similarity in conifers [26, 31, 37, 38].

2.2 The Growing Bulk of Information About Coordinated Gene Expression

Only a few available reviews [28, 39–43] aimed at unraveling the complex regulatory gene network expressed in conifers, from embryogenesis induction to early embryogenesis (embryonic phase), late embryogenesis (up to the cotyledonary embryo stage)

and the subsequent maturation steps from the acquisition of desiccation tolerance to the establishment of dormancy and accumulation of storage reserves needed for germination. The range of genes transcribed in conifer EM is apparently 30–40 % larger than in any other tissue [18, 28]. Our knowledge is currently highly fragmented because most studies performed during the last decade were targeting few genes (Table 1). Little could be learnt until recently about the expression of gene cohorts with similar transcript signatures during embryo development. For this purpose, the comparative analysis of favorable and unfavorable SE maturation conditions, as well as embryogenic and non-embryogenic material, appeared critical to identify differentially expressed genes among developmental stages [1, 21, 23, 46, 89, 90]. Most available transcriptomic data (Table 2) are from *P. abies* because SE development is tightly controlled in this species with clear, synchronized transition between developmental stages promoted by specific plant growth regulator (PGR) treatments. Transcript profiles were described from early embryogeny at the time of EM proliferation to the cotyledonary stage using macroarrays [23, 90], microarrays [2], or sequencing methods [18]. The latter study specifically reported on temperature-dependent differential transcriptomes in proliferating EMs that may be associated with the formation of an epigenetic memory with a delayed impact on seedling development. In the closely related species *P. glauca*, Rutledge et al. [21] provided the first results of transcriptome analysis (microarray) of early molecular events involved in the induction of somatic embryogenesis in conifers. Stasolla et al. [92] performed a macroarray analysis of the effect of polyethylene glycol (PEG) during SE maturation in *P. glauca*. Several macroarray studies also investigated gene expression during early cotyledonary SE development in *P. radiata* [1, 89], and from early embryogenesis to cotyledonary SE and/or ZE development in *P. taeda* [26, 27]. In *P. pinaster*, Morel et al. [25] described differences in both gene expression (sequencing) and proteome during early cotyledonary SE development in favorable (high gellan gum concentration) and unfavorable (low gellan gum) maturation conditions, whereas de Vega-Bartol et al. [19] performed the first microarray-based transcriptomic profiling of ZE in a conifer, from early embryo to the cotyledonary stage. Such transcriptomic studies excluded most small RNAs for technical considerations. However, both microRNAs (miRNAs) and other small noncoding RNAs are part of the epigenetic regulation complex of gene expression, which has a crucial role in regulating development, including embryogenesis [85, 106]. A high-throughput sequencing strategy was used in *L. kaempferi* [50] to identify miRNAs involved in regulation of target genes at specific SE stages. More than 100 predicted genes were found to be putative targets of 60 miRNAs.

Table 1

Recent targeted gene expression studies of embryo development in conifer species

Species	Embryogenesis step	Gene investigated	Reference
Araucaria angustifolia	Early embryogenesis (SE)	SERK 1 (*AaSERK1*)	[44]
	Early to late embryogenesis (SE)	Argonaute (*AaAGO*), CUC 1 (*AaCUC*), WOX (*AaWOX*), LEC1-like (*AaLEC*), vicilin 7S (*AaVIC*), S-locus lectin protein kinase (*AaLecK*), reversible glycosylated polypeptide (*AaRGP*), scarecrow-like (*AaSCR*)	[45]
Larix kaempferi	Early embryogenesis (SE)	miRNA (*miR159*, *169*, *171*, *172*)	[46]
	Early to late embryogenesis (SE)	MYB-like (*MYB33*), *miR159*	[47]
		Scarecrow-like (*SCL6*), *miR171*	[48]
		Superoxide dismutase (*SOD*), catalase (*CAT*), ascorbate peroxidase (*APX*)	[49]
		Trans-acting small interfering RNA *TAS3/miR390*, dicer-like 1 *DCL1/miR162*, *laccase/miR317*, *plastocyanin/miR398*, *ARF/miR160*, *167*, *class III HD-ZIP/miR166*, *miR156*, *159*, *168*, *171*, *397*	[50]
Larix x *marschlinsii*	Early to late embryogenesis, germination (SE)	Germin-like protein 1 (*LmGER1*)	[51]
		Apetala 2-like (*LmAP2L1,2*)	[52]
Picea abies	Early to late embryogenesis (SE)	Type II plant metacaspase subfamily (*mcII-Pa*)	[53]
		Actin isoformes (*Pa1,2,3,4*)	[54]
		KNOX1 (*HBK3*), argonaute (*PgAGO*)	[55]
		KNOX1 (*HBK1,2,3,4*)	[56]
		Aquaglyceroporin (*PtNIP1;1*)	[57]
		HD-GL2 homeobox (*PaHB1*)	[58]
		CUC (*PaNAC01/02*)	[59]
		ABI3/viviparous 1 (*PaVP1*)	[60]
		ABI3/viviparous (*PaVP1*), LEC1-like (*PaHAP3A*)	[61]
	Early to late embryogenesis (SE/ZE)	WOX (*PaWOX2*, *PaWOX8/9*)	[17]
	Early to late embryogenesis, germination (SE)	Lipid-transfer protein (*Pa18*)	[62]
	Early to late embryogenesis (SE), germination (seeds)	Class IV chitinase (*Chia4-Pa1*)	[63]
	Early to late embryogenesis (SE), vegetative tissue (seedlings and/or older trees)	Lipid-transfer protein (*Pa18*)	[64]
		HD-GL2 homeobox (*PaHB2*)	[65]
		WOX (*PaWOX2*, *3*, *4*, *5*, *8A*, *8B*, *8/9*, *13*)	[66]
		WOX (*PaWOX2*)	[67]
		PIN family of auxin efflux transporter (*PaPIN1*)	[68]
		PIN1-like auxin transport protein (*PIN1-like*)	[69]

(continued)

Table 1
(continued)

Species	Embryogenesis step	Gene investigated	Reference
Picea glauca	Initiation and early embryogenesis (SE)	Apetala 2-like (*AP2-L2*), LEC1-like (*CHAP3A*), *IAA2-like*, babyboom (*SAP2C*), *SERK1-like*, KNOX (*SKN1*,*2*,*3*,*4*), ABI3/viviparous (*VP1*), WOX (*WOX2*)	[70]
	Early to late embryogenesis (SE)	Argonaute (*PgAGO*)	[71]
		HD-ZIP (*PgHZ1*)	[72]
		SABATH methyltransferase (*PgIAMT1*)	[73]
	Late embryogenesis	ACS (*ACS1*,*2*,*3*,*4*)	[74]
	Early to late embryogenesis, germination (SE)	Wuschel homeobox (*WUS*), LEC1-like (*CHAP3A*)	[75]
Pinus contorta	Early embryogenesis (SE), vegetative tissue (somatic seedlings, mature tree)	WOX (*PcWOX2*), LEC (*PcHAP3A*)	[76]
Pinus oocarpa	Early to late embryogenesis (SE)	26S proteasome subunit S2 (*RPN1*), HD-ZIP (*HD-Zip1*), receptor protein kinase clavata-like (*CLV*), LEA, legumin- and vicilin-like proteins	[77]
Pinus pinaster	Early to late embryogenesis (SE/ZE)	Glutamine synthetase (*GS1a*, *GS1b*)	[78]
	Early to late embryogenesis (SE), adventitious caulogenesis (ZE)	Receptor protein kinase clavata 1-like (*PipsCLV1L*)	[79]
	Early to late embryogenesis (ZE)	Nonspecific lipid-transfer protein (*PpAAI-LTSS1*)	[80]
	Early to late embryogenesis (ZE), germination (seedlings)	Rab-related small GTP-binding protein (*PpRab1*)	[81]
Pinus pinea	Early to late embryogenesis (SE), adventitious caulogenesis (ZE)	Receptor protein kinase clavata 1-like (*PipiCLV1L*)	[79]
Pinus radiata	Early embryogenesis (SE/seedlings)	Otubain-like cysteine protease (*PrOTUBAIN*)	[82]
	Early to late embryogenesis (SE)	GRAS family (*PrSHR*, *PrSCL1*, and 13 other GRAS genes)	[83]
Pinus sylvestris	Early to late embryogenesis (SE/ZE)	Glutamine synthetase (*GS1a*, *GS1b*)	[78]
		ACS (*PsACS1*,*2*)	[84]
		LEC1-like (*PsHAP3A*), ABI3/viviparous (*VP1*)	[61]

Pinus taeda	Early to late embryogenesis (SE)	Class III HD-ZIP (*HB15L*), ARF (*ARF8L*), argonaute-like (*AGO9L*), MYB factor (*MYB33*), scarecrow (*SCRL*), apetala 2-like (*AP2L*), *miR159*, *166*, *167*, *171*, *172*	[85]
	Early to late embryogenesis (ZE)	LEA (*LPZ202*, *216*, *LSP094*), *dehydrin*	[86]
	Early to late embryogenesis (SE/ZE)	26S proteasome regulatory subunit S2 (*RPN1*), HD-ZIP (*HD-Zip 1*), receptor protein kinase clavata-like (*CLV*), LEA, legumin- and vicilin-like proteins	[77]
	Early to late embryogenesis (SE/ZE), vegetative tissue (seedlings)	Aquaglyceroporin (*PtNIP1;1*)	[87]
	Late embryogenesis (ZE)	ABA responsive (*ABI3*,*4*,*5*), root development (*woodenleg*, *short root*, *scarecrow*, *hobbit*, *bodenlos*, *monopteros*)	[88]
	Late embryogenesis (SE/ZE)	Starch synthase (*LPZ049*), small HSP (*LPZ091*), HSP70 (*LPZ270*), LEA (*LPZ202*, *216*), XETG-like (*LPZ060*), cyclic phosphodiesterase (*LPZ016*), 40S ribosomal protein (*LPZ206*)	[86]

SE somatic embryo, *ZE* zygotic embryo, *ABA* abscisic acid, *ABI* ABA-induced, *ACS* 1-aminocyclopropane-1-carboxylic acid synthase, *ARF* auxin response factor, *CUC* cup-shaped cotyledon, *GTP* guanosine triphosphate, *HD-GL2* homeodomain-glabra2, *HD-ZIP* homeodomain-leucine zipper, *HSP* heat shock protein, *IAA* indole-3-acetic acid, *KNOX* knotted-like homeobox, *LEA* late embryogenesis abundant protein, *LEC* leafy cotyledon, *miRNA* microRNA, *SERK* somatic embryogenesis receptor kinase, *WOX* wuschel-related homeobox, *XETG* xyloglucan endo-transglycosylase

Table 2
Genome-wide molecular profiling of embryo development in conifer species

Species	Embryogenesis step	Method	Reference
Transcriptomics			
Araucaria angustifolia	Early to late embryogenesis (SE/ZE)	NGS (Illumina)	[91]
Larix kaempferi	Early embryogenesis (SE)	NGS (454 sequencing)	[24]
	Early to late embryogenesis (SE)	NGS (Illumina), sRNA library	[50]
Picea abies	Early embryogenesis (SE)	cDNA array (373 cDNAs)	[90]
		17 K cDNA microarray	[2]
		NGS (Illumina)	[18]
	Early to late embryogenesis (SE)	2 K cDNA array (2178 cDNAs)	[23]
Picea glauca	Initiation and early embryogenesis (SE)	32 K oligo-probe microarray	[21]
	Early to late embryogenesis (SE)	2 K cDNA array (2178 ESTs)	[92]
Pinus pinaster	Early embryogenesis (SE)	NGS (Illumina)	[25]
	Early to late embryogenesis (ZE)	25 K cDNA microarray	[19]
Pinus radiata	Early to late embryogenesis (SE)	cDNA-AFLP	[1]
		Screening of cDNA library	[89]
Pinus taeda	Early to late embryogenesis (SE/ZE)	cDNA array (326 cDNAs)	[26]
		cDNA microarray (326 cDNA)	[27]
Metabolomics			
Picea abies	Early to late embryogenesis (SE)	GC/MS	[93, 94]
Picea glauca	Early and late embryogenesis (SE)	NMR spectroscopy	[5]
Pinus taeda	Early embryogenesis (SE)	GC/MS	[6]
Proteomics			
Araucaria angustifolia	Early embryogenesis (ZE)	2D-PAGE + LC-MS/MS	[95]
	Early embryogenesis (SE)	2D-PAGE + MS	[96]
	Early to late embryogenesis (ZE)	2D-PAGE + MS	[97]
	Late embryogenesis, germination (ZE)	2D-PAGE + LC-MS/MS	[98]
Cunninghamia lanceolata	Early to late embryogenesis (ZE)	2D-DIGE + LC-MS/MS	[99]
Cupressus sempervirens	Early to late embryogenesis (SE)	2D-PAGE	[100]
Larix x *eurolepsis*	Early to late embryogenesis (SE)	2D-PAGE + LC-MS/MS	[101]
	Late embryogenesis (SE)	2D-PAGE + LC-MS/MS	[102]
Larix principis-rupprechtii	Early embryogenesis (SE)	1D SDS-PAGE + iTRAQ protein labeling + LC-MS/MS	[103]
Picea abies	Late embryogenesis, germination (SE)	2D-PAGE + GC-MS	[93]
Picea glauca	Early to late embryogenesis (SE)	2D-PAGE + LC-MS/MS	[4]
Pinus massoniana	Early embryogenesis (ZE)	2D-DIGE + ESI-MS/MS	[104]
Pinus pinaster	Early embryogenesis (SE)	2D-PAGE + LC-MS/MS	[25]
	Late embryogenesis (SE/ZE)	2D-PAGE + LC-MS/MS	[105]

SE somatic embryo, *ZE* zygotic embryo, *AFLP* amplified fragment length polymorphism, *cDNA* complementary DNA, *DIGE* difference gel electrophoresis, *2D-PAGE* two-dimensional polyacrylamide gel electrophoresis, *ESI-MS/MS* electrospray ionization coupled with tandem mass spectrometry, *GC-MS* gas chromatography coupled with MS, *iTRAQ* isobaric tags for relative and absolute quantitation, *LC-MS/MS* liquid chromatography coupled with MS/MS, *NGS* next generation sequencing, *NMR* nuclear magnetic resonance, *SDS* sodium dodecyl sulfate, *sRNA* small RNA

2.3 Putative Regulated Genes During Embryogenesis Induction

In both conifers and angiosperms, little is known about gene expression during the early stages of embryogenesis, which is recognized to be critical for subsequent embryo development [1, 18]. The embryonic phase is described as the dedifferentiation process of mature, totipotent cells from competent explant (i.e., responding to stress or PGRs) to embryogenic cells (embryogenesis induction), giving rise to rapidly proliferating new early SE resulting in the establishment of embryo-generating culture [20, 42]. In conifers, the competent explants are restricted to ZE with limited progress from seedlings and from juvenile or adult trees (Klimaszewska et al., Chapter 7). SE initiation may apparently proceed through cell dedifferentiation within a competent explant. When cleavage polyembryony occurs within seed (e.g., in *Pinus*), "initiation" may be merely the prolongation of this process in vitro [42].

It is a particularly difficult task to identify genes underlying somatic embryogenesis induction as the resulting initiated early SEs may rapidly express additional confusing genes involved in proliferation, maintenance of embryogenic potential [20, 21, 48, 50], and early embryo development [24]. Recently, Elhiti et al. [20] reviewed the "omic" data available in plants and provided a short list of 12 genes that are most likely to be involved in embryogenesis induction, from cell dedifferentiation (*ARF19/auxin response factor19*, *PRC1/polycombrepressive complex 1*, *RGP-1/reverse glycosylating protein 1*, *HSP17/heat shock protein 17*), expression of totipotency (*SERK1/somatic embryogenesis receptor-like kinase 1*, *LEC1/leafy cotyledon 1*, *GLB1/plant hemoglobin*, *WUS/wuschel*, a member of the *WOX* gene family, *CLF/curly leaf*), and commitment to embryogenesis (*CDKA/cyclin-dependent kinase A*, *PRZ1/*adaptor protein involved in *CDK* regulation, and STM/shoot meristemless, a gene encoding *KNOX1/*homeodomain protein of the *KNOTTED1-like* class). Homologous genes were found in conifers but it is still unknown if they have similar expression patterns and functions [28]. Using the *A. thaliana* protein interactome database, Elhiti et al. [20] further identified 51 proteins that may be functionally associated with the expression of these 12 genes.

Strikingly, there is currently no molecular study in conifers dedicated to the early steps of somatic embryogenesis initiation from juvenile explants (ZE). Only the identification of *P. glauca* somatic trees which shoot buds have been responsive to initiation treatment has provided a unique opportunity to gain insights into the molecular aspects of embryogenesis induction in conifers [21, 70]. In addition to conifer homologs of important genes for embryogenesis induction discussed above, i.e., *SERK1*, *LEC1*, *WOX2*, and *SKN1*,*2*,*3*,*4* (*KNOTTED* genes), Klimaszewska et al. [70] studied the expression of genes with a recognized function during early embryogenesis, including *AP2-L2* (*apetala*), Auxin/*IAA2* (*indole-3-acetic acid-like 2*), *SAP2C* (*babyboom*), and

ABI3/VP1 (*viviparous*). After 3–6 days of induction, competent bud explants were downregulated for *AP2-L2*, *SERK1*, and *SKN1-4* and upregulated for *IAA2* and *SAP2C*. After initiation, most of these genes were expressed (*SAP2C*, *SERK1*, *SKN1*, *2*, and *4*) or upregulated in early SEs (*LEC1*, *WOX2*, and *VP1*). *SERK1*, *LEC1*, and *WOX* genes were similarly found expressed in early SE of other conifers [2, 17, 44, 45, 61, 66, 67, 76]. *WOX2* has been suggested as a possible marker of effective initiation in conifers [67, 70]. Further transcriptomic comparison of responsive and nonresponsive genotypes could be performed during somatic embryogenesis induction in *P. glauca* [21]. Surprisingly, only a few of the 12 candidate genes described by Elhiti et al. [20] for cell dedifferentiation, totipotency, and commitment to embryogenesis were found to be regulated, i.e., several *ARF* genes (including *ARF19*), *HSP17* and various *CDK* genes. It is suggested that effective SE initiation requires not only the activation of embryogenesis-related genes, but also a moderate activation of genes typical of adaptive stress response of explant to induction treatment. Some of the most highly expressed genes during SE induction in nonresponsive genotype encoded proteins (apoplastic class III peroxidase, cell wall invertase, serine protease inhibitors) possibly involved in biotic defense activation. Interestingly, the jasmonic acid pathway involved in biotic defense elicitation seems to be activated during both cell dedifferentiation and totipotency acquisition in plants [20]. Gene activation in bud explants from the responsive genotype was comparatively lower in magnitude and/or only transient. Among the most upregulated genes, such an expression pattern was observed for a gene (*DHN1*) encoding a conifer-specific group 2 of late embryogenesis abundant proteins (group 2 LEA), in accordance with a well-supported role for dehydrins in adaptation to environmental stress. An *apoplastic class III peroxidase* gene was also activated following a similar transient pattern, suggesting that, in contrast to nonresponsive genotype, cellular redox homeostasis was rapidly restored after the initial oxidative burst promoted by the induction treatment. Peroxidases are also involved in various physiological processes associated with cell dedifferentiation and totipotency (e.g., auxin metabolism, cell wall modification). They were shown to accumulate early during the induction phase of somatic embryogenesis in *Picea* species [107]. Two unknown genes encoding proteins containing repetitive segments rich in threonine–glutamine (QT-repeat) or proline (Proline-rich) were also persistently upregulated in responsive genotypes. As these genes appeared to be conifer-specific, no general conclusion could be drawn as to their putative role in somatic embryogenesis induction. Additional transcriptomic studies are therefore required to increase our understanding of the basic mechanisms governing the highly complex embryonic phase in conifers.

2.4 Developmental Switch from Embryonic to Vegetative Growth

As previously observed in *A. thaliana*, transcriptomic profiling of both SE [1, 2, 23, 50, 90, 92], and/or ZE [19, 26, 27] in conifers has revealed global characteristic changes in gene expression during transition to successive developmental stages. In *P. abies*, comparative studies of normal and developmentally arrested embryogenic lines [23, 90], revealed a transcriptional repressive state during EM proliferation in the presence of PGRs, followed by more active gene expression at the onset of embryo transdifferentiation from EM, and again a repression state at the time of embryo development. The number of differentially expressed genes increased as embryos were developing [2]. Most transcripts (92 %) were unique, suggesting that different sets of genes are regulated at the proliferation/early embryo development and early/late embryo transitions. Important changes in gene expression between consecutive SE stages were similarly detected in *P. radiata* [1] and *P. glauca* [92]. Considering ZE, general variations over multiple embryo stages were revealed in *P. taeda* [26, 27]. Both similarities and differences were observed between somatic and zygotic patterns, suggesting that transcriptomics could be a useful tool to check SE quality [26, 27, 77]. In *P. pinaster*, de Vega-Bartol et al. [19] revealed a large set of differentially expressed sequences from early to cotyledonary embryo stages. Functional categories associated with these genes clustered into nine different profiles, each suggesting a high level of gene co-expression at the same developmental stage. As in *P. abies*, there is an apparent general trend during *P. pinaster* embryogenesis towards massive gene regulation at the transitions from early to pre-cotyledonary embryos and from cotyledonary to fully mature embryos. Such a pattern may originate from both transcriptional (especially transcription factors, TFs) and posttranscriptional regulation through various epigenetic mechanisms, including transposable element-mediated DNA methylation and heterochromatin maintenance (histone deacetylase genes) at early stages, large chromatin-remodeling events during late embryo development, and ubiquitous small RNA-mediated regulation (especially miRNAs) across all developmental stages.

Below we review major processes that have been highlighted in transcriptomic studies for their crucial roles in the developmental switch from early to mature embryo, including programmed cell death (PCD), megagametophyte function and signaling, cell wall modification, auxin signaling and other developmental regulators, abscisic acid (ABA)-mediated processes, changes in metabolisms (especially carbohydrates and proteins) and stress-related genes. This is in close agreement with embryogenesis-related functions supported by proteomic studies (*see* Subheading 3).

2.4.1 Developmentally Regulated PCD During Somatic Embryogenesis

As part of initial embryo polarization, suspensor cells and EM differentiate very early in *P. abies*. Expression of a transmembrane protein C gene (*TMP-C*) encoding an aquaglyceroporin, known to

be predominantly localized in suspensor cells in both *P. taeda* [87] and *P. abies* [57], had already increased 24 h after early somatic embryos were stimulated to develop [2]. *TMP-C* expression continued to increase with concomitant enlargement of suspensor size up to the onset of exposure to ABA. Two waves of overlapping, apoptotic and autophagic types of PCD are required for the appropriate development of SE, including degradation of proliferating early SEs at the time of EM-to-SE transition and elimination of terminally differentiated suspensor cells during early embryo maturation [108–110]. PCD is also activated during germination [108, 111]. Upregulation of a cyclin-dependent kinase gene (*cdc2Pa*) involved in the progression of cell division was associated with these periods of PCD. Kinase activity may initiate apoptosis by phosphorylation of pro-apoptotic proteins [111]. Reorganization of cytoskeletal structures also has an important role in PCD [112], and both actin and tubulin genes are regulated during conifer embryogenesis [23–25]. The actin cytoskeleton was reported to differ between EM and suspensor cells with specific expression in suspensors of different actin isoforms [54]. Application of low doses of latrunculin B (an actin depolymerizing drug) during SE maturation predominantly degraded suspensor cells, which in turn accelerated and synchronized the development of high-quality embryos [54]. Actin depolymerisation has been shown to induce PCD associated with caspase-like activities in plants [113]. A gene encoding a putative actin depolymerizing factor is upregulated in *P. abies* at the early stage of embryo development [23]. PCD was found to be activated as soon as PCD-related genes encoding transient VEIDase/caspase-like activity, such as cathepsin B-like cysteine protease or metacaspase, were significantly upregulated at the EM-to-SE transition. Aquea and Arce-Johnson [1] similarly found an *uridylate kinase* gene and a *type-II metascaspase* gene upregulated during early embryo development in *P. radiata*. Metacaspase genes are recognized candidates for performing the role of cysteine protease genes [53], whereas specific alterations in the balance of pyrimidine nucleotide synthesis, involving uridylate kinase, may represent an early signal for PCD [1]. Endochitinase genes have also been associated with PCD in plants [63] and were found to be upregulated in *L. kaempferi* embryogenic cultures [24], during embryo development in *P. abies* (*Chia4-Pa*) [63], and at the onset of embryo development in *P. pinaster* [25]. *Chia4-Pa* expression was found to be restricted to the EM (embryo proper) base, and chitinase accumulated to form a covering film on the whole EM surface. As observed in *Pinus caribaea* [114], chitinases apparently target specific arabinogalactan proteins located in the epidermal cell wall [63]. In maturing EMs of *P. pinaster*, regulation of PCD was also suggested by the increased expression of a *disulfide isomerase* gene encoding a protein known to interact with specific cysteine proteases [25].

2.4.2 Genes with Megagametophyte Function and/or Signaling

Large suspensors are formed by conifer embryos [26]. Although it remains a transient organ, the suspensor is central to embryo development, which includes physical support, and the translocation and synthesis of nutrients and signaling molecules. Transcriptomic analysis of pre-cotyledonary ZEs in *P. taeda* revealed that various genes, encoding proteins normally associated with late embryo development (e.g., storage proteins, LEAs), are upregulated in suspensor tissue when compared to EM. This expression pattern has significant similarities with that of the megagametophyte, suggesting that the suspensor may be involved in the production of storage and other compounds to be mobilized during embryo development [26]. Various homologous genes of putative signaling factors, normally expressed in the female gametophyte, were found to be upregulated (*ATHB22/MEE68*, *MEE49*) or downregulated (*MEE66*) from early-embryo to late-embryo development in *P. abies* [2]. These genes are known to affect both endosperm and early embryo patterning in *A. thaliana*, suggesting that some somatic cells in proliferating EMs may have a megagametophyte signaling function in conifers. Endosperm properties are similarly recognized for both class IV chitinase (*Chia4-Pa*) [63] and *NARS2* [2] genes upregulated during early embryogenesis (*see* Subheadings 2.4.1 and 2.4.4), as well as for genes highly expressed at the onset of late embryo development in *P. pinaster* and involved in posttranscriptional regulation of gene transcription (small ubiquitin-related modifier/SUMO- or ubiquitin-conjugating enzyme) [25]. From in situ observations indicating that *Chia4-Pa* genes are expressed in subpopulations of cells in both proliferating EMs and early embryos [63], it is speculated that "nurse" cells expressing developmental regulators with megagametophyte signaling functions are required in conifers [2, 63].

2.4.3 Genes Related to Cell Wall Modification

The auxin-mediated effect on reorganization of cell wall architecture, possibly through alterations in cytoskeleton structure, is well established and has implications in cell fate and differentiation [23]. Many developmental regulators involved in embryo patterning proceed through cell wall modifications. Most transcriptomic studies have revealed that these modifications are developmentally regulated from embryogenesis induction to early and late embryogenesis, thus supporting their role in proper embryo development. In *P. abies*, several genes encoding enzymes involved in the synthesis of hemicellulose and pectin (UDP-glucose dehydrogenase), low molecular weight galactosides and cell wall polymers (UDP-galactose 4-epimerase like) were downregulated, especially at the time of effective early embryo development. A *laccase* gene involved in lignification and thickening of the cell wall was also downregulated during the transition from pre-cotyledonary to cotyledonary embryo [23]. A similar expression pattern was observed in *L. kaempferi* [50] and correlated with expression of *miR397*,

supporting a posttranscriptional regulation of *laccase* during somatic embryogenesis. Additional genes involved in cell wall loosening and reorganization are regulated at early stages in *P. radiata* [1] and *P. abies* [2], such as genes encoding α-d-galactosidase and myo-inositol oxygenase, expansin, and pectinesterase. Moreover, a *β-expansin* gene was found specifically expressed in EM [89], as well as genes encoding cellulase and apoplastic germin-like protein (GLP). At the switch from early- to late-embryo stages in *P. abies*, different *expansin* and *pectinesterase* genes, as well as a *xyloglucan:xyloglucosyl transferase* gene, significantly changed their expression level [2]. Transcriptomic and proteomic analyses at the onset of late embryogenesis in *P. pinaster* [25] similarly revealed overexpression of cell wall-related *expansin* genes and also a drastic upregulation of a putative gene encoding extensin-like protein.

2.4.4 Auxin Response Machinery and Embryo Patterning

Auxin biosynthesis and relocalization by polar auxin transport has a crucial function in the activation of the auxin response machinery during plant embryogenesis that results in setting up (1) apical–basal patterning (meristematic poles) and (2) radial embryo patterning (adaxial/abaxial organization). Various reports suggested that auxin-mediated events are of similar high importance in both angiosperms and gymnosperms to establish a roughly similar basic body organization. A recent transcriptome comparison of early SE and ZE performed in *A. angustifolia* suggested that incomplete SE development resulted from an auxin signaling failure in embryogenic cultures [91]. Endogenous auxin biosynthesis (especially IAA) is activated early during SE development in *P. abies* [69], whereas putative auxin transport proteins are upregulated [23]. Concomitantly, auxin starts to be relocalized by polar transport as observed in both *P. abies* [115] and *P. sylvestris* [116]. Disruption of polar auxin transport by *N*-1-naphthylphthalamic acid (NPA) affected early embryo polarization, promoted aberrant development, such as no or poor shoot apical meristem (SAM) and fused/aborted cotyledons, and resulted in abnormal germination [69, 115, 116]. Upregulation of auxin-responsive gene (*SAUR*) and downregulation of auxin biosynthesis competition gene (*SUR1*, involved in glucosinolate synthesis, a sister branch of IAA biosynthesis) were indicative of increased auxin synthesis during development of early stage embryos in *P. abies* [2]. IAA homeostasis may be modulated by additional mechanisms such as methylation of the free carboxyl group by methyltransferases of the plant SABATH family. Such a gene showing high catabolic activity with IAA was expressed in *P. glauca* during early embryo development, and then downregulated towards later stages [73]. Expression of *LEC1* during early embryogenesis in *P. abies* and *P. sylvestris*, followed by a strong decrease at the switch to late embryogenesis, was also observed [2, 61]. The activation of genes involved in localized auxin biosynthesis has been linked with expression of both *LEC1*

and *LEC2* [20, 75]. In *P. pinaster* ZE, a TF gene orthologue to the auxin response factor (*ARF16*) involved in regulation of auxin-modulated genes (e.g., *WOX5*: maintenance of pluripotent cells in root quiescent center) was drastically downregulated at the transition to pre-cotyledonary embryos [19]. In *L. kaempferi*, expression of different *ARF* genes, possibly regulated by *miR160 or miR167*, increased up to the early SE cotyledonary stage and then significantly decreased [50]. In the same species a putative trans-acting small interfering RNA (siRNA) gene (*TAS3*), regulated by *miR390* and known to target several *ARF* genes, was differentially expressed from early to late embryogenesis. *TAS3* has been involved in the juvenile-to-adult phase transition through the negative regulation of *ARF* genes [117]. Zhang et al. [50] similarly observed the upregulation at the cotyledonary stage of *miR156*, targeting a crucial gene for the juvenile-to-adult transition (*SPL3*). It is strongly suggested that auxin-mediated, early cell fate decisions, such as root apical meristem (RAM) delineation, are contributing to apical-basal embryo polarization. During the same transition, upregulation of a putative TF gene from the KANADI family (*KAN2*) involved in the regulation of polar expression of auxin efflux-facilitating proteins genes from the PIN-FORMED family (PIN), as well as concomitant regulation of *PIN3* and a gene involved in the recycling of PIN proteins (*GNOM*), indicated active modulation of auxin flow. Interaction of *KAN* genes and *class III HD-Zip* (class III homeodomain leucin zipper) TFs with auxin has been involved in abaxial pattern formation, especially during emergence of cotyledon primordia. Accordingly, a *HD-Zip III* gene target of *miR166* was found to be upregulated at the early cotyledonary stage in *L. kaempferi* [24]. Furthermore, TF genes known as primary coordinators of polar auxin transport (auxin influx carrier, *AUX1*) and modulation of auxin transport (*NDL1*), possibly through regulation of *AUX1* and other PIN protein genes (*PIN2*), were also upregulated at early stages [19]. If such important TFs have conserved functions in plants, they may contribute to auxin-related spatiotemporal regulation of genes that are involved in the establishment of early embryo patterning, as well as activation of the auxin response machinery later during development. In conifers, most TF genes are probably regulated by miRNA themselves [24].

Expression of auxin-induced genes significantly increased from early cotyledonary to cotyledonary embryos in *P. glauca* [92], as was *ARF16* in *P. pinaster* [19]. Vestman et al. [2] found that the putative conifer *SUR1* is upregulated at the onset of late embryo development, while expression of *SAUR* was maintained at high levels, suggesting an increase in glucosinolate biosynthesis together with maintenance of a high IAA level. Upregulation of genes encoding the auxin-induced protein (IAA11), auxin receptor (TIR1), TF regulator of auxin-responsive gene

(MYB77) and a positive regulator of brassinosteroid signaling suggested that auxin-responsive gene expression is being activated. At the transition from pre-cotyledonary to early cotyledonary embryos in *P. pinaster* ZE, a putative TF gene regulated by auxin and involved in SAM function (*ANT*) was significantly overexpressed. At later stages, upregulation of a putative TF gene from the YABBY family (*YABBY2*) with polar expression resulting from interplay with *KAN* and *phabulosa* genes is consistent with determination of adaxial–abaxial cell fate [19]. Similarly, downregulation in cotyledonary embryos of a putative TF gene, required for the establishment of leaf primordia adaxial–abaxial polarity (*AS2/LOB*) and repression of meristem-related homeobox genes of the KNOTTED1-like class (*KNOX1*), indicated that the formation of SAM and organ boundaries had started. Expression of some *KNOX1* genes in *P. abies* (*HBK2*, *HBK4*) is specific of competent EM to form cotyledonary embryos [56]. Delayed expression of *HBK2* and *HBK4* in NPA-treated lines resulted in embryos lacking SAM. Similarly, KNOTTED-like genes upregulated during early embryogenesis are downregulated at later stages in *P. glauca* [92].

Upregulation of a putative member of the NAC domain (specific DNA-binding domain in the N-terminal region) TF family, which is involved in downstream auxin signaling (*NAM/NARS2*), further suggested that SAM formation was initiated. *NARS2* is also upregulated in *P. abies* from early embryo development to the onset of late embryogenesis [2], indicating that delineation of important tissue might be ongoing as embryos start to develop. *NAM* and also other members of the large NAC domain TF family regulated by PIN1 (auxin carrier proteins), such as *CUC* (cup-shaped cotyledon) genes 1 and 2, are crucial for the initiation of SAM, as well as the formation and separation of aerial organs. A member of the polycomb group (Pc-G) protein (curly leaf, *CLF*), a part of the polycomb repressive complex 2 (PRC2) involved in chromatin remodeling, was increasingly upregulated towards the mature ZE stage in *P. pinaster* [19]. Both *CUC2* and *PIN1* genes have been described as target genes for Pc-G proteins. Expression of a NAC homologue of *CUC1* and *CUC2* in *P. abies* (*PaNAC01*) was regulated by polar auxin transport and was associated with SAM differentiation and formation of separated cotyledons [59]. A *CUC1*-like gene was similarly regulated during maturation of SE in *Araucaria angustifolia* [45], as was a *PIN1*-like gene from pre-cotyledonary to cotyledonary SE stages in *P. abies* [68, 69]. The embryo apical parts accumulated more IAA, especially in the protodermal cell layer where *PIN1*-like expression was high. NPA treatment of embryos before cotyledon initiation disrupted this pattern and resulted in deregulation of both *PIN1*-like [69] and *WOX2* [118], one of the WUS/WOX TF family members activated during embryo development [17, 67]. It is suggested that

correct auxin transport is crucial at the transition from early to precotyledonary embryos and is involved in the coordinated regulation of *WOX2* and *PIN1*. Polar auxin transport may proceed through actin-dependent PIN proteins cycling between cytoplasmic membrane and the endosomal compartment. A putative gene encoding a small Rab-related GTP-binding protein (*PpRab1*) involved in ER-to-Golgi vesicle transport was differentially expressed throughout embryo development in *P. pinaster* [81]. Differential expression of *WOX* members, as a function of auxin flow and through a regulatory loop with CLAVATA1 (*CLV1*), has been proposed as one mechanism contributing to delineation of different embryo domains [2, 17, 68]. CLV1-like genes are apparently expressed from early to late SE development in *P. glauca* [92], and *P. pinaster* and *Pinus pinea* [79].

2.4.5 Other Important Regulators for Early Establishment of Embryo Body Plan

Several genes known as important developmental regulators during early somatic embryogenesis, such as *SERK1* (cell reprogramming) and *WOX2* (cell fate decision, domain delineation), were found continually expressed during SE development in *P. abies* [2]. A similar pattern was observed with genes involved in the organization of cell division (*FK/fackel*, *RBR1/retinoblastoma-related1*) and SAM formation (*PNH/pinhead*), suggesting that embryo patterning is to some extent organized from the early embryo stages. *PNH* is a member of the Piwi Argonaute ZWILLE family known to act together with *AGO1*, a member of the argonaute family taking part in the RNA-induced silencing complex. In conifers, *AGO* genes are required for embryo development [19, 71, 85] and are themselves regulated by miRNA (e.g., *miR168*) [50]. In *P. pinaster* ZE, *AGO* genes were highly represented in late embryos and may be mediators of either 24-nt-long siRNA (*AGO9*, silencing of transposable/repetitive elements) or miRNA and other siRNAs (*AGO1*). Several other genes were regulated towards late stages, such as upregulated dawdle (*DDL*) and hyponastic leaves1 (*HYL*) or downregulated dicer-like1 (*DCL1*) and flowering locus CA (*FCA*) [19]. A decrease of *DCL1* expression was similarly observed in *L. kaempferi* with feedback regulation by *miR162* [50]. High expression of *DCL1* at early stages may prevent precocious expression of important TFs through TAS-derived, siRNA-triggered DNA methylation [19]. *AGO-like* genes were upregulated at early embryo stages in both *P. glauca* [92] and *A. angustifolia* [45]. In *P. glauca AGO* genes were preferentially expressed in SAM and RAM and deregulation resulted in severe embryo abnormalities [71]. Overexpression of a KNOX1 gene (*HBK3*) in *P. abies* proliferating EMs resulted in the upregulation of *AGO* and accelerated SE development with enlarged SAM areas [55]. Proper SAM formation in *P. glauca* SE was also suggested by overexpression of related *ZWILLE* (stem cell maintenance within SAM along with *WUS/WOX*), *KNOTTED*-like (*see* Subheading 2.4.4) and

FIDDLEHEAD genes (*FDH*, cell division and differentiation). Developmental regulators differentially expressed in *P. abies* [2], and supporting that embryo pattern formation starts very early, include homologues of *PDF2* and *LUG*, *LBD12/ASL5*, *LBD15*, and *LBD40*. Expression of a *PDF2-like* gene (protodermal factor 2, an HD-GL2 homeobox gene) in *P. abies* protodermal cells (*PaHB1* gene) is an indicator that SE protoderm will be formed [58]. The expression pattern of a lipid transfer protein (LTP) gene (*Pa18*) was also associated with differentiation of protoderm and adjacent outer cell layers [64]. A similar nonspecific *LTP* gene (*PpAAI-LTSS1*) was upregulated from pre- to early-cotyledonary ZE in *P. pinaster* [80]. Deregulation of *Pa18* in proliferating EM negatively impacted SE morphology and growth [62]. A different HD-GL2 gene (*PaHB2*) is also expressed in proliferating EMs and early SE but its pattern becomes restricted to the subepidermal cell layer in the mature embryo [65]. This gene could be involved in specification and maintenance of the cortex identity. Both *PaHB1* and *PaHB2* are suggested markers to monitor radial pattern formation in *P. abies*. Another important gene in early delineation of radial patterning (embryonic root) is *SCR* from the GRAS TF family (*scarecrow*). *SCR* expression was upregulated in *P. glauca* EMs shortly after transfer to maturation conditions [92], as well as in cotyledonary embryos from *P. taeda* (ZE) [88] and *P. radiata* (SE) [83]. *SCR-like* members appeared to be regulated by *miR171* in *L. kaempferi* [46, 48, 50]. Genes encoding LOB domain-containing (LBD) and/or asymmetric leaves-like (ASL) proteins (LBD12/ASL5, LBD15, LBD40) are identified as regulators of the formation of lateral shoot boundary regions ([2], and references therein). *LUG* may have an early role in the specification of EM cells by contributing to prevent ectopic expression of homeotic *AGAMOUS* gene. *ROXY1* is another negative regulator of *AGAMOUS*, downregulated at the switch from the early to late embryo stages [2]. Expression of *LEC* genes can directly induce *AGAMOUS* during early embryogenesis, which in turn upregulates gibberellin 2-oxidase (*GA2OX* gene) and decreases gibberellic acid (GA) synthesis [20]. Downregulation of *LEC1-like* genes was observed at the transition from early to late embryo [2]. Increased GA biosynthesis was concomitantly supported by downregulation of *GA2OX*, one gene involved in GA catabolic process and upregulation of negative regulators of GA signaling (*SPINDLY*, cotyledon formation) and response pathways (*RGL1*). As *LEC* genes are also associated with auxin biosynthesis [20, 75], it became apparent that expression of *LEC* and other regulators of *AGAMOUS* are involved in spatiotemporal modulation of auxin- and GA-mediated responses. The temporal and organ-specific expression of homeotic genes such as the coordinated *AGAMOUS* and *APETALA2* [52] therefore appeared to have direct implication in embryo patterning. Both genes are likely to be controlled

by epigenetic regulators, such as *MSI1* encoding a core protein of the PRC2 complex similarly regulated in *A. thaliana* and *P. pinaster* [19]. Part of the complex machinery involved in embryo patterning is therefore conserved between angiosperms and gymnosperms [2, 19].

2.4.6 ABA-Mediated Developmental Switch from Embryonic to Vegetative Growth

Global alteration in gene expression was observed in EM matured on ABA-containing medium in conifers [1, 25, 27, 92]. In combination with other triggers (e.g., sucrose, PEG, gellan gum), ABA stimulates the development of late stage embryos. ABA may alter EM responsiveness to PGRs (auxin, GA) and may promote establishment of the embryo body plan [92]. An exogenous supply of ABA is needed in vitro as it is essentially provided by the megagametophyte [77].

As previously discussed, the key TF developmental regulator-*LEC1* is expressed during early embryogenesis [2, 45, 61, 70, 75, 76], and then becomes significantly downregulated at the onset of late embryo development promoted by exogenous ABA [2, 61]. The resulting putative modulation of both auxin- and GA-mediated signaling pathways could be involved in the developmental switch from embryonic to vegetative growth. *LEC1* (HAP3 subunit) and *LEC2* (B3-domain) TF genes are part of a complex regulatory network with additional B3-domain genes, such as *ABI3* (ABA insensitive 3) and *FUSCA/FUS3* (fused cotyledon 3), resulting in direct or indirect ABA-dependent regulation of genes [2, 28, 119]. These four genes are known as the master regulators of late embryogenesis in *A. thaliana* [120]. They act synergistically to regulate the expression of important downstream pathways, e.g., carbohydrate metabolism, biosynthesis of storage proteins, LEAs or fatty acids. *FUS3* is a central regulator promoting increased endogenous ABA synthesis while decreasing GA levels. The ABA signal transduction cascade involved inactivation of ABA-insensitive (ABI) protein phosphatases 2C (PP2C), promoting phosphorylation of serine/threonine residues and activation of Sucrose non-fermenting 1 (Snf1)-related protein kinases 2 (SnRK2) and other calcium-dependent kinases. SnRK2 subsequently activates downstream targets, especially ABA-response elements binding the HD leucine zipper (B-ZIP) TF from the ABF/AREB/ABI5 clade [120, 121]. Expression of *ABI3* and *FUS3* is further triggered by exogenous sugar [120]. A *FUSCA/FUS3* homologue was differentially expressed in *P. glauca* during late SE development [92]. Several *PP2C* and *SnRK2* transcripts were significantly expressed in *P. pinaster* and endogenous ABA level increased after 4 weeks of maturation, suggesting an ontogenetic signal for SE development [25]. *ABI3* was upregulated at the transition to late SE development in *P. abies* [2] as well as *ABI4* (sugar signaling), a *B-ZIP* TF, several genes encoding LEAs, and a heat shock TFs. Homologues of *ABI3*, *4*, and *5* were similarly regulated in *P. taeda* [88].

Upregulation of LEA and HSP genes was also observed at late SE stages in *P. abies* [23] and *P. glauca* [92] during SE and ZE development in *P. taeda* and during SE maturation in *Pinus oocarpa* [77, 86]. Both LEAs and HSPs have been associated with the acquisition of embryo desiccation tolerance [92] (*see* Subheading 3). The fatty acid elongase gene *FDH* (epidermal cell differentiation) and an extracellular dermal glycoprotein (*EDGP*) were also downregulated [2], as was a *B-ZIP* gene at the transition to early ZE in *P. pinaster* [19]. Similarly, one *HD-ZIP I* gene gradually decreased with maturation of late SEs and ZEs in *P. taeda* [77] and *P. glauca* [72]. In contrast, a lower but sustained *HD-ZIP I* expression was observed in maturing *P. oocarpa* SEs [77]. Upregulation of an *ABI3* homologue (*PaVP1*) was similarly reported in *P. abies* and *P. sylvestris* [60, 61, 111]. In *P. abies*, *PaVP1* expression was specific to EM and was maintained at a high level in productive lines until the cotyledonary stage [60]. Both *LEC1* and *PaVP1* expression are affected by the histone deacetylase inhibitor trichostatin A, suggesting possible control by chromatin remodeling [61]. Several histone deacetylase genes were revealed as important epigeneticregulators regulated from early (*HD2C*, *HDA2*) to late embryogenesis (*HDA8/9*) in *P. pinaster* [19]. Other differentially expressed genes involved in H3K9 methylation (*SUVH1*) or encoding chromatin-remodeling ATPases may have a similar role in the organization of transcriptionally repressive chromatin. In *P. abies*, Vestman et al. [2] also revealed the downregulation of a gene from the WRKY TF family, known to act downstream of the PP2C-ABA receptor complex and to target ABA-responsive genes (e.g., *ABF2-4*, *ABI4-5*, *MYB2*, *DREB1a-2a*, *RAB18*) [122]. Accordingly, several genes activated by ABA are upregulated in *P. abies* [2], including putative DREB TFs (response to dehydration), Angustifolia 3 (*AN3*) and its target growth regulating factor 1 (*GRF1*), both involved in initial leaf morphogenesis, and a NAC domain-containing protein gene (*ANAC009*) expressed in growing tissues. Expression of the TF gene encoding *MYB33* homologue was shown to decrease during late embryo development [47]. *MYB33* and other *GAMYB-like* genes are positive regulators of ABA response and are essential for the temporal regulation of development. Interestingly, *MYB33* was found to be regulated by *miR159* in *L. kaempferi* as in *A. thaliana*. Expression of this miRNA is induced by ABA in an ABI3-dependent mode ([47], and references therein).

2.4.7 Changes in Metabolisms and the Link with Important Processes

During the transition from early to late SE development in *P. abies*, Vestman et al. [2] observed an increase in carbohydrate metabolism as revealed by the upregulation of phosphofructokinase 2 (*PFK2*) involved in glycolysis and sucrose synthase 3 (*SuSy3*) genes. In *P. glauca*, many genes involved in sucrose catabolism (glycolytic and tricarboxylic acid pathways) were downregulated at the SE

cotyledonary stage [92]. Conversely *SuSy* was upregulated from pre- to early-cotyledonary embryos. Activation of the glycolytic pathway in unfavorable conditions for *P. pinaster* SE maturation was revealed by transcriptomic, proteomic, and carbohydrate analyses [25]. Active carbohydrate catabolism at the onset of SE maturation may preclude embryo development. In favorable maturation conditions, various ubiquitine protein ligases genes were overexpressed, as well as ubiquitin-/small ubiquitin-related modifier (SUMO)-conjugating genes [25]. Ubiquitine protein ligases are associated with SUMO activation (a chromatin modifier), suggesting that maturing early SEs were subjected to global modifications of gene expression. Ubiquitin protein ligases are also activators of the PGR-regulated ubiquitin/26S proteasome pathway resulting in controlled proteolysis with increased supply of amino acids. Accordingly, upregulation of a 26S proteasome subunit gene (*RPN1*) was observed early in *P. taeda* [77], suggesting that controlled proteolysis is active at the developmental switch from early to late embryo. This pathway is also activated in *P. radiata* early SE and later embryo stages, as revealed by the expression of a gene from the OTUBAIN family of cysteine proteases involved in removing the ubiquitin chain of protein destined for degradation [82].

Transcripts, as well as corresponding storage proteins of the legumin- and vicilin-like classes, were shown to increase with a similar pattern in developing SE and ZE in *P. taeda* [77, 123] (*see* also Subheading 3). Interestingly, suboptimal conditions for SE maturation in *P. oocarpa* and *A. angustifolia* resulted in lower expression of genes encoding legumin- and/or vicilin-like storage proteins [45, 77]. In *P. glauca*, deposition of storage proteins was increased in cotyledonary SEs obtained on PEG-containing maturation medium [92], in accordance with the upregulation of two genes encoding glutamine or glutamate synthase (GS/GOGAT cycle). Activation of nitrogen assimilation through the GS/GOGAT cycle may result in increased available glutamine for storage proteins synthesis. Expression of the two glutamine synthase genes, associated with green (*GS1a*) or vascular tissues (*GS1b*), was studied in *P. pinaster* and *P. sylvestris* embryos. *GS1a* was expressed in SE but not in ZE cotyledons, suggesting precocious SE germination [78]. Expression of *GS1b* was detected in proliferating early SE and in procambial cells of both cotyledonary SE and ZE, suggesting that glutamine biosynthesis is effective a long time before differentiation of mature vascular elements. *GS1b* expression was proposed as a marker of SE quality [78]. An increase in endogenous polyamines levels (spermine, spermidine), another important class of nitrogen compounds, was observed during SE maturation in *P. glauca* ([92], and references therein). Polyamines synthesis requires decarboxyl-SAMet (*S*-adenosyl-methionine). SAMet originates from methionine (SAMet synthase activity) and is also a direct precursor of ethylene (ACC synthase/oxidase activities) and

a methyl donor in transmethylation mechanisms resulting in DNA or histone methylation. Methyl residue transfer from SAMet results in the production of S-adenosylhomocysteine (SAH), which is recycled into methionine through production of adenosine (SAH hydrolase). AMP is further produced from adenosine by adenosine kinase (AK). Stasolla et al. [92] found that an *ACC oxidase* gene was upregulated at the SE cotyledonary stage in *P. glauca* whereas several *AK* genes were downregulated, indicating active ethylene synthesis. High expression of *ACC synthase* genes was also observed in this species (*PgACS1*) [74] and in *P. sylvestris* (*PsACS2*) [84], especially at the early cotyledonary stage. *PsACS2* expression was proposed as a marker of competent embryo development in *P. sylvestris* since it was positively correlated with both ethylene production and embryogenic potential. Vestman et al. [2] similarly reported a strong increase in *ACC oxidase* expression in *P. abies* at the developmental switch to late embryogenesis. Upregulation of *SAH hydrolase and methionine synthase* and expression of *SAMet synthase* genes were also observed after induction of embryo development [23, 90]. Expression of these genes supported active transmethylation events resulting in DNA or histone methylation that may contribute to the global transcriptional repression state observed at specific embryo stages [23]. In *P. pinaster*, both DNA methylation and heterochromatin maintenance were important processes at the onset of embryo maturation through transposable element-specific DNA methylation resulting in heterochromatin formation (*DDM1*), RNA-directed methylation of various transposable elements (*FCA*), and regulation of DNA methylation involved in regulation of chromatin structure (*ORTH2/VIM1*) [19]. In accordance with the increased level of SAMet synthase proteins from early to late embryo stages in conifers (*see* Subheading 3), normal embryo development appeared associated with the modulation of polyamine and ethylene synthesis, as well as with epigenetic regulation of gene expression.

2.4.8 Defense Genes and Maintenance of Redox Homeostasis

As also revealed from proteomic and metabolomic studies (*see* Subheadings 3 and 4, respectively), a general trend towards regulation of genes involved in response to stress was observed at the transition to early embryo development and also during late development. The modulation of gene response to stress is required for proper embryo development and could be of practical interest to improve maturation protocols [2, 21]. In addition to genes encoding proteins related to embryo tolerance to desiccation (LEA, HSP) (*see* Subheading 2.4.6), various genes involved in defense and maintenance of redox homeostasis (oxidative stress) were differentially expressed [2, 19, 23, 25, 42, 92]. Defense genes were activated in suboptimal maturation conditions promoted by abiotic stresses, such as anoxia. Both *alcohol dehydrogenase* and *pyruvate decarboxylase* genes were upregulated in *P. pinaster* EMs during maturation in unfavorable conditions [25]. Regulation of

these genes was in agreement with activation of the glycolytic pathway, but they might also be involved in anoxia tolerance through alcoholic fermentation as suggested by increased expression of *SuSy3* gene. *SuSy* genes are responsive to low oxygen level and promote adequate sugar supply under anaerobic conditions. A *bifunctional enolase 2* gene related to stress-regulated transcriptional networks was concomitantly upregulated, as also observed during early embryogenesis in unproductive *P. abies* lines [23]. In contrast, *enolase* genes were upregulated in productive lines of *P. radiata* [1]. *Enolase* gene expression may reveal suboptimal maturation conditions (oxygen or other abiotic stress) or, alternatively, enhanced carbon metabolism as enolase is also involved in glycolysis/gluconeogenesis. Additional regulated genes with a likely defense function to overcome culture constraints include a putative *cytochrome P450* gene and genes related to biosynthesis of secondary metabolites. *Cytochrome P450* was expressed during early embryo development in *P. radiata* [89] and downregulated in *P. abies* cotyledonary SE [23]. This gene encodes monooxigenases involved in plant responses to PGRs (e.g., ABA, IAA) and environmental stresses such as osmotic stress. Pathways related to secondary metabolism such as phenylpropanoids or flavonoids were overrepresented in the transcriptome of *L. kaempferi* proliferating EMs [24]. Flavanone 3-hydroxylase gene (*F3H*) involved in flavonoid biosynthesis was downregulated in *P. abies* during EM proliferation [23], whereas *F3H* and several genes related to flavonol metabolism were strongly upregulated at the onset of SE maturation in *P. pinaster* [25]. Activation of flavanone hydroxylation and the subsequent production of condensed tannins have been associated with stress resistance in plants. Surprisingly, genes involved in response to pathogens are also regulated during embryo development in *P. abies*, particularly during transition from early to late embryogenesis [2]. A positive regulator (*SN1I*) of the systemic acquired resistance (SAR) response was expressed during early somatic embryogenesis in *P. radiata* [1]. This gene contributes to the regulation of pathogenesis-related (PR) protein genes and salicylic acid-mediated transduction of the SAR signal.

The maintenance of an efficient cellular homeostasis by redox antioxidant metabolites, such as glutathione, is critical to regulate oxidative stress and the associated production of reactive oxygen species (ROS), free radicals and hydrogen peroxides resulting from aerobic metabolism [2, 19, 23, 42, 92]. Redox homeostasis may represent a generic sensor for controlling embryo development [23] through PCD activation by ROS [49] or interplay of glutathione with NADP-linked thioredoxin in the frame of auxin transport and signaling. Both SE yield and quality are affected by deregulation of glutathione metabolism [124]. Expression of *cytosolic ascorbate peroxidase* and *thioredoxin H* genes, involved in both detoxification and control of the cellular redox state, were downregulated during EM proliferation in *P. abies* productive lines [23].

It was suggested that high oxidative stress could alter the activity of ascorbate peroxidase in unproductive lines and preclude embryo development [125]. Metabolic activity in developing *P. pinaster* ZE was reflected by a general overrepresentation of oxidation–reduction processes with a prevalence of glutathione metabolism (glutathione thiolesterase activity, expression of *glutathione transferases*), especially during early development [19]. Related genes were regulated in *P. glauca* [92] during early SE development (*glutathione-S-transferase*, *glutathione peroxidase*) at the transition from pre- to early-cotyledonary embryo (*glutathione reductase*) and at the cotyledonary stage (*glutathione peroxidase*, *ascorbate peroxidase*). Genes encoding another well-known antioxidant enzyme (*superoxide dismutase*, *SOD*) were similarly upregulated at the cotyledonary stage. In *L. kaempferi* expression of a *Cu/Zn SOD* gene (plastocyanin) putatively regulated by *miR398* increased at the pre-cotyledonary stage [24]. *SOD* genes were not differentially expressed at the onset of late SE development in *P. pinaster*, but resulted in enhanced SOD protein production (*see* Subheading 3). Differential expression of SOD proteins may relate with the concomitant upregulation of *GLP* genes. Germins and GLPs are usually located in the extracellular matrix where they can have both enzymatic (oxalate oxidase, SOD) and nonenzymatic activities (auxin-binding protein, serine protease inhibitor), associated with either response to stress or developmental regulation ([51], and references therein). Germin and GLP enzymatic activities result in the production of hydrogen peroxide that may be involved in cell wall remodeling during stress responses and/or development. Two *GLP* genes most similar to a germin with putative oxalate oxidase activity were specifically expressed in proliferating EMs of *P. radiata* [89]. In *L.* x *marschlinsii*, expression of a *GLP* gene in proliferating EMs (*LmGER1*) was associated with SOD activity in apoplastic proteins extracted from early SE [51]. *LmGER1* expression was located in suspensor cells and at the junction with EM during proliferation and persisted at the embryonal root cap after transfer to maturation medium. Interestingly, *LmGER1* expression corresponded to the pattern of active PCD during embryo development in conifers. Downregulation of *LmGER1* in proliferating EM resulted in reduced SE yield, asynchronous development, and precluded plantlet regeneration.

3 Proteomics of Conifer Somatic and Zygotic Embryo Development

The development of high-resolution, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), coupled with chromatographic separation and identification through mass spectrometry (MS), has allowed increased and untargeted qualitative proteome coverage, together with quantitative measurements of proteins

involved in plant development [126, 127]. Various proteome variations can be expressed by the same or different genomes according to ontogenetic programs or as a major component of phenotype plasticity. Many fundamental activities performed by proteins (especially enzymes) are involved in most metabolic and signaling pathways. Proteomics therefore aims to identify and assign physiological functions to "candidate proteins," contributing to developmental processes and valuable traits.

Despite significant inputs of quantitative proteomics to molecular identification and functional characterization of embryogenesis-related genes in model and crop plants [20, 36], there are relatively few recent contributions in conifers (Table 2) focused on somatic [4, 25, 93, 96, 100–103] and/or zygotic embryogenesis [95, 97–99, 104, 105]. First-generation approaches are efficient, but have high experimental and technical requirements [126, 127] and can be biased towards hydrophilic proteins [103]. New methods such as 2D difference gel electrophoresis (2D-DIGE) and unbiased 1D SDS-PAGE combined with isobaric tags for relative and absolute quantitation (iTRAQ) will considerably facilitate the identification of differentially expressed proteins and will offer a more global view of the proteome dynamics [103, 104]. Moreover, the identification rate of multiple proteins in large proteomic datasets is still challenging because it largely depends upon the availability of exhaustive genome resources [4, 7, 101, 127]. This information is expected to substantially expand according to completed, publicly available genome sequences [11–13].

Published proteomics studies of embryo development in conifers are currently restricted to nine species from the *Araucaria*, *Cunninghamia*, *Cupressus*, *Larix*, *Picea*, and *Pinus* genera (Table 2) and are mainly focused on late SE or ZE development. Proteomics considerably enhanced the sensitivity and scale (up to 1000 spots detected per gel) of protein expression studies during embryo development [4, 100, 102]. Previous qualitative or semi-quantitative methods were often restricted to a few major proteins, especially storage proteins [123, 128]. Pioneering investigations of temporal protein changes in *P. abies* [129] and *Cupressus sempervirens* [100] revealed the large sets of protein expression patterns that can be associated with embryo developmental stages. The paradigm shift in technology resulting from proteomics was further illustrated in *P. glauca* [4]. Most differentially expressed proteins (79 %) identified in this work were indeed new proteins not previously associated with embryo development. Biological and functional relevance of new candidate proteins may be elucidated and ultimately provide opportunities for refining the somatic embryogenesis process.

Here, we briefly review the importance of specific protein functional classes that were either validated (storage proteins) or reinforced (metabolic/cellular processes, stress-response proteins) by proteomics studies of embryo development.

3.1 Storage Proteins

Developing SEs and ZEs in conifers have been shown to accumulate major storage proteins of the globulin (legumin, vicilin) and albumin families based on electrophoretic mobility patterns. These assumptions were validated by MS approaches in *P. strobus* [128], *P. pinaster* [105], *P. glauca* [4], *P. abies* [93], and *L.* x *eurolepis* [102]. Expression pattern of storage proteins was similar in SE and ZE [4, 102, 105, 128], reaching a maximum at the cotyledonary stage. Storage proteins were already detected at the late stage of early embryogenesis (*P. glauca*) or at the pre-cotyledonary stage (*L.* x *eurolepis*). Protein accumulation and SE growth are affected by maturation duration and cultural conditions [93, 128]. Routine tracking of the main storage proteins may be valuable for assessing the quality of matured embryos. The most dominant vicilin-like storage proteins have been proposed as markers of SE development in *P. glauca* [4] and *L.* x *eurolepis* [102]. Similarly, three vicilin- and legumin-like proteins as well as two cupin domain-containing storage proteins were identified in *P. pinaster* as candidate biomarkers for the late cotyledonary SE/ZE stage [105].

3.2 Proteins Involved in Metabolism and Cellular Processes

The activation of various metabolic and cellular processes during SE development could be emphasized by proteomic data. A comparison of immature and mature embryos in *L.* x *eurolepis* showed an increase in proteins involved in primary metabolism (glucose, pentose, starch), suggesting active glycolysis, nucleotide metabolism, and accumulation of storage carbohydrates [102]. However, in both *L.* x *eurolepis* [101] and *P. pinaster* [25], the glycolytic pathway appeared to be reduced under favorable maturation conditions (high gellan gum concentration). Reduced water availability induced by high gellan gum may promote a decrease in carbon catabolism through downregulation of key proteins involved in glucose or pentose metabolisms. The decreased level of glycolysis in EM cultivated on favorable maturation medium has been associated with increased embryo dry weight (*L.* x *eurolepis*, *P. pinaster*) and enhanced starch accumulation (*P. pinaster*) at both cytological and proteomic levels (e.g., upregulation of glucose-1-phosphate adenylyltransferase). Upregulation of proteins involved in amino-acid metabolism was also highlighted in *Larix* [102] at the mature embryo stage, and is indicative of active protein synthesis as observed during zygotic embryogenesis in other conifers [95, 98, 99, 104]. Accordingly, the maturation treatment was reported to induce changes in nitrogen metabolism in mature embryos of *P. abies* [93] through differential expression of key enzymes for glutamine, glutamate and arginine synthesis. In accordance with gene expression studies (*see* Subheading 2.4.7), differential expression of various proteasome subunits in *P. pinaster* [25] and *P. abies* [4] as well as elongation factor II protein during EM proliferation in *A. angustifolia* [96] supported the importance of controlled proteolysis and protein synthesis when embryos are stimulated to

develop. Lippert et al. [4] proposed the proteasome complex as a source of protein markers to evaluate embryo development. Teyssier et al. [102] also suggested that various differentially expressed proteins from the primary and amino-acid metabolisms are suitable targets for marker validation.

Other metabolic pathways with important roles in embryogenesis were also suggested to be activated as a result of enhanced amino-acid metabolism, especially methionine and SAMet synthesis. A SAMet synthetase was found to be upregulated in *L.* x *eurolepis* mature SE [102], expressed at various developmental stages of *P. glauca* SE [4] and expressed from proembryogeny to late ZE stages in *A. angustifolia* [95]. This protein is involved in DNA methylation, polyamines and ethylene biosynthesis. Proteome analysis in *A. angustifolia* revealed a set of ten proteins unique to eight responsive or two recalcitrant lines to maturation treatment [96]. It is suggested that embryogenic potential could be associated with upregulation of SAMet synthetase during EM proliferation. Interestingly, Jo et al. [96] provided data showing increased ethylene release and lower putrescine content in responsive lines.

Other important cellular processes upregulated during normal embryo development included cell wall deposition and cell expansion in *P. pinaster* (e.g., expansin S2/B14) [25], *L. principis-rupprechtii* (e.g., α-1,4-glucan protein synthase) [103], and *P. abies* (e.g., reverse glycosylating protein RGP-1) [4], nucleocytoplasmic transport (e.g., tubulin beta-2 chain, GTP-binding nuclear proteins Ran-A1) in *P. pinaster* [25], regulation of membrane trafficking (e.g., ADP-ribosylation factor GTPase-activating proteins) in *L. principis-rupprechtii* [103], or energy metabolism in *A. angustifolia* (e.g., mitochondrial ATPase beta subunit) [96, 97] and *P. abies* (e.g., ATP synthase, H+ transportin) [4]. ATP production and catabolism have been associated to competent embryo maturation and structural reorganization via PCD. In *P. pinaster*, active PCD was revealed by combined analysis of transcriptomic and proteomic datasets, showing upregulation of both chitinases and disulfide isomerase [25]. A nondefensive role of chitinase IV in early SE development was also supported in *L. principis-rupprechtii* [103].

3.3 Stress-Related Proteins

Available proteomics studies emphasized the omnipresent "background" expression of stress-related proteins during SE development and maturation. This is in accordance with the datasets provided by transcriptomic and metabolomic studies (*see* Subheadings 2 and 4, respectively). Such proteins represented up to 6.7 % of differentially expressed proteins in *P. abies* [93] and are mainly involved in response to oxidative stress, anoxia, prevention of apoptosis, and tolerance to cellular dehydration. Oxidative stress may be induced by water and/or osmotic stress and it results in production of ROS, ATP depletion and, ultimately, in apoptosis [130]. The SOD enzyme involved in detoxification processes

through regulation of oxidative stress was found overexpressed in early developing SE in *P. pinaster* [25] and in mature SE in *L.* x *eurolepis* [102]. A similar pattern was observed during ZE development in *P. massoniana* [104]. A GLP was also overexpressed in *P. pinaster* [25], suggesting active antioxidant protein production. The interest of GLPs as predictive markers of embryo development is well supported by proteomics ([25], and references therein). Accordingly, suboptimal conditions for embryo maturation in *L.* x *eurolepis* resulted in upregulation of SOD and activation of secondary metabolism enzymes, possibly to cope with increased production of free radicals [101]. Similarly, Jo et al. [96] revealed that NADH dehydrogenase in *A. angustifolia* was upregulated in one recalcitrant line to a maturation treatment, thus suggesting a disturbed cell redox system. NADH dehydrogenase is a component of the plant energy-dissipating mitochondrial system preventing excessive ROS production. ROS were recently revealed as important signaling molecules for activation of PCD and normal SE development in *L. leptolepis* [49]. Overexpression of catalase (antioxidative enzyme) in non-embryogenic callus compared to EMs provided indirect evidence in *L. principis-rupprechtii* for excessive ROS generation in response to culture conditions [103].

Abiotic stress may also result from anaerobic conditions during in vitro culture. As previously discussed, enolase is involved in glycolysis/gluconeogenesis pathways but can also be induced by abiotic stresses such as oxygen levels. Enolase accumulates in *P. glauca* mature embryos and has been proposed as a putative protein marker of normal embryo development [4]. In *L.* x *eurolepis*, two enolase isoforms were found overexpressed after a suboptimal maturation treatment [101]. Overexpression in *P. glauca* of a submergence-induced protein at the early SE stages was interpreted as a possible response to oxygen stress promoting cell elongation in developing embryos. Several enzymes involved in the glycolytic pathway (e.g., alcohol dehydrogenase, pyruvate decarboxylase) were similarly activated under unfavorable SE maturation conditions in *P. pinaster* [25]. Interestingly, both alcohol dehydrogenase and pyruvate decarboxylase expressions were recently reported to be involved in tolerance to anoxia [131].

Various protein families with important protective roles during abiotic stresses resulting in cellular dehydration were confirmed to accumulate in mature embryos, including LEAs and group 2 LEAs (dehydrins), HSPs and small HSPs. Members of these protein families were upregulated in *P. abies* embryos matured with sucrose as a carbon source [93]. The presence of 3 % sucrose significantly improved SE germination rate by promoting the acquisition of desiccation tolerance. LEA and dehydrins were reportedly shown to accumulate in plants during late embryogenesis. In *L.* x *eurolepis* [102], a set of 21 proteins annotated as belonging to HSPs or related to protein folding were found differentially expressed in

developing versus mature embryos. Most were upregulated at the mature stage in accordance with the proposed role of HSPs in cellular protection (protein stabilization and refolding). HSPs and small HSPs were similarly detected in mature SE of *P. abies* [93] and were overexpressed in both cotyledonary SE and maturing ZE in *P. pinaster*, together with various LEAs [105]. HSPs were also found to accumulate at early SE stage in *L. principis-rupprechtii* [103], from early to mature SE stages in *L.* x *eurolepis* [102] and *P. glauca* [4], as well as during ZE development in *Cunninghamia lanceolata* [99]. HSP expression is known to be induced by ABA and there is strong evidence that these proteins are required throughout embryogenesis from initiation to early seedling growth ([102], and references therein). HSPs and other stress-related proteins are also overexpressed during maturation in suboptimal conditions [101]. Proteomics therefore strengthened both the protective function of HSPs in response to abiotic stress and their ubiquitous role in protein folding, assembly translocation and degradation during embryo development.

4 Metabolomics of Conifer Somatic and Zygotic Embryo Development

Metabolite profiling can be achieved in plants with high resolution and good sensitivity by using gas chromatography coupled with mass spectrometry (GC/MS) or nuclear magnetic resonance (NMR) spectroscopy. Both GC/MS and NMR spectroscopy are high-throughput techniques for unbiased acquisition of quantitative and qualitative data on multiple metabolites. These technologies are also suitable for time-series studies. Among all "omics," metabolomics is considered to provide the most functional information since metabolites are the end products of the cellular machinery. Multivariate data analyses are required to determine whether combined abundances of a set of metabolites can be associated with a specific physiological state. Metabolomics is an effective and increasingly popular approach in conifers for monitoring physiological responses to environmental variation [132]. Applications to embryo development are currently scarce (Table 2) because of the technical requirements involved and the integrated proteomics information (identification of enzyme substrates) needed to fully interpret the data [133].

Interestingly, metabolic profiling already provided relevant information in *Picea* species about the biochemical status of EMs at, or during, transition between different embryo developmental stages and in response to different maturation conditions [5, 93, 94]. The metabolic signature has also been demonstrated in *P. taeda* to accurately predict the ability of proliferating EMs to regenerate SE [6]. Therefore, it is expected that these studies will provide not only a better understanding of SE development, but

also tools for monitoring early metabolic events determining SE physiology. Metabolite profiling can be used to analyze intracellular metabolites (metabolic fingerprinting) or, alternatively, the metabolite composition of fresh and spent culture medium (metabolic footprinting). The latter noninvasive method is not affected by rapid turnover of intracellular metabolites and is likely to yield valuable information about critical metabolites, especially for the complementation and interpretation of metabolic fingerprints [6, 37]. Metabolic footprinting was performed in *P. glauca* (NMR spectroscopy) to identify significant metabolites (35 compounds detected) involved in SE proliferation and maturation [5]. Strong evidence for divergent metabolic processes and different EM physiological state in proliferation and maturation media was obtained within 48–72 h. Major sources of metabolic variation in culture media over time included carbohydrates, amino acids (consumption of medium compounds), and also processed metabolites excreted by the cultured cells. Early sucrose hydrolysis and preferential use of glucose over fructose by embryogenic cells was apparent in both conditions. Most other discriminating metabolites were overrepresented in the proliferation medium and were indicative of storage protein synthesis and regulation, nitrogen transport and ammonium assimilation (5-oxoproline, glutamine, BCAA/branched chain amino acids), response to various stresses and intracellular/inter-organ signaling (GABA/γ-aminobutyric acid), biosynthesis of phenylpropanoid compounds (phenylalanine) and cell expansion (malate). BCAA and GABA profiles are particularly suggestive of a metabolic imbalance as a result of altered coenzyme A biosynthesis during maturation. Such a metabolomic-generated hypothesis paves the way for expression studies of specific genes involved in this pathway in conifers.

In *P. abies*, metabolic fingerprinting (GC/MS) was used to study metabolic events involved in normal SE development [94]. Three different embryogenic lines with blocked, aberrant, or normal phenotype were investigated. Significant metabolites were identified from EM in proliferation through to cotyledonary embryos. Sucrose was revealed as the main carbohydrate in proliferating EM, whereas maltose was significant during late embryogenesis in the normal line. In contrast, a preponderance of fructose was observed in lines with abnormal phenotypes. Metabolite profiling therefore confirmed previous data showing that supplementation of maturation medium with maltose to promote nutritional stress (cellular carbon restriction) could improve embryo development ([134], and references therein). This hypothesis could be partially verified in *P. abies* as maturation yield increased when sucrose was replaced by a combination of maltose and PEG [93]. However, the latter formulation was detrimental to SE germination. Metabolic fingerprinting could separate samples according to maturation condition (45 compounds detected) and revealed that

SE treated with maltose and PEG accumulated less raffinose. The metabolite signature therefore suggested that poor germination rate results from reduced content in raffinose family oligosaccharides (RFOs) that are involved in the acquisition of desiccation tolerance together with sucrose and LEA.

Evidence suggesting that metabolic response to osmotic stress may be a key factor involved in normal embryo development was gained through metabolic fingerprinting of *P. taeda* proliferating EMs [6]. In this large study that detected 208 metabolites, embryogenic culture's regenerative capacity was not only influenced by the genetic background and maturation conditions, but also by the metabolic status of the proliferating culture at the time of sampling. It appeared that a culture containing developmentally advanced immature embryos is more likely to produce cotyledonary SE, as previously observed in other pine species [134]. Among the 47 identifiable metabolites selected to build a descriptive model of cell line ability to regenerate SE, several were related to osmoprotectants. Proline, serine and arabitol contents may be indicative of biological stress during proliferation as a negative relationship with culture productivity was observed. In contrast, a positive correlation was found with sorbitol accumulation, suggesting that some osmoprotective compounds may also play a role in preventing biological stress and preserving culture responsiveness to maturation treatments. The method was therefore highly efficient at identifying both informative metabolites and their relationships to gain insights into the transition from immature to mature SE. It is based on the multivariate analysis of a metabolite subset selected through a stepwise modeling procedure following the Bayesian information criterion. In addition, the model was demonstrated to accurately predict the regenerative capacity of proliferating EM in a genotype-independent manner. A robust assay based on multiple predictor metabolites accounting for genetic variability could prove invaluable in pine as the regenerative capacity is invariably, although erratically, decreasing as a function of line aging [15].

5 Conclusion and Future Directions

The molecular biology of conifer embryo development has begun to benefit from genome-wide approaches. Technical requirements are still high [22, 132, 135] and there are also strong limitations to the interpretation of these large datasets. The development of comprehensive genome resources [10–13] is expected to considerably increase the identification rate of differentially expressed genes. The "holy grail" will then be to characterize the function and molecular regulation of important genes in metabolic networks to model embryo development through integration of transcriptomic, proteomic and metabolomic data [20, 37, 38, 133].

Such a systems biology approach is likely to provide tested clues for the development of somatic embryogenesis in plants, including conifers.

"Omics" has started to improve our knowledge of conifer embryo development. Transcriptome profiling of embryogenesis-related genes in conifers has shown high homology with model angiosperms. It is suggested that differences in the molecular regulation of embryogenesis may mainly arise from spatiotemporal variation in gene expression. Several important processes are apparently conserved in plants [2, 19], in particular early organization of apical-basal embryo patterning driven by polar auxin transport and activation of the auxin-mediated response machinery during late embryogenesis (radial embryo patterning). Conserved expression profiles were also revealed for important epigenetic regulators (chromatin remodeling) involved in temporal and organ-specific expression of homeotic genes [19]. Transcriptomic studies have highlighted the complexity of processes and genes involved in the spatiotemporal development of embryos, from embryogenesis induction [20, 21] to the switch from embryonic to vegetative growth [2, 23, 25, 92]. A reference gene regulation network has been proposed for embryogenesis induction in plants [20], but there is a need for dedicated studies in conifers to further elucidate these pathways [21, 70, 76]. Transcriptome profiling and, to a lesser extent, proteomics have revealed multiple genes associated with early embryo and late embryo development. An impressive picture of coordinated functions and genes has been obtained during development of SE in *P. abies* [2, 23, 92], and of ZE in *P. pinaster* [19]. TFs genes appeared to have central roles in spatiotemporal modulation of both auxin- and GA-mediated responses, especially during early embryogenesis (e.g., *LEC* and *AGAMOUS*). Later during development, *LEC* genes and other master regulators revealed in *A. thaliana* (*ABI3* and *FUS3*) are likely to have similar roles in conifers, i.e., induction of ABA-dependent response that may modify EM responsiveness to auxin and GA, but also to other signaling molecules (polyamines, ethylene). Regulation of these pathways could be involved in the developmental switch from embryonic to vegetative growth. Various additional processes have been suggested to have general functions in development stages such as PCD, megagametophyte signaling, cell wall modification, epigenetic regulation (DNA methylation, small RNAs), carbohydrate, protein or energy metabolisms, and response to stress. Opportunity for modulation of any of these pathways could be of practical interest to refine specific steps of seed production or somatic embryogenesis in conifers.

Genome-wide profiling offers the possibility to check the quality of proliferating EMs and developing SE at the molecular level with unprecedented accuracy and throughput, showing that omics is already providing some important clues to improve conifer

embryo development. Early molecular screening can help preventing unnecessary expenses associated with EMs cultivated in unfavorable conditions and/or with low ability to be converted into high quality plantlets. There is a choice of proposed marker genes revealing specific processes and adaptation at each developmental stage or transition, from embryogenesis induction and initial demonstration of embryogenecity [21, 26, 70, 76, 89], to early embryo patterning [1, 2, 58, 65, 90] and late embryo development [19, 23, 25, 52, 77, 78, 84, 89]. Substantial support has been obtained for a few proteins proposed as robust markers of embryo development in *P. glauca* (vicilin, enolase, proteasome subunit) [4], *L.* x *eurolepis* (vicilin) [102] and *P. pinaster* (GLP, ubiquitin-protein ligase) [105]. More pragmatically, a selected subset of metabolites has been demonstrated in *P. taeda* [6] to accurately predict in a genotype-independent way the ability of proliferating embryogenic lines to regenerate cotyledonary SE.

There is also a large set of genes involved in epigenetic regulation that were repeatedly highlighted in transcriptomic studies with high relevance for proper embryo development [1, 19, 23, 50, 92]. In particular, expression of various miRNAs with stage-specific modulation was associated with the regulation of important genes during somatic embryogenesis in *L. kaempferi* [50], including genes involved in the regulation of auxin-mediated response, cell wall modification, embryo pattern formation, ABA response, oxidative stress and miRNA biogenesis. Some miRNA appeared to have functions in maintaining the embryogenic potential (*miR159*, *miR171*). It would therefore be interesting to infer the general significance of embryogenesis-related miRNAs in conifers.

Although SEs develop without true megagametophyte [2], there is a consensual trend toward approaching "substantial equivalence" of SEs with ZEs [26, 42, 77, 78, 91, 92]. Comparative "omics" of SE and ZE is a promising tool to elaborate new strategies to reach the performance standard of seedlings. Transcriptomic profiling in *P. taeda* [27] and proteomic analysis in *P. pinaster* [105] gave strong evidence that gene expression in cotyledonary SE obtained after "appropriate" culture time in "refined" maturation conditions did not conform to that of fully mature ZE, but to that of earlier, immature cotyledonary stages. Similar conclusions were made in *P. pinaster* after a study of genes involved in nitrogen metabolism and chloroplast development [78]. Data analysis suggested specific protocol refinements at either the maturation or post-maturation step. Optimization of SE maturation in *P. taeda* resulted in similar expression patterns of genes involved in controlled proteolysis and synthesis of storage proteins compared with ZE [77]. In *A. angustifolia*, comparative transcriptomics of SE and ZE revealed auxin signaling failure during SE development [91].

Another way to estimate if embryo quality could be enhanced is to compare different maturation conditions. In *P. glauca*, the beneficial effect of PEG in the maturation medium (improved SE yield and quality) could be demonstrated using a cDNA array strategy [92]. This study provided the first evidence that transcriptome profiling could predict embryo quality, as many regulated genes between PEG-treated and control lines were identified in early developing embryos. A similar approach integrating both transcriptomic and proteomic profiling was implemented in *P. pinaster* to study the early molecular events involved in SE development promoted by high gellan gum [25]. Differential expression of genes associated with embryo development or culture adaptive responses, as early as 1 week after exogenous ABA treatments, supported integrated genome-wide profiling as a robust diagnostic and predictive tool for detecting disruption of critical pathways for normal SE development. Interestingly, gene expression studies in *P. abies* have already influenced protocol improvement (accelerated and synchronized SE development) through either modification of maturation conditions (latrunculin B treatment affecting actin gene expression) [54] or genetic engineering of proliferating EM (*HBK3* overexpression promoting *AGO* upregulation) [55].

It is foreseeable that genome-wide profiling will be further implemented in both important species (to achieve cost-effective SE variety deployment) and orphan species (to save labor and associated cost of development). Integrating transcriptomic and proteomic approaches may inherently offer robust tools to assess embryo development [25]. Metabolomics may also provide unique opportunities for constructing genotype-independent, predictive models of embryogenesis-related traits. Interpretation of "omic" data may help identify new directions for gene expression profiling of selected metabolic pathways underpinning embryo development.

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Chapter 9

Androgenesis in Solanaceae

Jose M. Seguí-Simarro

Abstract

The Solanaceae is one of the most important families for global agriculture. Among the different solanaceous species, tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and pepper (*Capsicum annuum*) are five crops of outstanding importance worldwide. In these crops, maximum yields are produced by hybrid plants created by crossing pure (homozygous) lines with the desired traits. Pure lines may be produced by conventional breeding methods, which is time consuming and costly. Alternatively, it is possible to accelerate the production of pure lines by creating doubled haploid (DH) plants derived from (haploid) male gametophytes or their precursors (androgenesis). In this way, the different steps for the production of pure lines can be reduced to only one generation, which implies important time and cost savings. This and other advantages make androgenic DHs the choice in a number of important crops where any of the different experimental in vitro techniques (anther culture or isolated microspore culture) is well set up. The Solanaceae family is an excellent example of heterogeneity in terms of response to these techniques, including highly responding species such as tobacco, considered a model system, and tomato, one of the most recalcitrant species, where no reliable and reproducible methods are yet available. Interestingly, the first evidence of androgenesis, particularly through in vitro anther culture, was demonstrated in a solanaceous species, *Datura innoxia*. In this chapter, we report the state of the art of the research about androgenic DHs in Solanaceae, paying special attention to datura, tobacco, potato, tomato, eggplant, and pepper.

Key words Anther culture, Datura, Doubled haploids, Eggplant, Microspore culture, Microspore embryogenesis, Pepper, Potato, Tobacco, Tomato

1 Introduction

It is well known that crop productivity can be increased through the use of hybrids, made by crossing homozygous (pure) lines with defined traits. These lines are traditionally generated by techniques based on classical breeding, through successive rounds of selfing and selection. This requires a considerable amount of time and resources. However, in recent years alternative techniques, by far more advantageous than traditional methods, are being used in some species. These techniques, based on androgenesis, produce pure, doubled haploid (DH) lines through in vitro regeneration of plants from microspore/pollen-derived embryos or callus.

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This experimental pathway, alternative to normal pollen development, was discovered 45 years ago by Guha and Maheshwari [1]. In this route, the pollen grain precursors deviate from the gametophytic pathway and are in vitro induced to form haploid embryos or calli [2]. Then, plants can be directly regenerated by microspore-derived, haploid embryogenesis, or indirectly from an intermediate haploid callus phase. These plants will be DH if they duplicate their original haploid genome, or just haploid as the original microspore. In the latter case, additional treatments to promote genome doubling are needed [3]. In both cases, the resulting plants will have a genetic background exclusively coming from donor (male) plant, and 100 % homozygous. In other words, they will be pure lines. From the standpoint of plant breeding, this alternative reduces the typical 7–9 inbreeding generations necessary to stabilize a hybrid genotype to only one. It is therefore much faster and cheaper, and obviously this is the main advantage of DH technology in the context of plant breeding. Within this same context of plant breeding, DHs (homozygous for all of their loci) constitute a valuable tool for the study of the genetic basis of quantitative traits, including the genetic mapping of complex characters such as production or quality, the most agronomically interesting, and difficult to be addressed by other approaches. In fact, they have been successfully used in several crops for breeding plants with useful agronomic traits such as high yield, earliness, abiotic stress tolerance, and disease resistance, among others. DHs are also a powerful tool in transgenesis, to avoid hemizygotes and save time and resources in the production of plants transformed with the transgene in both homologous chromosomes. Moreover, from a scientific point of view, these lines are also very useful for basic studies of linkage and estimation of recombination fractions. They are also an extremely useful tool for genetic selection and screening of recessive mutants, because the phenotype of the resulting plants is not affected by the effects of dominance, and recessive phenotypes can be unmasked. For example, recessive embryo-lethal genes would be expressed in haploid embryos, and thereby eliminated for future generations.

Microspore-derived embryos (MDEs) or calli can be obtained using two main technical approaches based on in vitro culture: anther culture and isolated microspore culture. Anther culture is the easiest option. It consists on the excision of anthers from the flower bud, followed by their in vitro culture in a generally semi-solid, agar-based culture medium. After few weeks, microspores in the pollen sac transform into MDEs or calli and emerge out of the anther, which becomes necrotic. This approach is relatively fast and inexpensive, compared to the other option. This is why anther culture is the most adopted method to produce androgenic DHs. However, this method does not exclude the occasional appearance of somatic embryos from anther tissues, or the uncontrollable secretory effect of the tapetal layer surrounding the pollen sac,

which prevents us from having a strict control of the culture conditions. In addition, anther cultures need weeks-months to produce MDEs, and have a limited efficiency, generally producing only a few embryos per cultivated anther. All these limitations can be overcome by the direct isolation and culture of microspores, which is a more technically demanding method, but it is faster and more efficient. In some herbaceous species, where isolated microspore cultures are well set up, in 1–3 weeks it is possible to get hundreds or even thousands of embryos from the microspores contained in a single anther. It is evident that, where possible, microspore cultures are largely preferred over anther cultures. Using either anther or microspore cultures, at present there are systems for DH production in few hundreds of species of agronomic interest, from herbaceous crops such as wheat, barley, rice, rapeseed (canola), tobacco, or corn to trees such as clementine, mandarin, or cork, among others (reviewed in refs. [4–7]). However, except for model species such as rapeseed, barley, or tobacco, the efficiency is still very low. This is even more critical in horticultural crops of high agronomic interest, like those belonging to the Solanaceae family.

The Solanaceae family of flowering plants comprises between 3000 and 4000 species in about 90 genera [8]. Among these genera, the largest is *Solanum* L., estimated to contain 1500 species [9], and nearly 50 % of the diversity of the Solanaceae family. This family is one of the most important in terms of agricultural interest, and includes five major cultivated crop plants [10], namely potato (*Solanum tuberosum*), pepper (*Capsicumannuum*), eggplant (*Solanum melongena*), tobacco (*Nicotiana tabacum*) and tomato (*Solanum lycopersicum*). This family also includes the wild relatives of these five species, as well as many other plants belonging to the genus *Datura*, ornamentals such as those of the genus *Petunia*, or toxic, poisonous plants such as mandrake (*Mandragora officinarum*), henbane (*Hyoscyamus niger*), or deadly nightshade (*Atropa belladonna*). In Solanaceae, flowers are typically conical or funnel-shaped, with five petals, usually fused, persistent sepals, and a general floral formula of K(5)[C(5)A5]G(2). Stamens are bithecate and usually longitudinally or poricidally dehiscent. The ovaries are superior and biloculate. Seeds are usually small, round and flat. Fruits are in general berries, as in tomato or wolfberry, or drupes or dehiscent capsules, as in the genus *Datura*. Most Solanaceae have a basic chromosome number of $x=12$, being most of them diploid ($2n=2x=24$). Examples of this include tomato, eggplant, and pepper. However, there are some cases where this number has increased due to polyploidy. For example, some wild potato relatives range from diploid to hexaploid ($3n=6x=72$), while the cultivated species of *S. tuberosum* is allotetraploid ($2n=4x=48$), and the cultivated species of *N. tabacum* is autotetraploid. The Solanaceae are a typical example of an ethnobotanical family, meaning that it is extensively exploited and utilized by humans since the beginning of the agricultural age. It is an important source of food

and spices, mostly from the agricultural crops, but it is also a source of medicines and bioactive compounds of pharmaceutical interest, due to the presence of alkaloids in most plants of this genus. For example, nicotine in tobacco, or scopolamine, atropine, and hyoscyamine in species of the genus *Hyoscyamus*, *Datura*, and *Atropa*. From a nutritional point of view, the most important crop of this family is the potato (*S. tuberosum*), where the carbohydrate-rich tubers are used as food for human and animal nutrition, and as a source of starch for industrial purposes. In many other Solanaceae, the fruits are the interesting, edible part of the plant (for example tomatoes, tomatillos, eggplants, uchuva, sweet and hot peppers). In view of this, it is not surprising that from an agronomical point of view, the solanaceous crops are among the most important in the world.

Despite the tremendous importance of this family for world's agriculture, DH technology is not yet efficiently implemented in some of these interesting crops. Curiously, the first observation of in vitro, microspore-derived androgenesis was reported in a solanaceous plant by Guha and Maheshwari [1], who described the formation of microspore-derived plants within in vitro-cultured anthers of *Daturainnoxia*. However, of the five major solanaceous crops (pepper, tobacco, potato, eggplant, and tomato), at present only in tobacco enough progress has been made to consider this species as a model system for the study of microspore embryogenesis. The rest of interesting solanaceous crops (potato, tomato, eggplant, and pepper) are still far from the efficiency achieved in tobacco or in rapeseed, another model dicot species. In potato, eggplant, and pepper, only anther cultures seem to work efficiently in certain cultivars, but, up to now in tomato, not a single method has been demonstrated to work efficiently. Despite of the genetic proximity of these five species, they seem to respond to induction very differently. In this chapter we revise the most relevant work performed in the last four decades pertaining to the study of the experimental induction of androgenesis through anther cultures and isolated microspore cultures and the development of the DHs embryos, calli and plants in datura, the "pioneer" of microspore embryogenesis, and in the five most important solanaceous crops, tobacco, potato, tomato, eggplant, and pepper, illustrating when needed with examples of the practical applications of this technology in genetic breeding.

2 Datura

Within the family Solanaceae, the genus *Datura* comprises several species (commonly *daturas*), widely distributed throughout the globe, and characterized by their erect or spreading, trumpet-

shaped flowers, and by their spiny capsular fruits that open at maturity to release numerous seeds. *Datura* species are herbaceous, leafy annuals and short-lived perennials which can reach up to 2 m in height. Daturas belong to the classic "witches' weeds", along with deadly nightshade, henbane, and mandrake, because they all contain, primarily in their seeds and flowers, toxic and hallucinogenic tropane alkaloids such as scopolamine, hyoscyamine, and atropine. Because of the presence of these substances, Datura has been used for centuries in some cultures as a poison and as a key ingredient of love potions and brews. Nowadays, the controlled use of some of these alkaloids at low doses has been adopted by medicine as treatments for a wide range of diseases. Aside of their traditional and medical uses, datura plants deserve a honorific position in the field of haploid research for two main reasons. First, *Datura stramonium* was the first flowering plant for which cytological proof of the discovery of a haploid individual was obtained [11]. Second, and most importantly, *Datura innoxia* was the first plant producing embryos from the microspores contained in their anthers, when inoculated in a culture dish [1]. Sipra Guha and Satish C. Maheshwari, two Indian researchers, at that time working at the University of Delhi (India), first performed such culture and now they are considered as the founding fathers of haploid and DH technology [12] (*see* **Note 1**).

Since *D. innoxia* was the first species to produce haploid MDEs, it is easy to conceive that this experimental system was one of the first used to investigate such an emerging experimental process. Thus, some researchers focused on improving culture conditions in order to increase the "embryogenic power" of pollen in *Daturainnoxia* [13–15]. Other groups focused on the study of the changes undergone by the microspore within the anther as a consequence of the induction [16–20]. Later on, an established protocol for microspore embryogenesis in this species permitted the combination of this technique with others, such as *Agrobacterium-mediated* genetic transformation [21], or plant regeneration through embryogenesis from cultured cells coming from androgenic calli [22]. Aside of *D. innoxia*, other members of the *Datura* genus have been used to successfully induce microspore embryogenesis, or to study the changes associated to the induction. These species include *D. ferox* [23], *D. metel* [24–29, 20, 30] and *D. meteloides* [20, 31]. However, daturas are far from the economic importance for global agriculture that other Solanaceae have (tomato, potato, pepper, etc.). This is the reason why the number of articles and discoveries produced in the last decades using daturas as experimental system is limited. At present, the trend in DH research is to use model species (rapeseed, barley, tobacco, etc.) to study fundamental aspects of the process, and to use recalcitrant crops to try to make them responsive.

3 Tobacco

After more than 500 years of cultivation, tobacco (*Nicotiana tabacum*) is considered the most valuable non-food crop in the world. Among the 178 primary crops listed in the 2012 FAOSTAT database [32], tobacco ranks 49th in area harvested (4,291,014 Ha) and 82nd in production, with 7,490,661 t. Its main utility is the production of cigars, cigarettes and other derivatives used by the tobacco industry. Nowadays, the health problems associated to the habit of smoking are causing a decrease in the traditional uses of tobacco. However, tobacco is especially suitable for genetic transformation, which makes this crop a good candidate to be exploited as a biofactory. Indeed, tobacco can be used to produce starch for bioethanol or for industrial purposes [33, 34], to produce vaccines [35–37], and a large list of other pharmaceuticals [38–41].

Few years after Guha and Maheshwari milestone report, several groups published in a time range of 2 years the production of haploid plants from tobacco anthers [42–45]. A representative example was the work of Nitsch and Nitsch [42], who published on *Science* a paper presenting a method "by which hundreds of haploid plants of various species of Nicotiana can be raised from pollen grains". Soon after them, several researchers studied this phenomenon in tobacco from different experimental approaches [46–53]. In addition to *N. tabacum*, pioneering researchers also explored and successfully achieved the induction of haploidy in other *Nicotiana* species, including *N. sylvestris*, *N. affinis*, *N. rustica*, *N. attenuata*, *N. knightiana*, and *N. raimondii* [42, 54]. Since then, tobacco has been considered for long as a model species where to induce microspore embryogenesis efficiently. Indeed, there are different well set up protocols currently available to obtain DHs from anther and isolated microspore cultures with an acceptable efficiency [55–58]. For anther culture, most of the protocols include a cold treatment of excised flower buds prior to anther excision and culture on a charcoal-containing medium [55]. However, its relative simplicity makes isolated microspore culture the method of choice. For microspore culture, the most common way to stress the microspores is to starve them from carbon and nitrogen sources while applying a inductive, mild heat shock [55, 56, 59]. After induction, embryogenic microspores are transferred to a carbon and nitrogen-containing medium where they continue dividing and grow into haploid embryos. These embryos are transferred to a low-sucrose, agar-based solid medium for germination, and are finally treated with colchicine solutions for genome doubling [55]. Aside of these standard methods, the flexibility of tobacco microspores allowed the application of different types of stresses to induce the androgenic switch. Although these are not the most efficient ways to induce tobacco microspores, successful induction to embryogenesis has been achieved,

for example, by the application of basic pH (8–8.5), lithium (5 mM $LiNO_3$), abscissic acid (0.01 mM), reduced atmospheric pressure (12 mmHg) or centrifugation at 10,000–11,000 g (reviewed in ref. [60]). As in other model species like rapeseed, tobacco microspores also offer the possibility to reproduce microgametogenesis in vitro, provided that microspores are cultured in a rich, non-starving medium, with no stress sources [61, 62]. In this way, mature and fertile tobacco pollen can also be obtained in a petri dish. Thanks to these well-established protocols, tobacco embryogenic cultures and microspore-derived DH plants can now be routinely generated, and serve as excellent tools for the study of many different basic and applied aspects of the process of microspore induction and embryo development. In the last 15 years, the majority of the studies published on tobacco microspore embryogenesis used this species as a model system where to study the changes undergone by the induced microspore at the physiologic, transcriptional, metabolic, or ultrastructural levels, among others. In particular, tobacco microspore embryogenesis was used to decipher the cellular and ultrastructural changes undergone by the induced microspore [20, 48, 49, 51, 63, 64] and specifically by plastids [20], as well as to discover specific mRNAs [65, 66], MAP kinases [67], phosphorylated proteins [68–70], metabolites [66], and heat shock gene expression [71] associated with the induction. These studies have contributed significant insights in the understanding of how and why microspores are reprogrammed towards embryogenesis.

Aside from basic studies, tobacco has also been used to explore the advantages of DH technology in plant breeding. In addition to the production of pure lines for hybrid seed production, another advantage of DH technology is the avoidance of hemizygous transformants when combined with genetic transformation. Such a combination of both technologies was used in 2007 to produce an innovative breeding technology. Ribarits et al. [72] produced reversible male-sterile tobacco plants by firstly introducing mutated tobacco glutamine synthetase genes fused to the tapetum-specific TA29 and the microspore-specific NTM19 promoters, and secondly producing a non-segregant, male-sterile DH population through microspore culture. In this population, male sterility could be overcome at will by the exogenous addition of glutamine to plants or to in vitro maturing pollen. This is an interesting example of how this technology can help in plant breeding beyond the mere production of DH pure lines.

In conclusion, tobacco has served during many years as a useful model system to advance in the knowledge of microspore embryogenesis. In the last years, it appears that its role as a prominent dicot model has been taken by rapeseed (*Brassica napus*), which is the dicot model species used for most of the recent studies at the cellular, molecular and genetic levels. From the applied point of view,

the decrease in tobacco consumption worldwide may have an impact on tobacco breeding programs and therefore on the use of tobacco DHs in such programs. However, the suitability of tobacco for both genetic transformation and microspore embryogenesis could open the door for a promising future of doubled haploidy in this crop as a tool to be combined with transformation.

4 Potato

Potato (*Solanum tuberosum*) is a solanaceous crop originally from South America. It is believed that the first places where potato was cultivated were the region of the Titicaca Lake, in the north of Bolivia, and the highlands of the Andes [73]. Andian populations of the north of Peru and the south of Bolivia were the first to eat wild potatoes around 3000–4000 years bc. It was introduced to Europe by Spanish expeditions through Seville in 1570, and later on it was extended to the rest of Europe [73]. The edible part of potato plants are their tubers, which are nowadays extended worldwide as an essential part of many cuisines, as well as of a wealth of processed foods. Indeed, potato ranked eighth in production (365,365,367 t) and 18th in area harvested (19,278,549 Ha) among the 178 different crops analyzed in the 2012 FAOSTAT database [32]. These data illustrate to what extent potato is important in the current world's economy.

The major cultivated species of potato (*S. tuberosum* ssp. *tuberosum*) is an autotetraploid. Other interesting potatoes include cultivated species like the tetraploid *S. tuberosum* ssp. *andigena*, the diploid *S. stenotomum* and *S. phureja*, and the diploid wild potato relative *S. chacoense*. These species are principally used for genetic studies and for plant breeding, for example as sources of resistance for certain diseases. Despite that potato is a sexual species, it is often difficult to have it sexually reproduced. Sexual crosses are usually restricted to breeding centers which use them to generate new varieties, taking advantage of their germplasm collections from different potatoes and related species [73]. However, cultivated potatoes are vegetatively propagated, using the adventitious buds formed on the tubers. In this way, propagation is easier and the populations obtained are homogeneous. However, this has generated a very low level of genetic variability among the different potato cultivars. This is why it is highly desirable to obtain reduced, dihaploid and even monohaploid plants for potato breeding at the haploid level and for genetic analysis [74–76]. For example, genetically heterozygous potato dihaploids may be used as parents to hybridize with other *Solanum* species in order to obtain diploid, tetraploid, or even higher ploidy individuals with new genetic combinations. Sometimes, these hybrids are sterile and haploidization is employed as a possible strategy to overcome reproductive barriers, as recently

reported in hybrids between *Solanum bulbocastanum* and *S. tuberosum* [77]. Examples on the use of androgenic approaches in potato breeding also include *S. chacoense* x *S. phureja* clones [78] and *S. brevidens* x *S. tuberosum*somatic hybrids [79]. Other examples of this and other approaches to obtain potatoes with new genetic backgrounds can be found in a review by Tai [80].

Different techniques for dihaploidization have been described in the literature. These include interspecific hybridization of cultivated potato and related *Solanum* species with certain haploid inducer clones of *S. phureja*, and microspore embryogenesis (reviewed in refs. [81, 82]). With respect to the latter, potato cannot be considered as a model system, but at present there are several methods capable to induce androgenesis from anther cultures [80, 83, 84]. The main difference between them consists on the use of different culture media, either liquid [85–87] or semisolid [79]. In a technical review of 2003, Rokka [84] described a method for potato anther culture based on the Murashige and Skoog (MS) basal medium [88], capable of inducing microspore embryogenesis in a wide range of genetically diverse potato species, including interspecific and intergeneric hybrids between them. Aside of *S. tuberosum*, other papers have reported induction of androgenesis by anther cultures of *S. phureja* [89–93], and other potato relatives such as *S. acaule* [94], or *S. chacoense* [95–98].

The effect of colchicine in anther cultures was also evaluated [89], concluding that it did not affected neither the efficiency of induction nor the percentage of monoploid plants obtained. Anther cultures have been described to produce microspore-derived embryos and calli. Direct embryogenesis is preferred over callus formation due to the lower occurrence of somaclonal variation [82]. However, a RAPD analysis of the plants obtained from *S. phureja* anther cultures revealed the occurrence of genetic clones, possible originated by secondary embryogenesis during anther culture [89]. Other studies, also based on molecular markers, have revealed interesting results. For example, Sharma et al. [99] used SSRs to demonstrate that anther culture-mediated dihaploidization of *S. tuberosum* tetraploids involves extensive changes and genetic rearrangements. In addition, they demonstrated the occurrence of somatic embryogenesis from anther walls, and of somaclonal variation in the tetraploid somatic regenerants. Birhman et al. [97] studied by means of RFLPs the genetic architecture and the origin of S. *chacoense* plants produced through anther culture. They showed that some of the plants obtained had different ploidy levels but their genetic constitution was identical, which suggested the occurrence of microspore-derived, mixoploid calli developing clonal plants from their ploidy-different cells. They also showed that some of their plants, although regenerated from the same callus, had different genetic constitutions, which led them to conclude that they might come from two microcalli

derived from two different microspores, but proliferating together within the anther.

Although not abundant, reports exist on potato isolated microspore cultures, too [100–103]. Through the application of cold and starvation treatments, one of these reports [103] described a performance better than anther cultures and, as expected, a dependence on the genotype of the donor plant. They also observed that microspores of a dihaploid genotype divided symmetrically after 3 days from isolation, giving rise to suspensorless embryos. Symmetric divisions and suspensorless MDEs have also been observed in the rest of reports dealing with potato microspore cultures. However, microspores of "Albina," a tetraploid potato, divided later (8 days after isolation) and asymmetrically, giving rise to a large and a small cell, which led to the formation of a zygotic-like suspensor and an embryo proper, respectively [103]. This observation is interesting, since the number of inducible species that develop suspensor-bearing MDEs is very limited, being *Brassica napus* the most prominent example. However, none of the above mentioned reports have demonstrated the successful production of viable potato plants from isolated microspore cultures. Therefore, there is still work to do in order to provide a reliable and complete protocol.

5 Tomato

Within the genus *Solanum*, the section *Lycopersicon* includes tomatoes and their wild relative species. The native distribution of this section ranges across the west-central part of South America, from the high Andes to coastal Ecuador, Peru and the north of Chile, although it seems that it was in Mexico where tomato was domesticated, and then introduced to Europe and Asia by the Spanish and Portuguese [73]. Although the taxonomy of the section *Lycopersicon* has been subjected to a long-lasting discussion that has not yet reached a widely accepted consensus, the most recent classification [104] divided the section into four groups: the Lycopersicon group (*S. lycopersicum*, *S. pimpinellifolium*, *S. cheesmaniae*, and *S. galapagense*), the Neolycopersicon group (*S. pennellii*), the Eriopersicon group (*S. peruvianum*, *S. corneliomulleri*, *S. huaylasense*, *S. habrochaites*, and *S. chilense*), and the Arcanum group (*S. arcanum*, *S. chmielewskii*, and *S. neorickii*). From an economic point of view, cultivated tomato (*S. lycopersicum*) is by far the most important of the section. Tomato is the first vegetable crop worldwide, both in terms of production (161,793,834 t in 2012) and cultivated area (4,803,680 Ha in 2012). Among the 178 different crops analyzed in the 2012 FAOSTAT database [32], tomato ranks 16th in production and 47th in area harvested. These data give an idea of the tremendous importance that tomato has for global agriculture.

However, and despite of its importance, little is known in the tomato DH field, with no reliable and standardized methods available so far. Over the past 40 years, a significant number of media types and conditions, as well as combinations of nutrients, vitamins, growth factors and supplements, have been assessed (reviewed in ref. [105]). During these years most of the published papers reported just on induction of calli [106, 107] or multicellular structures [108], or regeneration of roots [109] or apical shoots [110]. Only two laboratories have published the complete regeneration of entire tomato plants with a demonstrated haploid or DH origin [111–115], although with different levels of morphological variability ranging from high [116] to low [114]. In all of these published studies, mixoploidy in a percentage of individuals and a low general efficiency were common features [114, 115]. Aside of these reports, a third group reported in 2001 the production of androgenic plants, but not in tomato. Gavrilenko et al. [117] obtained plants by culturing anthers of a somatic hybrid between *S. lycopersicum* and *S. etuberosum*, a wild non-tuberous *Solanum* species with several desirable agronomic traits. Again, the androgenic efficiency obtained from this intergeneric hybrid was extremely low, with 3.4 % of responding anthers and five plants from one of the hybrid donor plants, and 1.2 % of responding anthers and only one plant from the other hybrid donor plant used. The embryogenic response of anther cultures of *S. lycopersicum* x *S. peruvianum* hybrids was tested by Cappadocia and Ramulu [118]. Although they reported the observation of the first stages of embryogenesis, the plants obtained derived from anther tissues. Out of the *S. lycopersicum* species, anther cultures have also been performed in *S. peruvianum* [119, 120]. Reynolds [121] reported the production of callus, embryoids and regenerated plants from cultured anthers of wild tomato (*S. carolinense*), a pasture weed from North America. In this work, the occurrence of microspore-derived callus or embryoids depended on the hormonal composition of the culture medium. Other reports on *S. carolinense* anther culture can be found in refs. [122–126]. It can be deduced from all these works results that the state of the art on DH research in tomato is far from being considered minimally useful to be applied for DH production on a routine basis. Indeed, tomato can be regarded as one of the major examples of species recalcitrant to androgenesis induction. More efforts are needed to obtain successful results.

As in other in vitro morphogenic processes, the most critical factors to obtain androgenic DHs from tomato are the genotype and the developmental stage. As for the genotype, male-sterile mutant lines have been shown to be especially sensitive to being induced [112, 114, 115, 127, 128]. Usually, male-sterility is associated to defects at the late meiocyte stage, which ends up degenerating and dying. This was demonstrated for the *ms1035*

male-sterile genotype, where most of the work on tomato DHs has been developed [115]. Aside of male-sterile mutants, all of the works published to date on the evaluation of androgenic competence in commercial tomato cultivars have reported null or very few positive results. It is important to highlight that this information comes from published papers, but it might not be the only one. Considering the extraordinary importance of this crop for breeding companies, some of them have set up specific research programs and collaborations to find the key switch for this species. Indeed, some methods claiming the development of protocols to obtain haploids in tomato have been patented [5, 129, 130]. Thus, it might be possible that some companies would have found a way to obtain androgenic DHs in their particular tomato cultivars, but it is unlikely that this proprietary information will see the public light in the short-mid-term. The second key factor is the identification of the right stage for the tomato gametophyte (or its precursor) to be reprogrammed. This has been a matter of debate along 40 years of research on tomato DHs. A number of works have been sporadically published [109, 111, 127, 131–135], but a clear consensus could not be obtained from them. Perhaps, the most suggestive work of the first years of tomato haploidy research was by Dao and Shamina [135]. They induced the formation of callus-like structures from anthers with meiocytes at the tetrad stage, just after walling. On the other hand, they also obtained embryoids directly from anthers containing late, vacuolate microspores or young, bicellular pollen grains. This work, not further continued, pointed to the notion that, as also suggested by Chlyah et al. [136], tomato could follow the trend observed in other species, where different stages may be responsive under different treatments [137–139]. In the last decade, several studies have reinforced this idea. On the one hand, the optimal stage for anther culture was narrowed to the meiocyte just before compartmentalization of the tetrad [111, 113], which is a quite infrequent feature of tomato, together with some species of the genus *Vitis* [140]. This developmental window implies that recombination must be successfully finished without disruption, but microspore formation (tetrad compartmentalization) has to be prevented. On the other hand, the formation of few-celled structures from isolated microspore cultures was described upon exposure to a combined treatment of starvation, cold and colchicine [108]. In 2007, it was finally demonstrated that in tomato, gametophyte precursors can be induced to divide at two different stages: meiocytes and vacuolated microspores [114]. However, both possibilities still have a great number of limitations, as explained in the next paragraphs.

Haploid and DH plants can be obtained from in vitro cultured anthers containing meiocytes (Fig. 1), but they are not the only products of this process. Plants originate from meiocyte-derived calli, which may in turn come from two different origins: (1) from

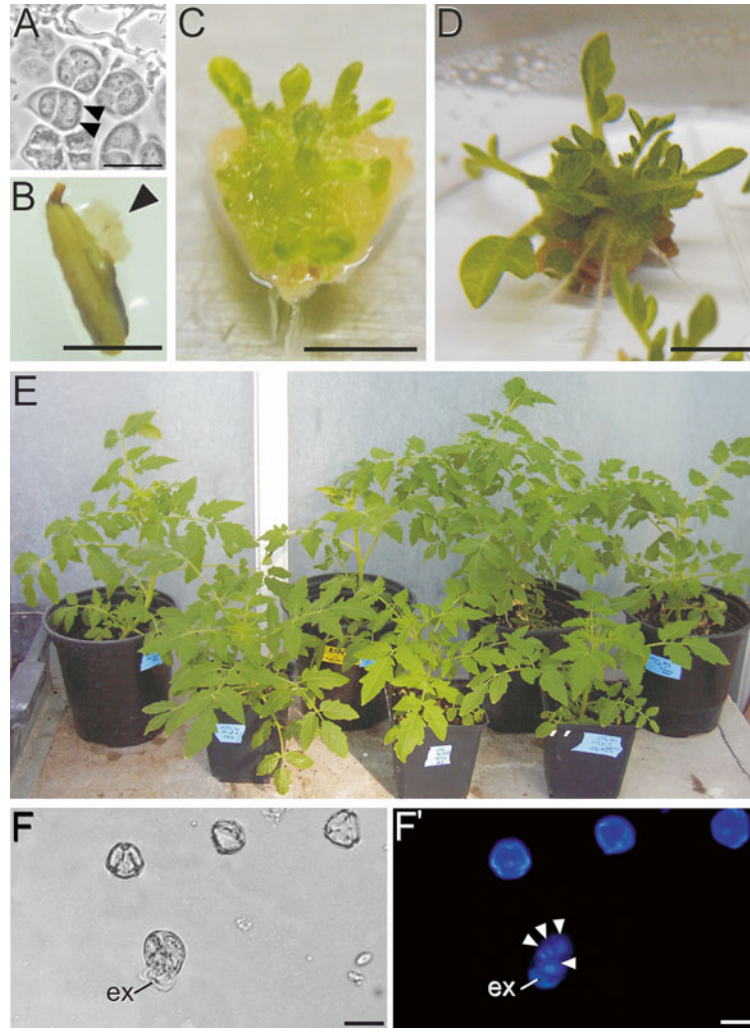

Fig. 1 Tomato anther and microspore culture. (**a**) Meiocytes within a cultured anther. *Arrowheads* point to two nuclei coalescing in the same cytoplasm. (**b**) Callus (*arrowhead*) emerging from a cultured anther. (**c**) Cultured callus with shoot-forming organogenic buds. (**d**) Plantlet regenerating from a callus. (**e**) Haploid and doubled haploid tomato plants. (**f**, **f′**) Isolated microspore culture (*arrowheads* point to the nuclei of a dividing microspore). *ex* exine. Bars: a, 20 μm; b, 2 mm; c, d, 1 cm; f, **f′**, 15 μm Images adapted from [114].

haploid meiotic products still enclosed within the tetrad (the future microspore), that stop their gametophytic program and start proliferation, or (2) from proliferating diploid cells, produced from the fusion of two different meiotic products. As for the first origin, it was demonstrated that stress-induced meiocytes (Fig. 1a) undergo a series of defects in tetrad compartmentalization (formation of defective, incomplete, or absent cell walls) that facilitate the fusion of nuclei between adjacent, not well separated cells [114, 115], in a way similar to that described for the tomato mutant

pmcd1 (*pre-meiotic cytokinesis defect 1*), which generates diploid gametes due to the fusion of two haploid meiotic products before microspore release [141]. When callus proliferation starts first and nuclear fusion occurs later on, the new cell formed will have a diploid nuclear content, formed by two identical haploid genomes. In other words, it will be a DH cell that can potentially give rise to a DH callus (Fig. 1b–d) and a DH plant (Fig. 1e). Alternatively, nuclear fusion may occur late in callus proliferation, giving rise to mixoploid calli where some cells remain haploid, and others, derived from those undergoing nuclear fusion, will be DH [114, 115]. A similar mechanism for mixoploid (haploid + DH) callus formation was proposed to explain the occurrence of genetically identical (clonal) plants with different ploidy levels from microspore-derived calli of *S. chacoense* [97]. However, nuclear fusion of haploid tetrad cells may occur first, followed by proliferation. This will lead to the second of the two possible origins mentioned above. In this case, the two fusing nuclei will also be haploid, but genetically different. Since they come from meiotic recombination at prophase I, fusion of two reduced meiotic products would generate new allele combinations not necessarily homozygous. Therefore, the callus and plant coming from this cell would not be DH. Interestingly, Birhman et al. [97] demonstrated by RFLP genetic analysis in *S. chacoense* that plants regenerated from the same callus may be genetically different. As explained in the section devoted to potato, they explained this result as the coupled proliferation of two calli originated from two different microspores. However, in light of what occurs in tomato, their results might be alternatively interpreted as the proliferation of a single callus coming from a diploid microspore, whose single diploid nucleus comes from the fusion, during meiosis, of two different haploid nuclei of a non-well compartmentalized tetrad. In addition to these two possible origins, callus and plants may also come from the proliferation of somatic, anther tissues. Indeed, filament tissues typically exhibit a high proliferative response when cultured in vitro [142], and it is believed that tomato anther tissues at meiotic stages are more sensitive to tissue culture than those of older stages [105]. All this considered, it seems that DHs are not the only individuals that can arise from tomato anther cultures. So the question is: how frequently do anther-derived tomato DHs arise? In a recent study, it was shown that the first possibility (proliferation and then fusion) accounts for only 7 % of the plants regenerated, which would be haploid or DH [115]. This study also revealed that the second possibility (fusion first and then proliferation) accounted for 10 % of the plants, and the third possibility (somatic origin) accounted for 83 % of the plants. In other words, more than 90 % of the plants obtained have a non-haploid origin, and only 7 % would be useful to obtain DH plants. It is evident that non-DH plants represent the vast majority of plants produced. It is also evident that this

method requires the genetic evaluation of each single regenerant to determine their origin and ploidy, and that most of them are useless and should be discarded. In other words, this technology, at its current state of the art, has few chances to be implemented by breeding companies to produce DHs at large scale.

Microspore culture is the second option to explore to obtain androgenic DHs in tomato. When microspores at the vacuolated stage are isolated and grown in liquid medium, it is possible to induce proliferative divisions in these microspores (Fig. 1f, f′), generating callus-like [108] and embryo-like structures [114]. This alternative would prevent all the problems mentioned above as derived from using anthers and meiocytes. However, this approach is still at its infancy. Up to now very few genotypes have been assessed using this method, and in the very few positive results obtained, it has not been possible to go beyond the first divisions of the developing MDEs [114]. After these divisions, embryos arrest and never progress beyond the early globular stage [114]. Perhaps, culture conditions were not well optimized to allow for in vitro embryo development, with no zygotic endosperm to provide nutrients and developmental cues to ensure proper embryo development. It could also be possible that haploidy would unmask embryo-lethal genes in the dividing haploid cells, which would preclude further development.

As seen in this section, current research in tomato haploidy is still far from providing a reliable and efficient protocol to produce androgenic DHs. After more than 40 years of tomato DH research, little is still known about the origin of the recalcitrance shown by cultivated tomato. In this respect, one interesting hypothesis was that suggested by Sangwan and Sangwan-Norreel [20], who related recalcitrance with plastid differentiation. These researchers studied the ultrastructure of plastids in an extensive number of species that, according to their own results, they divided into *androgenic* and *recalcitrant*. They found that in the androgenic group, proplastids do not differentiate into amyloplasts until the mid or late bicellular pollen stage of microgametogenesis, whereas in the recalcitrant group, such a differentiation occurs soon, during microspore development, or even sooner, before tetrad formation. In other words, they suggested that the differentiation of proplastids into amyloplasts marks the end of a favorable period for androgenesis induction. This has been widely acknowledged later on, accepting that starch accumulation is indicative of commitment towards gametogenesis (reviewed in refs. [143–145]). Interestingly, their investigations showed that, as opposed to the rest of the Solanaceae studied, tomato plastids soon differentiated into amyloplast, being present in microspores before the first pollen mitosis. Thus, the difficulty of induction shown by tomato microspores might be related to the fact that their plastids accumulate starch unusually soon, as compared with other, more sensitive species like

rapeseed, or even within their same genus, tobacco or *Datura*. This possibility is also consistent with the wide consensus about the difficulty of inducing in vitro redifferentiation or organogenesis from starch-rich somatic tissues, compared to tissues where plastids are still in the form of proplastids or chloroplasts. As an alternative to the androgenic approach, a limited number of works have been published on the development of ways to haploidy such as gynogenesis [117, 146], ovary culture, pollen irradiation [147], or wide hybridization, and little positive results have been published (reviewed in ref. [105]). Perhaps, the future of tomato DH research should focus on exploring the possibilities of microspore culture, and in parallel, of new, alternative pathways to doubled haploidy not related to androgenesis. An example of alternative ways to approach tomato DHs might possibly be the one demonstrated by Ravi and Chan [148] in *Arabidopsis*: the production of haploid plants produced by uniparental centromere-mediated genome elimination. By manipulating a single centromere-specific histone (CENH3), it is possible to produce *cenh3* null mutants expressing altered CENH3 proteins. When crossed to wild type plants, chromosomes from the mutant are eliminated, thereby producing haploid and DH progenies. However, this approach has still a number of technical challenges still to be overcome in order to make it possible in tomato.

6 Eggplant

Eggplant (*Solanum melongena* L.), also known as aubergine, brinjal, or Guinea squash [149], is another of the most important vegetables worldwide. In 2012, eggplant ranked the sixth and eighth among all the vegetable crops for production and area harvested, respectively [32]. In 2012, 1,853,023 Ha of eggplant fields were cultivated, and a total of 48,424,295 t were produced in the world [32]. From these, nearly 85 % were produced in China (59.5 %) and India (25.2 %), the two main producers worldwide. Eggplant is thought to have its origins in Asian tropical and subtropical regions, where it extended to Africa and then to the Mediterranean area of Europe [73]. Eggplant has a remarkable importance in economic terms in China, India, Africa, and some subtropical, Central American countries. It is also grown in some warm, temperate regions of the south of the USA, and in the Mediterranean basin, in countries such as Italy, Greece, or Spain, where eggplant constitutes a classic ingredient in the renowned "Mediterranean diet."

With respect to haploidy induction, eggplant appears to be between tomato and tobacco in terms of recalcitrance. It is possible to induce the deviation of the eggplant microspore within the anther towards an embryogenic route, thus generating a haploid or DH embryo that will eventually germinate into a haploid or DH

plant. However, we still are far away from the efficiency shown by tobacco. The first report of plant regeneration from anther cultures dates from 1973 [150]. Here, authors were able to induce callus proliferation and emergence from anthers, and plant regeneration upon treatment with colchicine. However, according to the authors' own interpretation, it is likely that the generated calli were produced from the connective tissue of the anther, having therefore a non haploid origin. Few years later, other articles reported the production of eggplant haploid individuals [151, 152], or the induction of callus derived from microsporogenous tissue within the eggplant anthers [153]. From these calli, they were able to regenerate shoots and roots. Then, Robert Dumas de Vaulx and Daniel Chambonnet established the basis for a general, reliable and reproducible protocol for haploid embryo and DH production from eggplant anther cultures [154, 155]. This method, in its original formulation, consisted on the incubation of anthers in a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, at 35 °C in darkness during 8 days to induce embryogenesis. Then, cultures are moved to 25 °C and exposed to light in order to promote the development of MDEs within the cultured anthers. At day 12th, anthers are moved to a second medium with a reduced level of 2,4-D and kinetin. This has been the basis of many protocols, now routine methods, adapted to particular eggplant varieties that have been, or are currently, used in breeding programs. At present, pure DH lines of some varieties and hybrids have already been developed [156–159], based on modified versions of this protocol. As it happens in all other inducible species, the efficiency of the embryogenic response of the microspores in eggplant is greatly influenced by the genotype of the donor plant [159] and by the stage of microspore development when anthers are excised. Related to the latter, a recent report highlighted two particular features of eggplant that may have a significant impact on the efficiency of induction. The first is the particular thickness of eggplant anther walls, which seems to delay the access of inductive factors to the anther locule, thus reducing their effect over inducible microspores [158]. Therefore, the culture of anthers with younger microspores was proposed to allow for younger microspores to grow up to the inducible stages while factors are entering the locule. The second is the heterostyly, present in certain eggplant cultivars, which might have an influence in the embryogenic response. Salas et al. [158] studied the embryogenic response of short and long-styled buds present in a heterostylic cultivar, and demonstrated that each floral morph produced buds and anthers of different lengths, but equally useful for anther culture, since both morphs produced similar embryogenic responses. Microspore embryogenesis depends on culture conditions, too, including temperature, type, and concentration of growth regulators [156, 157, 160, 161]. Genetic variability was

also observed in plants regenerated from anther cultures [162]. These results confirmed that in general, eggplant microspore embryogenesis in cultured anthers behaves as in other better-studied species, although with some particularities inherent to this species. Overall, eggplant anther cultures can be and are being used to obtain DHs for breeding purposes [156–159, 163–165]. Two examples can illustrate the potential of this technology in eggplant. First, in 2002 Rizza et al. [166] generated a population of androgenic dihaploid plants, derived from somatic hybrids between *S. melongena* and a wild eggplant relative, *S. aethiopicum*, and demonstrated that they constitute a useful source for the introduction into cultivated eggplant varieties of the *Fusarium oxysporum* resistance typical of *S. aethiopicum* group Gilo. Second, a similar approach was used to reduce to the dihaploid status the ploidy of eggplant somatic hybrids between *S. melongena* and other wild relative, *S. integrifolium*, in order to facilitate their crossability with cultivated eggplant varieties for the introgression of *Fusarium* resistance [167].

Despite that eggplant embryos can be successfully induced from microspores cultured within the anther, the development of a method for embryogenesis induction from isolated eggplant microspores would be highly desirable. Although the occurrence of somatic regenerants derived from anther walls seems not to be a big issue in eggplant [159], isolated microspore culture would avoid the other problems mentioned in the introduction: the uncontrolled contribution of tapetal cells to culture conditions and the low efficiency. Related to this, it is striking that very few papers have been published on the successful production of DH plants from isolated microspores [168–170]. Miyoshi described the proliferation of eggplant microspores to form calli, from which he obtained DHs through organogenesis. He obtained 20–65 calli per anther, and 0.001–0.02 plantlets were regenerated from each callus. As mentioned by Miyoshi, this efficiency was clearly beyond that of anther cultures. In the last 2 years, two papers have contributed to the progress in this field, confirming the applicability of the studies mentioned above to different eggplant genotypes, and developing a protocol that further enhances the efficiency of microspore induction [169, 170], well above that previously published by Miyoshi [168]. Like in the above mentioned paper, the plants apparently did not come from embryos, but were regenerated through a callus phase instead. However, a careful study of the process of microspore proliferation showed that, actually, microspore embryogenesis seems to initiate as in other inducible species (Fig. 2a–c), but arrests at the globular embryo stage (Fig. 2d). Instead of experiencing the radial-to-bilateral symmetry transition typical of zygotic embryos, eggplant MDEs enter a proliferative phase of undifferentiated growth and become callus-like structures (Fig. 2e) [169]. Haploid and DH plants regenerate from these calli

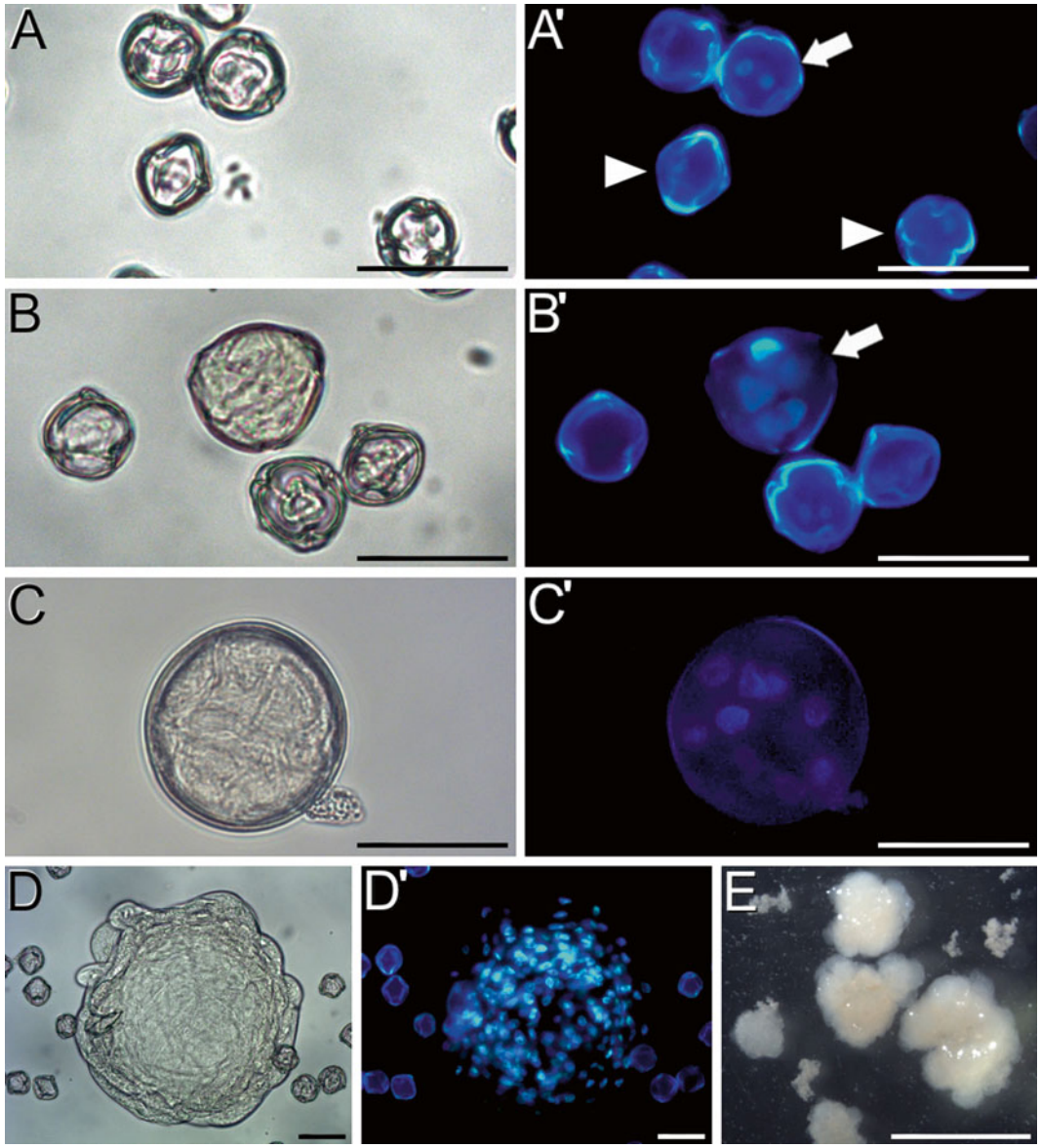

Fig. 2 Microspore cultures in eggplant. (**a**, **a**′) Two-celled, induced microspore (*arrow*) together with unicellular, non-induced microspores (*arrowheads*). Note the *blue*, DAPI-stained nuclei in **a**′. (**b**, **b**′) Four-celled, dividing microspore (*arrow*). (**c**, **c**′) Multicellular microspore. (**d**, **d**′) Globular embryo. (**e**) Embryo-derived calli (**a**–**d** are phase contrast images, **a**′–**d**′ are fluorescence images of DAPI-stained samples). Bars: **a**–**d**, **a**′–**d**′, 50 μm; **e**, 1 mm

through organogenesis, as first described by Miyoshi. In order to further increase the efficiency of induction, but principally to avoid the transformation of MDEs into calli, a number of experimental factors were tested, including polyethylene glycol (PEG), mannitol, abscisic acid, epibrassinolide, naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP), arabinogalactan proteins (AGPs), and different combinations of them [169, 170]. It was found that

certain combinations of these factors increased the efficiency of microspore induction towards embryogenesis, but only one (AGPs) permitted the development of bipolar embryos [170]. These embryos exhibited anatomically normal hypocotyls and radicular poles. However, their shoot apical meristems and cotyledons were either absent or severely distorted, which precluded a normal germination. Together, these results extended the knowledge, still scarce, regarding microspore culture in eggplant. In addition, they clearly pointed to deficiencies in the composition of the culture medium where the post-inductive phases take place, as the responsible of this abnormal pattern of embryogenesis. Thus, further studies to optimize eggplant microspore cultures should focus principally on the elimination of the bottleneck by which eggplant MDEs are not capable of progressing beyond the globular stage to produce mature, well-formed embryos ready for germination. In this way, the phase of plant regeneration from calli through organogenesis would be eliminated, and the method would be considerably faster, cheaper, and, therefore, efficient.

7 Pepper

Peppers (genus *Capsicum*) are plants native of America, where they were cultivated for thousands of years by the people of the tropical regions of South America [73, 171]. At present, they are cultivated worldwide. The genus *Capsicum* consists of approximately 20–27 species, five of which are domesticated: *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens* [171]. The fruit of *Capsicum* plants has a variety of names, depending on the region and the type of pepper, including chili pepper, red or green pepper, bell pepper, sweet pepper, just "capsicum" in Australian and Indian English, or paprika in Hungary. Some of the members of *Capsicum* are used in fresh as vegetables, and others are dried and used as spices. With a total world production in 2012 of 31,171,567 t in 1,914,685 Ha, peppers ranked 44nd in terms of production and 69th in terms of area harvested among the 178 primary crops listed in the FAOSTAT 2012 database [32]. China was by far the leader in production of freshly edible peppers with 16,000,000 t, around 51 % of the total production [32]. In dry pepper, India was the world leader with 1,299,940 t, almost 39 % of the total world production.

Pepper is the third solanaceous crop that could be defined as difficult with respect to the induction of androgenesis. In this species, the production of haploids and DHs has been assessed by different means including the use of both the male and the female gametophyte and their precursors (reviewed in ref. [172]). Apart from the spontaneous occurrence of some cases of in vivo androgenesis of no practical relevance [173], haploids in pepper were

firstly obtained through parthenogenesis (reviewed in refs. [172, 174]). However, after the discovery of in vitro anther culture as a way to induce androgenesis [1], this has been the most successful technique so far. The first reports on the production of haploid-*Capsicum* plants by anther culture involved the use of Asian varieties [175–177]. A year after, anther culture of European varieties was reported [178, 179]. Thereafter, many *C. annuum* cultivars and interspecific crosses have been tested for responsiveness to anther culture [180–190]. To our knowledge, the most extensive of this type of studies was that of Gémes-Juhász et al. [191], who tested over 2000 genotypes, including Hungarian sweet, Hungarian spice, Dutch blocky, Spanish, and Turkish pepper types. Anther cultures have been used as a tool for genetic- [192] and cell biology-based studies [193–201], as well as for dissecting the genetic basis of resistance to pests [202] and diseases [203]. However, the most important application of anther-derived, androgenic cultures in pepper has been their use in breeding programs, finally aimed to produce commercial hybrids with maximum heterosis and new or improved traits [172, 204–216]. Although the vast majority of the studies mentioned above have been conducted in *C. annuum*, anther culture has also been explored in other domesticated species, such as *C. frutescens* [217, 218].

As for many other androgenic systems, microspore embryogenesis in pepper strongly depends on three critical factors. The first relates to the donor plants, their endogenous and exogenous conditions, their growth environment (photoperiod, temperature, fertilization, irrigation, use of pesticides, age, season), and principally the genotype, as it occurs in all other responsive species. However, it must be mentioned that for the particular case of some pepper cultivars, it is necessary to use antibiotics to prevent contamination due to the presence of endogenous bacteria in flower buds, extremely difficult to eradicate by the conventional bud surface sterilization. This has also been observed by other researchers in pepper [219] as well as in other species [220], which led to the routine addition of cefotaxime to prevent bacterial growth. The second critical factor is the optimal microspore stage to apply the inductive treatment. In pepper, the discrepancies about the optimal stage have never been as remarkable as in tomato. The literature shows examples of papers claiming that, for certain genotypes, the most inducible stage is the vacuolated microspore [199], whereas others support the early bicellular pollen as the best stage [193]. In general, the most accepted notion is that the inducible stages revolve around the first pollen mitosis, as usual for other species. Perhaps, the fact of defining one stage as the most suitable largely relies on the precision and correctness of the method used to select anthers with microspores at the right stage. A recent paper describes a comparison of four of these methods, including morphological markers such as bud length, anther length, anther pigmentation,

and calyx/bud ratio [221]. This work proposes a combination of calyx/bud ratio and anther pigmentation (once the bud is open under the flow hood), as the most convenient, fast and accurate way to identify anthers containing vacuolated microspores and young bicellular pollen. The third critical factor is the culture environment, including the stress used for induction (reviewed in ref. [174]). As for many other androgenic systems, the first protocols used to culture pepper anthers generated callus, from which plants were regenerated through organogenesis [175–177]. However, further refinement of the experimental procedures allowed the group of Dumas de Vaulx to obtain the direct induction of embryogenesis without an intervening callus phase. This, in turn, permitted a significant improvement in the efficiency of the process of DH production [180, 222]. The improvements introduced by this method were principally based on the use of a high temperature treatment (35 °C) to induce microspore divisions, and two different medium compositions and growth conditions for induction and embryo development, in a way similar to that developed by the same group for eggplant. Thus, a general, reliable method for anther culture in pepper was established. Thereafter, this general protocol has been applied, with slight modifications, to many different pepper varieties. For example, it was recently shown that different durations (4, 8, 12, and 16 days) of the 35 °C heat shock applied to different commercial F1 hybrids of the Lamuyo and California types had significant effects in embryo production, but also in callus generation, which increased with prolonged exposures in a genotype-dependent manner [190]. Nowadays, these and other particular adaptations of the general protocol are still being used to generate DHs for breeding purposes in a number of varieties [174, 184, 189, 194, 196, 206, 217, 223–225]. Similarly, several hybrid seed companies use this technique to obtain pure lines worldwide.

Few years after the Dumas de Vaulx method was developed, the addition to the culture medium of activated charcoal was proposed to adsorb toxic, metabolism-derived compounds [223, 226]. Based on the work of Morrison et al. [182], Dolcet-Sanjuan et al. [227] proposed a biphasic medium consisting of a liquid medium phase poured over a semisolid, agar-based phase with activated charcoal, and added a significant improvement with the implementation of a carbon dioxide environment. This method allowed for the production of embryos of varieties that did not respond to the method of Dumas de Vaulx, and increased the efficiency of other, poorly responding peppers. However, the technical difficulty of having a growth chamber with a carbon dioxide supply has precluded many laboratories from adopting this method on a routine basis. At present, the simplicity of the Dumas de Vaulx method makes it the most popular and routinely used. Notwithstanding this, the anther culture technique also carries the

drawbacks described in the introduction, affecting other Solanaceae such as eggplant or tomato. Thus, in the case of pepper it would be equally desirable to have a protocol for isolated microspore culture. This method, although technically much more complex than anther culture, would provide significant advantages: a higher efficiency, the avoidance of the uncontrollable secretory effect of the tapetum surrounding the pollen sac of the anther, and especially the possibility of occurrence of calli/embryos derived from sporophytic tissues. In the last decades, several research groups have explored the isolated microspore culture pathway. Although many of them described the formation of MDEs [174, 195, 196, 199], in most cases embryo development beyond the globular embryo stage could not be promoted. One exception to this rule was the work of Supena et al. [187, 188], who were able to regenerate haploid plants from microspores isolated from the anther by non-mechanical means. The technique described by Supena et al. consisted of the preparation of a biphasic medium, different from that of Dolcet-Sanjuan et al. [227], and the inoculation of anthers floating on the surface of the liquid phase. Under the right conditions, a few days after inoculation the anthers enter into dehiscence, open and pour their microspore contents into the liquid medium. Embryogenic microspores sink and accumulate at the bottom of the plate, over the semisolid phase. Thus, microspores are isolated from the anther and allowed to form embryos out of the influence of anther tissues. The main problem of this method, as admitted by the authors, was the low percentage (20 %) of normal-looking embryos. However, in 2011 they published a refinement of their own protocol whereby the modification of several physical and chemical parameters raised the percentage of normal-looking embryos beyond 50 % [228]. The shed-microspore method has been tested in many different varieties of Indonesian hot pepper and worked in all of them with different efficiencies, much higher to those obtained by other culture techniques. Indeed, the shed-microspore method was tested against the most popular methods for anther culture, including that of Dumas de Vaulx and of Dolcet-Sanjuan [188]. The shed microspore method proved to be better that the other two. Recently, it was applied to some sweet pepper types. More exactly, the performances in terms of callus and MDE production of the shed-microspore method and of the Dumas de Vaulx method were evaluated in four sweet pepper cultivars [190]. For all genotypes tested, the protocol of Dumas de Vaulx promoted the induction and development of MDEs, but also the growth of callus derived from anther walls. Instead, the shed-microspore method produced no callus but only embryos. However, the embryo responses of the cultivars to each treatment was strikingly different, indicating that there seems not to be a universally useful method to induce androgenesis in pepper.

Aside of the shed microspore culture for microspore isolation, the mechanical isolation of microspores has also been assessed. Supena et al. [187] reported a high rate of microspore induction in the same Indonesian hot types evaluated for the shed microspore method. However, a very low percentage of microspores transformed into MDEs, yielding a maximum of 0.1 regenerated plants per bud. Higher efficiencies were obtained in hot pepper types by Kim et al. [229], using a different protocol defined by a pretreatment of microspores at 32 °C in sucrose-free medium, the use of sucrose as a carbon source for embryo growth, and the use of an optimal microspore plating density. However, the quality of the embryos obtained still needed to be improved. Very recently, the same group published a refinement of their previous protocol, whereby high-quality embryos could be obtained. Based on a two-step culture system, they produced MDEs that germinated into haploid or DH plants at a rate higher than 95 % [230]. With respect to sweet pepper types, in the last years several reports showed that it is also possible to produce DHs through microspore culture, although not as easy as it seems to be in hot types ([191, 219, 231] and our unpublished results). However, the problem of embryo quality and ability to germinate still appears as a major bottleneck to be overcome.

In summary, at present there are four main types of protocols shown to experimentally induce microspore embryogenesis with an acceptable, although variable efficiency: (1) the Dumas de Vaulx method, (2) the biphasic method of Dolcet-Sanjuan, (3) the shed microspore method of Supena, and (4) the isolated microspore method. The four have proven useful in obtaining DH pepper plants. However, not all of them are in principle applicable to all genotypes. In fact, the genotype of the donor plant is one of the most decisive factors in the induction of pepper androgenesis [180, 182–186, 189, 224, 227]. In some cases, the optimization of growth conditions for a given genotype is not sufficient to overcome the barriers imposed by the genotype itself [186]. However, the possibility of applying different types of inductive protocols allows for the choice of the most convenient for each variety. For example, the cultivars “Quito” and “Piquillo” show a null/very low response, respectively, to the method of Dumas de Vaulx [189, 190], but “Quito” shows a fairly acceptable response to the shed-microspore method [190] and “Piquillo” shows a positive response to the biphasic method of Dolcet-Sanjuan [227]. Therefore, before starting a breeding program based on DH production it is advisable to assess the response of each variety to the different types of induction protocol available. The development of the haploid embryo is a second major drawback for an efficient DH production in pepper. It seems that for most of the published methods, a significant amount of MDEs are lost during the transition of a proliferating, yet undifferentiated globular embryo into a heart-shaped

bilateral one. Problems in the formation of the shoot apical meristem and the cotyledons sometimes constrain a proper germination of the embryo. It would be advisable to devote more efforts to the knowledge of the particular developmental requirements of these embryos, in order to facilitate their transition towards a mature embryo.

8 Other Solanaceae

Aside of *Datura* and the five main solanaceous crops, attempts have been made in other Solanaceae to obtain haploids and DHs via anther or microspore culture. The following list illustrates some representative examples: *Atropa belladonna* [232–234], *Hyoscyamus niger* [235–241], *Hyoscyamus muticus* [242], *Petunia* sp. [233, 243–251], *Physalis ixocarpa* [252, 253], *Solanum bulbocastanum* [254], *Solanum dulcamara* [255, 256], *Solanum iopetalum* [257], *Solanum surattense* [258], *Solanum torvum* [259], and *Solanum viarum* [260]. Although more studies will surely be published in the future in these and other solanaceous species, the lower importance of them, compared to the five major solanaceous crops, makes it unlikely to expect major achievements related to these other Solanaceae.

9 Notes

1. Due to the significance of their finding, both in fundamental and applied terms, it is interesting to add some bits of history to this review about the "making off" of this discovery. According to a recent e-mail exchange that the author had with Dr. Maheshwari, his discovery, as many others in experimental sciences, came quite by an accident. The story starts with the father of Satish Maheshwari, named Panchanan Maheshwari. In his young days at Harvard in 1945, Panchanan Maheshwari came into contact with A.F. Blakeslee, who inspired him to investigate on haploid production. At that time it made no sense to think of pollen as a source of haploid embryos, so all efforts were directed to ovule culture. Indeed, Dr. Maheshwari's wife, Nirmala, was a former Ph.D. student at the lab of his father (Panchanan Maheshwari), where they established a technique for ovule culture. In 1960, Satish Maheshwari and his wife were working as postdoctoral researchers at the California Institute of Technology (USA). In the James Bonner laboratory, they participated in the discovery of the existence of RNA polymerases in plant nuclei and chloroplasts. Then, he returned to India, to the Botany Department at Delhi University, headed at that time by his father. Satish Maheshwari did not know

exactly what to do, since the continuation of his previous work on nucleic acids was very difficult in a Department which by that time had only microscopes, microtomes, and tissue culture facilities. Following the advice of his former mentor (James Bonner), he wanted to focus on fundamental research. In an attempt to combine his research interests with the available equipment, he decided to investigate the control of meiosis in anther cultures. The idea was to culture anthers, then isolate young microspore mother cells and see whether hormonal or physical treatments were able to turn meiosis into mitosis or vice versa. At this point Sipra Guha joined his group as a post-doctoral researcher. They chose *Datura innoxia* for two very simple reasons: by the time he started this project, there were *Datura* plants flowering in the Botanical Garden of his university, and due to the large size of the anthers, this seemed to be the right material for his research. When they started the project, accidentally, they discovered microspore embryogenesis, publishing their results in *Nature* in 1964. In words of Dr. Maheshwari, "we were naïve to try to study molecular biology of meiosis this way, but then it led to the happy accident of embryos popping out of anthers". After the discovery, it was very difficult for Dr. Maheshwari to believe that the embryos were originated from pollen grains, and that they were haploid. Sipra Guha had to convince him providing the scientific evidences that led to the publication of their second *Nature* paper in 1966 [12].

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Chapter 10

Bioreactors for Plant Embryogenesis and Beyond

Liwen Fei and Pamela Weathers

Abstract

A variety of different bioreactors have been developed for use in initiating and cultivating somatic embryos. The various designs for embryogenesis and culture are critically evaluated here. Bioreactor optimization and operation methods are also described along with recommendations for use based on desired outcome.

Key words Balloon-type bubble reactor, Mist reactor, RITA®, Temporary immersion system, TIS, Wave reactor

1 Bioreactor Types for Initiating and Cultivating Somatic Embryos and Resulting Plantlets

There are many types of bioreactors that have been designed and used for cultivating plant cell, tissue and organ cultures as illustrated schematically in Fig. 1. These include: (a) classic stirred tank (STR); (b) bubble column reactor (BCR); (c) balloon-type bubble reactor (BTBR); (d) airlift reactor; (e) temporary immersion system (TIS) reactor; (f) RITA® (a variation of TIS); (g) rotating drum; (h) life reactor; (i) bag lined BCR; (j) bag lined STR; (k) wave reactor; (l) undertow reactor; (m) box-in-a-bag; and (n) mist reactor. A number of these reactors also have disposable culture bags whereby both contamination risk and initial capital cost are reduced. In Fig. 1, reactors with disposable culture bags include: (h) life reactor; (i) bag lined BCR; (j) bag lined STR; (k) wave reactor; (l) undertow reactor; (m) box-in-a-bag; and (n) mist reactor. Bioreactors are generally divided into two main groups, liquid phase and gas phase, and have been used with varying success. For a more in depth description of the utility of these different types of reactors for in vitro cultivation of a variety of different plant species, see recent reviews by Paek et al. [1] and Weathers et al. [2, 3]. Here we focus on the key conditions of concern to embryo culture and characteristics of these different types of reactors, as they have been used specifically for somatic embryo (SE) initiation and cultivation.

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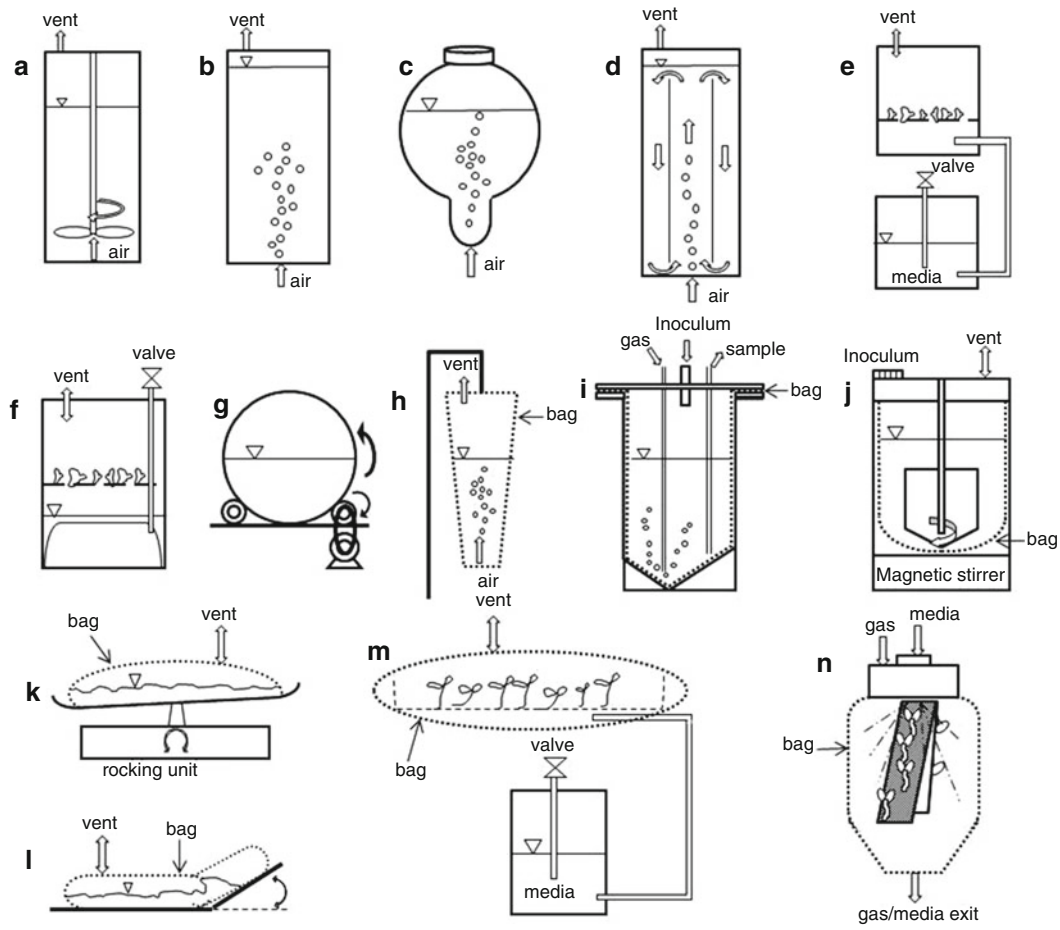

Fig. 1 Various bioreactors used for plant cell, tissue, organ and somatic cell embryogenesis

2 Conditions Critical to Formation and Culture of SEs in Reactors

All plant cell cultures require media selection and optimization per species, and embryogenic cultures are no different. Bioreactors offer some distinct advantages in the larger volume cultivation of in vitro cells, tissues, or organs in that gases and nutrients can be added or removed at various stages of cultivation. However, the type of reactor will also dictate how such environmental factors are controlled. For example, a liquid-based system like a BCR is usually run in a batch mode and the nutrient medium is not externally altered. On the other hand, gas composition and delivery rate in a BCR are critical and must be optimized to maximize SE generation and development. Operating parameters, beyond nutrient medium constituents, that are of considerable concern and that can be most fully controlled in bioreactors are: gases and gas exchange, shear stress, and light.

2.1 Gases

There are three main gases of concern for any plant culture: O_2, CO_2, and ethylene (C_2H_4). In liquid cultures, gases have limited solubility, so efficient gas transfer is challenging. Gas solubility is a function of temperature, pressure and solutes such as salts and sugars [4, 5]. For the gases most important to plant cultures, solubility decreases as temperature and solutes increase. At 25 °C the water solubility of CO_2 and C_2H_4 is about 25 and 4 times higher, respectively, than O_2, so in liquid phase reactors the amount of gas available to a growing tissue culture is quite restricted. Oxygen affects differentiation of embryogenic cells. Although callus formation and explant viability is not affected, anoxia almost completely inhibits embryogenesis [6], suggesting oxygen is required for embryogenesis. Low O_2 concentration may enhance embryo formation by simulating the in ovule environment normally encountered during zygotic embryo development [7]. The overall demand on O_2 then increases during subsequent maturation to the cotyledon stage [8–10].

Elevating CO_2 level in the culture container often improves somatic embryogenesis [11, 12] and effective concentration varies from 0.3 to 5 % with species and cultivars [11–16]. An extremely high CO_2 concentration (10 %), however, is toxic to embryo proliferation [17]. Of course O_2 and CO_2 are not present, nor do they affect, plant cells in isolation. They function in combinations that may fluctuate at different optimal concentration, depending on the developmental stage. Thus, while the early stage of embryogenesis may prefer relatively low O_2 and high CO_2 levels [6, 18–20], there is considerable species and cultivar variation. For example, embryo initiation of celery is favored under 30 % dissolved oxygen (DO, about 5 mL/L) plus 3 % CO_2 [15]. On the other hand one cultivar of *Cyclamen persicum* had significantly more embryos formed at 40 % DO (about 7 mL/L) than another cultivar where there was better embryo formation at lower oxygen levels [21]. Embryo differentiation is also affected as shown in *Coffea arabica* where a DO of 80 % (about 14 mL/L) generated more total embryos, but many fewer at the torpedo stage than at 50 % DO (about 8.4 mL/L) [19].

Ethylene also seems to be required for early differentiation during somatic embryogenesis [15, 22–28]. However, there are some conflicting reports on the effect of headspace C_2H_4 on somatic embryogenesis [29, 30]. It is thus likely that species and cultivars vary in their endogenous production of C_2H_4 and optimal C_2H_4 concentration for embryo development. Sub-optimal-producers may need an exogenous supply of C_2H_4, while over-producers may require removal of C_2H_4 [18, 31, 32]. In sealed containers C_2H_4 generally accumulates to toxic levels for subsequent embryo maturation [33]. Embryo development can be improved, however, by increasing ventilation, using a C_2H_4 trap (e.g., potassium permanganate), adding inhibitors of C_2H_4 biosynthesis (e.g., aminooxyacetic acid, aminoethoxyvinylglycine), or action (e.g., silver nitrate, CO_2) [34–39].

Although O_2, CO_2, and C_2H_4 are the key metabolic gases of concern for plant and SE culture, other gases may also be important. For example, ozone (O_3) is a strong oxidant used to disinfect water. O_3 readily decomposes to O_2 with no toxic by-product, so it could conceivably be used in reactors as a periodic in situ disinfectant to help maintain SE or other plant tissues in axenic culture. Indeed, *Aloe barbadensis* grown in a 4 L BTBR with 1–15 min daily treatment of O_3 over 4 weeks of culture responded quite favorably [40]. Although O_3 can be toxic to plants, limited intermittent use may be a reasonable process option.

2.2 Gas Exchange Rate

The gas environment inside a culture container, as well as in a bioreactor, is highly dependent on the gas exchange rate (ventilation) of the headspace gas. Increasing gas exchange benefits growth by increasing CO_2 level and reducing relative humidity, as well as toxic volatiles (i.e., C_2H_4) in the headspace of a culture container [41–43]. In practice, the gas exchange rate can be increased by increasing passive ventilation in culture containers or integrating a system of forced ventilation. In passive ventilated culture containers, gas exchange rate is increased by using porous closures or gas permeable membranes on the closure [44–47]. By using these strategies, the gas exchange rate can be elevated from 0.04 times/h (0.00066 vvm) under non-ventilated conditions to around 5 times/h (0.083 vvm) [43, 48, 49]. Forced ventilation is best described as vvm, which is defined as the number of volumetric exchanges of headspace gas per unit time (e.g., per minute) within the culture vessel. Use of vvm allows for comparison between differently ventilated culture systems. Except for small culture containers, the gas exchange rate under passive ventilation may still be limited, so even in spite of CO_2 enrichment, CO_2 concentration inside the container is challenging to maintain at ambient levels (0.039 %) [42]. Increasing ventilation in gelled medium is also limited by water potential, as the medium can desiccate when gas exchange is increased even via passive ventilation, and in vitro growth may therefore become limited [50, 51]. Forced ventilation, on the other hand, is more effective than passive ventilation in terms of gas exchange rate for promoting photosynthesis of cotyledonary stage embryos and their subsequent germination, as well as the conversion to plantlets, in bioreactors [52–54]. It is also a more reliable means for controlling the gas environment inside culture container than passive ventilation [55]. Forced ventilation is essential for maintaining efficient gas exchange for photoautotrophic growth in large culture containers, i.e., bioreactors [56]. Forced ventilation is achieved by flushing humidified air via an air pump, connected to a sterile air filter, into the culture container [57]. The gas exchange rate under forced ventilation can be adjusted to more than 10 times/h (0.16 vvm), which efficiently replenishes CO_2 for photosynthesis in bioreactors [42, 58–60] and provides more uni-

form gas distribution in the headspace and, therefore, in the culture medium. In forced ventilation, the greater the ventilation rate, the more the gaseous environment in vitro is similar to that of the ex vitro environment and, as a consequence, the more closely in vitro plantlets resemble ex vitro plants in their morphophysiological traits [60].

2.3 Shear Stress

To obtain adequate mass transfer of gases into large volumes of liquid, usually requires significant agitation of the culture medium, and this can induce hydrodynamic shear stress on the growing cells or tissues. SEs are subject to shear stress and, to reduce shear forces, a slow-speed "string bioreactor" with bubble-free aeration, delivered by thin silicone tubing hanging inside the periphery of the reactor, has been developed. This modified stirred tank reactor (Fig. 1a) has been used to propagate somatic embryos of carrot, Norway spruce, birch, cyclamen, as well as shoots of Christmas begonia with minimum or no damage from shear [61]. There are other stirred reactors similar to the stirred tank (Fig 1a), but they use a spinning filter or cell lift impeller and aeration tubes to provide low shear and bubble free aeration culture of somatic embryos [62–64]. Low-shear mixing and aeration in reactors can also be achieved by bubbling and these bioreactors include bubble column (BCR; Fig. 1b), balloon type bubble (BTBR; Fig. 1c) and air-lift (Fig. 1d) reactors. These reactors use air sparged into the bottom of the reactor to create rising bubbles for mixing and gas diffusion. Compared to bubble reactors, the air-lift reactor has an additional draft tube. However, the major disadvantage of air-lift and bubble column reactors is foaming induced by large volumes of air bubbling, growth of plant tissue in the head space and loss of culture medium volume. To overcome the foaming problem, various reactor designs with a larger top-section diameter have been developed, and the BTBR appears to yield the best biomass due to its high oxygen transfer coefficient [65]. Volume loss is minimized through humidification of the incoming gas by use, for example, of Nafion tubing (Perma Pure, Toms River, NJ, USA).

2.4 Light Effects

Although seemingly sparsely studied, light quality can also play a role in embryogenesis. For example, compared to darkness, far-red, or red-far-red exposure, red light induced a fourfold increase in SEs of quince (*Cydonia oblonga*) [66]. In contrast, initiation of SEs from *Agave tequilana* showed no dependency on light quality, but later development into the cotyledon stage was maximized after exposure to either red or white light [67]. In carrot cell suspensions, darkness produced the most SEs, and did not differ from cells exposed to red or green light [68]. On the other hand, both white and blue light inhibited SE formation [68]. Michler and Lineberger also showed that, in carrot, red light enhanced heart stage SEs [68]. Although light quality may have beneficial effects

on embryogenesis, once the cell density in a liquid-phase reactor increases, light penetration into reactors becomes a nontrivial task, so darkness would certainly be the preferred condition for cultivation [68]. Indeed, the box-in-a-bag reactor (Fig. 1m) was developed because of the challenge in getting adequate light into cylindrical TIS reactors [53, 54, 69].

3 Liquid Phase Bioreactors for Embryos

Most bioreactors used for SE formation are liquid phase wherein the embryos are immersed in liquid medium all the time or intermittently. Here we describe those that have been used successfully for somatic embryogenesis and are also commercially available (Table 1). Although shake flasks can be used as small-scale bioreactors, the ability to control gas delivery and shear can enhance productivity of SEs. These reactors range in size from 15 mL to 20,000 L. Similar to a stirred tank (Fig. 1a), the miniPerm® is a small-volume mechanically driven modular reactor that offers multiple, tiny culture systems, each of which can be fed a stream of gas. Modularity provides the flexibility of testing multiple cell lineages under simultaneous conditions but with small volumes and inoculum. The wave reactor (Fig. 1k) is basically a horizontal, transparent plastic bag, residing on a slowly rocking platform that provides agitation of the liquid medium. As a result of the large surface area of the liquid in the bag, there is relatively good gas exchange potential; gases are provided either passively or actively via sterile permeable filters or membranes. Unfortunately the wave reactor scales laterally, requiring a considerable footprint, instead of less costly vertical space. To our knowledge there are few, if any, reports of either the miniPerm® or the wave reactor being used for SE production.

Table 1
Commercial bioreactors currently available for embryo cultivation

Reactor type	Current manufacturer	Max. vol (L)
Mechanically driven membrane bioreactor—miniPerm®	Sartorius AG, GDR and USA	0.015
Wave Bioreactor	GE Healthcare Life Sciences, USA	0.3–500
BIOSTAT (wave type)	Sartorius AG	1–600
Balloon-type bubble reactor	Samsung Science Co., Seoul, So. Korea	4–20,000
TIS (RITA®, Plantima, Plantform)	Vitropic, France; Plantima A-tech; Toronado, Netherlands; Plantform, Sweden	1–4

TIS temporary immersion system

Although not commercially available, the bubble column reactor (BCR; Fig. 1b) is easy to construct in-house. It is comprised of a cylinder of glass or plastic (autoclavable) with a bottom frit attached via tubing to a gas supply, e.g., air, which passes through the frit, subsequently forming small bubbles that rise through the column of liquid medium, thereby aerating and mixing the culture. Gas vents via a sterile filter at the top. Many different species of SE-forming plants have been grown in BCRs, with some examples reported in Table 2. Unfortunately one of the problems with BCRs is foaming, and because of it the balloon-type bubble reactor (BTBR) was developed (Fig. 1c). The broad surface area of the culture liquid alleviates foaming and provides even better gas exchange than the BCR. A variety of different plant species have produced SEs in the BTBR at a variety of volumes (Table 2; Fig. 2). Although the BTBR has been scaled to 500 L, it is constructed of glass and at large scale must be provided with a stainless steel superstructure, which adds to capital costs. Smaller scale versions (e.g., 4 L) of the BTBR are, however, quite competitive with other smaller bioreactors.

The TIS (Fig. 1e) allows for periodic wetting of the inoculum with nutrient medium. Liquid is pressure fed via tubing from the bottom chamber into the top growth chamber to the level of the inoculum that is located on a porous platform. Gas vents with sterile filters are used to equalize pressure. The liquid is held in the top chamber for a short period of time and then drained back to the bottom chamber until the next filling. This can occur at any regularly set interval, which is often species-specific. TIS can easily be built in-house using Nalgene bottles or other small vessels. A similar version is the "twin flasks" system (not shown) where liquid is passed between two flasks horizontally, instead of vertically; this system is also easy to build in-house. A commercially available example of a TIS is the RITA® system (about 1 L of total volume). To initiate an immersion cycle, pressure is applied to the lower chamber, pushing the medium into the upper chamber. This way plant material is immersed in the bubbling medium, so providing gentle mixing and headspace gas renewal. When the pressure is released, the medium drains back to the lower chamber to complete the immersion cycle. There is a diffusive aeration outlet on the top of the apparatus to balance pressure.

As SEs mature and become chlorophyllous in the cotyledonary stage, light transmittance becomes important for germination of SEs and their subsequent plantlet development [70]. However, the cylindrically shaped vessels in most TIS restrict light penetration into their center, thus also restricting SE development in the center of the culture vessel [54]. To obtain uniform light transmittance and, therefore, more or less synchronized SE maturation, the box-in-a-bag TIS was developed for production of pre-germinated embryos using torpedo stage embryos as inoculum [53, 71].

Table 2
Some examples of SEs successfully cultivated in bioreactors.

Bioreactor type	Species	Volume (L)	Ref.
BTBR	*Eleutherococcus senticosus*	500	[65]
	Transgenic *E. senticosus* SEs	130	[77]
	Panax notoginseng	3	[78]
	Musa acuminata cv Berangan (AAA)	5	[79]
	Santalum album	10	[80]
TIS	*Coffea arabica*	1	[76]
		1–10	[54]
	Saccharum spp. cv Q165	≤1	[81]
	Hevea brasiliensis	1	[82]
	Theobroma cacao	1	[83]
	Bactris gasipaes Kunth	1	[84]
BCR	*Castenea dentate* x *mollisima*	0.1–1.0	[85]
	Eleutherococcus senticosus	10	[86]
	Lilium x *formolongi* (5 cvs)	2	[87]
	Picea sitchensis	2	[88]
Mist	*Daucus carota*	4	[73]
Shake flasks	*Quercus suber* L.	0.1–0.25	[75]

BCR bubble column reactor, *BTBR* balloon type bubble reactor, *SE* somatic embryos, *TIS* temporary immersion system

The growth chamber of this bioreactor is made of a transparent disposable plastic bag, fitted outside a box with a lateral screen on which SEs reside and grow. Despite the high light transmittance, this bioreactor has a large footprint; it also has a problem in medium mixing and the sterile vent connector parts on the bag are too costly to be disposable [69]. RITA® and other types of TIS have been used to culture SEs of many plant species, as noted in Table 2.

4 Gas Phase Bioreactors for Embryos

The mist reactor is, to our knowledge, the only gas-phase reactor. In this system, nutrient medium is provided to cells, explants tissues or organs via an ultrasonic nozzle that yields a fine mist, coalescing and dripping back into the medium reservoir. Although not commercially available, the mist reactor can readily be constructed in-house and used for experiments [72]. It has a disposable transparent plastic bag that is used as a culture chamber. Recently, the mist reactor was used to generate carrot SEs [73]. Cells were manually inoculated onto poly-l-lysine (PLL)-coated nylon mesh that was then hung inside the reactor bag and grown in misted medium for several weeks. Carrot cells were attached to the PLL strips and formed SEs that developed into small rooted

Fig. 2 Large scale cultivation of Siberian ginseng in a balloon type bubble column reactor. Suspension cultures of somatic embryos of *Eleutherococcus senticosus*. (**a**) Embryogenic cells in MS liquid medium supplemented with 30 g/L sucrose and 1 mg/L 2,4-dichlorophenoxyacetic acid. (**b**) Embryogenic suspension in 3-L capacity balloon-type airlift bioreactor containing 2-L MS medium with 30 g/L sucrose. (**c**) Embryogenic suspension in 500 L balloon-type airlift bioreactor. (**d**) Biomass harvested from 500 L balloon bioreactor after 30 days of culture (reproduced from [65] with permission from Springer Science + Business Media)

plantlets, while still hanging on the strips inside the reactor. Subsequently, inoculation was attempted by spraying the cells through the ultrasonic nozzle such that they landed on the hanging PLL-coated nylon mesh. Cells attached, remaining for several weeks while they developed into rooted plantlets. This method suggested that fully developed plantlets, ready for planting into soil, can be obtained in a single step in a bioreactor from cells that underwent somatic embryogenesis.

5 Scaling Up Somatic Embryogenesis and Somatic Embryo Cultivation

Before scaling up, all conditions, especially soluble components like nutrients and plant growth regulators, should first be optimized in small scale shake flasks. The move to larger scale reactors should be made with the following important caveat: not all responses can be directly scaled from shake flasks to any reactor system, mainly because flasks and reactors usually differ significantly in design and operation. There are several reports that document simple, yet effective, approaches for optimizing inoculum, DO, and shear for scaling up SEs. For example, shake flasks were used to optimize inoculum for SE formation in *Coffea* sp. [54]. Inoculum densities of 0.1–3.0 g FW/L of suspension cells were grown in 25 mL of medium in 250 mL Erlenmeyer flasks. If the medium was refreshed with new medium each week, maximum SEs were obtained at the 1.5 g inoculum level, with 25 % reaching the torpedo stage; when medium was not refreshed the number of SEs was much less. Although fewer SEs were obtained at 1.0 and 0.5 g FW/L inoculum, conversion rate to the torpedo stage was considerably greater than at the higher inoculum density. At 3.0 g FW/L, only a few SEs appeared, suggesting that high inoculum density was inhibitory. In another study using cell suspension inoculum in 25 mL of medium in 250 mL Erlenmeyer flasks, banana (hybrid FHIA-18 AAAB) also had an optimum inoculation density. Both inoculum density and culture medium were optimized for somatic embryo production [74].

In another instance, in *Quercus suber* three different shake flasks were used to determine the optimum DO and shear conditions for SE formation [75]. The three types of shake flasks were: a 100 mL Erlenmeyer (EF100), a baffled 150 mL Erlenmeyer (BEF150), and a 250 mL Erlenmeyer (EF250). In conjunction with three orbiting speeds of 60, 110, and 160 rpm, they were able to vary the oxygen volumetric mass transfer coefficient ($K_L a$/h) more than tenfold, from 0.11 in the EF100 at 60 rpm to 1.47 in the BEF150 at 160 rpm with an oxygen transfer rate that increased sixfold at the higher orbit speed. Similarly the shear force index (SFI, cm/min) was altered from 1.4×10^3 in the EF100 at 60 rpm to 8.8×10^3 in the BEF150 at 160 rpm. These simple shake flask studies provided statistically significant differences that enabled relatively easy optimization of *Q. suber* for embryogenesis, with the largest number of embryogenic cell clumps obtained in the BEF150 at 160 rpm.

In contrast to the production of plantlets, hairy roots or production of secondary metabolites, scaling up embryogenesis for cells has the end goal of working with smaller volume reactors. A considerable number of viable embryos can be produced within a relatively small reactor volume, even in a shake flask. For example, 1,600 *Coffea* embryos were obtained in the 1 L volume of one

RITA® container [76]. However, for large plantations of elite genotypes, larger volumes may be required, and thus far the balloon-type bubble reactor has shown considerable promise.

Recently, in South Korea, Shohael et al. [65] scaled up production of Siberian ginseng SEs (*Eleutherococcus senticosus*) to 500 L in a balloon-type reactor (Fig. 2). They inoculated somatic embryos grown first in shake flasks into a 3 L balloon feeder reactor that then was fed into the final 500 L reactor. Using four different 3 L scale (2 L working volume) reactor designs, the balloon reactor that performed best, in terms of biomass and eleutheroside yield, was determined, showing that a stepped aeration protocol of 0.05–0.3 vvm and 5 g cells/L of inoculum density was optimal. Finally, they compared large scale production in 500 L rotating drum and balloon reactors, and the balloon outperformed the rotating drum.

6 Conclusions

There are a number of different reactor types that can be used for the cultivation of embryogenic cell lines. Some involve use of a disposable culture bag, while several are simple and easy to construct in-house. However, only a few are commercially available. Thus, although large scale production of somatic embryos in bioreactors is certainly possible, the possibility of commercial exploitation will clearly be the driving force in the further development of the technology. Furthermore, each plant species needs to be separately optimized for reactor design and production operating parameters.

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Part II

Protocols of Somatic Embryogenesis in Selected Important Horticultural Plants

Chapter 11

Somatic Embryogenesis and Genetic Modification of *Vitis*

Sadanand A. Dhekney, Zhijian T. Li, Trudi N.L. Grant, and Dennis J. Gray

Abstract

Grapevine embryogenic cultures are ideal target tissues for inserting desired traits of interest and improving existing cultivars via precision breeding (PB). PB is a new approach that, like conventional breeding, utilizes only DNA fragments obtained from sexually compatible grapevine plants. Embryogenic culture induction occurs by placing leaves or stamens and pistils on induction medium with a dark/light photoperiod cycle for 12–16 weeks. Resulting cultures produce sectors of embryogenic and non-embryogenic callus, which can be identified on the basis of callus morphology and color. Somatic embryo development occurs following transfer of embryogenic callus to development medium and cultures can be maintained for extended periods of time by transfer of the proliferating proembryonic masses to fresh medium at 4–6-week intervals. To demonstrate plant recovery via PB, somatic embryos at the mid-cotyledonary stage are cocultivated with *Agrobacterium* containing the desired gene of interest along with a, non-PB, enhanced green fluorescent protein/neomycin phosphotransferase II (e*gfp/nptII*) fusion gene. Modified cultures are grown on proliferation and development medium to produce uniformly modified somatic embryos via secondary embryogenesis. Modified embryos identified on the basis of green fluorescence and kanamycin resistance are transferred to germination medium for plant development. The resulting plants are considered to prototype examples of the PB approach, since they contain *egfp/nptII*, a non-grapevine-derived fusion gene. Uniform green fluorescent protein (GFP) fluorescence can be observed in all tissues of regenerated plants.

Key words *Agrobacterium*, Culture medium, Embryogenic cultures, Growth regulators, Plant tissue culture, Precision breeding, *Vitis*

1 Introduction

Grapevine is highly prized and grown worldwide for consumption as fresh fruit and processed products, including jam, jelly, juice, raisin, and, particularly, wine. Grape and its products contain a number of flavonoid and non-flavonoid phenols that act as antioxidants and impart health-beneficial properties. Resveratrol and proanthocyanidins present at high levels in wine possess anti-inflammatory activities and are responsible for cardioprotection [1]. A number of grapevine cultivars have been cultivated for centuries and are greatly valued for their specific fruit/enological characteristics. Only 35 elite, mostly ancient, grapevine cultivars account for 66 % of acreage

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worldwide, as consumers continually seek out the wines produced from them [2]. However, the elite cultivars, having been selected in antiquity with no directed genetic improvement possible since, are susceptible to a number of fungal, bacterial, and viral diseases; they require substantial chemical control in traditional production areas and cannot be grown at all in regions with extreme climatic conditions. Improving abiotic and biotic stress tolerance of these elite cultivars by conventional breeding is impossible because their key sensory attributes are invariably lost. For example, a prized strain of "Pinot Noir" cannot be improved by conventional breeding to create a disease resistant "Pinot Noir." This is because grapevine, like many woody-perennial crops, are out-crossers, exhibiting self-incompatibility and inbreeding depression. New conventionally bred varieties, despite being resistant and even producing acceptable wine, never correspond to their elite counterparts, since existing enological characteristics are disrupted, thereby meeting consumer recalcitrance [3–5]. Inserting desired traits via precision breeding technology is a viable alternative for improving elite grapevine cultivars without altering their highly prized enological characteristics [6, 7].

Recent advances in grapevine genome sequencing have fostered discovery of desirable traits to instill into elite cultivars, while still maintaining their unique varietal characteristics. Grapevine improvement via precision breeding (PB) involves using DNA sequences found solely in the grapevine genome and it is a logical refinement of conventional breeding, only recently made possible by advances in cell culture, gene insertion, and computational technology [7–9]. Grapevine embryogenic cultures have long been the targets of choice for inserting genes encoding desired traits, since single cells on their surface can be prompted to develop into complete plants. Hence gene insertion into such totipotent cells results in plants that stably express the desired trait [10, 11]. An embryogenic response in a grapevine cultivar involves a complex interaction of the genotype with explant, culture medium and culture conditions [4, 12]. This necessitates protocol optimization for each grapevine cultivar grown in specific regions of the world. We have studied the embryogenic response of leaf and floral explants at various developmental stages, various media compositions, and culture conditions for a large number of cultivars over the last three decades [13, 14]. Embryogenic cultures are maintained on development medium for extended periods of time by careful selection and transfer of proliferating embryonic masses. Following somatic embryo development and germination, regenerated plants are hardened in a growth room and transferred to a greenhouse. We also continue to optimize our previous protocols for gene insertion by improving culture development, cocultivation procedures, reducing culture necrosis, and increasing plant recovery.

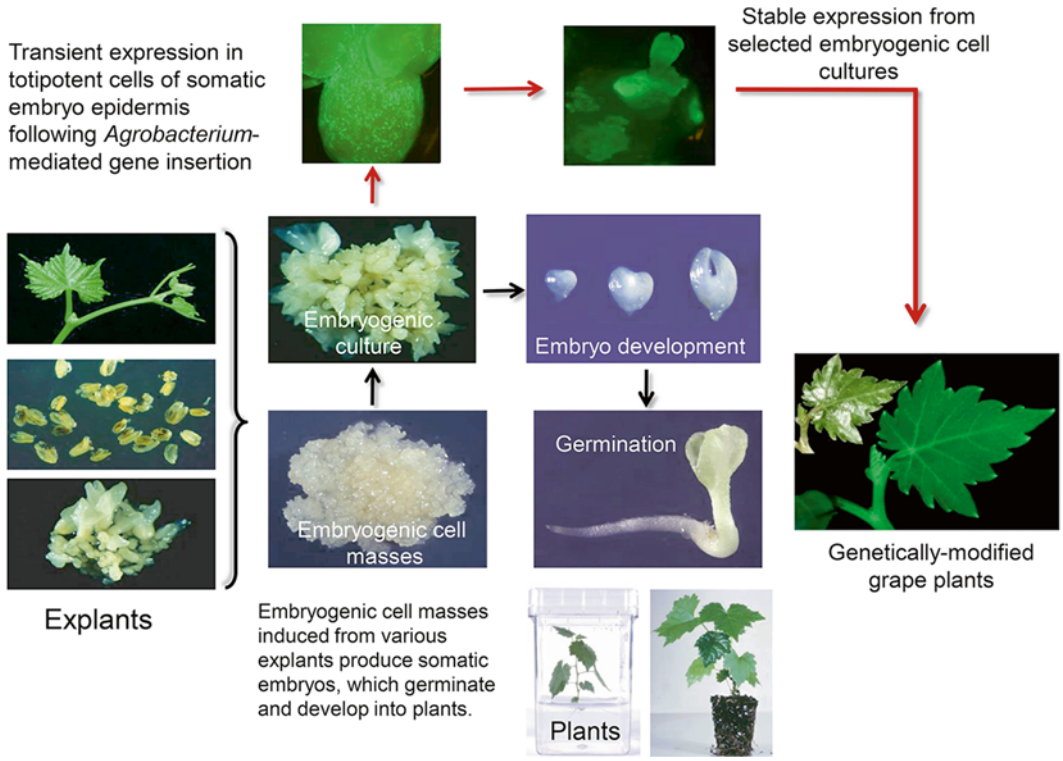

Fig. 1 Somatic embryogenic culture and genetic modification system for grapevine (reproduced from Ref. 26 with permission from Nature Publishing Group)

Genetically modified plants have been recovered from a wide array of *Vitis* species, cultivars, and interspecific hybrids [15–19].

This chapter describes specific methods for the induction, maintenance, and genetic modification of grapevine embryogenic cultures to insert desired traits of interest in order to produce precision-bred versions of elite cultivars (Fig. 1). Cultures are initiated from leaves and/or floral explants on a wide array of culture media. Rapid proliferation of embryogenic cultures is obtained by growing them in liquid medium [20] and long term maintenance is achieved by culture on specialized X6 medium [12]. Somatic embryos at the mid-cotyledonary stage of development are cocultivated with disarmed (non-disease causing) *Agrobacterium* harboring the desired genes of interest. For this demonstration, modified cultures are identified on the basis of non-precision-bred green fluorescence and kanamycin resistance. Plants obtained following germination of embryos are hardened in a growth room and transferred to a greenhouse. The genetically modified status of regenerated plants is confirmed by the uniform expression of the green fluorescence protein gene in various plant tissues.

2 Materials

2.1 Supplies and Equipment

1. Autoclave.
2. Bead sterilizer.
3. Stereomicroscope.
4. Fiber-optic illuminator.
5. Forceps.
6. Scalpels.
7. Clorox® bleach or equivalent.
8. Sterile Whatman 3MM filter paper.
9. Tween 20.
10. Sterile distilled water.
11. 100 × 15 mm plastic Petri dishes.
12. GA7 Magenta culture vessels.
13. Laminar airflow sterile culture hood.
14. Growth chamber.
15. pH meter.
16. Micropipettes and micropipette tips.
17. Spray bottles.
18. 125 mL Erlenmeyer flasks.
19. 960 μM sieves.
20. Rotary shaker.
21. Leica MZFLIII stereomicroscope or equivalent equipped for epi-fluorescence with an HBO 100 W Mercury lamp illuminator and a green fluorescent protein (GFP) filter set composed of an excitation filter (470/40 nm), a dichromatic beam splitter (485 nm), and a barrier filter (525/50 nm) (Leica Microscopy System Ltd., Heerbrugg, Switzerland).

2.2 Explant Sources

1. One-year-old, dormant grapevine cuttings (*see* **Note 1**).
2. Established micropropagation cultures.

2.3 Culture Medium Composition

1. Embryogenic culture induction from leaf explants (NB2 medium): Nitsch and Nitsch [21] macro-, micronutrients and vitamins, 0.1 g/L myoinositol, 20 g/L sucrose, 1.0 μM benzyl amino purine (BAP), 5.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 7.0 g/L Tissue culture (TC) grade Agar (Phytotechnology labs), pH 6.0 (*see* **Note 2**).
2. Embryogenic culture induction from stamen and pistil explants (MSI medium): Murashige and Skoog [22], macro-, micro-nutrients and vitamins, 0.1 g/L myo-inositol, 20 g/L

sucrose, 4.5 μM BAP, 5.0 μM 2,4-D, 7 g/L TC agar, pH 6.0 (*see* **Note 3**) [13].

3. The following media may variously be used for embryogenic culture induction from anther and pistil explants:
   (a) PIV medium: Nitsch and Nitsch macro- and micronutrients, B5 vitamins, 60 g/L sucrose, 8.9 μM BAP, 4.5 μM 2,4-D, 3.0 g/L Phytagel, pH 5.7 [23].
   (b) X1 medium: Modified MS macro-, micronutrients and vitamins, which lack glycine and consisting of modified MS nitrate (X nitrate) consisting of 3.033 g/L KNO_3 and 0.364 g/L NH_4Cl, 0.1 g/L myoinositol, 20 g/L sucrose, 5.0 μM BAP, 2.5 μM 2,4-D and 2.5 μM beta-naphthoxy-acetic acid (NOA), 7 g/L TC agar, pH 5.8 [13].
   (c) X2 medium: Modified MS macro-, micronutrients and vitamins, which lacks glycine and MS nitrate being replaced with X nitrate consisting of 3.033 g/L KNO_3 and 0.364 g/L NH_4Cl, 0.1 g/L myoinositol, 20 g/L sucrose, 5.0 μM BAP, 15.0 μM 2,4-D and 2.5 μM NOA, 7 g/L TC agar, pH 5.8 [13].
   (d) NI medium: Nitsch and Nitsch macro-, micronutrients and vitamins, 0.1 g/L myoinositol, 20 g/L sucrose, 5.0 μM BAP, 2.5 μM 2,4-D and 2.5 μM NOA, 7 g/L TC agar, pH 5.8 [13].
   (e) NII medium: Nitsch and Nitsch macro-, micronutrients and vitamins, 0.1 g/L myoinositol, 20 g/L sucrose, 5.0 μM BAP, 15.0 μM 2,4-D and 2.5 μM NOA, 7 g/L TC agar, pH 5.8 [13].
4. Embryogenic culture maintenance in liquid medium (B5/MS medium): B5 macro-nutrients, MS micronutrients and vitamins, 0.4 g/L glutamine, 60 g/L sucrose, 4.5 μM 2,4-D, pH 5.8 [19].
5. Embryo development and maintenance medium (X6 medium): Modified MS macro-, micronutrients and vitamins, which lacks glycine and MS nitrate being replaced with X nitrate consisting of 3.033 g/L KNO_3 and 0.364 g/L NH_4Cl, 60.0 g/L sucrose, 1.0 g/L myoinositol, 7.0 g/L TC agar, 0.5 g/L activated charcoal, pH 5.8 (*see* **Note 4**).
6. *Agrobacterium* solid culture medium (YEP medium): 10 g/L yeast extract, 10 g/L peptone, 5.0 g/L NaCl, 20 g/L agar, pH 7.0.
7. *Agrobacterium* liquid culture medium (MG/L medium): 5.0 g/L mannitol, 1.0 g/L L-Glutamate, 5.0 g/L tryptone, 2.5 g/L yeast extract, 5.0 g/L NaCl, 150.0 mg/L KH_2PO_4, 100.0 mg/L $MgSO_4 \cdot 7H_2O$, 2.5 mL/L Fe–EDTA, pH 7.0 (*see* **Note 5**).

8. *Agrobacterium* liquid transfer medium (X2 medium): X6 medium modified to contain 20.0 g/L sucrose without TC agar and activated charcoal, pH 5.8.
9. Liquid cocultivation medium (DM medium): DKW basal salts [24], 0.3 g/L KNO_3, 1.0 g/L *myo*-inositol, 2.0 mg/L each of thiamine–HCl and glycine, 1.0 mg/L nicotinic acid, 30 g/L sucrose, 5.0 μM BAP, 2.5 μM each NOA and 2,4-D, pH 5.7.
10. Callus induction medium (DM medium): DKW basal salts, 0.3 g/L KNO_3, 1.0 g/L *myo*-inositol, 2.0 mg/L each of thiamine–HCl and glycine, 1.0 mg/L nicotinic acid, 30 g/L sucrose, 5.0 μM BAP, 2.5 μM each NOA and 2,4-D, 7.0 g/L TC agar, 200 mg/L each of carbenicillin and cefotaxime, and 100 mg/L kanamycin, pH 5.7.
11. Embryo germination medium (MS1B): MS macro-, micronutrients and vitamins, 0.1 g/L myoinositol, 30.0 g/L sucrose, 1.0 μM BAP, 7.0 g/L TC agar, pH 5.8.

2.4 Antibiotic Stock Solutions

1. Rifampicin: Filter-sterilized stock solutions containing rifampicin at 20 mg/mL (*see* **Note 6**).
2. Kanamycin sulfate: Filter-sterilized stock solutions containing kanamycin sulfate at 100 mg/mL.
3. Carbenicillin and cefotaxime: Filter-sterilized stock solutions containing either carbenicillin or cefotaxime at 200 mg/mL.

2.5 Agrobacterium Culture

1. Binary vector containing the gene of interest and an egfp/nptII fusion gene (reporter marker fusion) under the control of a constitutive promoter.
2. *Agrobacterium* stock (containing the binary vector) stored in glycerol at −70 °C.

3 Methods

Carry out all surface sterilization, explant isolation, and transfer procedures using established aseptic techniques in a laminar airflow hood. Clorox®. Wrap all dishes with Parafilm®.

3.1 Embryogenic Culture Induction from Leaf Explants

1. Initiate in vitro micropropagation cultures from field- or greenhouse-grown grapevine shoot tips (*see* **Note 7**).
2. Excise unopened leaves, 1.5–5.0 mm in size, from in vitro-grown micropropagation cultures and transfer them to Petri dishes containing NB2 medium (*see* **Note 8**).
3. Incubate cultures in darkness at 26 °C for 5–7 weeks for the induction of embryogenic callus.

4. After 5–7 weeks, transfer cultures to light (65 μM m^{-2} s^{-1} and 16 h photoperiod) at 26 °C for 5 weeks. Screen callus cultures for growth and possible contamination at weekly intervals.
5. Explants will produce callus cultures, which can be distinguished into cream-colored embryogenic callus and dark brown non-embryogenic callus.
6. Carefully transfer the cream colored embryogenic callus to X6 medium for proliferation of proembryonic masses (PEM) and development of somatic embryos (SE).

3.2 Embryogenic Culture Induction from Stamen and Pistil Explants

1. Obtain grapevine inflorescences from field-grown grapevines or one year old dormant cuttings.
2. Surface-sterilize dormant cuttings in 25 % Clorox® solution with constant agitation for 5 min, followed by two washes with sterile distilled water.
3. Make fresh cuts at the top and base of the cuttings and transfer 30 cm long cuttings to 500 mL conical flasks containing 250 mL sterile distilled water.
4. Transfer flasks under light (65 μM m^{-2} s^{-1} and 16-h photoperiod) at 26 °C for 3–5 weeks for inflorescence growth and development.
5. Determine development stages of stamens and pistils using a stereomicroscope to identify the optimum stage for the specific cultivar (*see* **Note 9**) (Fig. 2).
6. Surface-sterilize inflorescences by rinsing briefly in 70 % ethanol followed by washing them in 25 % Clorox® solution containing a small drop Triton X-100 for 5 min with a periodic manual high degree of agitation. Following washing with Clorox® solution, treat explants with three 5-min washes in sterile distilled water.
7. Using a stereomicroscope, carefully excise intact stamens by separating them from the calyptra and pistil. Place stamens from five inflorescences as a clump in the center of the Petri dish containing induction medium and corresponding pistils with the filament stubs at the perimeter. Seal Petri dishes and place in the dark at 26 °C for 5 weeks (*see* **Note 10**).
8. After 5 weeks of incubation in the dark, transfer Petri dishes to light (65 μM m^{-2} s^{-1} and 16-h photoperiod) at 26 °C. Screen developing cultures using a dissecting microscope for the presence of embryogenic callus at weekly intervals for 12–16 weeks (*see* **Note 11**).
9. Induction of embryogenic callus is observed either from the filament tip, connective tissue or in some cases from pistil explants.
10. Transfer the embryogenic callus to X6 medium for SE development and proliferation.

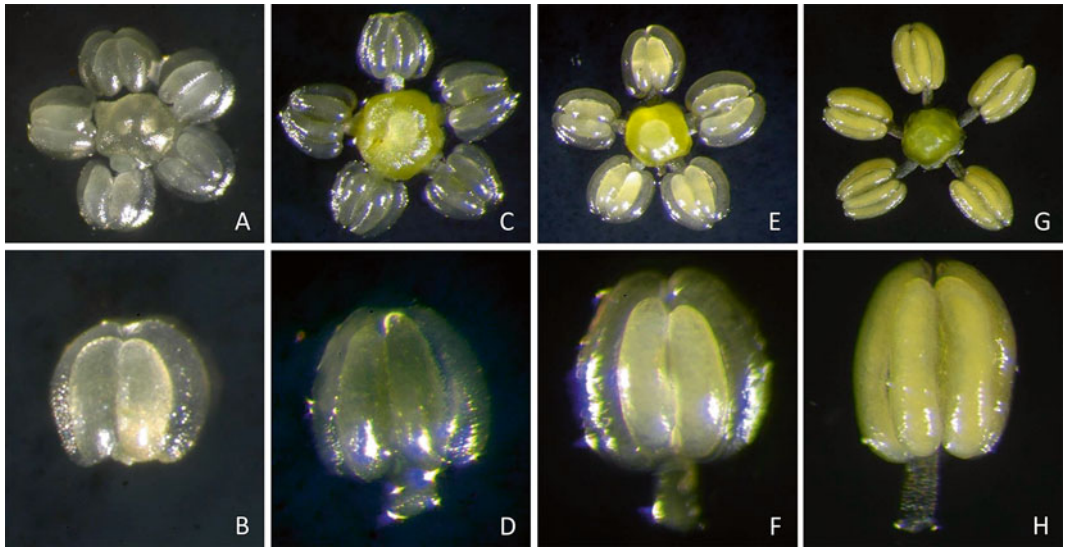

Fig. 2 Stamen and pistil explant developmental stages in *Vitis*. Four stages, I (**a**, **b**), II (**c**, **d**), III (**e**, **f**), and IV (**g**, **h**) can be identified on the basis of inflorescence size, stamen color and size, and microspore development stage. Stage I flower clusters are about 2.5–3.0 cm long, individual flower buds 0.5–0.7 mm in diameter, anthers 0.1–0.2 mm in length, white in color and clear in appearance. Stage II flower clusters are about 6–8 cm long with individual flower buds being approximately 1.5 mm in diameter. Anthers are 0.8–1.0 mm long, yellowish in color, and appear translucent with clear walls. Stage III flower clusters are 9–10 cm long and individual flower buds 1.5–2.0 mm in diameter. Anthers are 1.0 mm in length, yellowish in color, and cloudy in appearance with clear walls. The locule appears cloudy and yellowish in color. Microspore walls are thicker and well developed. Stage IV flower clusters are greater than 10 cm in length and individual flower diameter similar to Stage III. Anthers are 1.0 mm long and yellowish in color with completely opaque walls. The locule appears yellow in color and opaque. Microspore walls are thicker and pores in the cell wall are evident (reproduced from Ref. 13 with permission from American Society of Horticultural Sciences)

3.3 Embryogenic Culture Proliferation in Liquid Medium

1. Transfer 1.0 g rapidly growing embryogenic culture to sterile 125 mL Erlenmeyer flasks containing 40 mL autoclaved liquid medium. Cover the flasks with aluminum foil and seal the neck with Parafilm. Transfer the flasks to a rotary shaker and incubate in diffused light (15 μM m^{-2} s^{-1} and 16-h photoperiod) at 120 rpm.
2. After 2 weeks, separate differentiated somatic embryos by filtering cultures through a sterile 960 μM stainless steel and collecting the fine fraction. Transfer the fine fraction to fresh liquid medium and differentiated SE to X6 medium for embryo development.
3. Maintain suspension cultures by transfer to fresh liquid medium at 2–3-week intervals (*see* **Note 12**).

3.4 Embryogenic Culture Maintenance

1. Transfer embryogenic cultures obtained from induction medium to X6 medium for development and proliferation of SE (Fig. 3a).

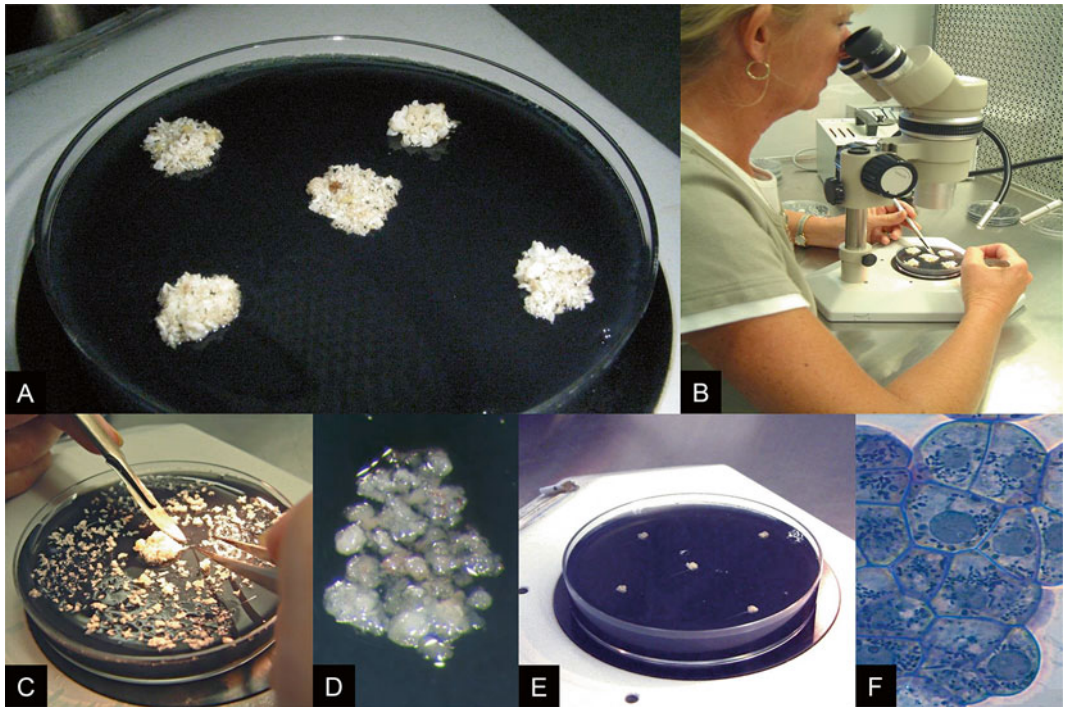

Fig. 3 Embryogenic culture maintenance in *Vitis*. Actively proliferating proembryonic masses contained within embryogenic culture masses grown on X6 medium (**a**) are identified, sub-cultured, and manipulated using a stereomicroscope placed in a laminar flow culture hood and illuminated with a fiber optic light source (**b**, **c**). Only microscopic proembryonic tissue masses are selected (**d**) and these are accumulated so as to create five cultures in each Petri dish containing 30 mL (*thick*) of freshly made X6 medium (**e**) and the cycle is repeated at 4–6-week intervals. Proembryonic masses are uniformly composed of small, densely cytoplasmic embryogenic cells (**f**). It is important to keep a uniform subculture time and a stable incubation temperature to avoid precocious germination

2. Maintain embryogenic cultures by precisely separating PEM from differentiated SE using a stereomicroscope as described below (Fig. 3b, c) and selectively transfer only PEM to fresh X6 medium at 4–6-week intervals (*see* **Note 13**) (Fig 3d, e).

3.5 Agrobacterium Culture Initiation for Plant Genetic Modification

1. Streak *Agrobacterium* culture stock containing the binary plasmid onto a Petri dish containing solid YEP medium with 20 mg/L rifampicin and 100 mg/L kanamycin. Incubate dishes in the dark at 26 °C for 2–3 days until single bacterial colonies are visible.
2. Transfer a single bacterial colony to a 125 mL conical flask containing 30 mL MG/L medium with 20 mg/L rifampicin and 100 mg/L kanamycin. Seal the flask with Parafilm and incubate on a rotary shaker at 180 rpm and room temperature for 16–20 h.

3. Transfer the overnight culture to a 50 mL centrifuge tube and spin at 4200 × *g* for 8 min at room temperature. Discard the supernatant and resuspend the pellet in 20 mL liquid X2 medium. Transfer the culture to a 125 mL conical flask and incubate for an additional 3 h under the same conditions as above. Use this culture for cocultivation.

3.6 Gene Insertion into Embryogenic Cells

1. Carefully transfer cotyledonary-stage SE to sterile Petri dishes. Avoid wounding of embryos during transfer to prevent culture browning (*see* **Note 14**).
2. Add 5.0 mL *Agrobacterium* culture to the SE and mix thoroughly by swirling. Incubate for 7–10 min and then remove the bacterial solution completely using a micropipette.
3. Transfer SE to a Petri dish containing two layers of filter paper soaked in liquid DM medium. Seal the Petri dish with Parafilm® and cocultivate in darkness at 26 °C for 72 h (*see* **Note 15**).
4. Following cocultivation for 72 h, observe SE for transient GFP expression using a stereomicroscope equipped for epi-fluorescence.
5. Transfer cocultivated cultures to a 125 mL conical flask containing liquid DM medium with 200 mg/L each of carbenicillin and cefotaxime, and 15 mg/L kanamycin.
6. Transfer the flask to a rotary shaker at 110 rpm and wash SE for 3 days to inhibit remnant bacterial growth.
7. Transfer washed cultures to each 100 × 15 mm Petri dish containing 25 mL solid DM medium with 200 mg/L each of carbenicillin and cefotaxime and 100 mg/L kanamycin.
8. Place Petri dishes in dark at 26 °C for 4 weeks to permit callus development and proliferation.
9. After 4 weeks, transfer callus cultures to 100 × 15 mm Petri dishes containing 30 mL X6 medium with 200 mg/L each of carbenicillin and cefotaxime and 70 mg/L kanamycin for secondary embryo development. Place Petri dishes in dark and screen at weekly intervals for the presence of modified SE lines.
10. Independent SE lines are identified by bright GFP fluorescence and kanamycin resistance (*see* **Note 16**).
11. Transfer independent genetically modified embryo lines to individual Petri dishes containing X6 medium with 200 mg/L each of carbenicillin and cefotaxime and 70 mg/L kanamycin.
12. Screen cultures for the development of modified embryo development and proliferation.

3.7 Somatic Embryo Germination and Plant Regeneration

1. Transfer cotyledonary-stage SE to MS1B medium and culture under light (65 μM m^{-2} s^{-1} and 16 h photoperiod) at 26 °C for embryo germination (*see* **Note 17**).
2. After 3 weeks, transfer well-developed plants with a robust shoot and root system to plastic pots containing sterile Pro-Mix BX potting mix (Premier Horticulture Inc., Red Hill, PA) and acclimate in a growth room in light (65 μM m^{-2} s^{-1} and 16-h photoperiod) at 26 °C.
3. After 4 weeks, transfer well acclimated, vigorously growing plants to a greenhouse.
4. Confirm gene expression in regenerated plants by observing various plant tissues using a stereomicroscope equipped for epi-fluorescence (*see* **Note 18**).

4 Notes

1. Dormant cuttings are obtained by pruning annual wood from grapevines during the winter season. Alternatively, certified cuttings can be obtained from grapevine germplasm repositories such as the University of California, Davis, or the USDA cold-hardy grapevine repository in Geneva, NY.
2. An embryogenic response from unopened leaf explants on NB2 medium is observed from all seedless cultivars tested, whereas a majority of seeded cultivars will only respond using the stamen/pistil procedure. This factor must be considered prior to embryogenic culture initiation from leaf explants.
3. Production of embryogenic responses from stamen and pistil explants varies widely with *Vitis* species and cultivar. Hence untested individual cultivars must be evaluated on each induction medium listed above to obtain an embryogenic response. A list of responsive varieties and their optimum induction media can be found in our reference publication [13].
4. The use of TC agar (Phytotechnology Laboratories, LLC, Shawnee Mission, KS, USA, Catalog No. A 175) or an agar brand of equivalent purity, is paramount for successful induction and maintenance of embryogenic cultures. Use of other gelling agents in X6 medium results in a rapid decline in embryogenic potential and eventual culture death. A simple observation to judge agar purity is the relative translucence of poured dishes: the more translucent, the better.
5. To make a stock solution of Fe–EDTA, dissolve 7.44 g of $Na_2EDTA{\cdot}2H_2O$ and 1.86 g $FeSO_4{\cdot}7H_2O$ in sterile distilled water and make final volume to 1 L. Although a number of bacterial media were used for *Agrobacterium* culture, MG/L medium provides better cell quality by avoiding overgrowth and assists in maintaining bacterial virulence.

6. Rifampicin is dissolved in methanol or DMSO for making stock solutions. Carbenicillin, cefotaxime and kanamycin sulfate are dissolved in distilled water and then filter sterilized. All antibiotic solutions are stored at −20 °C and thawed just prior to use. Antibiotics are added to culture medium after autoclaving and cooling the medium to 55 °C. Rifampicin is light labile; preparation of stock solutions must be accomplished in very dim light with storage in the dark.
7. Micropropagation cultures are initiated from the earliest sprouting shoots of previously dormant field-grown plants when they reach approximately 10 cm in length. Microdissected shoot apical meristems are used as explants. We previously determined that shoot apical meristems taken at this stage and from the field consistently yield the most sterile and vigorous micropropagation cultures. Cultures are initiated on C2D4B medium, with five meristems per dish [25] and are ultimately used for obtaining unopened leaf explants.
8. It is critical to use only unopened leaves of specific size. Use of larger leaf explants will produce solely non-embryogenic cultures with no regeneration ability.
9. Stamen and pistil explants can be divided into 4 developmental stages based on size of inflorescences and individual flowers, anther size and anther color. Stage II and III explants are known to produce an embryogenic cultures in a large percentage of cultivars tested [12].
10. It is critical to carefully excise intact stamens (anther with attached filament) and place all stamens from five flowers in a clump/group to obtain an optimal embryogenic response. No embryogenic response will be obtained with damaged or detached filaments.
11. Embryogenic response from stamen and pistil explants is genotype dependent. In general, a greater number of cultivars produce an embryogenic response from stamen explants [13].
12. A difference in culture proliferation rates and persistence is observed among various cultivars in both solid and liquid medium. This factor must be considered to ensure transfer to fresh medium at the right interval and avoid culture browning.
13. The use of a stereomicroscope in order to select proper tissue for transfer is an absolute requirement to accomplish this procedure (Fig. 3b) and cannot be stressed enough. It is important to selectively transfer rapidly proliferating PEM to fresh X6 medium using a microscope at 4–6-week interval (Fig. 3c, e). Failure to do so will lead to asynchrony of cultures, precocious SE germination, decrease in embryogenic competence and eventual termination of cultures.

14. It is important to use rapidly growing embryogenic cultures for gene insertion. Use of older cultures can result in significantly lower-to-none insertion frequency and poor plant regeneration.
15. Cocultivation of SE on filter paper dramatically improves gene insertion efficiency while preventing bacterial overgrowth and culture necrosis [19].
16. Proliferation of grapevine embryogenic cultures occurs by direct secondary embryogenesis with new embryos arising from the surface cells of existing SE or pre-existing embryogenic calli (Fig. 3f). Thus, surface cells of cotyledonary-stage SE are ideal targets for gene insertion and plant regeneration.
17. Plant recovery from germinated somatic embryos can be enhanced by trimming enlarged, fleshy cotyledons. This response is species and cultivar dependent and needs to be tested for specific cultivars [16]. A newly published two-step culture procedure dramatically improves plant recovery [26]. This includes culturing embryos on C2D4B medium for a 3-week period followed by transferring the germinated embryos to MSN medium.
18. Uniform GFP expression is observed in plant tissues including leaves, roots, flowers, stamens, and pistils (Fig. 4). Gene insertion efficiency varies widely with *Vitis* species and cultivars [16].

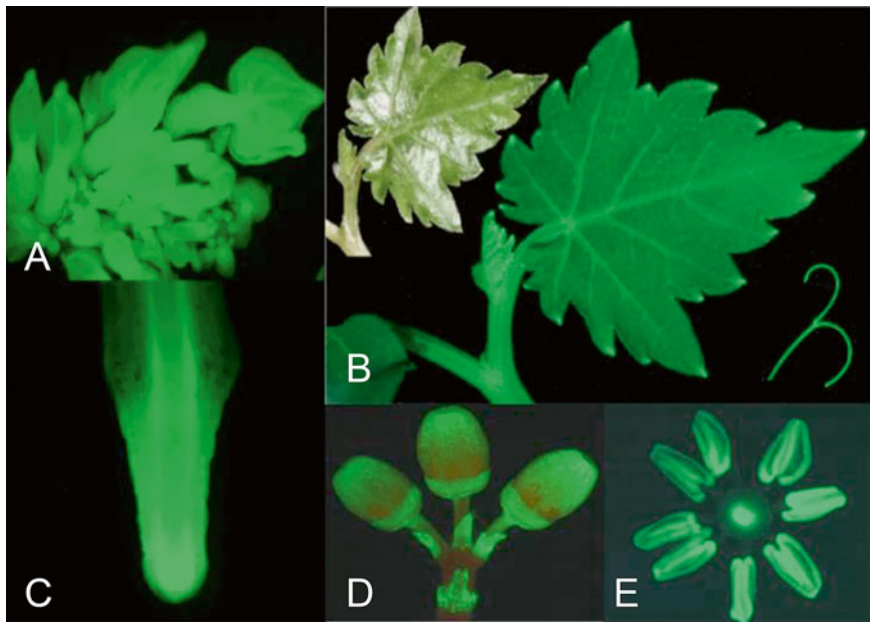

Fig. 4 GFP expression in a genetically modified grapevine. Uniform expression is observed in somatic embryos (**a**), leaves and tendrils (**b**), roots (**c**), inflorescences (**d**), stamens, and pistils (**e**). Note that the *central glowing spot* in (**e**) represents the stigma (reproduced from Refs. 18 and 15 with permission from Springer)

Among the various species and cultivars tested, "Thompson Seedless" (syn. "Sultania") will initiate embryogenic cultures from leaves, stamens and pistils at very high efficiencies and produces the highest number of modified embryo and plant lines [16]. Cultures are readily initiated from both leaves and floral organs. Thus, it is an ideal model with which to learn the procedures.

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Chapter 12

Somatic Embryogenesis in Peach-Palm (*Bactris gasipaes*) Using Different Explant Sources

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Abstract

Peach palm (*Bactris gasipaes* Kunth) is a member of the family Arecaceae and is a multipurpose but underutilized species. Nowadays, fruit production for subsistence and local markets, and heart-of-palm production for local, national, and international markets are the most important uses of this plant. Conventional breeding programs in peach palm are long-term efforts due to the prolonged generation time, large plant size, difficulties with controlled pollination and other factors. Although it is a caespitose palm, its propagation is currently based on seeds, as off-shoots are difficult to root. Hence, tissue culture techniques are considered to be the most likely strategy for efficient clonal plantlet regeneration of this species. Among various techniques, somatic embryogenesis offers the advantages of potential automated large-scale production and putative genetic stability of the regenerated plantlets. The induction of somatic embryogenesis in peach palm can be achieved by using different explant sources including zygotic embryos, immature inflorescences and thin cell layers from the young leaves and shoot meristems. The choice of a particular explant depends on whether clonal propagation is desired or not, as well as on the plant conditions and availability of explants. Protocols to induce and express somatic embryogenesis from different peach palm explants, up to acclimatization of plantlets, are described in this chapter.

Key words Clonal propagation, Conservation programs, Heart-of-palm, Large-scale production, Pejibaye palm, Somatic embryo

1 Introduction

The peach palm (*Bactris gasipaes* Kunth, Arecaceae) is a Neotropical palm whose origin is still uncertain. Authors hypothesizing a single origin point out to the western Amazon Basin, while those supporting a multiple origin suggest the western and northwestern sides of the Andes and lower Central America, in addition to the western Amazon Basin, to be the centers of origin [1]. It is considered a multipurpose tree and plays an important role in agroforestry in several Latin American countries [2]. The production of fruits and heart-of-palm for the national markets is one of its most important uses, becoming peach palm the main source for

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cultivated heart-of-palm [3]. Historically, this species has been considered recalcitrant to in vitro culture. During the last few years, however, several advances have been achieved with the successful application of these techniques in this species; nevertheless, a commercial protocol does not exist yet [4].

Conventional breeding programs of peach palm are long-term efforts due to long generation times of at least 6 years, large plant size, difficulties with controlled pollination, and other factors. Therefore, in vitro clonal propagation has the potential to reduce the time necessary for establishing elite plant orchards by capturing and fixing the genetic gain expressed by selected plants for breeding purposes. Peach palm conservation programs may also profit from the use of in vitro regeneration protocols since germplasm banks could be cloned and backups kept in other institutions for safekeeping. Furthermore, somatic embryogenesis has the possibility to be coupled to conservation programs through, for instance, the cryopreservation of somatic embryos [5], as well as the production of synthetic seeds for plantlet exchange. Hence, a reliable in vitro regeneration protocol for peach palm is important and the development of efficient methodologies is considered a necessity to support use, conservation and breeding programs of this species [1]. Among the available techniques for in vitro plant generation, somatic embryogenesis offers advantages such as large scale automated production, cycling cultures through secondary embryogenesis [6] and genetic stability of the regenerated plantlets [4]. For palm species, somatic embryogenesis has been considered the preferred in vitro regenerative pathway because of the larger number of regenerated plantlets that can be produced compared to organogenesis [7–9].

Tissue culture of palms is generally time consuming and the biological events in each step of the process progress very slowly [4, 10]. In peach palm, our experience shows that in vitro regeneration of a reasonable number of plantlets takes about 2 years and one of the critical aspects regarding the protocol is the choice of the explants. Successful induction of somatic embryogenesis has already been reported from different tissues, such as leaf primordia from adult plants [11], shoots and leaf primordia from in vitro-grown plantlets [9], immature inflorescences [4], mature zygotic embryos [12] and immature zygotic embryos [13]. The choice of the explant source in this species depends also upon the aim pursued and explant availability. For instance, the use of zygotic embryos as explants might have limited applications in conservation programs; however, they may serve as an interesting model to study peach palm somatic embryogenesis because a relatively high induction rate has been observed within few months of culture [12] and the morpho-histological responses from zygotic embryos were very similar to those observed from shoot meristems and leaf sheaths [9, 12]. However, for the clonal propagation and conservation of selected genotypes, the development of protocols that allow regeneration from explants obtained from adult plants is necessary.

2 Materials

1. Zygotic embryos as explants: Seeds from mature fruits, about 4 months after pollination, from one selected open pollinated palm (Fig. 1) (*see* **Note 1**).
2. Inflorescences as explants: Immature inflorescences from open pollinated plants (*see* **Note 2**).
3. 70 % (v/v) ethanol.
4. 40 % bleach solution containing at least 5 % of available chlorine with one drop of the surfactant Tween 20® for each 100 mL.
5. Sterile distilled water
6. Culture tubes (10 × 25 mm).
7. Disposal Petri dishes (90 × 15 mm).
8. Basal culture medium containing MS salts [14], Morel vitamins [15], 3 % (w/v) sucrose, 500 mg/L glutamine, with pH adjusted to 5.8 prior to adding the gelling agent and autoclaved for 15 min at 1 kgf cm^{-2}.
9. Pretreatment liquid culture medium based on basal culture medium supplemented with 1.5 g/L activated charcoal and 200 μM 2,4-dichlorophenoxyacetic acid (2,4-D).
10. Induction culture medium I based on basal culture medium gelled with 2.5 g/L Gelrite and enriched with 1 μM $AgNO_3$ and 10 μM Picloram (4-amino-3,5,6-trichloropicolinic acid) [12].
11. Induction culture medium II based on basal culture medium supplemented with 1.5 g/L activated charcoal and 300 μM Picloram gelled with 2.5 g/L Gelrite.
12. Growth culture medium based on basal culture medium supplemented with 1.5 g/L activated charcoal and gelled with 7 g/L Agar.

Fig. 1 Fruits collected during ripening used as explant source. (*left*) Ripening fruits, showing characteristic color. (*middle*) Seeds inside the fruits. (*right*) Mature and well-developed zygotic embryo used as explant for induction of somatic embryogenesis

13. Maturation culture medium based on basal medium supplemented with 2,4-D (40 μM), 2-isopentenyl adenine (2-iP, 10 μM), activated charcoal (1.5 g/L), and increased glutamine (1 g/L) plus hydrolyzed casein (0.5 g/L) as organic nitrogen source and gelled with 2.5 g/L Gelrite.
14. Conversion culture medium constituted by basal media containing 24.6 μM of 2-iP plus 0.44 μM of naphthalene acetic acid and gelled with with 2.5 g/L Gelrite.
15. Expanded polystyrene trays, containing 5 × 5-cm cells.
16. Commercial substrate (e.g., PlantMax® Paulinia, SP, Brazil, electrical conductivity 1.5–2.0 dS/m) and carbonized rice straw (1:1)

3 Methods

3.1 Somatic Embryogenesis from Zygotic Embryos

1. Remove the hard endocarp of the seeds to obtain the kernels (i.e., zygotic embryos enclosed in the endosperm). Endocarp can be easily removed without damaging the embryo by allowing the former to dry slightly. Afterwards, let the seeds to rehydrate in distilled water for further use.
2. Wash the kernels with running tap water and surface-sterilize the hard endosperm with enclosed embryo by 1 min immersion in 70 % ethanol, followed by 40-min soaking in the 40 % bleach [12].
3. Rinse the kernels with distilled-autoclaved water for at least three times in the transfer hood.
4. Remove the zygotic embryos (Fig. 2a) from the endosperm in the transfer hood with the help of a stereoscope.
5. Transfer the embryos to the induction culture medium I in Petri dish.
6. After 1–2 weeks in culture, a swelling in the mesocotyl region of the zygotic embryo will be observed. Histological analysis has indicated that mitotic events occur in the subepidermic tissue, mainly in cells adjacent to vascular bundles in the mesocotyl of the zygotic embryo [12]. After 4 weeks, intense cellular proliferation occurs in the cotyledonary blade showing the first globular structures onto the primary callus (Fig. 2b). These initial globular structures will further develop into small clusters of somatic embryos (Fig. 2c).
7. After 3 months in culture, up to 27 % of primary calli develop embryogenic callus using this method [12] (Fig. 2d).
8. All steps of induction and expression of somatic embryogenesis are to be undertaken in the dark in a growth chamber at 25 ± 2 °C.

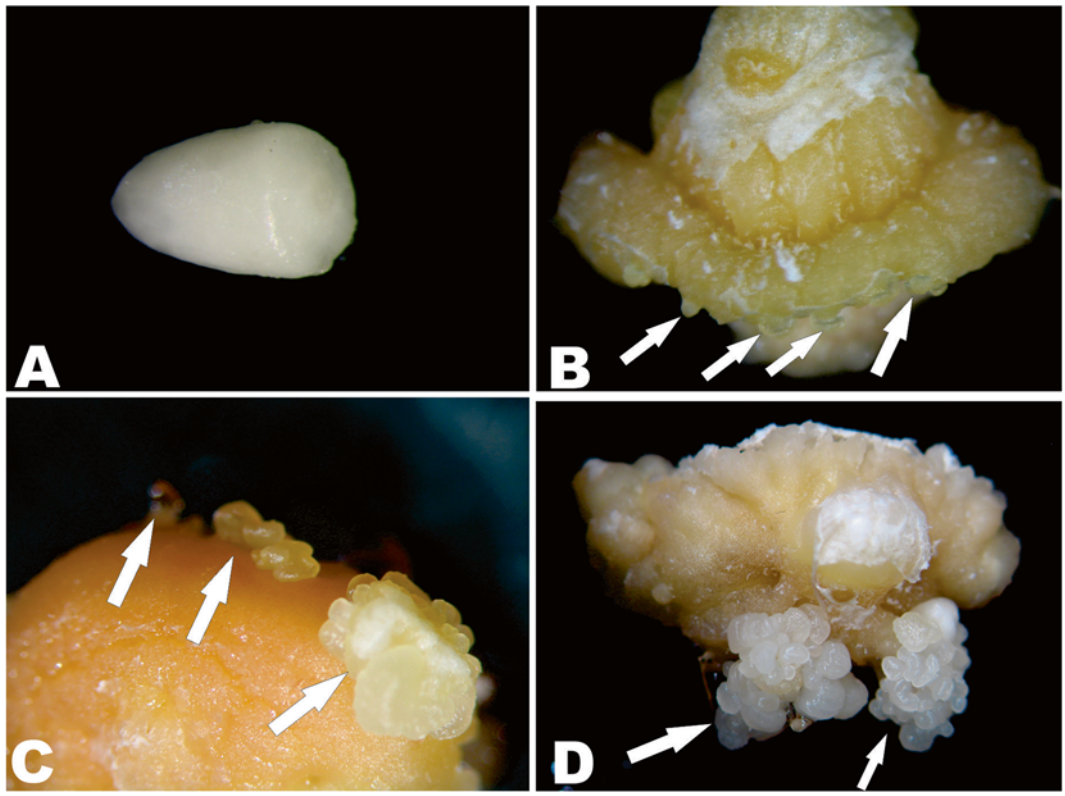

Fig. 2 Induction of somatic embryogenesis from peach palm zygotic embryos. (**a**) Zygotic embryos used as primary explant source. (**b**) Initial development of callus showing the initial development of globular structures (*arrow*). (**c**) Further development of the globular structures into small clusters after 6-week culture. (**d**) Development of a cluster of somatic embryos (*arrows*) on the callus after 12 weeks of culture (reproduced from Ref. 17 with permission from Oxford University Press)

3.2 Somatic Embryogenesis from Inflorescences

1. Collect inflorescences in the stage described above (Subheading 2) from adult plants. Care should be taken to avoid damaging the mother plant or the inflorescence. The inflorescences must be promptly transported to the laboratory.
2. Remove the external spathes. Surface-sterilize the inflorescences when they are still surrounded by the internal spathes by immersion in 70 % ethanol for 5 min, following by air-drying in aseptic conditions.
3. Remove the internal spathes (with a size of approximately 5–8 cm) obtaining the explant that is going to be dissected (Fig. 3a). Separate the isolated inflorescences in individual rachillae and use them as explants (*see* **Note 3**).
4. Place the dissected rachillae in culture tubes containing 25 mL of pre-treatment liquid culture medium for 4 weeks with occasional agitation.

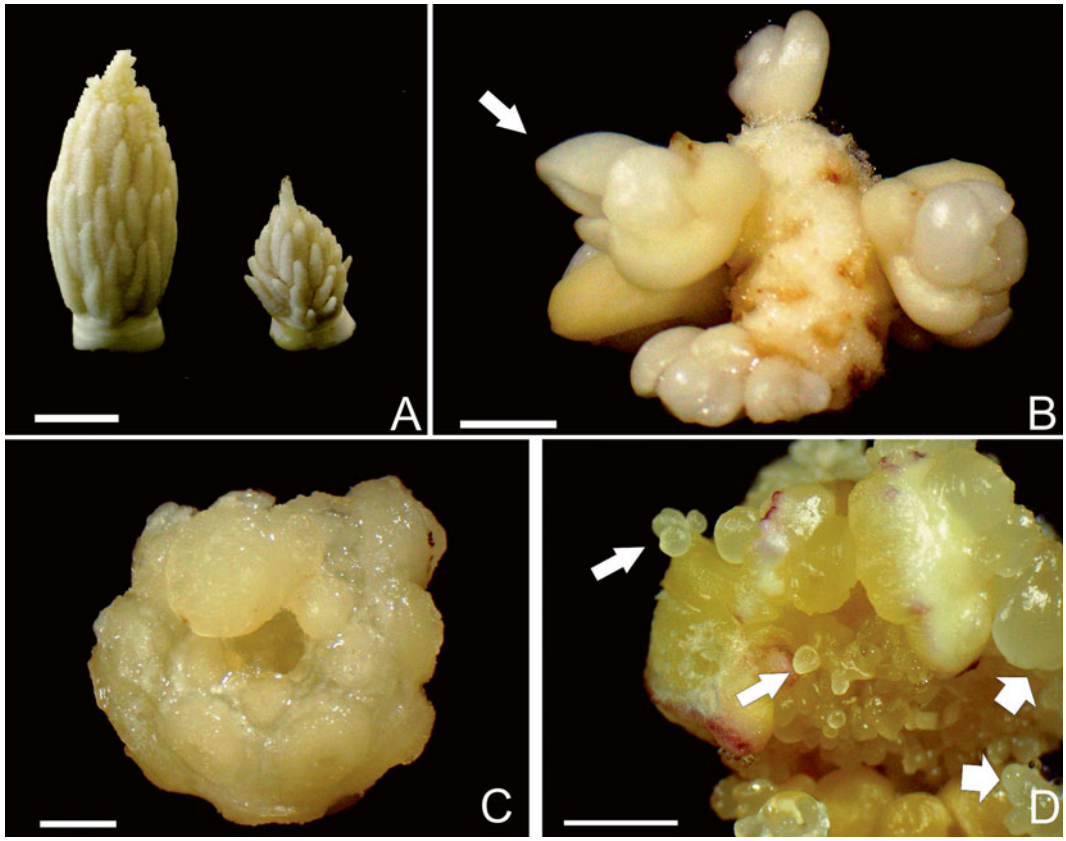

Fig. 3 Somatic embryogenesis and plantlet regeneration from immature inflorescences of peach palm. (**a**) Immature inflorescences utilized as explant source (bar, 1 cm). (**b**) In vitro development of flower bud (*arrow*) (bar, 1 mm). (**c**) Non-organized cellular proliferation of explants (bar, 2.5 mm). (**d**) Somatic embryogenic induction: note the development of globular somatic embryos (*thin arrow*) and nodular tissue (*thick arrow*) (bar, 2.5 mm) (reproduced from Ref. 4 with permission from Springer Science and Business Media)

5. Afterwards dissect the rachillae into slices 1–2 mm thick and inoculate them into Petri dishes containing induction culture medium II.
6. Distinct in vitro responses might be observed, including oxidation of the explants, development of flower buds (Fig. 3b), dedifferentiation into actively growing tissue (Fig. 3c), and development of clusters of somatic embryos (Fig. 3d).
7. Up to 8 % of the explants can develop embryogenic callus after 32 weeks without subculturing [4].
8. All steps of induction and expression of somatic embryogenesis are to be undertaken in the dark in a growth chamber at 25 ± 2 °C.

3.3 Use of Thin Cell Layers as Explants to Induce Somatic Embryogenesis

1. Remove the zygotic embryos as indicated in Subheading 3.1 and transfer them to culture tubes containing 10 mL growth culture medium. Keep cultures at 26 ± 1 °C in a 16-h photoperiod, with 50–60 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps, until the plantlets reach 5–8 cm in height.
2. Remove the leaves, roots, any haustorial tissue and the most external green leaf sheath of the plantlets. Section the remaining embryo axis transversely in 0.7–1.0 mm slices to obtain different histogenic layers (Fig. 4a) (*see* **Note 4**).
3. Inoculate the thin cell layers in Petri dishes containing induction culture medium II.
4. Subculture to the same culture medium only after development of callus is evident. Using this procedure, up to 43 % of the explants can develop embryogenic callus [10].
5. All steps for induction and expression of somatic embryogenesis are to be undertaken in the dark in a growth chamber at 25 ± 2 °C.

Fig. 4 Schematic diagram showing the origin of the explants utilized for the thin cell layer method (*dark scale bar*, 1.75 cm; *white scale bar*, 3 mm)

3.4 Conversion of Somatic Embryos and Plantlet Acclimatization

1. Embryogenic calli developed from any of the protocols described above are similar and result in the development of clusters of somatic embryos; therefore, all might be further cultured following the next steps.
2. Subdivide embryo clusters into smaller clusters of 5–8 somatic embryos before transferring to maturation conditions.
3. Transfer the embryo clusters to maturation culture medium and incubate under dark conditions.
4. These cultures are subculture every 4 weeks in new fresh culture medium.
5. To convert mature somatic embryos into plantlets, transfer them to conversion culture medium in Petri dishes. Keep the cultures at 25 ± 2 °C under a 16-h photoperiod (50–60 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps) for 4 weeks.
6. Subsequently, transfer the plantlets to growth culture medium until they are 6 cm tall, when they can be acclimatized.
7. For acclimatization remove plantlets from the culture vessel, wash all culture medium remnants thoughtfully and prune the root system to approximately 2 cm (Fig. 5a). Transfer the plantlets to a commercial substrate in expanded polystyrene trays.
8. Allocate the trays inside a plastic box covered with glass (Fig. 5b) to allow the entry of light and reduce water loss. Keep these plantlets under 16-h photoperiods with 100–130 μmol m^{-2} s^{-1} light intensity provided by fluorescent and sodium vapor lamps in a growth chamber.
9. After 4 weeks start gradually opening the glass cover to increase gas exchange and reduce relative humidity.
10. Transfer the plantlets to plastic bags containing the same substrate and move them to the greenhouse with shading.
11. This acclimatization system presented high survival (84.2 ± 6.4 %) after 16 week (Fig. 5c) [13].

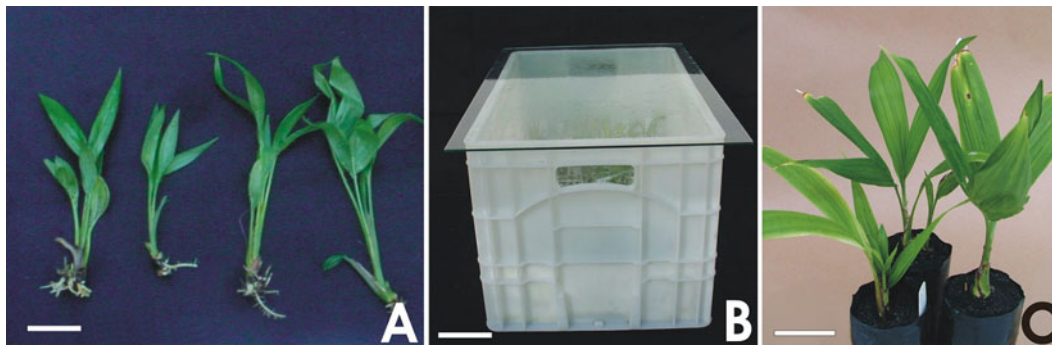

Fig. 5 Acclimatization of peach palm plantlets. (**a**) Plantlets used for acclimatization and with pruned roots (bar, 2.5 cm). (**b**) Apparatus utilized for acclimatization (bar, 12.5 cm). (**c**) Acclimatized plantlets (bar, 5 cm) (reproduced from Ref. 12 with permission from Springer Science and Business Media)

4 Notes

1. Our practical experience suggests that most appropriate stage is when fruits are changing from green color to its characteristic ripe color (yellow to red). During this stage, low contamination rate are observed and the explants respond promptly to the in vitro conditions.
2. The inflorescences' developmental stage on SE, these were classified as Infl1, Infl2, and Infl3, according to the external spathes' size from 5 to 8, 8 to 12, and 12 to 16 cm, respectively. According to [16], these inflorescences are formed in the axils of leaves 2–5, 6–9, and 10–15, respectively, where leaf 1 is the newest expanded leaf in the crown.
3. The rachis is naturally sterile inside when the internal spate is intact and healthy. If the rachis appears to be oxidized or contaminated, low success rate is observed.
4. This explant source might also be obtained from off-shoots of adult selected palm trees.

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Chapter 13

Somatic Embryogenesis: Still a Relevant Technique in Citrus Improvement

Ahmad A. Omar, Manjul Dutt, Frederick G. Gmitter, and Jude W. Grosser

Abstract

The genus *Citrus* contains numerous fresh and processed fruit cultivars that are economically important worldwide. New cultivars are needed to battle industry threatening diseases and to create new marketing opportunities. *Citrus* improvement by conventional methods alone has many limitations that can be overcome by applications of emerging biotechnologies, generally requiring cell to plant regeneration. Many citrus genotypes are amenable to somatic embryogenesis, which became a key regeneration pathway in many experimental approaches to cultivar improvement. This chapter provides a brief history of plant somatic embryogenesis with focus on citrus, followed by a discussion of proven applications in biotechnology-facilitated citrus improvement techniques, such as somatic hybridization, somatic cybridization, genetic transformation, and the exploitation of somaclonal variation. Finally, two important new protocols that feature plant regeneration via somatic embryogenesis are provided: protoplast transformation and *Agrobacterium*-mediated transformation of embryogenic cell suspension cultures.

Key words *Agrobacterium*-mediated transformation, Cell suspension, Cybridization, Polyethylene glycol (PEG), Protoplast fusion, Protoplast transformation, Somaclonal variation, Somatic hybrid

1 Introduction

Citrus spp., native of South East Asia and China, are cultivated in more than 100 countries, between approximately 40° N and 40° S around the world. The genus *Citrus* has been recognized as one of the most economically important fruit tree crops in the world. The most commercially important *Citrus* species are oranges (*Citrus sinensis* L. Osbeck), tangerines (*Citrus unshiu* Marc., *Citrus nobilis* Lour., *Citrus deliciosa* Ten., *Citrus reticulata* Blanco and their hybrids), lemons (*Citrus limon* L. Burm. f.), limes (*Citrus aurantifolia* Christm. Swing. and *Citrus latifolia* Tan.), and grapefruits (*Citrus paradisi* Macf.). *Fortunella*, *Poncirus*, *Microcitrus*, *Clymenia*, and *Eremocitrus* are other genera of the family *Rutaceae*, related to *Citrus*. The importance of *Citrus* spp. is linked to their economic value and to the nutritional proprieties of their fruits.

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Moreover, *Citrus* spp. are connected to the social background of the countries where they are grown, because many traditions, also those related to the cookery, involve the use of *Citrus* fruits. *Citrus* fruits are mostly eaten fresh, but a large part of the production, mainly of grapefruits and oranges, is also used for juice extraction. Furthermore, *Citrus* spp. are utilized in several fields, not only in the food industry, such as the production of marmalades, candies, etc., but also, due to their richness in essential oils and polyphenols, in the cosmetic, flavor, and pharmacy industries.

Although a high genetic variability is present in the genus *Citrus* and its wild relatives, improvement by conventional breeding is difficult because of various biological factors including sterility [1] self- and cross-incompatibility [2], widespread nucellar embryony [1, 3], and long juvenile periods resulting in large plant size at maturity. A consequence of these factors is the dearth of information on genetic control of economically important traits and rapid and effective screening procedures [4]. Sweet orange and grapefruit are important citrus species, and they are believed to be interspecific hybrids, not true biological species [5, 6]. All cultivars within these species have arisen via somatic mutation, either bud-sport or nucellar-seedling variants [7], and not sexual hybridization; intraspecific hybridization results in weak or inviable hybrid progeny (indicative of inbreeding depression) that generally produces fruit unlike those of the parents. The hybrid orange cultivar Ambersweet, which originated by hybridization of a mandarin × tangelo hybrid with sweet orange [8], may be the only exception.

Advances in in vitro tissue culture and improvements in molecular techniques offer new opportunities for developing novel citrus cultivars as some of these technologies can overcome the limitations of sexual hybridization. For example, somatic hybridization can create new combinations that were previously impossible because of sterility or sexual incompatibility. By using this technique, improved varieties of citrus and unique new breeding parents, for scion as well as for rootstocks, can be produced. This technique consists of combining complementary parents with the purpose of transferring desired traits to new plants such as resistance to *Phytophthora*, citrus canker, citrus greening (HLB), citrus variegated chlorosis, blight, and drought [9, 10]. Selecting somatic mutations or genetic transformation allow the modification of very few traits while retaining the essential characterization that typifies specific cultivar or cultivars groups. These techniques often require somatic embryogenesis for efficient plant recovery. This chapter will review somatic embryogenesis, and discuss applications of in vitro biotechnologies and their protocols by utilizing somatic embryogenesis in plant recovery, that can be used to obtain useful new genetic combinations for citrus improvement.

1.1 Somatic Embryogenesis in Plants

Somatic embryogenesis is defined as the differentiation of somatic cells into somatic embryos which show several distinct characteristics [11], including similarity to the developmental stages of zygotic embryogenesis: bipolar structure presenting shoot and root meristems, a closed tracheal system separated from the maternal tissue, and frequently single-cell origin with production of specific proteins. Somatic embryos play an important role in many fields, particularly for large-scale vegetative propagation. This morphogenic process, that can occur with the formation of embryos emerging directly from explants (direct somatic embryogenesis, DSE), or after the formation of callus (indirect somatic embryogenesis, ISE), has been reported in several species [12, 13]. Somatic embryos developing via DSE are formed from competent explant cells which, contrary to ISE, are able to undergo embryogenesis without dedifferentiation, i.e., callus formation. It is believed that both processes are extremes of one continuous developmental pathway [14]. Distinguishing between DSE and ISE can be difficult [15], and both processes have been observed to occur simultaneously under the same tissue culture conditions [16]. Secondary somatic embryos can arise cyclically from the surface of primary somatic embryos, often at a much higher efficiency for many plant species [17, 18]. Some cultures are able to retain their competence for secondary embryogenesis for many years and thus provide useful material for various studies, as described for *Vitis ruperis* [19]. It is possible to induce somatic embryogenesis using different types of culture media, environmental conditions and explants including seedlings and their fragments, petioles, leaves, roots, shoot meristems, seeds, cotyledons, anthers, pistils, and zygotic embryos. Immature zygotic embryos present the most frequently applied source of embryogenic cells which have been employed in most of the established protocols. Immature zygotic embryos made possible the induction of SE in plant species which, for many years, had been considered to be recalcitrant, viz grasses [20] and conifers [21]. By 1995 tissue culture conditions for SE induction had been described for over 200 plant species [17]; increasing numbers of protocols were published after that. The most frequent mode of embryogenesis is via callus formation, which is an indirect type of regeneration.

The interest in somatic embryogenesis is due to several factors such as high regeneration efficiency and the infrequent appearance of somaclonal variation [22]. Somatic embryogenesis has a key role in in vitro clonal propagation for plant mass propagation, as well as for germplasm conservation and exchange, cryopreservation to establish gene banks, sanitation, metabolite production, and synthetic seed production. The application of synthetic seed technology to *Citrus* has been reported for somatic embryos of *Citrus reshni*, *Citrus reticulata* Blanco (cv Avana and cv Mandarino Tardivo di Ciaculli), *Citrus clementina* Hort. ex Tan. (cv Monreal

and cv Nules), a lemon hybrid [23, 24], and Kinnow mandarin [25]. Moreover, in vitro conservation of several *Citrus* species using encapsulation–dehydration technology of cryopreservation has also been reported [26, 27].

Plant regeneration systems that limit or avoid genetic chimerism in regenerants are of special value for biotechnologies that combine tissue culture with genetic transformation or mutant induction and selection. Genetic modification is a unicellular event, and hence regeneration from multicellular centers frequently results in the formation of genetic chimeras. A high probability for the single cell origin of regenerants is what provides for ideal SE. The classical conception of SE is based on the unicellular origin of somatic embryos [28], and this mode of somatic embryo development was the most frequently noticed in embryogenic cell suspensions of *D. carota* [29]. However, single-cell origin of somatic embryos is not the rule, and even in a model system such as embryonic cell suspension of *Daucus carota*, development of embryos from a group of cells cannot be excluded [30]. Development of somatic embryos from more than one cell has in fact been reported in several plant systems. Moreover, both a multicellular and a unicellular origin of somatic embryos in the same regeneration system is quite a common phenomenon, as was observed in several species including *Musa* spp. [31], *Cocos nucifera* [32], *Santalum album* and *S. spicatum* [33], and *H. vulgare* [34]. It is believed that somatic embryos originated from a single cell displayed normal morphology of "single embryo" while aberrant, multiple embryos are derived from a group of cells [35–37]. Numerous published protocols on successful SE induction and plant regeneration in different plant species, suggest that SE could be achieved for additional plant species provided that appropriate explant and culture conditions are employed, although progress will probably remain slow with the more recalcitrant woody species.

1.1.1 Somatic Embryogenesis Protocol Development

The establishment of efficient embryogenic cultures has become an integral part of plant biotechnology as regeneration of transgenic plants in most of the important crops (such as canola, cassava, cereals, cotton, soybean, and various woody tree species) is dependent on the formation of somatic embryos. One of the most attractive features of embryogenic cultures is that plants derived from them are predominantly normal and devoid of any phenotypic or genotypic variation, possibly because they are often derived from single cells and there is stringent selection during embryogenesis in favor of normal cells [38]. Embryogenic cultures were first described in callus and suspension cultures of carrot by Reinert [39] and Steward et al. [40], respectively. In the following decades with increasing understanding of the physiological and genetic regulation of zygotic as well as somatic embryogenesis, embryogenic

cultures had been obtained on chemically defined media in a wide variety of species [38]. In most instances the herbicidal synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) was required for the initiation of embryogenic cultures; somatic embryos develop when such cultures are transferred to media containing very low amounts of 2,4-D or no 2,4-D at all.

During the 1950s a number of attempts were made to demonstrate the totipotency of plant cells. The first evidence of the possibility that single cells of higher plants could be cultured in isolation was provided by Muir et al. [41], who obtained sustained cell divisions in single cells of tobacco placed on a small square of filter paper resting on an actively growing callus, which served as a nurse tissue. Similar results were obtained by Bergmann [42] who plated single cells and cell groups suspended in an agar medium. Further progress was made by Jones et al. [43], who were able to culture single isolated cells in a conditioned medium in specially designed microculture chambers. Direct and unequivocal evidence of the totipotency of plant cells was finally provided by Vasil and Hildebrandt [44, 45], who regenerated flowering plants of tobacco from isolated single cells cultured in microchambers, without the aid of nurse cells or conditioned media. Up to date, in vitro culture techniques have enabled plant regeneration from over 1000 different species [46], following two alternative morphogenetic pathways, shoot organogenesis (SO) or SE. Both morphogenic pathways, SE and SO, may be induced simultaneously in the same tissue culture conditions [47]. Thus, differentiation between SE and SO can sometimes be difficult, and even a detailed comparative histological analysis of the morphogenic process can only suggest an embryo-like origin of developing structures [48]. However, SE and SO can be separated in space and time [49, 50] with the use of appropriate medium composition, mainly type or concentration of plant growth regulators (PGRs).

The application of in vitro systems based on SE for plant regeneration is determined not only by a high efficiency of somatic embryo formation, but frequently depends on capacity of the embryos for complete plant development. The process of developmental changes, which a somatic embryo undergoes, is called "conversion", and it involves the formation of primary roots, a shoot meristem with a leaf primordium and greening of hypocotyls and cotyledons [51]. In numerous systems, in spite of the high number of somatic embryos produced, problems with a lack or a low frequency of embryo conversion into plants has occurred. To stimulate embryo conversion, and to improve the efficiency of plant regeneration, a number of different strategies have been tested. Gibberellic acid (GA_3) is frequently employed in media used for somatic embryo conversion. It should be stressed that in some systems, abnormal morphology of somatic embryos did not decrease the chances of development into normal plants [52–54].

In a plant seed the embryo is generally formed following the fusion of gametes from two parents during fertilization. However, some species form embryos in the seed without fertilization. This kind of reproduction is termed apomixis by which somatic cell-derived embryos develop in a seed. Apomixis is a fairly uncommon trait in plants, but approximately 400 species exhibit this type of propagation in nature [55]. Apomixis is classified into apospory, diplospory, and adventitious embryony according to the developmental process of somatic embryo(s). In apospory and diplospory, apomictic embryo(s) develop megagametophytic structure without meiotic reduction, which is widely observed in grass species. On the other hand, in adventitious embryony as observed in citrus and mango (*Mangifera indica* L.), somatic embryos are directly initiated from nucellar cells in ovule tissue [56]. In citrus, polyembryony, specifically adventitious embryony, is a common reproductive phenomenon. Some cultivars develop many embryos in a seed, such as Satsuma mandarin (*Citrus unshiu*) and Ponkan (*Citrus reticulata*) which form 20 or more embryos in a seed. In contrast, monoembryonic cultivars (e.g., Clementine, *Citrus clementina*, and Kinokuni mandarin, *Citrus kinokuni*) form only a single, zygotically derived embryo in each seed [57]. Apomixis has great potential as a breeding technology because introduction of apomixis into non-apomictic plants enables clonal propagation with genetically true seeds from hybrids. The potential economic benefit of incorporation of apomixis in rice was estimated to exceed US $2.5 billion per annum [58]. Because of its economic potential as a breeding technology, genomics-based approaches have been applied to identify the gene responsible for apomixes [59, 60].

1.1.2 Somatic Embryogenesis in Citrus

Somatic embryogenesis is particularly attractive in citrus because many cultivars and accessions have the capacity for nucellar embryony [61]. Somatic embryogenesis has been induced directly in cultured nucelli [62] and undeveloped ovules [63, 64] or indirectly via callus formation [65–69]. Embryogenesis has also been induced from endosperm-derived callus [70], juice vesicles [71], anthers [72, 73], and styles [74–77].

In order to apply the techniques of modern plant biotechnology to citrus breeding, it is necessary to develop reliable and efficient plant tissue culture procedures for plant regeneration (Fig. 1). In citrus, the production of embryogenic callus lines have been reported from the culture of excised nucelli [78], abortive ovules [79], unfertilized ovules [80], undeveloped ovules [64], isolated nucellar embryos [81], Satsuma juice vesicles [71], anthers [82], styles and stigmas of different species of citrus [75, 83], as well as from leaves, epicotyls, cotyledons and root segments of in vitro grown nucellar seedling of *C. reticulata* Blanco [84]. The embryogenic potential of citrus varies with genotype and type of explant. One important application of this technique is the production of

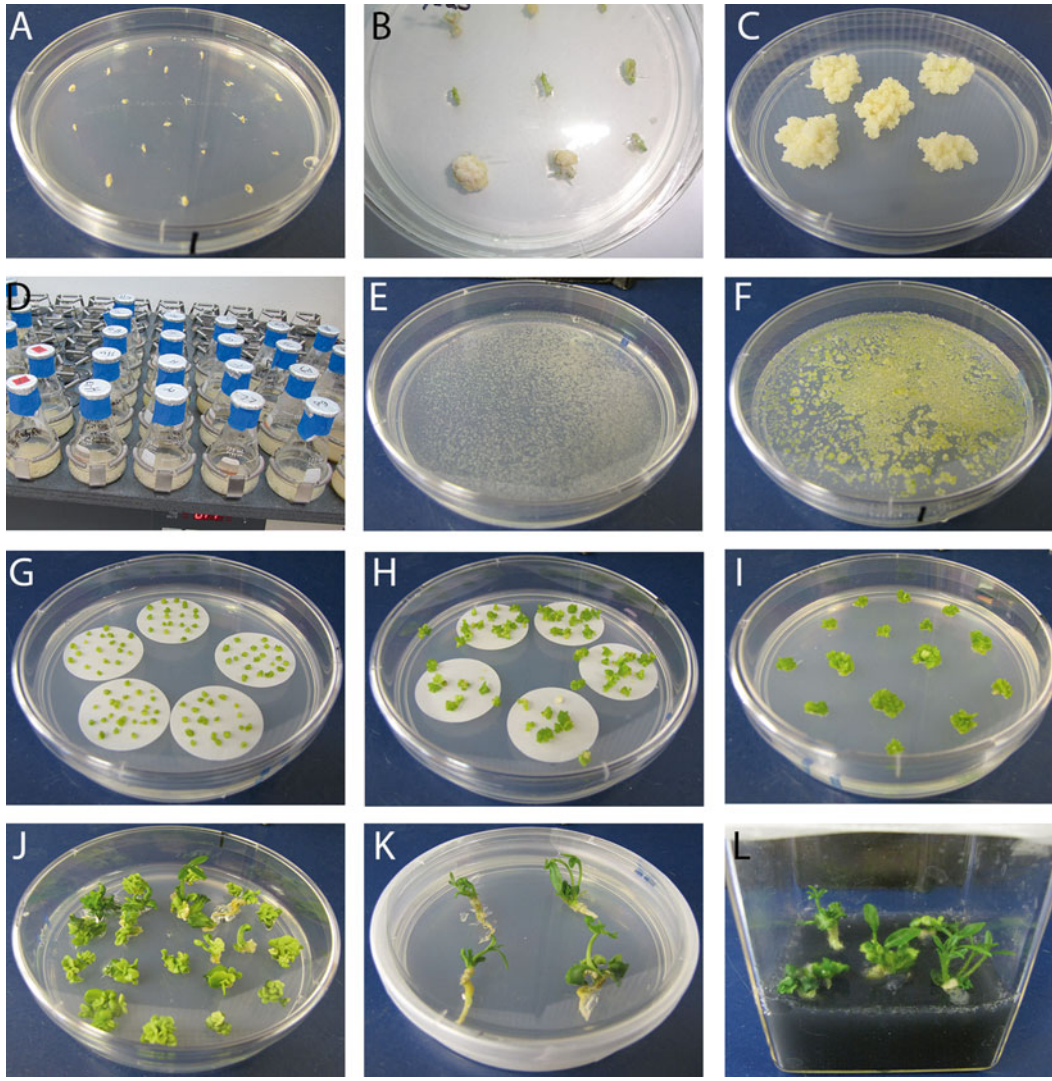

Fig. 1 Somatic embryogenesis in citrus. (**a**) Citrus ovules, (**b**) ovule derived embryogenic callus, (**c**) embryogenic callus, (**d**) embryogenic cell suspension cultures, (**e**) protoplast derived micro-calli, (**f**) callus derived somatic embryos, (**g**, **h**) small-medium somatic embryos on cellulose acetate filter papers, (**i**) enlarged embryos on EME-maltose medium, (**j**) enlarged embryos and shoots on 1500 medium, (**k**) small plantlet on B+ medium, (**l**) plantlets on rooting medium

virus-free citrus plants through somatic embryogenesis from undeveloped ovules of some citrus species [79, 85]. Somatic embryos, embryogenic callus and cell cultures recovered from in vitro cultured ovules have also been used to develop cryopreservation strategies for germplasm conservation [86], to generate somaclonal variation [87], and for protoplast fusion technologies to generate somatic hybrids and cybrids [4, 9, 88, 89]. Many citrus species are found responsive to culture on a basal medium supplemented with malt extract, but embryogenesis has been enhanced by the addition of other growth substances.

Anther culture is a commonly used method to produce haploids and doubled-haploids in *Citrus*, as well as in other fruit crops [90–92]. *Citrus* anther culture produced also somatic embryo-derived regenerants in *C. aurantium* [82, 93], *C. sinensis*, *C. aurantifolia* [94], *C. madurensis* [95], *C. reticulata* [93, 96], *Poncirus trifoliata*, the hybrid No. 14 of *C. ichangensis*× *C. reticulata* [97] and *C. paradisi*. While the somatic embryogenesis capacity of *Citrus* has been found to vary with the cultivar and type of explant, regeneration methods that involve the use of embryogenic callus of nucellar origin (polyembryonic types) generally provide the best results. Unfortunately, these systems either fail or provide only poor results with monoembryonic species that produce only zygotic embryos. Kobayashi et al. [98] cultured the ovules of 23 monoembryonic cultivars and never obtained nucellar embryos.

1.1.3 Source of Explants to Initiate Somatic Embryos in Citrus

The selection of elite citrus plants is essential for the development of efficient systems of somatic embryogenesis. For these purposes, explants should be collected from selected elite specimens that are visibly free from any symptoms of disease, stress or spontaneous mutations (i.e., variegated fruits and leaves, variation in color, size and shape of fruits, and various other plant abnormalities). Carimi [99] addressed several points to bear in mind when deciding upon the choice of explant, i.e., (1) callus formation appears to depend on the status of the tissue, (2) callus initiation occurs more readily in tissues that are still juvenile, and (3) explants must contain living cells. When floral tissues and fruits are old, chances of callus and embryo formation from undeveloped ovules, stigma, or style explants decrease. Stigma and styles derived from immature flowers and undeveloped ovules from unripe fruits have higher embryogenic potentials, although embryogenic callus lines have been successfully initiated also from the undeveloped ovules of mature fruits.

1.1.4 Somatic Embryo Induction and Growth Media in Citrus

The composition of the media used for in vitro regeneration of citrus somatic embryos is based on the inorganic salts recommended by Murashige and Skoog [100] and on the organic compounds suggested by Murashige and Tucker [101]. Sucrose (50 g/L) is usually used as the carbon source. When needed, growth regulators can be added directly to the medium before or after autoclaving. The pH of the medium is generally adjusted to 5.8. Normally, 8 g/L agar is used to solidify media for citrus tissue culture. After preparing the media, it could be stored at room temperature for several weeks before use. Starrantino and Russo [64] first reported somatic embryogenesis from undeveloped ovule culture. The percentage of embryogenic explants ranges from 0 % to 70 %, depending on the genotype. As mentioned, this regeneration procedure does not work with monoembryonic genotypes [102] (for more details about how to initiate somatic embryogenesis including embryogenic callus and suspension lines from undeveloped

ovule culture, see [9, 10, 103]). EBA (MT basal medium plus 0.01 mg/L 2,4-D and 0.1 mg/L 6-BAP) and DOG (MT basal medium plus 5 mg/L kinetin) media are often used for embryogenic callus induction [103].

1.2 Applications of SE in Cultivar Improvement of Citrus

1.2.1 Generation of Somaclonal Variation

Somaclonal variation, first defined and reviewed by Larkin and Scowcroft [104], is a commonly observed phenomenon in cell and tissue cultures of different species regardless of the regeneration system used [105]. This variation involves changes in both nuclear and cytoplasmic genomes, and their character can be of genetic or epigenetic nature [22]. Mechanisms which determine somaclonal variation [106–108], as well as the advantages and drawbacks of in vitro produced plant variants [109–111], have been widely discussed. The identification of valuable somaclonal variants holds great promise for cultivar improvement, especially for the citrus species that are difficult to manipulate by sexual hybridization [4]. Somaclonal variation has been observed in citrus plants regenerated from nucellar callus of monoembryonic "Clementine" mandarin [85]. Callus lines have been selected for salt tolerance [112, 113] and regenerated into plantlets; however, regenerated plantlets lacked internodes and hence could not be propagated further [114]. *C. limon* embryogenic culture lines resistant to "mal secco" toxin were selected. These lines produced somatic embryos, which retained resistance to the toxin [115]. "Femminello" lemon somaclones have also been evaluated for tolerance to mal secco by artificial inoculation [116]. Somaclones of "Hamlin," "Valencia," "Vernia," and "OLL" (Orie Lee Late) sweet oranges have been obtained via regeneration from callus, suspension cultures, and/or protoplasts, obtained via somatic embryogenesis, in efforts to improve processing and fresh market sweet oranges [87, 117]. Significant variation has been observed in fruit maturity date, juice quality, seed content and clonal stability. The University of Florida, Institute of Food and Agricultural Science (UF/IFAS), through Florida Foundation Seed Producers (FFSP), has released several improved sweet oranges regenerated using the somatic embryogenesis pathway, such as "Valencia protoclone SF14W-62" (Valquarius®-U.S. Patent PP21,535, selected for 6–8 weeks early maturity date), "Valencia protoclones N7-3" (U.S. Patent PP21,224 and T2-21, seedlessness and late maturity), "Hamlin protoclone N13-32" (improved juice color), and somaclones "OLL-4" and "OLL-8" (high yield and juice quality, clonal stability). We are also evaluating several hundred lemon somaclones (derived from multiple commercial lemon cultivars) for fruit rind oil content and seed content. We have identified several seedless somaclones and somaclones that consistently yield more oil per unit of rind surface area (Gmitter, Grosser and Castle, unpublished data). It is clear that useful genetic variation can be obtained from large enough populations of somatic embryogenesis-regenerated somaclones.

1.2.2 Protoplast Regeneration via Somatic Embryogenesis

As mentioned above, new cultivars of sweet orange have been developed from populations of plants regenerated from protoplasts via somatic embryogenesis (protoclones) (Fig. 1). In plant tissue culture history, embryogenic cell culture and the development of protoplast technologies that require plant recovery are closely linked. Although progress in the development of protoplast technologies has been made in other woody tree species, including the regeneration of somatic embryos from protoplasts isolated from embryogenic cells of *Pinus taeda* and *Picea glauca* [118–120], citrus has been the true model system in this regard primarily due to its robust ability for somatic embryogenesis. The limited range of the explant source from which morphogenetically competent tissues can be obtained has limited success with protoplast culture in other tree species. Methods for the isolation and culture of *Citrus* protoplasts from embryogenic callus and suspension cultures, and subsequent plant regeneration are well developed [9, 10, 89, 103, 121–123]. Protoplast fusion techniques have been used to generate somatic hybrid plants from more than 500 parental combinations, including more than 300 from our laboratory (for reviews, see ref. [4, 9, 10, 88, 124]). As a by-product of protoplast fusion, hundreds of diploid cybrid citrus plants have also been regenerated via somatic embryogenesis [125, 126]. Protoplasts have also been proven to be very useful in the genetic transformation of plants [127–130], including economically important cereals [131]. Once again, citrus has led the way with genetic transformation of protoplasts among woody fruit trees, with transformed plant recovery due to robust somatic embryogenesis [129, 132].

1.2.3 Protoplast Isolation and Culture

The complex 8P protoplast culture medium of Kao and Michayluk [133] has been used for successful protoplast culture and plant regeneration from embryogenic cultures of several plant species. The success of this complex medium is probably due to the appropriate concentrations of the multivitamin, organic acid, and sugar/alcohol additives that are combined with the basal medium formulation. These additives seem to provide additional buffering capacity and reduce the environmental stress on protoplasts by providing required metabolic intermediates needed to sustain adequate cell viability and totipotency. However, optimal basal tissue culture media have been developed for most plant genera, and an efficient protoplast culture medium may be developed for a particular genus by simply supplementing the optimal basal medium with 8P multivitamin, organic acid, and sugar/alcohol additives. This approach has been successful for *Trifolium* [134, 135] and *Citrus* [9]. Reducing or eliminating the ammonia content of the basal medium has also been useful. Most basal media contain high levels of NH_4NO_3 that can often be toxic to protoplasts. Glutamine or $Ca(NO_3)_2$ have been found to be good alternative sources of N in

embryogenic suspension culture and protoplast culture media, as demonstrated in H+H suspension culture medium and BH3 protoplast medium of citrus [9], as well as in *Populus* protoplast media [136]. Vardi et al. [137] reported the first example of successful citrus protoplast isolation and culture, followed by callus formation and embryo differentiation. Subsequently, numerous *Citrus* species have been regenerated from protoplasts via somatic embryogenesis [124]. Ohgawara et al. [138] obtained for the first time somatic hybrids of citrus regenerated via somatic embryogenesis, involving *Citrus* (*C. sinensis* and *Poncirus trifoliata*). Citrus protoplasts can be isolated from different sources including embryogenic cells (cultured on either solid or liquid media), non-embryogenic callus, and leaves. Embryogenic cell cultures (on solid or liquid media) yield protoplasts with the best potential for proliferation and embryo regeneration. Leaves are another often utilized source for protoplast isolation in *Citrus*, because leaf protoplasts are generally easy to isolate and large amounts of protoplasts are produced; however, they are not totipotent and do not develop into somatic embryos. In vitro cultured nucellar seedlings are becoming more commonly used as a source of leaf material for protoplast isolation, as this source eliminates the need for harsh decontamination. Leaf protoplasts are often used in somatic fusions with embryogenic culture protoplasts, where the latter provides the capacity for somatic embryogenesis and plant recovery in somatic hybrids and cybrids. Embryogenic callus or suspension cultures used for protoplast isolation should be in the log phase of growth at the time of isolation. Friable tissue with low starch content generally gives the best results. *Citrus* embryogenic cultures often require continual subculturing for long periods before they reach adequate friability and appropriate starch levels for protoplast manipulation. Transferring *Citrus* callus to glutamine-containing media can sometimes reduce the starch content of cells to appropriate levels for protoplast isolation [9, 10, 103]. A procedure for the induction of suspension cultures from embryogenic calli has been previously described [9, 10, 103, 139]. Suspension cultures offer several distinct advantages over stationary cultures, especially when conducting multiple experiments requiring large volumes of explant. Suspension cultures quickly generated needed volumes of explant for multiple experiments, and rapidly growing suspension cells have thinner cell walls that are more amenable to enzyme digestion. Combining an enzyme solution (generally containing cellulose and macerase) with a complex protoplast culture medium may reduce stress on protoplasts during isolation and thereby increase viability. We prefer maintaining suspension cultures on a 2-week subculture cycle, with optimum protoplast isolations occurring at days 4–12, when suspension cultures are in the log phase of growth.

1.2.4 Somatic Hybridization

Somatic hybridization allows production of somatic hybrids that incorporate genomes of the two parents with little or no recombination, but with increased heterozygosity in the resulting polyploidy hybrids [140]. Somatic hybridization in citrus relies on the process of somatic embryogenesis for plant regeneration. In citrus, this technology has been extensively used and has important applications in both scion and rootstock improvement [124]. The first successful protoplast isolations were reported as early as 1982 [123], and the first citrus somatic hybrid was obtained between *C. sinensis* and *P. trifoliata* [138]. These results encouraged the development and incorporation of somatic hybridization techniques into the citrus breeding programs in several countries [9]. Somatic hybridization has made it possible to hybridize commercial citrus with citrus relatives that possess valuable attributes, thus broadening the germplasm base available for rootstock improvement [141]. Somatic hybrids have been developed and established at the Citrus Research and Education Center, University of Florida, USA for three decades to improve citrus scions and rootstocks [9, 10, 124]. The most important contribution somatic hybridization can make to citrus breeding programs is the creation of unique tetraploid breeding parents.

Scion Improvement

We have used somatic hybridization to create new tetraploid somatic hybrids that combine elite diploid scion material as tetraploid breeding parents being used in interploid hybridization schemes to develop seedless and easy-to-peel new mandarin varieties [142], and in grapefruit/pummelo and acid fruit improvement (lemons/limes) [10, 143]. The first seedless triploid mandarin from this program (C4-15-19, from a cross of "LB8-9" with a somatic hybrid of "Nova" mandarin hybrid + "Succari" sweet orange), was recently released by UF/IFAS for commercialization. This is the first released triploid citrus cultivar fathered by a somatic hybrid. The majority of somatic hybrid breeding parents produced for scion improvement have been from fusions of two polyembryonic parents. In this case, the somatic hybrid can only be efficiently used as a pollen parent in interploid crosses. Using this approach, we have produced several thousand triploid hybrids fathered by somatic hybrids. Interploid crosses utilizing a monoembryonic diploid female parent and a tetraploid male parent require embryo rescue for triploid plant recovery because embryos do not complete normal development, presumably as a consequence of endosperm:embryo ploidy level balance. By contrast, interploid crosses utilizing a monoembryonic tetraploid females do not require embryo rescue [10]. Somatic hybrids produced by the fusion of a polyembryonic embryogenic parent with a monoembryonic leaf parent are frequently monoembryonic. We have recently efficiently recovered triploid progeny by simply planting fully developed seeds from interploid crosses involving the

following monoembryonic somatic hybrid females in our breeding program: "Succari" sweet orange + "Hirado Buntan" pummelo, "Murcott" + "Chandler" sdlg.#80, "Murcott" + "Chandler" sdlg. A-1-11 (grapefruit/pummelo improvement), "Santa Teresa" lemon + "Lakeland Limequat" (lemon improvement), and "W. Murcott" + UF03-B ("Fortune" × "Murcott") (mandarin improvement) (J.W. Grosser, unpublished information). Thus, our future somatic hybridization work will focus more on production of monoembryonic somatic hybrids. Creation of triploid citrus hybrids directly by electrofusion of haploid and diploid protoplasts is also promising [144].

Rootstock Improvement

Numerous allotetraploid somatic hybrids via protoplast fusion with plant recovery by somatic embryogenesis, which combine complementary diploid rootstocks, have been produced [9]. These hybrids have direct rootstock potential [145], but their most important contribution may be their use as breeding parents in rootstock crosses at the tetraploid level. We initiated tetraploid rootstock breeding around the year 2000, and since this time hundreds of zygotic allotetraploids ("tetrazygs") have been obtained. This approach is quite powerful genetically, because the alleles from four rootstock genotypes can be recombined simultaneously, creating a wealth of genetic diversity in progeny. Resulting allotetraploid rootstock candidates have been screened for tolerance to the *Diaprepes/Phytophthora* complex [117, 87], salinity [145], and now HLB (Huanglongbing or citrus greening), all with promising results. With the cost of citrus production and harvesting increasing over time, there has been greater emphasis on developing rootstocks to facilitate Advanced Citrus Production Systems (ACPS), that reduce tree size to make orchard management and crop harvesting more efficient and also to bring young trees into economically valuable production earlier. We learned early on that tetraploid rootstocks, especially allotetraploid somatic hybrids, always have some capacity to reduce tree size, even from somatic hybrids produced between two vigorous parents [10, 145]. Through multiple field trials, we have identified some somatic hybrid and "tetrazyg" rootstock hybrids that have combined desirable horticultural attributes, disease resistance and stress tolerance traits, and confer varying degrees of tree size control [10]. UF/IFAS has recently "fast track" released 17 new rootstock selections to the Florida industry for large scale evaluation that include one somatic hybrid and six "tetrazyg" allotetraploid hybrids. The release additional improved allotetraploid rootstocks can be expected in the near future.

1.2.5 Somatic Cybridization

Cybrids combine the nucleus of a species with alien cytoplasmic organelles [126, 146]. Cybridization could be a valuable method for improvement of various crops that would be in the non-regulated category of genetically modified organisms.

The first cybrids in citrus were created by the "donor–recipient" method [147]. The phenomenon of cybridization in citrus also occurs as an accidental by-product of somatic hybridization via protoplast fusion [125, 148]. The general somatic hybridization model of fusing embryogenic culture cell protoplasts with leaf protoplasts often yields diploid plants with the morphology of the leaf parent. These plants have always, without exception, been validated as cybrids, as citrus leaf protoplasts are not capable of plant regeneration. Such cybrids always have the mitochondrial (mt) genome of the embryogenic suspension/callus parent, whereas the chloroplast (cp) genome is randomly inherited. Thus, recovered cybrid plants are regenerated via somatic embryogenesis. Moreira et al. [148] found that embryogenic suspension culture cells generally have four times more mt per cell than do leaf cells and hypothesized that the extra mt acquired by the cybrid cells is necessary to satisfy the high energy demand of the somatic embryogenesis pathway of regeneration. This phenomenon has been exploited to produce targeted cybrids. One approach for cultivar improvement has been to transfer of cytoplasmic male sterility (CMS) from "Satsuma" mandarin to other elite but seedy scions via cybridization. This approach has the potential to make existing popular cultivars less seedy, without altering the cultivar integrity in any other way [126, 146]. This technique has only been partially successful in our experience; for example, we have produced cybrid "Sunburst" mandarin clones that have less than half the normal seed content of "Sunburst", but still too many seeds to label them as seedless (JW Grosser, unpublished information). However, these cybrid "Sunburst" trees produce a fruit that is easier to peel and with better flavor than traditional "Sunburst." Accidental cybrids of "Ruby Red" and "Duncan" grapefruit, both containing the mt genome from "Dancy" mandarin, have also been produced from separate experiments. In both cases, the fruit from cybrid trees has improved characteristics, including significantly higher brix and brix/acid ratios, and an extended harvesting season that extends well into the summer with no vivipary or fruit drying (Satpute et al., submitted). UF/IFAS has released the first cybrid citrus cultivar, namely the N2-28 cybrid "Ruby Red" grapefruit, from this work. We are also attempting to utilize cybrid technology for improving disease resistance in existing cultivars. The mt genome of kumquat (*Fortunella crassifolia* Swingle) is purported to contain a gene for citrus canker resistance. Citrus canker disease has caused significant damage to the Florida grapefruit industry. We have initiated an embryogenic suspension culture of "Meiwa" kumquat and performed fusions with leaf protoplasts of grapefruit cultivars "White Marsh," "Flame" (red) and a dark red somaclone N11-11.

Multiple diploid plants from each fusion combination exhibiting grapefruit morphology have been regenerated and their cybrid nature confirmed by mitochondrial intron marker analysis [149]. Cp genome inheritance analysis in these plants is currently underway. These cybrid grapefruit plants are being propagated for a canker challenge assay to determine if the transfer of the kumquat mt genome can indeed improve their resistance to citrus canker.

1.3 Citrus Transformation Involving Somatic Embryogenesis

Genetic transformation has become an attractive alternative method for improving plant species including citrus, because it is possible to maintain cultivar integrity while adding a single trait. Exploiting the process of somatic embryogenesis, citrus can be transformed either directly from embryogenic cell suspension cultures or indirectly from isolated protoplasts. Embryogenic cells are usually treated with an *Agrobacterium* culture followed by selection and regeneration of transgenic plants. Plant protoplasts are commonly transformed using the polyethylene glycol (PEG)-mediated DNA uptake process, and less frequently using electroporation. The PEG-mediated DNA transfer can be readily adapted to a wide range of plant species and tissue sources. In this chapter we describe an efficient, protoplast-based citrus-transformation system that could be routinely used to transform several important polyembryonic citrus cultivars that feature robust somatic embryogenesis, including important processing sweet oranges and the popular mandarin cultivar W. Murcott.

The first reports of citrus transformation began to appear more than two and half decades ago [150–152]. Over time, citrus transformation efficiency has been increased due to continual improvements in *Agrobacterium*-mediated methodology and protoplast transformation system, as well as the selection techniques of the transgenic events. In citrus, the common method of transformation is *Agrobacterium*-mediated transformation of stem pieces (mostly nucellar seedling internodes). This method works best with seedy polyembryonic cultivars and uses adventitious shoot induction (organogenesis) as the regeneration pathway. However, many important citrus cultivars are commercially seedless (zero to five seeds per fruit) or totally seedless, which makes it difficult or impossible to obtain adequate nucellar seedling explants for *Agrobacterium*-mediated transformation. Other limitations of *Agrobacterium*-mediated citrus transformation include inadequate susceptibility to *Agrobacterium* infection and inefficient plant regeneration via adventitious shoot-bud induction in certain commercially important cultivars, particularly mandarins. Finally, there are significant Intellectual Property issues with the use of the common *Agrobacterium*-mediated method.

1.3.1 Protoplast Transformation

Direct delivery of free DNA molecules into plant protoplasts has been well documented [153]. Several factors could affect the efficiency of free DNA delivery systems, including plasmid

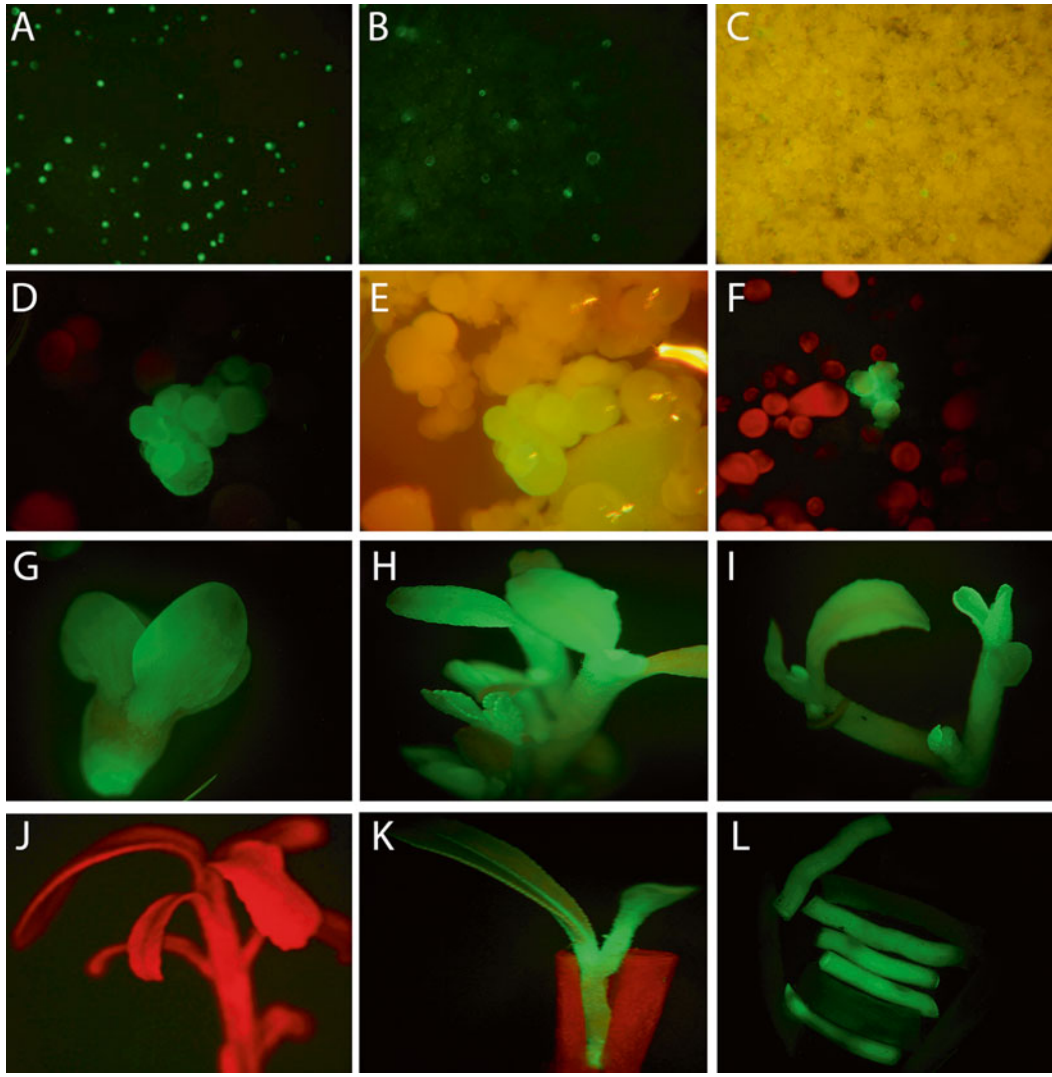

Fig. 2 GFP selection in protoplast/GFP transformation system. (**a**) protoplasts expressing GFP 24 h after transformation, (**b**) protoplast derived micro-calli (transformed and non-transformed) under *blue light*, (**c**) protoplast derived micro-calli (transformed and non-transformed) under *white light*, (**d**) transgenic (*green*) and non-transgenic (*red*) somatic embryos under *blue light*, (**e**) transgenic (*green*) and non-transgenic (*yellow*) somatic embryos under *white light*, (**f**) transgenic (*green*) and non-transgenic (*red*) somatic embryos under *blue light*, (**g**) enlarged transgenic embryo expressing GFP, (**h**, **i**) transgenic somatic embryo derived shoots, (**j**) non-transformed shoot, (**k**) micrografting of transgenic shoot onto non-transgenic rootstock, (**l**) GFP expression in root

DNA concentration and form, carrier DNA, and treatment and pretreatment buffers. The delivery of foreign genes into protoplasts is usually carried out by electroporation [154] or treatment with polyethylene glycol (PEG) [130, 155] (Fig. 2). The PEG-mediated transformation is simple and efficient, allowing a

simultaneous processing of many samples, and yields a transformed cell population with high survival and division rates [156]. The method utilizes inexpensive supplies and equipment, and helps to overcome an obstacle of host range limitations of *Agrobacterium*-mediated transformation, since DNA uptake by protoplasts is promoted by chemical treatment with PEG. Plant recovery is usually through the somatic embryogenesis pathway rather than through organogenesis. Moreover, the transformation method of choice for plant protoplasts is dependent on a number of factors, including efficiency of DNA delivery, toxicity to the cells, ease of use, and cost and availability of materials. In protoplast transformation systems, plating and selection methods are important considerations in the development of stable transgenic plants. The ideal system should permit easy identification of transformants without the complications of multiple recovery of single transformation events or recovery of "false-positives" due to inadequate selection pressure. Therefore using the GFP gene (green fluorescent protein) as a selectable marker essentially eliminates the problem of multiple recoveries of single events. Under optimal conditions, up to 50 transformed embryos can be recovered per million input of protoplasts (transformation frequency = 0.005 %). The low toxicity, simplicity, high efficiency, and low cost of the PEG transformation method make it an attractive alternative to electroporation as the method of choice for stable transformation of plant protoplasts.

PEG-mediated gene transfer to citrus protoplasts has proven to be efficient, reliable, inexpensive, and a simple method that works well when using relatively young embryogenic cultures with good totipotency [129, 132, 157]. In this system, large populations of protoplasts are isolated from embryogenic suspension cultures to increase the likelihood of obtaining an adequate number of stable independent transformation events. Regeneration of transgenic plants via somatic embryogenesis is possible under suitable in vitro conditions through selection at an early stage of development (usually the pro-embryo stage) using GFP gene as a reporter gene. However, the tissue-culture response may vary depending on the plant genotype, handling and the condition of the suspension cells. A major requirement for protoplast transformation system is the preparation of viable protoplasts. We have successfully used the procedure described below for gene transfer to citrus for several cultivars, including "Hamlin" and "Valencia" sweet oranges, and "W. Murcott" tangor [9, 10, 129, 132]. Cell suspensions provide an unlimited source of rapidly dividing protoplasts that can be obtained after 12–18 h incubation in enzyme solution and show a transient expression of introduced genes within 24 h after transformation. This protocol can be adapted to a wide range of plant species and tissue sources used for protoplast preparation.

1.3.2 Agrobacterium-Mediated Transformation of Embryogenic Cell Suspension Cultures

Genetic transformation using embryogenic cell suspension cultures offers a practical alternative to the transformation of epicotyl explants obtained from germinating seedlings, since almost all polyembryonic cultivars can be introduced in vitro as embryogenic cell suspension cultures [158]. Amenability of cell suspension cultures to transformation using *Agrobacterium* would allow the transformation of any cultivar that can be introduced as embryogenic cell masses, including specialty seedless sweet oranges or "Satsuma" mandarins and other difficult-to-transform cultivars of the mandarin or lemon group. Our protocol is based on a hygromycin selection regime, as it was observed that kanamycin selection resulted in erratic and low transgenic embryo production. Inefficient kanamycin selection was either due to cells overcoming the effects of the antibiotic or to the protection of cells from kanamycin by the surrounding cells [159, 160]. Successful callus transformation of sweet oranges and mandarins can be accomplished in a selected medium containing 25 mg/L of hygromycin B. Most material, stocks, and medium are similar to the protoplast transformation process. *Agrobacterium* mediated transformation relies on an active *Agrobacterium* culture instead of plasmid DNA as in the protoplast transformation process. Additional materials required in this protocol are indicated in the protocol section. A description of the transformation process can also be found in Dutt and Grosser [158].

2 Materials

2.1 Equipment

1. Fluorescence microscope with FITC filters: Zeiss SV11 epifluorescence stereomicroscope equipped with a 100 W mercury bulb light and a fluorescein-5-isothiocyanate/GFP (FITC/GFP) filter set with a 480 nm excitation filter and a 515 nm long-pass emission filter (Chroma Technology Corp., Brattleboro, VT, USA).
2. Temperature-controlled rotary shaker at 28 ± 2 °C.
3. Laminar flow cabinet.
4. pH meter.
5. Autoclave.
6. Sterilized paper plates.
7. Syringe filter units, 0.2 μm pore size.
8. Centrifuge with 100–400 × *g* capability.
9. 40 mL Pyrex tubes.
10. 15 mL Pyrex capped tube.
11. 15-mL round-bottom screw-cap centrifuge tubes.

12. 60 × 15 mm petri dishes.
13. 100 × 20 mm petri dishes.
14. 100 × 15 mm petri dishes.

2.2 Medium Stock Solutions

1. Sterilization solution: 20 % (v/v) commercial bleach solution.
2. BH3 macronutrient stock: 150 g/L KCl, 37 g/L $MgSO_4 \cdot 7H_2O$, 15 g/L KH_2PO_4, 2 g/L K_2HPO_4; dissolve in H_2O and store at 4 °C.
3. Murashige and Tucker (MT) macronutrient stock [101]: 95 g/L KNO_3, 82.5 g/L NH_4NO_3, 18.5 g/L $MgSO_4 \cdot 7H_2O$, 7.5 g/L KH_2PO_4, 1 g/L K_2HPO_4; dissolve in H_2O and store at 4 °C.
4. MT micronutrient stock: 0.62 g/L H_3BO_3, 1.68 g/L $MnSO_4 \cdot H_2O$, 0.86 g/L $ZnSO_4 \cdot 7H_2O$, 0.083 g/L KI, 0.025 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.0025 g/L $CuSO_4 \cdot 5H_2O$, 0.0025 g/L $CoCl_2 \cdot 6H_2O$; dissolve in H_2O and store at 4 °C.
5. MT vitamin stock: 10 g/L myoinositol, 1 g/L thiamine-HCl, 1 g/L pyridoxine-HCl, 0.5 g/L nicotinic acid, 0.2 g/L glycine; dissolve in H_2O and store at 4 °C.
6. MT calcium stock: 29.33 g/L $CaCl_2 \cdot 2H_2O$; dissolve in H_2O and store at 4 °C.
7. MT iron stock: 7.45 g/L Na_2EDTA, 5.57 g/L $FeSO_4 \cdot 7H_2O$; dissolve in H_2O and store at 4 °C.
8. Kinetin (KIN) stock solution: 1 mg/mL; dissolve the powder in a few drops of 1 N HCl; bring to final volume with H_2O and store at 4 °C.
9. BH3 multivitamin stock A: 1 g/L ascorbic acid, 0.5 g/L calcium pantothenate, 0.5 g/L choline chloride, 0.2 g/L folic acid, 0.1 g/L riboflavin, 0.01 g/L p-aminobenzoic acid, 0.01 g/L biotin; dissolve in H_2O and store at −20 °C.
10. BH3 multivitamin stock B: 0.01 g/L retinol dissolved in a few drops of alcohol, 0.01 g/L cholecalciferol dissolved in a few drops of ethanol, 0.02 g/L vitamin B12; dissolve in H_2O and store at −20 °C.
11. BH3 KI stock: 0.83 g/L KI; dissolve in H_2O and store at 4 °C.
12. BH3 sugar and sugar alcohol stock: 25 g/L fructose, 25 g/L ribose, 25 g/L xylose, 25 g/L mannose, 25 g/L rhamnose, 25 g/L cellobiose, 25 g/L galactose, 25 g/L mannitol; dissolve in H_2O and store at −20 °C.
13. BH3 organic acid stock: 2 g/L fumaric acid, 2 g/L citric acid, 2 g/L malic acid, 1 g/L pyruvic acid; dissolve in H_2O and store at −20 °C.

2.3 Plant Growth Regulator Stocks

1. Coumarin (stock solution, 1.46 mg/mL): Dissolve the powder in warm H_2O; store at 4 °C.
2. α-Naphthalene acetic acid (NAA; stock solution, 1 mg/10 mL): Dissolve the powder in a few drops of 5 M NaOH, bring to final volume with H_2O and store at 4 °C.
3. 2,4-Dichlorophenoxyacetic acid (2,4-D; stock solution, 1 mg/10 mL): Dissolve the powder in a few drops of 95 % (v/v) ethanol, bring to final volume with H_2O; store at 4 °C.
4. 6-Benzylaminopurine (BAP; stock solution, 1 mg/mL): Dissolve the powder in a few drops of 5 M NaOH, bring to final volume with H_2O; store at 4 °C.
5. Gibberellic acid (GA_3; stock solution, 1 mg/mL): Dissolve the powder in a few drops of 95 % (v/v) ethanol, bring to final volume with H_2O, filter-sterilize; store in small aliquots at 4 °C; add to the medium after autoclaving and cooling the medium to 55 °C in a water bath.

2.4 Enzyme Stock Solutions

The enzyme solution is filter sterilized.

1. Calcium chloride ($CaCl_2{\cdot}2H_2O$ stock solution, 0.98 M): Dissolve 14.4 g in 100 mL H_2O and store at −20 °C.
2. Monosodium phosphate (NaH_2PO_4 stock solution, 37 mM): Dissolve 0.44 g in 100 mL H_2O and store at −20 °C.
3. 2 (N-morpholino) ethanesulfonic acid (MES stock solution, 0.246 M): Dissolve 4.8 g in 100 mL H_2O and store at −20 °C.
4. Enzyme solution: 0.7 M mannitol, 24 mM $CaCl_2$, 6.15 mM MES buffer, 0.92 mM NaH_2PO_4, 2 % (w/v) Cellulase Onozuka RS (Yakult Honsha), 2 % (w/v) Macerozyme R-10 (Yakult Honsha), pH 5.6. To prepare 40 mL of enzyme solution, dissolve 0.8 g Cellulase Onozuka RS, 0.8 g Macerozyme R-10 and 5.12 g mannitol in 20 mL H_2O and add 1 mL of $CaCl_2{\cdot}2H_2O$, NaH_2PO4 and MES stock solutions; bring volume to 40 mL with H_2O, pH to 5.6 using KOH, filter-sterilize; store at 4 °C for up to 3 weeks.

2.5 CPW Solution

1. CPW salts stock solution 1: 25 g/L $MgSO_4{\cdot}7H_2O$, 10 g/L KNO_3, 2.72 g/L KH_2PO_4, 0.016 g/L KI, 0.025 ng/L $CuSO_4{\cdot}5H_2O$; dissolve in H_2O and store at −20 °C.
2. CPW salts stock solution 2: 15 g/L $CaCl_2{\cdot}2H_2O$; dissolve in H_2O and store at −20 °C.
3. 13 % CPW (13 %, w/v, mannitol solution with CPW salts): Dissolve 13 g mannitol in 80 mL H_2O, add 1 mL each of CPW salts stock solutions 1 and 2; bring volume to 100 mL with H_2O, pH to 5.8, filter-sterilize; store at room temperature.

4. 25 % CPW (25 %, w/v, sucrose solution with CPW salts): Dissolve 25 g sucrose in 80 mL H_2O, add 1 mL each of CPW salts stock solutions 1 and 2; bring to 100 mL with H_2O, pH to 5.8, filter-sterilize and store at room temperature.

2.6 Protoplast Transformation Solutions

1. PEG 8000 MW (stock solution, 50 %): Place the bottle of PEG in a water bath at 80 °C until it melts completely, take 250 mL and mix it with 250 mL H_2O, add 4 g of resin AG501-X8 (Bio-Rad), stir for 30 min, filter out the resin through a layer of cotton and allow to stand for several hours before use; store at 4 °C.
2. Polyethylene glycol (PEG) working solution: 40 % (w/v) PEG, 0.3 M glucose, 66 mM $CaCl_2 \cdot 2H_2O$, pH 6.0. To prepare 100 mL of PEG solution, dissolve 0.97 g $CaCl_2 \cdot 2H_2O$ and 5.41 g glucose in 10 mL H_2O, add 80 mL of PEG stock solution (50 %) and adjust the volume to 100 mL with H_2O, pH 6; filter-sterilize and store at 4 °C . Check the pH every 2–3 weeks, since this solution acidifies with time.
3. Elution solutions for PEG removal. Solution A: 0.4 M glucose, 66 mM $CaCl_2 \cdot 2H_2O$, 10 % dimethyl sulfoxide (DMSO), pH 6.0. Solution B: 0.3 M glycine adjusted with NaOH pellets to pH 10.5. Filter-sterilize both solutions; store at room temperature and mix together (9:1, v:v) immediately prior to use to avoid precipitation.

2.7 Agrobacterium Culture Medium

1. Any suitable binary vector containing the hygromycin selectable marker gene for selection in plants. We have had good success with the pCAMBIA 1300 series of plant transformation vectors (www.cambia.org).
2. *Agrobacterium tumefaciens* EHA105 stock containing the appropriate binary vector plasmid (stored in 20 % glycerol at −80 °C).
3. Solid bacterial growth medium: Yeast Extract Peptone (YEP) medium (10 g/L peptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0) supplemented with 15 g/L TC agar, 20 mg/L rifampicin, and 100 mg/L kanamycin.
4. Liquid bacterial growth medium: YEP medium supplemented with 20 mg/L rifampicin and 100 mg/L kanamycin.

2.8 Suspension Cell Transformation Stock Solutions

1. Rifampicin: 20 mg of antibiotic dissolved in 1 mL of DMSO.
2. Acetosyringone: 0.196 mg dissolved in 1 mL of DMSO to prepare a 100 mM concentration stock solution.
3. Hygromycin sulfate: 50 mg of antibiotic dissolved in 1 mL of water. The solution sterilized by filtration using a 0.2 μm membrane.

4. Timentin and cefotaxime: 400 mg of each antibiotic dissolved in 1 mL of water. The solution sterilized by filtration using a 0.2 μm membrane.

2.9 Callus-Induction Media

1. EME 0.15 M semisolid medium: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 50 g/L sucrose, 0.5 g/L malt extract, 8 g/L agar, pH 5.8; autoclave medium and pour into 100×20 mm petri dishes, 35 mL per dish.
2. DOG semisolid medium: Same as EME 0.15 M semisolid medium plus 5 mg/L kinetin (5 mL kinetin stock solution); autoclave medium and pour into 100×20 mm petri dishes, 35 mL per dish.
3. H+H semisolid medium: 10 mL/L MT macronutrient stock, 5 mL/L BH3 macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 50 g/L sucrose, 0.5 g/L malt extract, 1.55 g/L glutamine, 8 g/L agar, pH 5.8; autoclave medium and pour into 100×20 mm petri dishes, 35 mL per dish.

2.10 Cell Suspension Maintenance Medium

1. H+H liquid medium: 10 mL/L MT macronutrient stock, 5 mL/L BH3 macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 35 g/L sucrose, 0.5 g/L malt extract, 1.55 g/L glutamine, pH 5.8; pour 500 mL aliquots into 1000 mL glass Erlenmeyer flasks, autoclave and store at room temperature.

2.11 Protoplast Isolation and Culture Media

All protoplast liquid media are filter sterilized.

1. BH3 0.6 M liquid medium: 10 mL/L BH3 macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 2 mL/L BH3 multivitamin stock A, 1 mL/L BH3 multivitamin stock B, 1 mL/L BH3 KI stock, 10 mL/L BH3 sugar and sugar alcohol stock, 20 mL/L BH3 organic acid stock, 20 mL/L coconut water, 82 g/L mannitol, 51.3 g/L sucrose, 3.1 g/L glutamine, 1 g/L malt extract, 0.25 g/L casein enzyme hydrolysate, pH 5.8; filter-sterilize and store at room temperature.
2. EME 0.6 M liquid medium: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 205.4 g/L sucrose, 0.5 g/L malt extract, pH 5.8; filter-sterilize and store at room temperature.

2.12 Protoplast Culture and Plant Regeneration Media

1. EME 0.15 M liquid medium: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 50 g/L sucrose, 0.5 g/L malt extract, pH 5.8; filter-sterilize and store at room temperature.
2. EME–malt 0.15 M liquid medium: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 50 g/L maltose, 0.5 g/L malt extract, pH 5.8; filter-sterilize and store at room temperature.
3. EME–malt 0.15 M semisolid medium: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 50 g/L maltose, 0.5 g/L malt extract, 8 g/L agar, pH 5.8; autoclave medium and pour into 100 × 20 mm petri dishes, 35 mL per dish.
4. EME 1500 semisolid medium: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 50 g/L sucrose, 1.5 g/L malt extract, 8 g/L agar, pH 5.8; autoclave medium and pour into 100 × 20 mm petri dishes, 35 mL per dish.
5. B+ semisolid medium: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 25 g/L sucrose, 20 mL/L coconut water, 14.6 mg/L coumarin (10 mL coumarin stock), 0.02 mg/L NAA (200 μl NAA stock), 1 mg/L GA_3 (add 1 mL GA_3 stock after medium is autoclaved and cooled to 55 °C in water bath), 8 g/L agar, pH 5.8; autoclave medium and pour into 100 × 20 mm petri dishes, 35 mL per dish.
6. DBA3 semisolid medium: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 25 g/L sucrose, 1.5 g/L malt extract, 20 mL/L coconut water, 0.01 mg/L 2,4-D (100 μl 2,4-D stock), 3 mg/L BAP (3 mL BAP stock); 8 g/L agar, pH 5.8; autoclave medium and pour into 100 × 20 mm petri dishes, 35 mL per dish.
7. RMAN medium (Root induction and propagation): 10 mL/L MT macronutrient stock, 5 mL/L MT micronutrient stock, 5 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 25 g/L sucrose, 0.5 g/L activated charcoal, 8 g/L agar, 0.02 mg/L NAA (200 μl NAA stock solution), pH 5.8; autoclave medium and pour into sterile Magenta GA-7 boxes, 80 mL per box.

2.13 Suspension Culture Transformation and Plant Regeneration Media

1. EME–sucrose 0.15 M semisolid medium supplemented with Acetosyringone: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 50 g/L sucrose, 0.5 g/L malt extract, 8 g/L agar, pH 5.8; autoclave medium, add 1 mL/L acetosyringone stock solution to partially cooled medium and pour into 100 × 20 mm petri dishes, 35 mL per dish.
2. EME–sucrose 0.15 M liquid medium: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 50 g/L sucrose, 0.5 g/L malt extract. Pour into 250 mL bottles before autoclaving.
3. EME–maltose 0.15 M semisolid medium supplemented with antibiotics: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 50 g/L maltose, 0.5 g/L malt extract, 8 g/L agar, pH 5.8; autoclave medium, add 1 mL/L timentin, 1 mL/L cefotaxime and 500 mg/L hygromycin stock solutions to partially cooled medium, and pour into 100 × 20 mm petri dishes, 35 mL per dish.
4. EME 1500 semisolid medium supplemented with antibiotics: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 50 g/L sucrose, 1.5 g/L malt extract, 8 g/L agar, pH 5.8; autoclave medium, add 0.5 mL/L timentin, 0.5 mL/L cefotaxime and 500 mg/L hygromycin stock solutions to partially cooled medium, and pour into 100 × 20 mm Petri dishes, 35 mL per dish.
5. B+ semisolid medium supplemented with antibiotics: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 25 g/L sucrose, 20 mL/L coconut water, 14.6 mg/L coumarin (10 mL coumarin stock), 0.02 mg/L NAA (200 μl NAA stock), 1 mg/L GA_3 (add 1 mL GA_3 stock solution after medium is autoclaved and cooled to 55 °C in water bath), 8 g/L agar, pH 5.8; autoclave medium, add 0.5 mL/L timentin stock solution to partially cooled medium and pour into 100 × 20 mm petri dishes, 35 mL per dish.
6. DBA3 semisolid medium supplemented with antibiotics: 20 mL/L MT macronutrient stock, 10 mL/L MT micronutrient stock, 10 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 25 g/L sucrose, 1.5 g/L malt extract, 20 mL/L coconut water, 0.01 mg/L 2,4-D (100 μl 2,4-D stock solution), 3 mg/L BAP (3 mL BAP stock solu-

tion); 8 g/L agar, pH 5.8; autoclave medium, add 0.5 mL/L timentin stock solution to partially cooled medium and pour into 100 × 20 mm petri dishes, 35 mL per dish.

7. RMAN medium supplemented with antibiotics: 10 mL/L MT macronutrient stock, 5 mL/L MT micronutrient stock, 5 mL/L MT vitamin stock, 15 mL/L MT calcium stock, 5 mL/L MT iron stock, 25 g/L sucrose, 0.5 g/L activated charcoal, 8 g/L agar, 0.02 mg/L NAA (200 µl NAA stock solution), pH 5.8; autoclave medium, add 0.5 mL/L timentin stock solution to partially cooled medium and pour into sterile Magenta GA-7 boxes, 80 mL per box.

3 Methods

3.1 Protoplast Transformation [9, 129]: Initiation and Maintenance of Embryogenic (Callus and Cell Suspension) Cultures

1. Immerse harvested immature fruit in sterilization solution in a beaker for 30 min.
2. Using sterile tongs, place fruit on sterilized paper plates in a laminar flow hood.
3. Using a sterile surgical blade, make an equatorial cut, 1–2 cm deep, and open the fruit.
4. With sterile forceps, extract ovules and place them onto callus-induction medium (EME 0.15 M, H+H or DOG).
5. Incubate extracted ovules in the dark at 28 ± 2 °C and transfer them every 3–4 weeks to new callus-induction medium until embryogenic (yellow and friable) callus emerges from the ovules.
6. To maintain long-term cultures, transfer embryogenic undifferentiated calli (*see* **Note 1**) onto new medium every 4–6 weeks and incubate under the same conditions.
7. To initiate cell suspensions from embryogenic undifferentiated nucellus-derived callus, take approx. 2 g of calli from callus-induction medium and transfer to 125 mL Erlenmeyer flasks, each containing 20 mL of H+H liquid medium.
8. Shake the cell suspension cultures on a rotary shaker at 125 rpm under a 16 h photoperiod (70 µmol m^{-2} s^{-1}) at 28 ± 2 °C.
9. After 1 week, add 10 mL of new H+H liquid medium to Erlenmeyer flasks and return back to the shaker.
10. After one more week, add 20 mL of new H+H liquid medium to Erlenmeyer flasks and return back to the shaker.
11. Subculture established embryogenic cell suspension cultures every 2 weeks by removing 20 mL from the culture and replacing with 20 mL fresh aliquots of H+H liquid medium; shake at 125 rpm and incubate under the same conditions.

3.2 Protoplast Transformation: Preparation and Enzymatic Incubation of Cultures from Embryogenic Callus

1. Transfer 1–2 g of friable callus into a 60 × 15 mm petri dish. If using a suspension as a source for embryogenic cells (*see* **Note 2**) transfer approx. 2 mL of suspension (*see* **Note 3**) with a wide-mouth pipette.
2. Drain off the liquid medium using a Pasteur pipette.
3. Resuspend the cells in a mixture of 2.5 mL 0.6 M BH3 liquid medium and 1.5 mL enzyme solution (*see* **Note 4**).
4. Seal petri dishes with Parafilm and incubate overnight (15–20 h) at 28 °C on a rotary shaker at 25–30 rpm in the dark.

3.3 Protoplast Transformation: Protoplast Isolation and Purification [9, 129]

1. Following overnight incubation, pass enzymatic preparations through a sterile 45 μm nylon mesh sieve (*see* **Note 5**) to remove undigested tissues and other cellular debris; collect the filtrate in 40 mL Pyrex tubes.
2. Transfer the protoplast-containing filtrate (*see* **Note 6**) to a 15 mL calibrated screw-cap centrifuge tube.
3. Centrifuge at 900 rpm for 10 min.
4. Remove the supernatant with a Pasteur pipette and gently resuspend the protoplast pellet in 5 mL of 25 % CPW solution.
5. Slowly pipette 2 mL of 13 % CPW solution directly on top of the sucrose layer. Avoid mixing the two layers.
6. Centrifuge at 900 rpm for 10 min.
7. Only viable protoplasts (*see* **Note 7**) form a band at the interface between the sucrose and the mannitol layers.
8. Remove the protoplasts (*see* **Note 8**) from this interface with a Pasteur pipette and resuspend them in 10 mL of BH3 0.6 M liquid medium (using a new screw-cap centrifuge tube).
9. Centrifuge at 900 rpm for 10 min.
10. Remove the supernatant and gently resuspend the pellet in 10 mL of BH3 0.6 M medium (*see* **Note 9**).
11. Centrifuge at 900 rpm for 10 min.
12. Remove the supernatant and gently resuspend the pellet in 10 mL of BH3 0.6 M medium.
13. Centrifuge at 900 rpm for 10 min.
14. Remove the supernatant and resuspend the pellet into 5 mL BH3 0.6 M.
15. Determine protoplast density using a hemocytometer (*see* **Note 10**).
16. Centrifuge at 900 rpm for 10 min.
17. Remove the supernatant and resuspend the pellet into BH3 0.6 M to reach 4×10^6 protoplasts/mL.

3.4 Protoplast Transformation: Polyethylene Glycol (PEG)-Induced Protoplast Transformation [129] (see Note 11)

1. In a 15 mL round-bottom screw-cap centrifuge tubes (*see* **Note 12**) add 0.5 mL of protoplast suspension (2×10^6 protoplasts/mL).
2. Add 30–40 μg plasmid DNA (*see* **Note 13**) and gently mix well by gentle agitation.
3. Immediately add 0.5 mL of PEG solution directly into the center of the tube to give the desired final PEG concentration (20 %) (*see* **Note 14**), allowing the PEG to mix with the protoplast suspension by gentle agitation (*see* **Note 15**).
4. After 25–30 min, add 0.5 mL of A + B solution (9:1, v:v) into each transformation tube, but this time gently and slowly onto the inside edge of the tube, trying not to agitate the fragile transforming protoplasts.
5. After another incubation period of 25–30 min, gently add 1 mL of BH3 0.6 M medium onto the inside edge of the tube, again trying not to disturb the protoplasts.
6. After incubating for an additional 10 min, dilute the protoplast suspension with four 1-mL aliquots of BH3 0.6 M at 5 min intervals onto the inside edge of the tube, again trying not to disturb the protoplasts.
7. Cap and seal the tube with Parafilm.
8. Centrifuge at 700 rpm for 5 min.
9. Carefully, remove supernatant, add 2 mL BH3 0.6 M medium and gently resuspend the protoplast.
10. Centrifuge at 700 rpm for 5 min.
11. Carefully, remove supernatant, add 2 mL BH3 0.6 M medium and gently resuspend the protoplast (*see* **Note 16**).
12. Repeat **steps 10** and **11** one more time, carefully avoiding the loss of protoplasts.
13. Finally, add 1–1.5 mL of a 1:1 (v:v) mixture of BH3 0.6 M and EME 0.6 M liquid media to each tube, gently resuspend the protoplast.
14. Transfer the suspended protoplast into 60 × 15 mm petri dishes and spread into a thin layer by gently swirling the petri dishes (*see* **Note 17**).
15. Seal the dishes with Parafilm and culture in the dark at 28 ± 2 °C for 4–6 weeks (*see* **Note 18**).
16. Check *GFP* expression 48 h after transformation (*see* **Note 19**) using Zeiss SV11 epifluorescence stereomicroscope and return the dishes back in the dark at 28 ± 2 °C (Fig. 2).

3.5 Protoplast Transformation: Protoplast Culture and Plant Regeneration

1. After 4–6 weeks of incubation (*see* **Note 20**), supplement cultures of transformed protoplasts with new medium containing reduced osmoticum. Accomplish this by adding 10–12 drops of 1:1:1 (by volume) mixture of BH3 0.6 M, EME 0.6 M, and EME 0.15 M liquid media.
2. Incubate cultures for another 2 weeks in low light (20 μmol m^{-2} s^{-1} intensity) with a 16 h photoperiod at 28 ± 2 °C.
3. Accomplish another reduction of osmoticum in the cultures by the following steps: add 2 mL of 1:2 (v:v) mixture of BH3 0.6 M and EME-malt 0.15 M liquid media to each dish of transformed-treated protoplasts.
4. Immediately pour the entire contents onto petri dishes with agar-solidified EME-malt 0.15 M medium and swirl gently each dish in order to spread the liquid containing protoplast-derived colonies evenly over the entire semisolid agar surface.
5. Incubate cultures with a 16 h photoperiod (70 μmol m^{-2} s^{-1} intensity) at 28 ± 2 °C and, from this point until somatic hybrids are planted in compost, keep the cultures under the same growth conditions.
6. Transfer regenerated somatic embryos as soon as they appear from callus colonies to new agar-solidified EME-malt 0.15 M medium (*see* **Note 21**).
7. After 3–4 weeks, move small somatic embryos to semis solid EME 1500 medium for enlargement and germination.
8. Move the germinated embryos to semisolid B+ medium for axis elongation.
9. Dissect abnormal embryos that fail to germinate into large sections and place on DBA3 medium for shoot induction.
10. Transfer all resulting GFP positive shoots into RMAN medium to induce rooting (*see* **Note 22**) (Fig. 2).
11. Transfer rooted plants into peat based potting mixture in the greenhouse and cover with rigid clear plastic for 3–4 weeks maintaining high humidity.
12. Remove the plastic covers following this period of acclimatization.
13. After having an established plant with 3–4 leaves start molecular analysis (*see* **Note 23**).

3.6 Suspension Cell Culture Transformation [158]: Agrobacterium Preparation and Culture Transformation

1. Obtain *Agrobacterium* cultures kept in a −80 °C freezer and thaw.
2. Remove a loopful of bacteria from each thawed culture, and streak it on an individual YEP plate.
3. Incubate plates at 28 °C for 2 days.
4. Use a single bacterial colony and inoculate a flask of 25 mL liquid YEP medium containing appropriate antibiotics.

5. Culture for 24 h at 28 °C.
6. Transfer a 3–5 mL overnight aliquot into fresh 25 mL liquid YEP medium containing appropriate antibiotics.
7. Culture for 3–4 h at 26 °C.
8. Centrifuge cells at 6000 rpm for 8 min at 25 °C.
9. Resuspend cells in 25 mL liquid EME-sucrose medium.
10. Prior to use in transformation, measure the optical density (OD) of cultures and adjust to 0.3.
11. Transfer 20 mL of cell suspension cultures into a 100 × 15 mm petri dish. Drain off the liquid medium using a Pasteur pipette.
12. Transfer bacterial solution into the suspension cells for 20 min with frequent and gentle agitation.
13. Blot cell suspension cultures on sterile paper towels and transfer onto semisolid EME-sucrose medium supplemented with acetosyringone.
14. Incubate in the dark at 25 °C for 5 days.

3.7 Suspension Cell Culture Transformation: Selection of Putative Transformed Embryos and Regeneration of Transformed Plants

1. Transfer putative transgenic cells onto EME + maltose embryo production medium supplemented with appropriate antibiotics.
2. Maintain cultures either in the dark or under low light (20 μmol m^{-2} s^{-1} intensity) condition.
3. After 4–6 weeks in this medium, transfer cells into fresh medium. At this stage add 2 mL of 1:2 (v:v) mixture of BH3 0.6 M and EME-malt 0.15 M liquid media to each dish of transformed-suspension cells. Supplement the 1:2 mixture with 200 mg/L timentin and 25 mg/L hygromycin.
4. Transfer regenerated somatic embryos as soon as they appear from callus colonies to new agar-solidified antibiotic supplemented EME-maltose medium.
5. After 3–4 weeks, move small somatic embryos to semi solid EME 1500 antibiotic supplemented medium for enlargement and germination.
6. Move the germinated embryos to semisolid antibiotic supplemented B+ medium for axis elongation.
7. Dissect abnormal embryos that fail to germinate into large sections and place on antibiotic supplemented DBA3 medium for shoot induction.
8. Transfer all resulting shoots into RMAN medium to induce rooting.
9. Transfer rooted plants into a peat based potting mixture in the greenhouse and cover with rigid clear plastic for 3–4 weeks maintaining high humidity.

10. Remove the plastic covers following this period of acclimatization.
11. Established plants with 3–4 leaves can then be subjected to appropriate molecular analysis to determine gene insertion.

4 Notes

1. Since the nucellar callus has high embryogenic capacity, the best way to maintain the long-term callus in an undifferentiated state is to visually select and subculture only white/yellow friable callus. Differentiated callus types and organized tissues should be discarded.
2. Cultured embryogenic cells used for protoplast isolation should be in the log phase of growth. For consistent results, maintain uniform growth conditions for the cell suspension, because the physiological state of the suspension cells is an important factor influencing protoplast yield, quality and transformation efficiency. Use 5–12 day-old suspensions from a 2 week subculture cycle, or 7–21 day-old callus from a 4 week subculture cycle.
3. Cell suspension morphology differs from one genotype to another, thus we recommend using a volume of suspension that approximates 1 g fresh weight of callus.
4. Best release of protoplasts is obtained with freshly prepared digestion enzymes, do not store enzyme solution more than 2 weeks.
5. Nylon mesh is sealed to a 4 cm long plastic cylindrical tube made from a plastic syringe. In order to make a similar piece of equipment, take a 30 mL plastic syringe, cut it at the 25 mL mark and keep the upper part with wings. Place a nylon membrane on a preheated hot plate beneath the cylindrical tube and seal the two parts.
6. Protoplasts are fragile, thus take extra care when filtering the protoplast/enzyme solution and later when centrifuging and resuspending protoplasts. When being transferred from one tube to another it is important that the protoplasts are drawn gently into the Pasteur pipette and dispensed slowly down the inside wall of the receiving centrifuge tube. Also, when resuspending pellets of protoplasts with different solutions, ensure a gentle technique of breaking clumps by introducing small bubbles of air with a Pasteur pipette, instead of sucking suspensions in and out of the pipette. Mishandling of the protoplasts can affect their integrity and thereby affect the efficiency of the procedure.
7. If, after isolation and purification, a good yield of protoplasts ($5–10 \times 10^6$ protoplasts/incubation plate) is not obtained, it

may be necessary to vary both the enzyme concentration and length of incubation time to optimize digestion efficiency.

8. When recovering protoplasts from the sucrose-mannitol gradient take as little of the sucrose as possible with the protoplasts. Retention of too much sucrose makes it difficult to pellet the protoplasts at later steps.
9. The washing step that removes the enzymes seems to have a greater bearing on the transformation efficiency, because protoplast samples that have not been washed very well always yield lower transformation, division, and survival rates. It is recommended to repeat **steps 10** and **11** in method 3.3 until a tight clean pellet is obtained.
10. Determine the protoplast density using a hemocytometer. If the number of protoplasts exceeds 100 cells/square in the hemocytometer, dilute the protoplast suspension to obtain accurate counting.
11. Perform protoplast transformation (PEG-induced method) within 1–2 h (preferably immediately) after protoplast isolation, since protoplasts start to regenerate cell walls as soon as they are rinsed from the enzyme solution. Cell wall regeneration may hinder transformation.
12. The number of tubes is determined by the total volume of mixed protoplasts at 4×10^6 protoplasts/mL.
13. The DNA should be sterile (ethanol-precipitated and dissolved in sterile water). Do not incubate DNA for too long with the protoplasts because it may result in lower transformation efficiency due to nuclease digestion. The DNA concentration should be at least 1 μg/μl, to minimize the added volume. In the co-transformation you will add two DNA plasmids one for the gene of interest and the other for the reporter gene. In the direct transformation you will add one DNA plasmid which contain both the gene of interest and the reporter gene in one construct.
14. Using high PEG concentration could reduce the transformation frequency due to either PEG toxicity, lower final DNA concentration, or a combination of these two factors. Use only freshly prepared and filter-sterilized PEG solution (do not autoclave). Check the pH periodically. The PEG should be added immediately after DNA addition as protoplasts are a rich source of nucleases (secretion and release by breakage) that may hydrolyze the DNA.
15. The protoplast/PEG solution may be agitated gently every 5 min for 30 min. A certain proportion of protoplasts will invariably break during and following the PEG treatment. The debris of dead cells is detrimental for a continued liquid culture of surviving protoplasts, thus try to handle the protoplast/PEG culture very gently to reduce this phenomena.

16. Washing the protoplasts three times is very important to remove all the PEG and A:B solution.
17. In each transforming dish, protoplasts are plated at a density of approx. $1–1.5 \times 10^6$ protoplasts/mL of culture medium. In order to retain viability and induce cell division, transformed protoplasts have to be plated in thin-layer culture at high cell density. In the case of citrus protoplasts, the best results are obtained when the cell density exceeds 1×10^6 protoplasts/mL of medium. If necessary, determine and adjust protoplast density using a hemocytometer.
18. Check the cultures every week to evaluate the rate of colony development. In the event of fast colony development feeding can begin as early as 10–14 days after transformation as follows: add 6–8 drops of liquid 1:1:1 of BH3 0.6 M–EME 0.6 M–EME 0.146 M medium to reduce the osmotic pressure and incubate the cultures again in the dark at 28 °C without agitation. Ten to 14 days later add another 6–8 drops of liquid 1:1:1 of BH3 0.6 M–EME 0.6 M–EME 0.146 M medium. Depending on the quality of protoplast preparations, up to 75 % of the protoplasts survive and 20–40 % of cells will undergo divisions during the first 7–10 days of culture. After 14 days of culture, the dividing cells should form colonies of 2–16 cells.
19. Optimal fluorescence is observable only after 48 h, although some transformed protoplasts start exhibiting GFP fluorescence 24 h after transformation.
20. In the case of slow developing colonies, begin feeding 4–6 weeks after transformation.
21. Take care of those GFP positive embryos developing faster and separate them from the rest. Transfer them earlier for regeneration to plants. Faster-developing embryos can rapidly produce healthy (normal) plants. It is recommended to place a few (6–8) embryos together onto cellulose acetate filter paper for rapid and normal development.
22. In certain leaf pieces, the green fluorescence can be entirely masked by the chlorophyll pigment. In case of doubts about the transgenic nature of regenerated plants, a small portion of leaves may be used to make protoplasts in 1 mL of enzyme solution and the protoplasts may directly be observed as they are released. To accelerate the propagation of the transgenic shoots, you can use any available grafting technique, either shoot tip grafting onto a greenhouse growing rootstock or in vitro micro-grafting on seedling rootstock [129].
23. Molecular analysis (PCR, Southern and Western analysis) should confirm the integration and expression of the transgene in the citrus genome.

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Chapter 14

Somatic Embryogenesis Induction and Plant Regeneration in Strawberry Tree (*Arbutus unedo* L.)

João F. Martins, Sandra I. Correia, and Jorge M. Canhoto

Abstract

Somatic embryogenesis is a powerful tool both for cloning and studies of genetic transformation and embryo development. Most protocols for somatic embryogenesis induction start from zygotic embryos or embryonic-derived tissues which do not allow the propagation of elite trees. In the present study, a reliable protocol for somatic embryogenesis induction from adult trees of strawberry tree is described. Leaves from in vitro proliferating shoots were used to induce somatic embryo formation on a medium containing an auxin and a cytokinin. Somatic embryos germinated in a plant growth regulator-free medium.

Key words Epicormic shoots, Ericaceae, Fruit, Germination, In vitro, Neglected crops, Shoot proliferation, Somatic embryo

1 Introduction

Arbutus unedo L. is an Ericaceae species commonly known as strawberry tree. It grows on acidic, rocky, and well-drained soils [1, 2] and can withstand low (until −12 °C) temperatures [1] as well as dry conditions [1]. *A. unedo* individuals grow spontaneously (Fig. 1a) in several countries of the Mediterranean Basin, from Spain to Turkey, as well as in North Africa, Mediterranean Islands, and Atlantic Coast, including Ireland and Portugal [1, 2]. Strawberry tree is a small perennial shrub or tree (Fig. 1b) that usually grows up to 3 m [3] with a spreading habit and gray-brown bark. The edible fruit (Fig. 1c) is a spherical berry, with about 2 cm diameter, covered with conical papillae and enclosing 10–50 small seed whereas the flowers are hermaphrodite, bell shaped, whitish to slightly pink, and organized in hanging panicles (Fig. 1d), [4]. The reproductive cycle is long with fruits taking a year to ripe, and, during several months of the year, both flowers and fruits are present in the same tree, making the species a very attractive ornamental plant. From an ecological perspective, strawberry tree is a very important species in Mediterranean ecosystems avoiding erosion,

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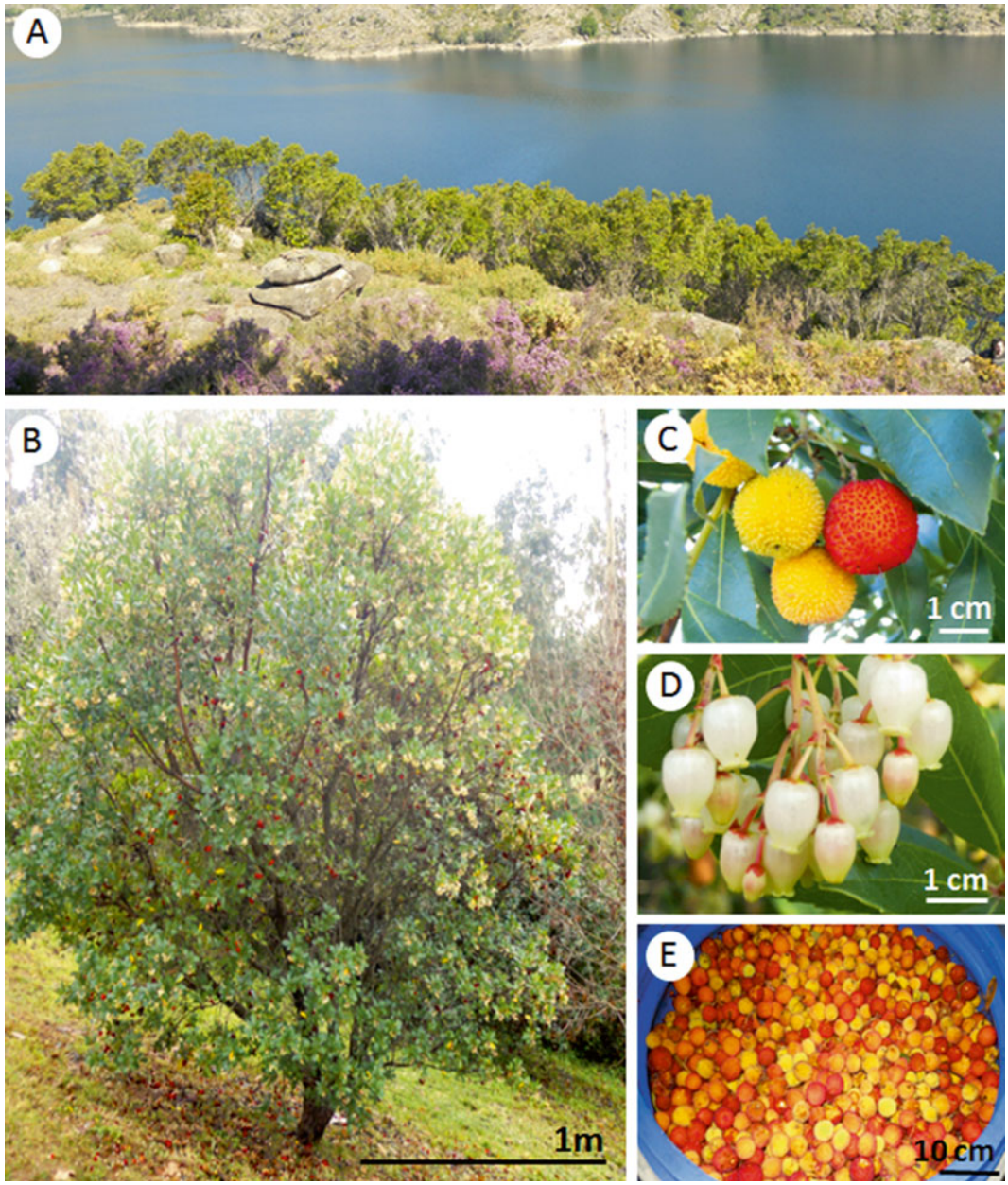

Fig. 1 Aspects of strawberry tree. (**a**) Field-growing trees in the North of Portugal, on the slopes of a hill near a dam (Google Earth information: 41°45′55″N, 8°11′89″, altitude 639 m). Adult tree (**b**), the fruits (**c**), and the flowers (**d**). Fruits in fermentation for the production of the spirit "medronheira" (**e**)

providing food for fauna and helping recover marginal lands [1, 4]. Its ability to regenerate after fires is a feature that makes the species interesting for reforestation programs, especially in southern Europe countries such as Portugal, Spain, Italy, and Greece, where forest fires are common [5]. Once considered a "Neglected or Underutilized Crop" (www.cropsforthefuture.org), the impor-

tance of strawberry tree is growing [3, 4]. In a context where some of the most important forest species in southern Europe, such as pine and eucalyptus, are suffering from several diseases, the demand for strawberry tree by producers and stakeholders is increasing, particularly in Portugal [3, 4]. The fruits are commonly used in the manufacture of traditional products such as jam and jelly [5]. However, its main application is for the production of an alcoholic distillate [6], known in Portugal as *medronheira* (Fig. 1e).

The propagation of strawberry tree can be achieved through the use of conventional methods of vegetative propagation such as cuttings [7] or by seeds [8, 9]. Seeds do not assure true-to-type propagation and particular characteristics can be lost. Assays of vegetative propagation can be made by conventional vegetative propagation methods such as rooting or grafting. However, the frequencies of rooting are quite low, especially when mature cuttings are used [10, 11], and elite genotypes are unavailable for grafting. In vitro tissue culture techniques have been applied to the propagation of strawberry tree, in particular axillary shoot proliferation [11, 12]. However, somatic embryogenesis has much more potential for cloning than other micropropagation techniques since somatic embryos are easier to handle than other propagules and can be obtained in large amounts from a single explant [13, 14]. Somatic embryogenesis has great potential for genetic transformation and cryopreservation of desirable selected lines [15, 16]. Moreover, somatic embryo induction and development serve as a model to understand the physiological and genetic factors controlling the different steps of embryo development [17, 18]. Previous works on *A. unedo* have shown that somatic embryogenesis can be achieved from adult material [19, 20].

Here, a protocol for somatic embryogenesis induction and plant regeneration from *A. unedo* trees is described. To overcome the lack of potential of adult tissues for somatic embryogenesis, an indirect approach was attempted in which shoots from selected adult trees were first established in vitro through axillary shoot proliferation and then somatic embryogenesis was induced in leaves from these shoots. This protocol of somatic embryogenesis induction and plant regeneration can also be applied to the propagation of *Arbutus canariensis* Duham, a species quite similar to *A. unedo*.

2 Materials

Somatic embryogenesis in *A. unedo* can be induced from leaves of in vitro propagated shoots, established from adult or juvenile (i.e., not yet in the reproductive phase) plants or from seedlings. The methodology is the same but shoots from adult trees are of known genotypes allowing the propagation of selected trees. Hence, the

following methodology is based on experiments with leaves from in vitro developing shoots established from adult plants.

2.1 Plant Material

1. Selected adult trees (Fig. 1a).
2. Semi-woody branches (*see* **Note 1**), collected from the selected plants (30–40 cm length).
3. Epicormic shoots (Fig. 2a) of 2–4 cm, collected from these branches.
4. Proliferating shoots (Fig. 2b).
5. Leaves from proliferating shoots (Fig. 2c).

2.2 Culture Media

1. Semisolid medium for culture establishment (SP medium): Major salts from the Anderson medium [21], micronutrients from the Murashige and Skoog [22] medium (without KI), and organic compounds of the De Fossard medium [23]. Add 0.087 M sucrose and 8.8 μM BA (6-benzyladenine, Sigma Chemical Company, St. Louis, MO, USA).
2. Induction medium (IM): This medium contains the same components of the SP medium and the following growth regulators: 8.8 μM BA and 26.8 μM NAA (1-naphthaleneacetic acid, Sigma Chemical Company, St. Louis, MO, USA).
3. Somatic embryo germination medium (GM): Knop [24] major salts, micronutrients of the Murashige and Skoog medium [22], vitamins (without riboflavin) of the De Fossard medium [23], 0.044 M sucrose and 1 % (w/v) activated charcoal (*Merck* KGaA, Darmstadt, Germany).
4. Add 0.6 % (w/v) agar (Panreac, Spain, or equivalent) to all media before autoclaving at 121 °C for 20 min (800–1100 g/cm gel strength after autoclaving). Adjust the pH of all media to 5.7 using KOH or HCl diluted solutions (0.01–1 M) before autoclaving and agar addition (*see* **Note 2**).

3 Methods

1. Remove the leaves from semi-woody branch segments and wash them in running water to remove major detritus. Spray washed branches with a fungicide solution (Benlate or equivalent, 6 % w/v), and set upright in jars containing water to allow the development of axillary shoots in a growth cabinet at 20 °C and 80–90 % relative humidity, under a 16 h daily illumination regime of 15–20 μmol/m^2/s photosynthetically active radiation (PAR, cool-white fluorescent lamps) (*see* **Note 3**). Change the water every 2 days to avoid fungi growth (*see* **Note 4**).

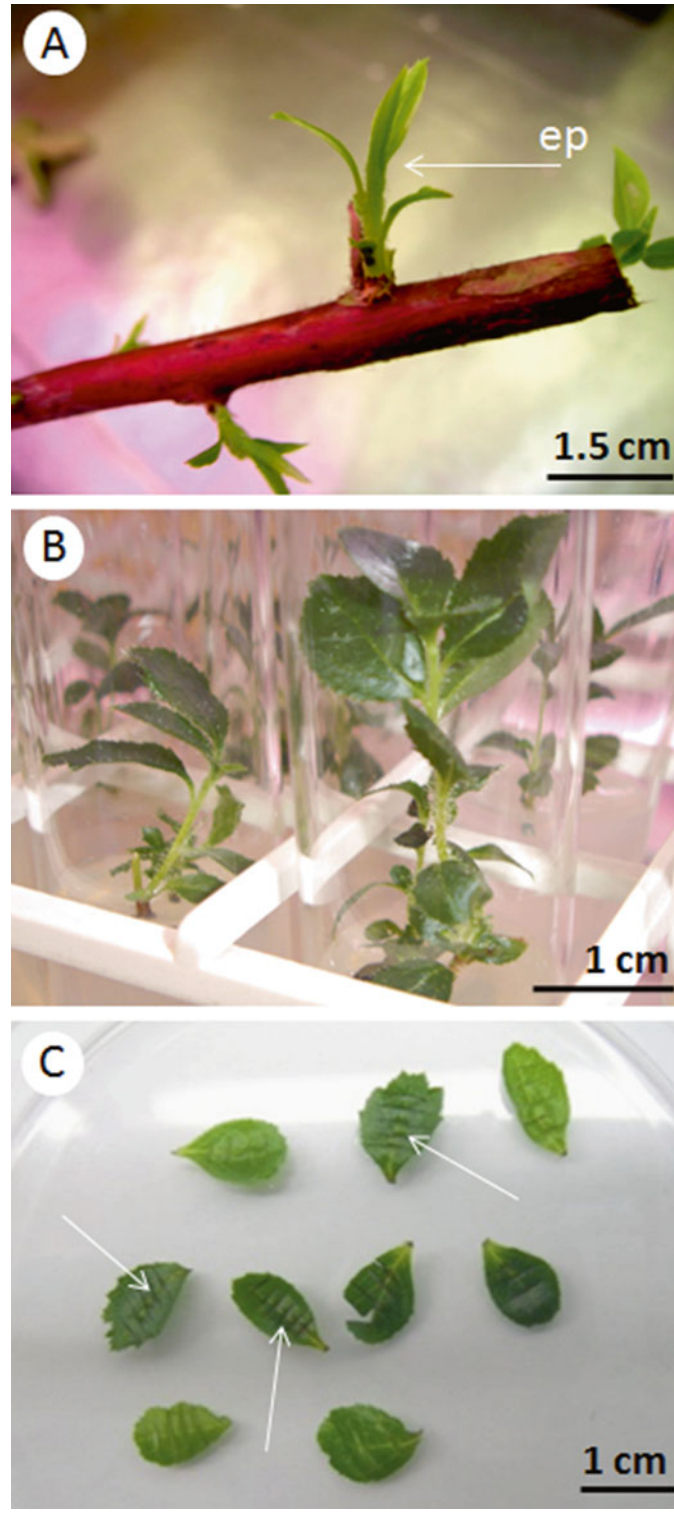

Fig. 2 Formation of epicormic shoots and shoot proliferation. (**a**) Epicormic shoots (ep) developing from a branch after 1.5 month. (**b**) Shoot proliferation on medium SP. (**c**) Wounded (*arrows*) leaves at the time of the culture for somatic embryogenesis induction

2. Isolate the shoot apex (0.5 cm) and nodal segments (*see* **Note 5**) from the epicormic shoots (3–4 cm length). Remove the leaves and wash with detergent (2–3 drops of Tween 20) in a volume of 100 mL of water.
3. Transfer the explants to an ethanol (*see* **Note 6**) solution (70 % v/v) during 30 s.
4. Wash three times with sterilized water, and transfer to a 5 % (w/v) calcium hypochlorite solution containing 2–3 drops of Tween 20 under stirring, during 15 min (*see* **Note 7**).
5. Wash three times with sterilized water to remove the excess of hypochlorite.
6. In a laminar flow chamber, transfer shoot apices and nodal segments of epicormic shoots to test tubes (15 × 2.2 cm) containing 15 mL/tube of SP medium (*see* **Note 8**). Place one apex or nodal segment per test tube for culture establishment (*see* **Note 9**).
7. Keep the cultures in a growth chamber under a 16 h photoperiod of 15–20 μmol/m^2/s (cool-white fluorescent lamps) at 25 °C (*see* **Note 10**).
8. Following establishment, shoots can be subcultured to obtain large amounts of leaves for further assays. Subcultures must be carried in the same conditions than in vitro establishment.
9. Leaves (0.4–0.8 cm length) from proliferating shoots (3–4 cm) are used for somatic embryogenesis induction. Remove the most apical expanding leaves (8–12 mm) from proliferating shoots and place them (abaxial side down) in test tubes (*see* **Note 8**) containing the IM medium. With a scalpel make 4–6 transverse cuts in the central part of the leaves (*see* **Note 11**).
10. Transfer the cultures to a growth chamber, in the dark, at 25 °C. Somatic embryos start to appear after 6–8 weeks of culture (Fig. 3a). About 2 weeks later, globular somatic embryos are formed (Fig. 3b) (*see* **Note 12**).
11. Transfer 2–4 mature cotyledonary somatic embryos (Fig. 3c) per test tube containing 15 mL of GM and keep the cultures under a 16 h photoperiod of 15–20 μmol/m^2/s (cool-white fluorescent lamps) at 25 °C (*see* **Note 13**). Germination can be seen after 10–15 days (Fig. 3d) of culture on GM medium.
12. Remove from the test tubes well-rooted (*see* **Note 14**) plantlets (Fig. 3e), measuring 3–4 cm in the aerial part, and wash the roots with tepid tap water to get rid of agar (*see* **Note 15**).
13. For acclimatization, transfer the plantlets to pots (250 mL volume) containing a mixture of sand and perlite (1:1), previously sterilized by autoclaving.
14. Place the containers in a greenhouse under 18–20 °C until plantlets reach 8–10 cm (*see* **Note 4**).

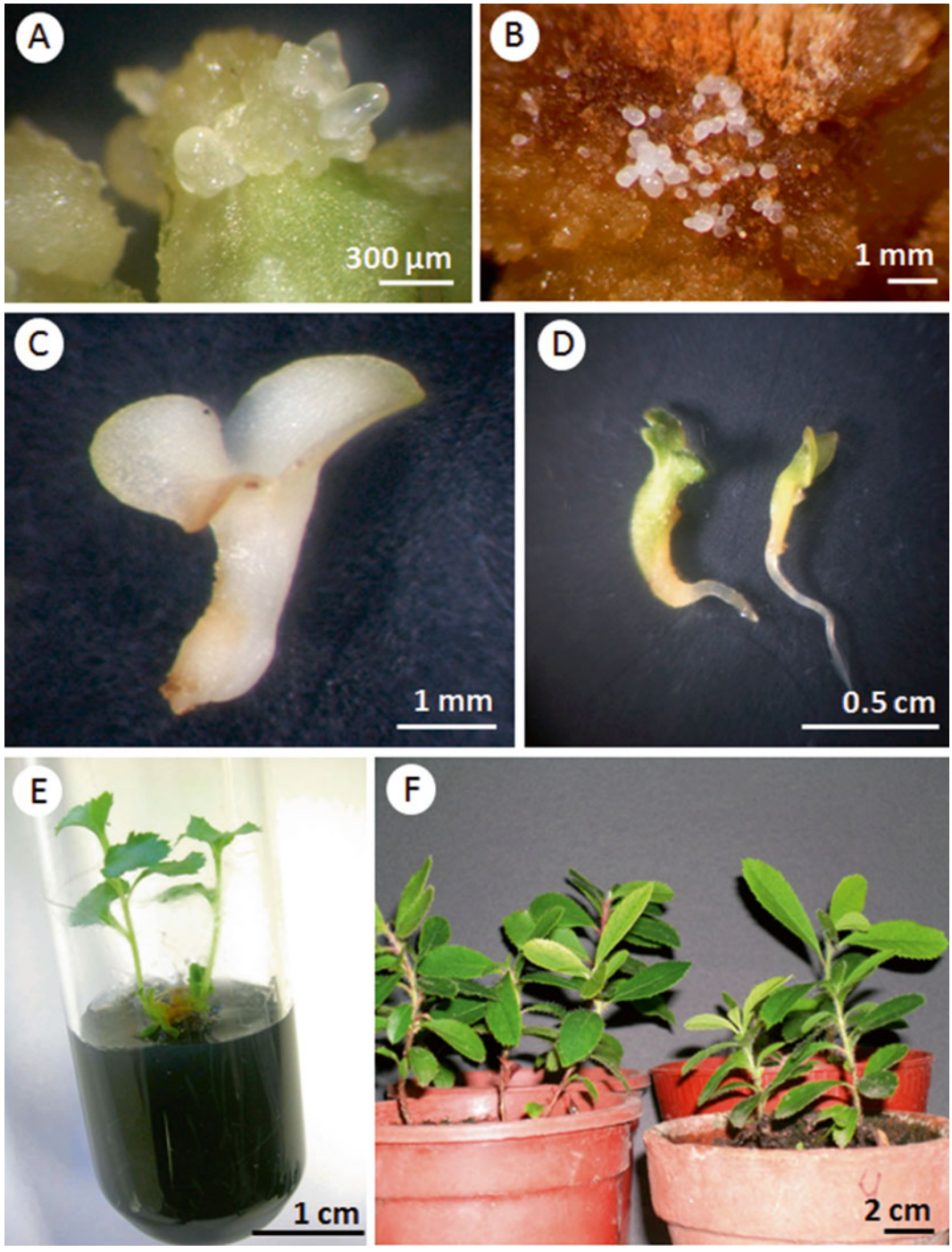

Fig. 3 Somatic embryo formation and plant regeneration. (**a**) Early stages of somatic embryo development. (**b**) Somatic embryos mostly at the globular stage. (**c**) Cotyledonary embryo. (**d**) Germinated somatic embryos after 12 days on GM medium. (**e**) Well-developed plantlets of somatic embryo origin just before to be potted. (**f**) Several plantlets obtained by somatic embryogenesis

15. Transfer the plantlets (Fig. 3f) to larger containers (1 L volume) containing a mixture of autoclaved substrate, composed of sand and peat (1:1).
16. When the plants reach 30–40 cm, transfer them to field conditions. Figure 4 summarizes the different steps of the methodology used to achieve plant regeneration through this protocol.

4 Notes

1. Branches must be healthy and from the upper part of the plant. Avoid using older woody branches. These branches sprout as well as younger branches, but the in vitro response of the explants is lower. Spring is the best time to initiate the cultures, but it was found that branches collected during other periods of the year can also be used without significant behavioral differences.
2. Recent data (unpublished) showed that the pH of the culture media strongly influences the in vitro response and that this effect is genotype dependent. Thus, it may be necessary to use different pH values for different trees but this has to be established experimentally.
3. Spraying (three times a week) the branches with a 9.0 μM solution of BA stimulates epicormic shoot development (*see* ref. 11) but is not absolutely necessary to induce it. Furthermore, it can affect further response of the apices and nodal segments in culture by reducing shot growth.
4. Covering the branches with polypropylene plastic bags maintains a higher humidity and may help to stimulate epicormic shoot development.
5. Although both shoot apices and nodal segments can be used for axillary shoot proliferation, the first gave better rates of proliferation.
6. Strawberry tree tissues are very sensitive to ethanol. Do not keep the explants in contact with this alcohol longer than suggested.
7. As a general procedure, we submit the explants to calcium hypochlorite for 15 min. The effect of this treatment can be genotype dependent, e.g., leaves of some trees are more sensitive than others. If the tissues start to bleach before the indicated time, immediately remove the explants and wash them.
8. Other containers such as Magenta boxes or glass boxes can be used instead of test tubes. From the different containers tested, it was found that test tubes covered with plastic caps are the most effective for shoot proliferation. For somatic embryogenesis induction, leaves can also be cultured in Petri dishes or other plastic or glass containers.

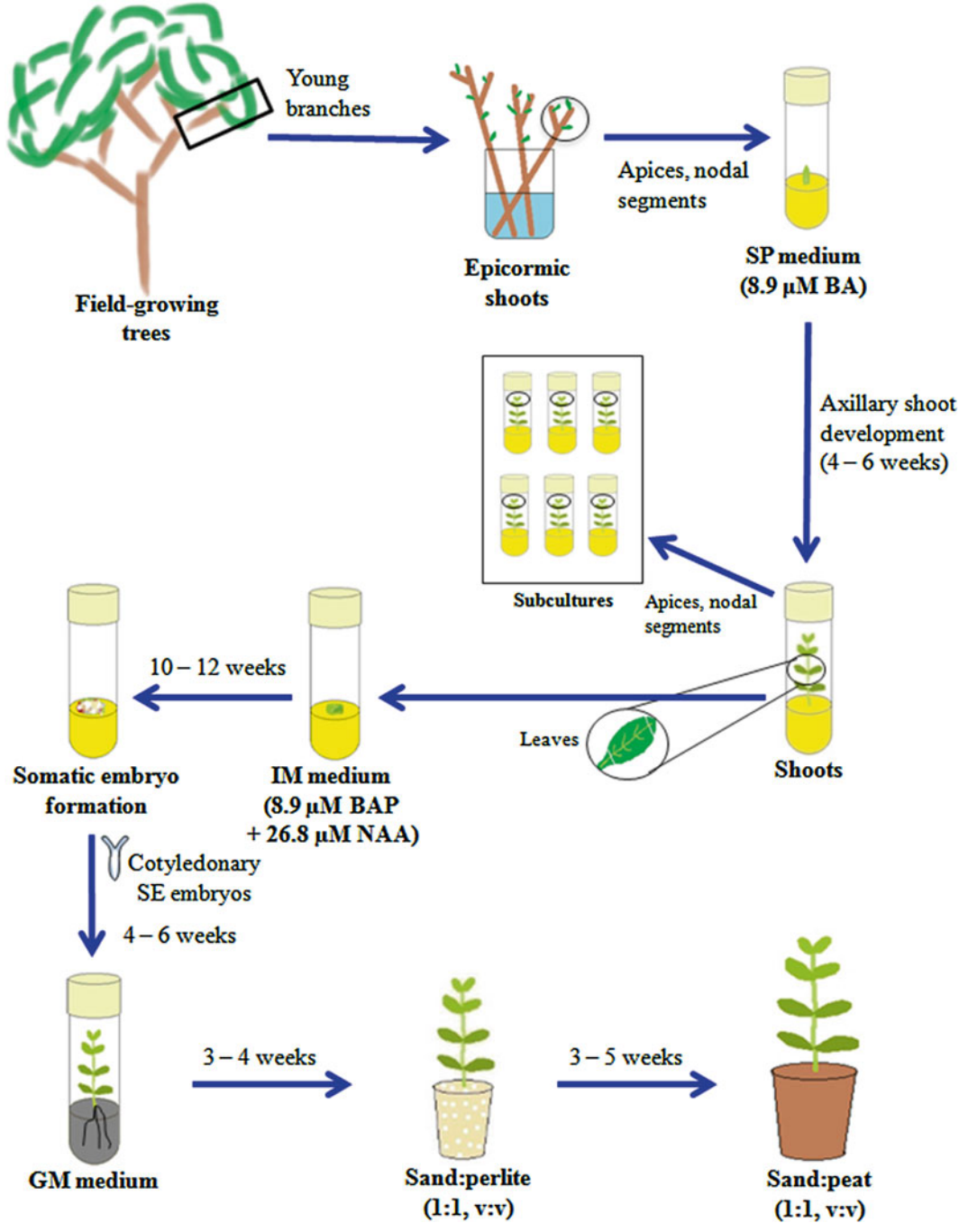

Fig. 4 General overview of the process of plant regeneration of *Arbutus unedo* through somatic embryogenesis

9. More than one explant can be placed per test tube; however, this way the probability of contamination is higher.
10. In general, using this protocol, *A. unedo* tissues do not release phenolics to the culture medium. However, we have found phenol exudation and oxidation when working with some genotypes. In this case tissue cultures must be placed in a growth chamber at 25 ± 1 °C under dark conditions for a week and then transferred to light conditions. The inclusion of 100 mg/L ascorbic acid in the medium also helps to reduce phenol oxidation and necrosis of the explants.
11. Somatic embryogenesis can be achieved even without wounding the leaves. However, this procedure increases the rates of somatic embryogenesis induction.
12. Somatic embryo formation is asynchronous and embryos at different developmental stages, from globular to cotyledonar stage, can be seen in the same explant. The rates of somatic embryogenesis induction are quite variable among different trees. In the tested conditions, recalcitrant genotypes as well as high responsive genotypes (i.e., with over 50 % of induction rates) were found. The number of somatic embryos per embryogenic explants is also variable, with some explants giving rise to only a few embryos, whereas others produce over 100.
13. Do not transfer to the germination medium the non-cotyledonar embryos. They will not germinate or will undergo precocious germination. Try to select morphologically normal embryos since off-type embryos usually give rise to abnormal plantlets.
14. Plantlets are considered well-rooted when showing at least two well-developed roots. In some cases, only the stem part of the plantlets develops after somatic embryo germination. In these cases, the shoots can be rooted following an IBA (indole-3-butyric acid) treatment followed by transfer to an auxin-free medium, as described in ref. 9.
15. Roots must be washed carefully not to break them, thus preventing hindrance of the success of the next steps.

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Chapter 15

Somatic Embryogenesis in Olive (*Olea europaea* L. subsp. *europaea* var. *sativa* and var. *sylvestris*)

Eddo Rugini and Cristian Silvestri

Abstract

Protocols for olive somatic embryogenesis from zygotic embryos and mature tissues have been described for both *Olea europaea* sub. *europaea* var. *sativa* and var. *sylvestris*. Immature zygotic embryos (no more than 75 days old), used after fruit collection or stored at 12–14 °C for 2–3 months, are the best responsive explants and very slightly genotype dependent, and one single protocol can be effective for a wide range of genotypes. On the contrary, protocols for mature zygotic embryos and for mature tissue of cultivars are often genotype specific, so that they may require many adjustments according to genotypes. The use of thidiazuron and cefotaxime seems to be an important trigger for induction phase particularly for tissues derived from cultivars. Up to now, however, the application of this technique for large-scale propagation is hampered also by the low rate of embryo germination; it proves nonetheless very useful for genetic improvement.

Key words Double regeneration technique, Immature and mature zygotic embryos, Mature tissues, *Olea europaea* L. var. *sativa*, *Olea europaea* L. var. *sylvestris*, Somatic embryogenesis

1 Introduction

Until recent years, olive breeding was limited, mainly due to the long juvenile phase of the seedlings and to the scarce knowledge related to genetic aspects, which discouraged breeders and public researchers. For this reason, biotechnology-mediated genetic improvement, which still requires efficient in vitro regeneration protocols from cells or tissues, is a precious tool in support of conventional breeding. Actually, it is possible to easily regenerate somatic embryos or adventitious shoots from juvenile material, such as zygotic embryos, due to the high morphogenetic ability of tissues collected from immature or mature seeds [1–10] in both *Olea europaea* var. *sativa* and *Olea europaea* var. *sylvestris*. However, these structures cannot be employed either for plant cloning or for breeding as, due to the high heterozygosis of the *Olea* spp.,

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they produce plants genetically different from the mother plant. On the other hand, somatic embryogenesis is very difficult to obtain from mature tissues of cultivars; moreover, this phenomenon is strongly genotype dependent. In corroboration of that, up to now somatic embryogenesis has been successfully reported only in the cultivars Canino, Moraiolo [11], and Dahbia [12, 13] of *O. europaea* L. var. *sativa* and in one genotype of *O. europaea* L. var. *sylvestris* [14]. The var. *sylvestris* has been taken into account in this chapter because it may contribute to improve the cultivated varieties of *O. europaea* L. var. *sativa*, since it belongs to the primary gene pool [15] of the genus. In our experience [11], regeneration of adventitious buds, which normally provokes rejuvenation of the regenerants, seems to be one of the key factors to obtain somatic embryogenesis from mature tissues of cultivars, together with the employment of a proper combination of basal medium and plant growth regulators (PGRs). However, the results obtained in the cv. Dahbia demonstrated that rejuvenation through adventitious shoot organogenesis is not essential for the induction of embryogenic response [13]. Regarding the PGRs, thidiazuron (TDZ) seems to be an important trigger for the induction phase, since it was used successfully for somatic embryogenesis induction. Recently, the development of early stages of embryogenic cell suspension culture from mature olive leaf-derived calli of the cv. Chetoui has also been reported, but the development of well-formed embryos was not achieved [16].

The ability of somatic embryos to form secondary embryos and, consequently, a cyclic embryogenesis can both be useful in the unconventional breeding of olive, although the derived plants acquire juvenility over time and, hence, delay their adult phase [17]. Concerning somaclonal variation, in our experience, an evident phenotypic variability was not observed yet, after many years of in-field observation of plants of cv. Canino derived from somatic embryogenesis (Fig. 1f–i). This fact was confirmed by Lopes et al. [18] in *Olea* spp., where the genome integrities have been maintained throughout the embryogenesis process. On the contrary, a report described a different vegetative behavior (bushy and columnar phenotype) in plants derived from somatic embryos, originated from one cotyledon of the cv. Frangivento [19]. These conflicting results suggest to pay attention to the use of somatic embryogenesis for propagating true-to-type olive plants. On the other hand, the high multiplication potential of cyclic somatic embryogenesis, originated from mature tissues of elite cultivars, makes it a very suitable technique to induce somaclonal variation under selective pressure of biotic and abiotic stresses, as well as to be applied in genetic transformation for the introduction of some agronomical useful genes, as reviewed by Rugini et al. [17].

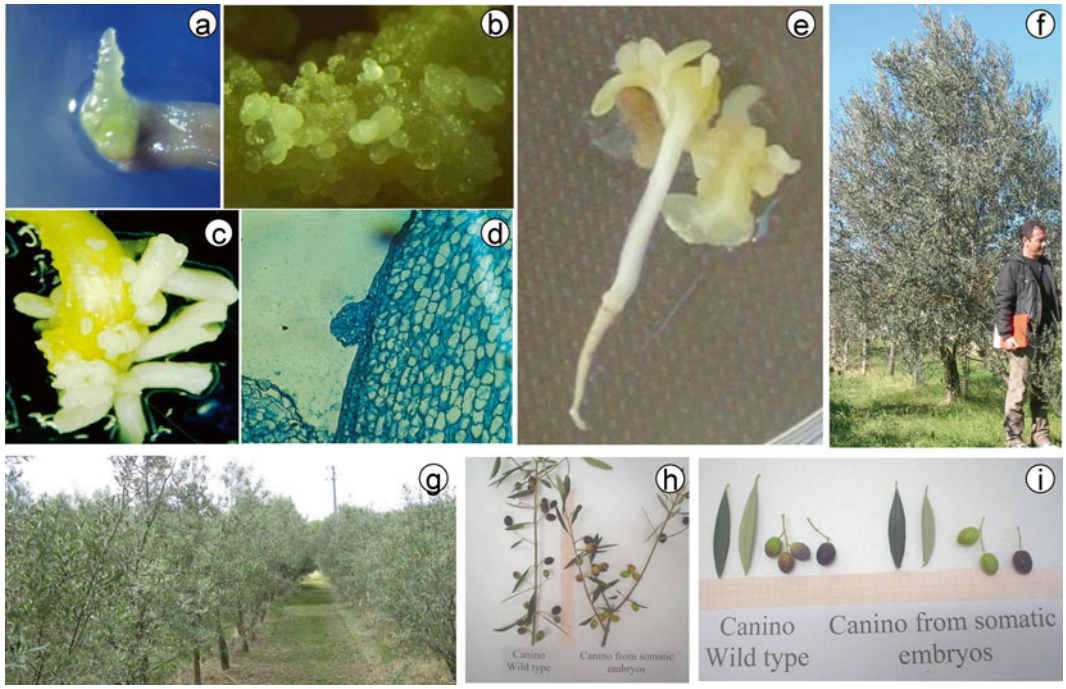

Fig 1 Somatic embryogenesis by using a double regeneration system from in vitro growing shoot (cv. Canino). (**a**) Adventitious bud petioles from in vitro growing shoots. (**b**) Somatic embryogenesis from leaflets of a small adventitious bud that was previously regenerated from the petiole. (**c**) Secondary somatic embryogenesis from epidermal cell layer of somatic embryos and teratoma (cyclic somatic embryogenesis). (**d**) Histology of secondary somatic embryogenesis from epidermal cell layer of somatic embryos. (**e**) Embryo conversion. (**f**) 14-year-old olive from somatic embryogenesis. (**g**) View of 7-year-old in-field plants derived from somatic embryos. (**h**) Comparison of young branches with leaves and drupes collected from somatic embryo-derived plants and the donor plant. (**i**) Detail of leaves and fruits from previous photos. Up to now, no evident variation has been observed in vegetative and reproductive habits

2 Materials

1. Material used to induce embryogenic callus: (1) Immature olive fruits harvested between 30 and 75 days after blooming, to be used both at harvest time or after storage at 12–15 °C for 2–3 months, before extracting the embryos; (2) mature olive fruits of *O. europaea* L. var. *sativa* and of *O. europaea* L. var. *sylvestris*; (3) in vitro shoots from mature trees of the cv. Canino, Moraiolo, or Dahbia, micropropagated on OM medium (Table 1; *see* **Note 1**) supplemented with 2190 mg/L L-glutamine, 1 mg/L zeatin riboside (ZR), 3.6 % mannitol (or 3 % sucrose), and 0.6 % agar; (4) adventitious buds regenerated from leaf petioles of the cv. Canino or Moraiolo; and (5) young plants of *O. europaea* L. var. *sylvestris* (about 2-year-old plants, grown in greenhouse and derived from field plants of over 30 years of age) used to collect young leaves.

Table 1
Rugini olive medium composition (as reported by Duchefa, the Netherlands)

Micro elements (*mg/L*)	
$CoCl_2 \cdot 6H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.250
FeNaEDTA	36.700
H_3BO_3	12.400
KI	0.830
$MnSO_4 \cdot H_2O$	16.900
$Na_2MoO_4 \cdot 2H_2O$	0.250
$ZnSO_4 \cdot 7H_2O$	14.300
Macroelements (*mg/L*)	
$CaCl_2$	332.16
$Ca(NO_3)_2$	416.92
KCl	500.00
KH_2PO_4	340.00
KNO_3	1100.00
$MgSO_4$	732.60
NH_4NO_3	412.00
Vitamins (*mg/L*)	
Biotin	0.05
Folic Acid	0.50
Glycine	2.00
Myoinositol	100.00
Nicotinic acid	5.00
Pyridoxine HCl	0.50
Thiamine HCl	0.50

2. Deionized water and sterile water.
3. Commercial bleaching solution.
4. 70 % ethanol.
5. MS medium [20], OM medium [21], zeatin riboside (ZR), benzylaminopurine (BAP), 3-indolbutyric acid (IBA), α-naphthaleneacetic acid (NAA), 6-γ-γ-(dimethylallylamino)-purine (2-iP), TDZ, Difco Bacto agar, plant agar, Tween 20.
6. Equipment: Laminar flow hood, growth chamber, dissecting microscope, pH meter, and autoclave.

7. Disposables: Petri dishes (25 mm × 90 mm), 25-well multiwell plates, pipettes, Whatman 3 mm filter paper, 0.22 μm Millipore filters, forceps, scalpels, Parafilm, and Jiffy pots.

3 Methods

1. Adjust the pH of the media at 5.8 with 0.1 N KOH or 0.1 N HCl and solidify with plant agar (unless otherwise stated).
2. Sterilize all the culture media by autoclaving at 121 °C for 20 min.
3. Add the filter-sterilized hormones after autoclaving.

3.1 Somatic Embryogenesis from Immature Zygotic Embryos

1. Break the stones and remove the seeds from the stony endocarp.
2. Surface sterilize the seeds with 10 % commercial bleaching solution for 10–15 min.
3. Rinse three times the seeds with sterile water.
4. Soak the seeds for at least 24 h in sterile water at room temperature.
5. With a scalpel, remove the zygotic embryos by either a longitudinal or transversal cut across the seed teguments and endosperm.
6. Place the embryos [10–15] horizontally in each Petri dish, containing 20 mL of half-strength MS medium [20] supplemented with 0.1–0.5 mg/L BAP, 2 % sucrose, and 0.6 % Difco Bacto agar; seal the dishes with Parafilm.
7. Keep the cultures in the dark at 23 ± 1 °C and subculture them in fresh medium every 30 days.
8. Subculture the embryogenic calli (*see* **Note 2**) in the same medium reducing BAP concentration at 0.05 mg/L or in hormone-free medium.

3.2 Somatic Embryogenesis from Mature Zygotic Embryo of Olea europaea var. sativa and var. sylvestris

1. Break the stones and remove the seeds from the stony endocarp.
2. Sterilize the seeds for 1 min with 70 % ethanol, and then soak them for 20 min in 10 % commercial bleaching solution plus 20 drops/L of Tween 20.
3. Rinse three times with sterile distilled water, and soak for 24 h in sterile distilled water in the dark, at 24 ± 1 °C.
4. Extract the embryos and dissect them in three parts: proximal and distal half of cotyledons and radicle (*see* **Note 3**).
5. Transfer the explants to Petri dishes containing a modified OM medium (OMc, *see* **Note 4**) with 0.5 mg/L 2-iP, 5 mg/L IBA, and 0.6 % Difco Bacto agar.

6. Maintain the Petri dishes in 16 h photoperiod, under 40 μmol/m^2/s of light intensity, at 24 ± 1 °C, for 21 days.
7. Transfer 25 callusing explants after 14–21 days to Petri dishes containing gelled 20 mL hormone-free OMc medium, at the abovementioned environmental conditions, to induce somatic embryogenesis (*see* **Notes 5** and **6**).

3.3 Somatic Embryogenesis from Mature Tissue Explants of In Vitro Grown Shoots

3.3.1 Cv. Canino and Moraiolo

1. Collect leaf petioles from shoots, micropropagated on OM medium supplemented with 1 mg/L ZR, 3.6 % mannitol (or 3 % sucrose), and 0.6 % agar.
2. Place the leaf petioles in Petri dishes containing 20 mL MS medium supplemented with TDZ, at concentration ranging between 2 and 10 mg/L, 2 % sucrose, 0.6 % Difco Bacto agar (*see* **Note 7**), and seal with Parafilm with the aim of regenerating adventitious buds (Fig. 1a).
3. Dissect the leaflets from the neo-formed adventitious buds when they are no longer than 1–3 mm. Place them individually in a 25 multiwell plate (Sterilin) with 3 mL of OMc medium supplemented with 1 mg/L hydrolyzed casein, 3 % sucrose, 200 mg/L cefotaxime, 0.1 mg/L BAP, 0.1 mg/L 2-iP, 0.5 mg/L IBA, and 0.6 % Difco Bacto agar.
4. Store then the cultures in the dark at 24 ± 1 °C.
5. After about 1 month, when morphogenetic masses are produced from petioles, transfer them to a filter paper (Whatman no. 3) of the same diameter of the Petri dishes, with 5 mL OMc liquid medium.
6. Every 3 weeks, remove the exhausted medium by pipetting and replace it with an equivalent volume of fresh liquid medium, until masses with pro-embryonic structures appear (they are recognizable by a small spherical callus, with diameter of 1–2 mm and a smoothly yellowish surface; the masses continue to enlarge in size and start to differentiate somatic embryos).
7. Place the neo-formed embryos (Fig. 1b) on hormone-free OMc medium with an agar content reduced to a value of 0.3 % (*see* **Note 8**).

3.3.2 Cv. Dahbia

1. Collect the young leaflets and petioles from in vitro micropropagated shoots, growing on OM medium supplemented with 1 mg/L ZR, 3.0 % sucrose, and 0.6 % agar.
2. Place the explants in Petri dishes on a shaker (regulated at 60 rpm), filled with liquid induction medium, consisting of half-strength MS medium supplemented with 6.60 mg/L TDZ and 0.1 mg/L NAA, for 4 days.
3. Transfer the explants to hormone-free half-strength MS medium, solidified with 0.6 % Difco Bacto agar for 8 weeks.

4. Transfer the explants to the expression ECO medium (*see* **Note 9**), supplemented with 0.1 mg/L BAP, 0.1 mg/L 2-iP, 0.05 IBA, and 0.6 % Difco Bacto agar.

3.4 Somatic Embryogenesis from Mature Tissue Explants of In Vivo Grown Plants of Olea europaea var. sylvestris

1. Collect young leaves formed on 2-year-old potted plants grown in greenhouse.
2. Sterilize the leaves for 1 min in 70 % ethanol, followed by 10 min immersion in 25 % commercial bleaching solution.
3. Rinse three times with sterile distilled water.
4. Dissect the leaves and place 10 explants (petioles, median, and distal portion of leaf blades) in Petri dishes, containing MS medium supplemented with 3 % sucrose, 1 mg/L ZR, 2.5 mg/L IBA, and 0.7 % Difco Bacto agar (*see* **Note 10**).
5. Store the cultures in the dark at 22 ± 1 °C for 3 months.
6. Transfer the cultures on hormone-free MS medium in order to achieve somatic embryogenesis expression.
7. Subculture the tissue explants every 4 weeks in the same medium to maintain somatic embryogenesis expression.

3.5 Embryo Maturation and Conversion to Plantlets

1. Separate embryos from calli or from original tissues.
2. Place 5–6 embryos in 6-well multiwells (Sterilin) with 2 mL OMc liquid medium, supplemented with 0.3 mg/L ZR; place the multiwells on a gyratory shaker at 80 rpm.
3. Place the cultures in a growth chamber at 16 h light photoperiod (40 μmol/m^2/s).
4. Transplant the young plantlets with 2–3 pairs of leaves to Jiffy pots for the acclimatization.
5. Transfer them to the greenhouse under high relative humidity (80 %), at a temperature of about 23 °C and gradually reduce the humidity.

4 Notes

1. Olive medium [21], commercialized in powder form by the company DUCHEFA, is normally used for long-term proliferation of olive shoots with 35-day subcultures. Olive medium is characterized by a high content of Ca, Mg, S, Cu, and Zn (Table 1). This medium usually induces tender shoots, with less basal callus, when subcultures are done regularly every month.
2. The embryos will be visible after 5–6 weeks in culture. Usually, the percentage of zygotic embryos forming somatic embryos directly from the tissues (direct somatic embryogenesis) is considerably higher than embryos originated from the neo-formed calli (indirect somatic embryogenesis), which lose their embryogenic ability after one to two subcultures.

Secondary embryogenesis normally takes place from epidermal tissues of neo-formed embryos or teratoma, which can be maintained for several subcultures in the same medium, allowing cyclic somatic embryogenesis.

3. The radicles show a somatic embryogenesis ability higher than that of cotyledon explants.
4. Modified OMc medium is a medium in which OM macroelements are replaced with those of BN [22], and the organic compounds (myoinositol, glycine, thiamine HCl, pyridoxine HCl, nicotinic acid, biotin, folic acid) are ten times higher than the ones of OM medium.
5. Embryo formation, although in lower percentage than in hormone-free medium, can be achieved also in modified OMc medium, supplemented with 0.5 mg/L IBA [23].
6. For *Olea europaea* L. var. *sylvestris*, transfer the explants to hormone-free OMc medium or supplemented with 0.1–1 mg/L IBA after 21 days, in order to get somatic embryos.
7. Rugini and Mencuccini [24] obtained shoot organogenesis by using both TDZ alone (at concentrations ranging between 2 and 10 mg/L) and in combination with auxin (2 mg/L TDZ plus 0.5 mg/L NAA).
8. Secondary somatic embryos (Fig. 1c) originate from the epidermis, or rarely from the first subepidermal layer, of the embryos (Fig. 1d) [25]. A limited number of cells of the primary explant are apparently involved in the formation of somatic embryos [26]. Embryogenic masses and normal embryos produce various embryo types, consisting of single embryos of normal shape, single embryos with developed cotyledons, single embryos with more than two cotyledons, and double or multiple embryos fused together [9]. Activated charcoal at 0.1 % can help the production of cyclic secondary embryogenesis, originating from normal and abnormal embryos or embryogenic masses.
9. ECO medium [8] consists of ¼ OM macroelements, ¼ MS microelements, and ½ OM vitamins supplemented with 550 mg/L L-glutamine. Embryo "germination" (i.e., the conversion of embryos to plantlets, Fig. 1e) is difficult, although the hypocotyl elongation is usually successful, while epicotyl development does not always occur, due to the abnormal embryo structure. However, 1 week at cold treatment (4 °C) often increases embryo germination.
10. Somatic embryogenesis from petioles collected from in vivo grown plants has also been achieved, although in low efficiency [14], in the medium proposed by Rugini and Caricato [11], consisting of OM medium supplemented with 0.1 mg/L BAP, 0.05 mg/L IBA, and 0.1 mg/L 2iP.

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Chapter 16

Somatic Embryogenesis in *Crocus sativus* L.

Basar Sevindik and Yesim Yalcin Mendi

Abstract

Saffron (*Crocus sativus* L.) is one of the most important species in *Crocus* genus because of its effective usage. It is not only a very expensive spice, but it has also a big ornamental plant potential. *Crocus* species are propagated by corm and seed, and male sterility is the most important problem of this species. Hence, somatic embryogenesis can be regarded as a strategic tool for the multiplication of saffron plants. In this chapter, the production of saffron corms via somatic embryogenesis is described.

Key words *Crocus sativus* L, Ornamental saffron, Somatic embryogenesis, Tissue culture

1 Introduction

The Iridaceae family contains 65 genus and 2025 species. It has a big ornamental potential because of its leaf and flower characteristics [1]. Generally, this family includes geophytes and monocotyledon [2]. *Crocus* is the most important genus of the Iridaceae family. It includes plants with rhizomes, corms, and bulbs. This genus expands in tropical and subtropical regions of the Northern Hemisphere, particularly in Southeastern Europe, North Africa, and temperate Asia (Western and Central Asia) [3–5]. Many countries, such as Iran, India, Greece, Spain, Italy, Turkey, France, Switzerland, Israel, Pakistan, Azerbaijan, China, Egypt, the United Arab Emirates, Japan, Afghanistan, and Iraq (and, recently, also Australia in the Southern Hemisphere), produce saffron [4]. *Crocus* has invariably been cultivated by means of traditional labor-intensive methods which contribute to its very high price.

Crocus belongs to subfamily *Crocoideae*, one of the most crowded subfamily of the Iridaceae family [6]. The genus includes nearly 100 species which are commonly used as popular ornamental plants [7]. All the members of the genus are geophytes and perennial plant species with an underground storage organ and renewable buds. They are generally propagated not only by

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seeds but also by specialized underground storage organs, such as bulbs, corms, tubers, or rhizomes [8]. *Crocus* is highly prized as garden plants for their colorful flowers and as horticultural varieties for industrial applications. *Crocus* genus includes native species, especially in Greece, Turkey, Iran, and India, while other countries (Italy, Hungary, and Spain) have representatives of *Crocus* species [6]. This genus presents a wide variety of chromosome numbers ($2n = 6, 8, 10, 12, 14, 16, 18, 20, 22, 23, 24, 26, 28, 30, 32, 34, 44, 48$) [9].

Crocus sativus L., commonly known as saffron, is the most common cultivated plant, used for different purposes (ornamental, medicinal, and as spice). It is unknown as a wild plant, representing a sterile triploid derived from the naturally occurring diploid *C. cartwrightianus* Herbert. Some archaeological and historical studies indicate that domestication of saffron dates back to 2000–1500 years B.C. [4]. Presently, saffron is commonly cultivated for its stigmas (used dried to produce the spice), while other parts of the plant (such as leaves or petals) are useless; moreover, a toxic effect of the bulbs on animals has been described [10]. Because of its autotriploidy, saffron is multiplicated through the formation of daughter corms from the mother corm, and its breeding is very difficult. The saffron plant produces about 160 compounds, and crocin, safanal, picrocrocin, and crocetin are the most valuable. Crocin, typically deep red in color, quickly dissolves in water to form an orange-colored solution, thereby making crocin widely used as a natural food colorant. The second most abundant component is picrocrocin that gives the taste of saffron [11]. These components provide different usage both in medicine industry and in alternative medicine. Recently, several studies showed the anticarcinogenic and antitumor activity of saffron. As ornamentals, although *Crocus* species have flowers with different shapes and colors and different plant morphology, as well, they are still not exploited in gardening as it is in their potential.

Tissue culture is an effective method for producing plants from species with propagation problems. Tissue culture provides many benefits for rapid multiplication, and somatic embryogenesis is one of the most useful technique, as it can produce high numbers of plants from embryos originating from somatic cells of various tissues and organs. Somatic embryos can be also used for the production of synthetic seeds, for genetic transformation and other biotechnological applications. This chapter deals with the induction of somatic embryogenesis from *C. sativus* L. corms. It lists the required equipment and describes a stepwise protocol useful to sterilize explants and equipments, prepare somatic embryogenesis medium, and induce somatic embryo maturation and conversion to plantlets.

2 Material

2.1 Plant Material

C. sativus L. is a perennial herb, and the plants used in this study were collected in a small area in the Northwest of Turkey (Safranbolu district, in the province of Karabük). Here, blooming of saffron starts in October and goes on up to the middle of November. Plants should be collected early in the morning in the middle of October, during the flowering period. Plant material is kept in pots until the use of explants for in vitro culture.

2.2 Laboratory Equipments

1. Vertical fume hood, laminar flow cabinet.
2. Autoclave for sterilization.
3. pH meter.
4. Tissue culture facilities: magnetic stirrers, magnetic bars, forceps, scalpels, and micropipettes.

2.3 Preparation of Culture Media

1. Salts and vitamins from MS medium ([12]; Table 1).
2. Sucrose.
3. Gelrite (Duchefa, NL).
4. 2,4-Dichlorophenoxyacetic acid (2,4-D).
5. N^6-(2-isopentenyl)adenine (2iP).
   (a) 1 N KOH solution.
   (b) 1 N HCl solution.
6. 250 and 500 mL glass flasks.
7. 500 mL beakers.
8. 1000 and 500 mL cylinders.
9. Sterile Petri dishes (9 cm in diameter).
10. Magenta boxes (77 mm × 77 mm × 97 mm).

2.4 Explant Sterilization

1. Tap water.
2. 0.1 % $HgCl_2$ solution.
3. 70 % ethyl alcohol (ETOH).
4. Distilled water.
5. 500 mL beakers.
6. 250 and 500 mL cylinders.
7. 20 % sodium hypochlorite (Domestos®, commercial bleach solution at 4.5 % NaOCl).

2.5 Acclimatization of Plantlets

1. Turf.
2. Perlite.
3. Vials (3.5 cm × 3.5 cm).
4. Fungicide (Captan 50 %).

Table 1
MS formulation [12]

Macronutrients	*mg/L*
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \times 2H_2O$	440
$MgSO_4 \times 7H_2O$	370
KH_2PO_4	170
Micronutrients	*mg/L*
H_3BO_3	6.2
$MnSO_4 \times 1H_2O$	16.9
$ZnSO_4 \times 7H_2O$	10.6
KI	0.830
$Na_2MoO_4 \times 2H_2O$	0.25
$CuSO_4 \times 5H_2O$	0.025
$CoCl_2 \times 6H_2O$	0.025
Amino acid and Vitamins	*mg/L*
Glycine	2
Nicotinic acid	0.1
Thiamine HCl	0.5
Pyridoxine HCl	0.5

3 Methods

3.1 Preparation of Somatic Embryogenesis Induction (SEI) and Somatic Embryo Maturation (SEM) Media

1. To prepare the SEI medium, prepare the stock solutions of macro- and microelements from MS medium. Alternatively, use ready-to-use MS powder preparation (4.4 g/L) by Sigma-Aldrich (M5519).
2. Prepare 2,4-D and 2iP stock solutions at 100 mg/100 mL concentration by dissolving 2,4-D in 2–3 mL ETOH and 2iP in 1 N NaOH.
3. Add 2 mL of 2,4-D stock solution to 1 L of MS medium to have a final concentration of 2 mg/L, and 1 mL 2iP stock solution to have a final concentration of 1 mg/L.
4. Add 30 g/L sucrose, add distilled water up to 1 L, optimize the pH between 5.6 and 5.8 with 1 N KOH and 1 N HCl, and add 4 g/L Gelrite (Duchefa, NL) (*see* **Note 1**).

5. For media sterilization, autoclave MS medium at 121 °C for 15 min and 1.05 atm pressure.
6. Pour the medium into the Petri dishes (diameter, 90 mm) in the laminar flow cabinet.
7. To prepare the SEM hormone-free medium, follow the same procedure reported above, with the exception of **steps 2** and **3**.

3.2 Explant Sterilization

1. Before surface sterilization, tunics are separated from the corms. Wash tunic-removed corms under tap water for 30 min to eliminate soil traces from corm surface (Fig. 1a).
2. Treat the corms with 0.1 % $HgCl_2$ for 30 min under the vertical fume hood (*see* **Note 2**), and then wash the corms with sterile distilled water for 6–7 times.
3. Treat the explants with 70 % ETOH (*see* **Note 3**) under the laminar flow cabinet for 1 min. Wash the explants for three or four times with sterile distilled water.
4. Treat the explants with 20 % NaOCl solution in the laminar flow cabinet for 20 min (*see* **Note 4**), and then wash them with sterile distilled water for six or seven times (Fig. 1b).

3.3 Induction of Somatic Embryogenesis and Somatic Embryo Maturation

1. Under the laminar flow cabinet, cut the explants into small pieces of 4 mm, on average (Fig. 1c).
2. Plate them on the SEI medium (four explants per Petri dish).
3. Culture the explants in the climate chamber, at 25 °C in darkness (Fig. 1d–f).
4. After 6 weeks, somatic embryos are observed from both direct and indirect somatic embryogenesis.
5. For maturation, transfer the somatic embryos on the SIM medium to get germination of embryos (Fig. 1g, h).

3.4 Plantlet Acclimatization

1. Autoclave the 1:1 turf/perlite mix at 121 °C, 1.05 atm of pressure, for 15 min; put the mix into vials (5.3 cm in diameter).
2. Separate the microcorms from the hormone-free MS medium and wash them under tap water.
3. Solubilize 1 g fungicide (Captan 50 %) in 1 L of distilled water.
4. Dip microcorms with emblings into the fungicide and put them into the vials.
5. Put the vials into the climate chamber at 25 °C, photoperiod 16 h and about 38 μmol m^{-2} s^{-1} of light intensity; close the vials with transparent nylon tarpaulin. Two weeks later, transfer the plantlets in 500 cc pots, inside a greenhouse.

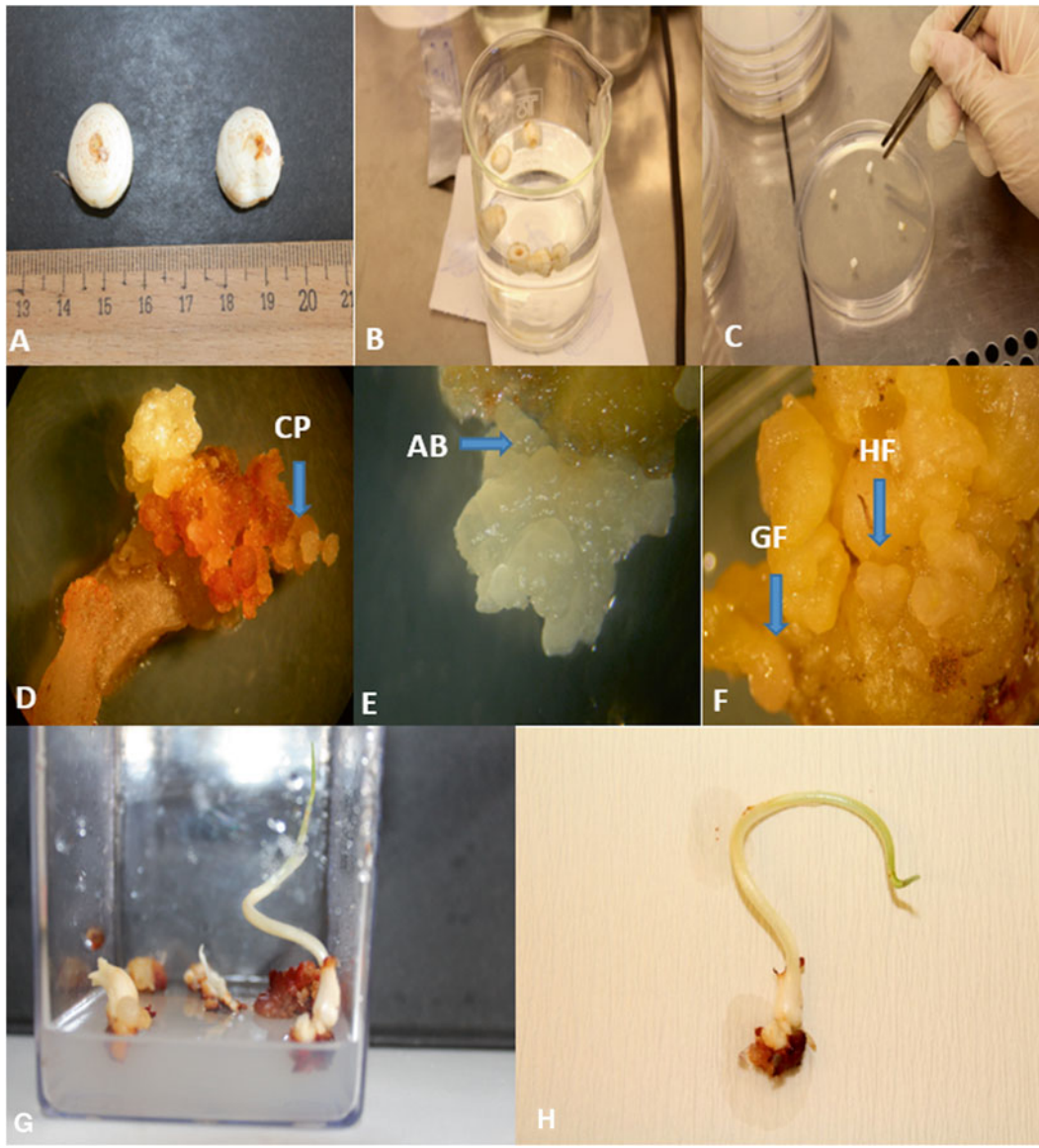

Fig. 1 Different stages of somatic embryogenesis in saffron. (**a**) Removal of tunics from the corms. (**b**) Surface sterilization of the corms. (**c**) Culture of the explants containing apical buds on MS medium. (**d**) Callus stage (CP) in indirect somatic embryogenesis. (**e**) Differentiation of callus into somatic embryos from apical buds (AB). (**f**) Different stages of somatic embryos (HF, heart stage; GF, globular stage). (**g** and **h**) Embling elongation from somatic embryos

4 Notes

1. Optimize the pH using 1 N KOH and 1 N HCl.
2. Be careful when managing $HgCl_2$, a carcinogenic compound. Work in the vertical fume hood, use the safety mask, and do not

breathe the solution. Weigh 1 g $HgCl_2$, put in 1 L of distilled water, and mix until dissolved.

3. Put 700 mL of ETOH into the cylinder measure and supplement with 300 mL of distilled water.
4. Put 200 mL of commercial bleach solution (Domestos®, containing 4.5 % NaOCl) into a cylinder measure and supplement with 2–3 drops of Tween20 (nonionic detergent, Sigma-Aldrich, USA) and add distilled water up to 1 L.

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Chapter 17

Somatic Embryogenesis in Lisianthus (*Eustoma russellianum* Griseb.)

Barbara Ruffoni and Laura Bassolino

Abstract

Somatic embryogenesis is, for the main floricultural crops, a promising system for commercial scale-up, providing cloned material to be traded as seedlings.

Somatic embryos, having the contemporary presence of root apical meristem and shoot apical meristem, can be readily acclimatized. For Lisianthus it is possible to induce embryogenic callus from leaf fragments of selected genotypes and to obtain embryos either in agarized substrate or in liquid suspension culture. The production of somatic embryos in liquid medium is high and can be modulated in order to synchronize the cycle and the size of the neoformed structures. The possibility to use the liquid substrate with high propagation rates reduces labor costs and could support the costs of eventual automation. In this paper we report a stepwise protocol for somatic embryogenesis in the species *Eustoma russellianum*.

Key words Cell suspension, Conversion, 2,4-D, Lisianthus, Somatic embryos, Somatic embryo maturation

1 Introduction

Lisianthus (*Eustoma russellianum* Griseb. or *Eustoma grandiflorum* (Raf.) Shinn.) belongs to Gentianaceae family and is a moderately cold-tolerant annual or biennial plant native to the southern part of the United States and Mexico [1]. It is commonly known as "Texas bluebell" and "prairie gentian." Lisianthus gained popularity in the international flower market due to its roselike flowers, excellent postharvest life (the cut inflorescences typically have a vase life of 3–6 weeks) [2], and its attractive range of colors. In nature, the phenology of the species provides for the initial development of a rosette that grows very slowly during winter, stems elongation in spring, and blooms in summer [3]. The domesticated varieties can be adapted for the production of flowers throughout the year in protected cultivation. In recent decades, breeders have developed a variety of cultivars with respect to many traits such as uniform flowering (it can produce up to 20–40 flow-

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ers per plant), lack of rosetting, heat tolerance, flower color, and size and form, including double flowers [4]. Currently, it is among the top ten cut flowers in the international Holland market.

Eustoma is commonly propagated by seed or cutting. A large number of seedlings can be produced by seed propagation, but the quality is not uniform due to variations in flowering time, plant height, and the number of flowers. In some cultivars with marginal variegation, or doubled petals, the seedlings show a wide range of variation because of their heterozygous character [5]. Methods for micropropagation of *Eustoma* have been developed by several authors and shoots can be easily regenerated from stem and leaf. The shoot regeneration from petals was studied by Ruffoni et al. [6] in relationship to the stage of the flower maturation. Large-scale multiplication of selected plants with superior characters is possible for all the cultivars showing for this trait poor genotype variation [7]. The effective multiplication rates depend on several factors like genotype, culture media, plant growth regulators (PGR), and type of explants [8–10]. Somatic embryogenesis was first reported in agarized substrate [11] and, afterwards, in liquid substrate [12]. Attempts to obtain artificial seeds after encapsulation in alginate were also performed [13], as well as the use of the somatic embryogenesis protocol for genetic transformation [14, 15]. Data about the productivity of several Lisianthus genotypes were presented in 2006 [16]; in the same year, somatic embryogenesis protocols were also reported by various authors [16, 17].

2 Materials

2.1 Surface Sterilization of In Vivo Grown Source Material

1. 70 % ethanol prepared with 70 mL 99.8 % ethanol and 29 mL distilled water.
2. Aqueous NaOCl solutions of active chlorine (e.g., commercial bleach ACE, Procter & Gamble, USA) plus two drops of surface active agent Tween 20®.
3. Autoclaved reverse osmosis water, 200 mL aliquots in 500-mL culture vessels.
4. Magnetic stirrer, 1000-mL flask or beaker.
5. Tissue culture facilities: Instruments (scalpel, forceps, spirit burner to flame sterilize instruments), laminar flow hood, culture room.
6. Lisianthus potted plants as explant source.

2.2 Culture Media for Micropropagation and Callus Development

1. Media based on the formulation of Murashige and Skoog (MS; [18]) for shoot propagation from apical and axillary buds of young branches and root induction (Table 1).
2. Glass culture vessels (500 mL) with transparent caps.

Table 1
Chemical composition of the substrates for somatic embryogenesis in Lisianthus in agarized or liquid medium (f.s., full strength)

	A) Callus establishment	B) Cell suspension culture	C) Somatic embryos development	D) Somatic embryos conversion
MS salts	f.s.	f.s.	f.s.	f.s.
MS vitamins	f.s.	f.s.	f.s.	f.s.
2,4-D	9.05 μM	9.05 μM	–	–
Kin	–	–	–	1.5 μM
Sucrose	30 g/L	30 g/L	30 g/L	30 g/L
Technical agar	8 g/L	–	8 g/L	8 g/L
pH	5.7	5.7	5.7	5.7
P.P.F.D.	35 μmol/m^2/s	25 μmol/m^2/s	35 μmol/m^2/s	35 μmol/m^2/s

3. Petri dishes (9-cm diameter, 2-cm height Bibby Sterilin, Stone, UK).
4. Parafilm (Parafilm® M Barrier Film, SPI Supplies, West Chester, USA).
5. Duran glass flasks or beakers, 1000-mL capacity (Schott AG, Mainz, Germany).

2.3 Cell Suspension Culture

1. 100, 250, 500 mL Erlenmeyer vessels with screw caps sterilized in autoclave at 121 °C, 1 atm (1.01325 bar) for 20 min.
2. Steel 500-μm mesh sieve (Fig. 1b), combined with a beaker having the same diameter (Fig. 1d), enveloped together with autoclavable plastic bag or paper, sterilized in autoclave as in **step 1**.
3. Sterile disposable pipette (5, 10, 25, 50 mL).
4. Fresh weight determination: Sterile filter paper dishes, vacuum pump, sterile filtration device (all glass filter holder, Millipore®).
5. Fluorescein diacetate test (FDA [19]): Non-sterile tubes, Pasteur pipettes, inverse microscope with fluorescence equipment (filter set 09 ZEISS Co., l ex = 450–490 nm, l em = 520 nm).
6. Settled cell volume (SCV) determination: 100- and 250-mL cylinders sterilized by autoclaving (*see* **step 1**).

2.4 Acclimatization of Emblings to Ex Vitro Conditions

1. Plastic alveolary pots.
2. Potting medium consisting of peat (Trysubstrate Klasmann-Deilmann) and sterilized sand (1:1, v/v).

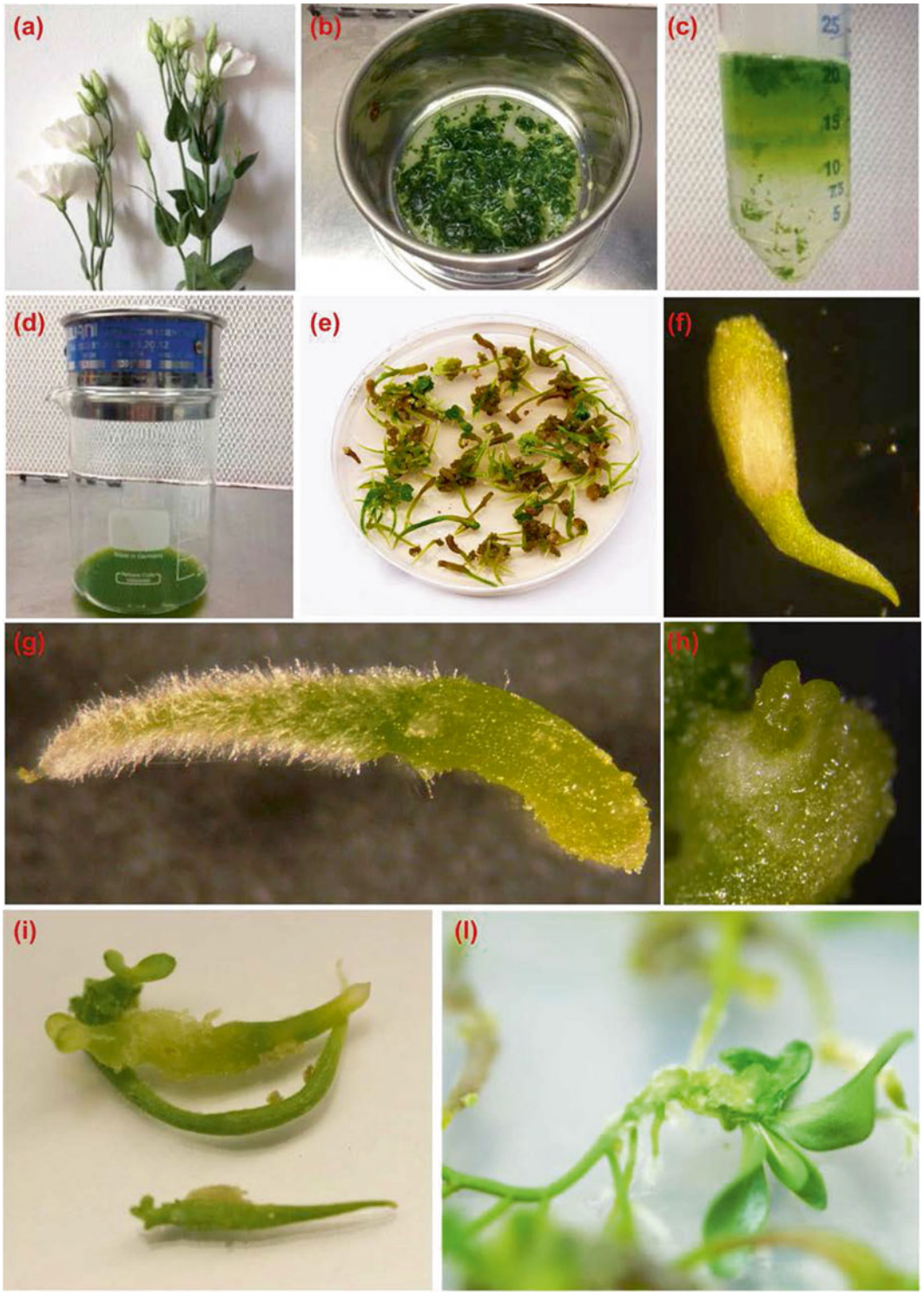

Fig. 1 (**a**) Lisianthus flower branches; (**b**) leaf fragments washed and filtered; (**c**) leaf debris and chlorophyll in the washing medium; (**d**) filtering apparatus, embryogenic cells synchronized for suspension culture; (**e**) somatic embryos developed in agarized medium lacking in 2,4-D; (**f**) somatic embryo developed in liquid hormone-free medium; (**g**) mature somatic embryo; (**h**) particular of the SAM of the somatic embryo (120×); (**i**) converted somatic embryos; (**l**) embling with true leaves ready for transfer to pot

3 Methods

3.1 Preparation and Sterilization of Culture Media

1. Prepare 10 L of a 10× stock solution by dissolving 43 g of MS (Duchefa Biochemie B.V., The Netherlands; cod M 0221.0010) powder, containing a micro- and macro-element complex, in 9 L of deionized water. While stirring the water, add the powder and stir until complete dissolution; bring the solution to a final volume (10 L) by adding water; use 1 L of this solution (4302.09 mg) for each liter of culture medium.
2. Prepare 250 mL of 1000x stock solution by dissolving MS vitamins (Duchefa Biochemie B.V., The Netherlands; cod M 0409.0250) powder, containing 25.8 g mixed vitamins, in deionized water, and stir until completely dissolved, eventually warming the solution up to 30 °C. Use 1 mL vitamin stock solution (103.1 mg) for each liter of culture medium.
3. Add any desired suitable supplement (commercial sucrose, usually 30 g/L) weighing the powder and dissolve by stirring.
4. PGR stocks: To prepare the two stock solutions at 1 mg/mL of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kin), or gibberellic acid (GA3), weigh 100 mg and add in a volumetric flask 20–30 mL ethanol (99.8 %). Gently stir until completely dissolved and bring slowly the volume to 100 mL with distilled water at room temperature. Store the stock solutions as recommended (4 °C in light for 2,4-D and Kin). Add the PGR as requested for each culture phase.
5. After adding all the components, while stirring, adjust the pH of the medium to 5.7 by using NaOH 1 N or HCl 1 M.
6. Add 8 g/L technical agar (e.g., Duchefa Biochemie B.V., The Netherlands); heat until clarity of the solution, stirring the medium on an electric plate or heating in a microwave.
7. Dispense 62.5 mL medium each into 16 culture vessels (500 cc).
8. Sterilize the medium in autoclave at 121 °C, 1 atm (1.01325 bar) for 20 min. Allow medium to cool and solidify prior to plant inoculation.
9. To pour culture medium in Petri dishes (9-cm diameter): Follow instructions from point 1 to 5, add 8 g/L technical agar, sterilize the medium in autoclave at 121 °C, 1 atm (1.01325 bar) for 20 min, move the flask (when cooled below 100 °C) under the laminar flow hood, and dispense 25 mL medium in each Petri dish to prepare 40 Petri dishes.
10. For liquid medium: Prepare the solution as previous **step** (**1**–**5**) without adding agar; sterilize the medium in autoclave at 121 °C, 1 atm (1.01325 bar) for 20 min; move the flask (when cooled below 100 °C) under the laminar flow hood. The liquid media can be stored at 5 °C in the dark up to 30 days. Warm the medium at room temperature (20 °C) before use.

11. For FDA stock solution: Dissolve 5 mg of fluorescein diacetate in 1 mL acetone, and store it at −18 °C. The stock solution can be used for several months.

3.2 Callus Establishment

1. Excise young leaves of Lisianthus-selected genotypes from mature greenhouse-grown plants (Fig. 1a), maintained at 20 °C in 14-cm diameter pots, under natural light conditions.
2. Rinse explants in a detergent warm aqueous solution with a few drops of liquid dish soap. Then, sterilize with 70 % ethanol for 30 s, treat with NaOCl solution (1.75 % active chlorine) for 15 min, and rinse twice with sterilized distilled water.
3. Chop accurately the leaf tissue, wash the material and collect the fragments over the steel filter (Fig. 1b), discard the liquid with chlorophyll and debris (Fig. 1c), and transfer the material onto the medium for in vitro callus induction (Table 1, A) for 30 days.

3.3 Embryogenic Callus Biomass Proliferation

1. Transfer the callus formed after 30 days, observe with a binocular microscope, accurately cut off the brown leaf tissue, and select the undifferentiated tissue. Then subculture the green callus every 30 days.
2. Grow callus in growth chamber at the following conditions: 23 ± 1 °C and 16 h photoperiod (light intensity 35 μmol/m^2/s photosynthetic photon flux density (PPFD)).
3. Transfer 1 g of the green friable callus, at 28–30-day intervals, in fresh medium in Petri dish (*see* **Note 1**).

3.4 Embryogenic Cell Suspension Culture

1. Transfer 1 g of friable callus in 100-mL sterile Erlenmeyer flask and insert with a sterile pipette 25 mL of liquid embryogenic medium (Table 1, B).
2. Put the flask in a gyratory shaker at 110 rpm in 16 h light photoperiod at 35 μmol/m^2/s PPFD (*see* **Note 2**).
3. Every 15 days renew the medium: Transfer the flask in the sterile bench, let settle the cells for 15 min, gently aspire with a sterile pipette the old liquid medium over the plant material and discard, and then add the same amount of new liquid medium previously stored for 1 h at room temperature.
4. Monitoring the growth by settled cell volume (SCV) (*see* **Note 3**): Pour the cell suspension in a graduated cylinder of adequate volume and allow the suspension to settle for 30 min and record the volume of fraction occupied by the cells. Take a second reading 30 min later; if the variation is higher than 5 %, take a third reading 30 min later.
5. Assessment of the cell viability, fluorescein diacetate test [19]: Dilute 1 mL of FDA stock solution with 2 mL of distilled water in a test tube (it turns white milky). Then, mix 1 mL of

the diluted FDA solution with 1 mL cell suspension and incubated for 5 min at room temperature. Put onto a slide a small amount of the solution containing FDA stained cells (one or two drops) and observe it under a fluorescence microscope by visualizing the greenish fluorescence of the cells at 100–400× magnification to calculate the percentage of viable cells (*see* **Note 4**).

6. Synchronization: After four subcultures pour out the suspension culture in the sieve (Fig. 1d), the fraction that remains over the sieve can be used for a second mother suspension culture; the fraction that pass through the grid contains cell aggregates <500 μm; gently aspire this material and re-suspend 2 mL of it in a 100-mL Erlenmeyer flask filled with 30 mL of fresh medium and put in agitation (Subheading 3.4, **step 2**) (*see* **Note 5**).
7. Evaluation of the growth curve: Prepare 30 Erlenmeyer flasks (100 mL) containing the same proportion of embryogenic synchronized cell culture (2 mL) and fresh liquid medium (30 mL); put in agitation as described and evaluate fresh weight, dry weight, and the pH of three samples separately every 2 days (*see* **Note 6**).

3.5 Somatic Embryo Development

1. In agarized medium: Transfer 500 mg of embryogenic callus from medium A (Table 1) in Petri with medium C (Table 1) in growth chamber at normal conditions (*see* Subheading 3.3, **step 2**); verify the somatic embryo development with binocular 25–30 days after transfer (Fig. 1e).
2. In liquid phase: Transfer 1 mL of embryogenic synchronized cell culture in medium C (Table 1) in Petri dishes (*see* Subheading 3.1, **step 9**); store in light, in stationary phase at 23 ± 1 °C for 4 weeks (*see* **Note 7**).

3.6 Somatic Embryo Conversion and Growth

1. In agarized medium: 28–30 days after transfer in medium C (Table 1), accurately select the neoformed structures from the remaining callus and transfer them separately onto fresh agarized medium D (Table 1) (*see* **Notes 8 and 9**). Let them grow for additional 30 days and then transfer to the greenhouse (Subheading 3.7, **step 1**).
2. In liquid medium: Filter the neoformed structures with a 500-μm mesh sieve, gently collect the structures that remain in the filter (Fig. 1f–h), and transfer them onto fresh agarized medium D (Table 1). Let them grow for additional 30 days (Fig. 1i) and then transfer to the greenhouse (Subheading 3.7, **step 1**).

3.7 Embling Acclimatization

1. Transfer the plantlets with root and several true leaves (Fig. 1l), 3.5-cm high, in alveolary pots prepared (Subheading 2.4); place for 21 days in the glasshouse with 70 % relative humidity,

maintained by mist system (10 s every 30 min); and then decrease the humidity up to 60 %

2. The mean temperature can vary between 20 and 25 °C, and for lighting, use natural light with 50 % shade provided by polyester-aluminum net.
3. After 2 months transfer the acclimatized plants to 11-cm diameter pots filled with the same substrate.

4 Notes

1. Experiments were carried out in order to determine the best 2,4-D concentration to use for embryogenic callus production. The primary callus was grown in the presence of 4.5, 9.05, or 18.10 μM 2,4-D for 30 days, in light (*see* Subheading 3.4, **step 2**) or in dark at the same temperature. The fresh weight of ten replications per each condition was evaluated in grams and compared (Fig. 2). The first two concentrations gave similar results while the higher 2,4-D concentration induced less amount of callus. Light conditions allowed a better development of green and friable callus.
2. The presence of light during the cell culture increases the embryogenic cell parameters as shown in Table 2: Both viability and SCV are low in darkness. The presence of a higher amount of small clusters is also remarkable.

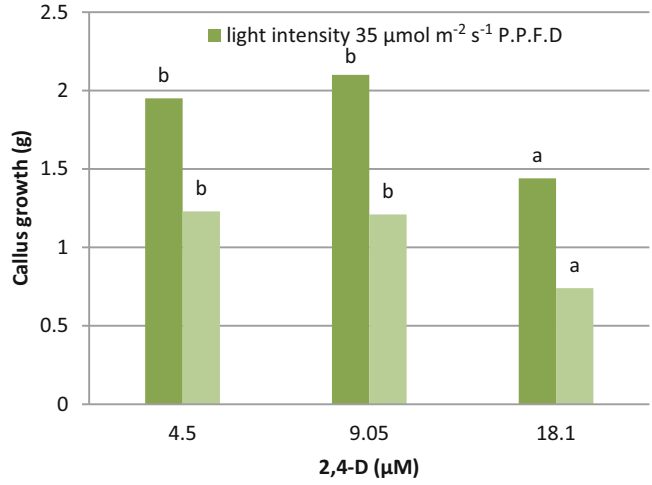

Fig. 2 Evaluation of the growth of embryogenic callus (fresh weight), depending on the presence or absence of light (16 h photoperiod at 35 μmol/m²/s PPFD). In each light condition, different letters indicate means differing at $p \leq 0.05$ by the Student-Newman-Keuls test

Table 2
General features of the embryogenic cell cultures grown in dark or in the light (16 h photoperiod, light intensity 35 μmol/m²/s PPFD). SCV, FDA test

	Dark	Light
Average viability (FDA test)	85 %	92.5 %
Shape of single cells	80 % isodiametric <30 μm 20 % elongated	Isodiametric <60 μm
Cell clusters (amount/type)	++/small	+++/small and medium
Average SCV	15.38 %	18.3 %

Table 3
Mean number of somatic embryos per milliliter of embryogenic cell culture after transfer in 10 mL of liquid medium C (Table 1), in stationary phase, in the dark, or in the light per 30 days, related to the cell size. Filtration made by a steel sieve (clone LT3; 12 replications ± standard error; mean separation by the Student-Newman-Keuls test; *, significant at $p \leq 0.05$, n.s., not significant)

	Sieve mesh size			
	>500 μm	<500 μm	<200 μm	$p(\leq 0.05)$
Light	16.7 ± 7.3b	13 ± 3.9ab	3.4 ± 1a	*
Dark	8.5 ± 1.4	4.4 ± 1.6	11.7 ± 3.9	n.s.

3. The SCV value differs from packed cell volume (PCV) for the sedimentation system; SCV uses gravity to sediment cells; PCV is determined using a centrifuge, but in the case of embryogenic cells, the centrifugation increases the risk of bacterial contamination without giving further evaluation elements on the cell growth.
4. The milky solution of FDA is active for 15–20 min; after this time the molecules crystallize and loose activity.
5. The mesh size is important to increase the cells and the cell aggregates of a similar diameter; it was established that 500 μm is a suitable grade that does not reduce the further somatic embryo development (Table 3); in the same table, it is possible to note that, at grade 200 μm, the number of somatic embryo *per* mL significantly decreases in light conditions.
6. Growth curve of embryogenic cell culture: Starting from the filtered material (*see* Subheading 3.4, **step 6**), 30 Erlenmeyer flasks were prepared with 3 mL of cells and 25 mL of medium B

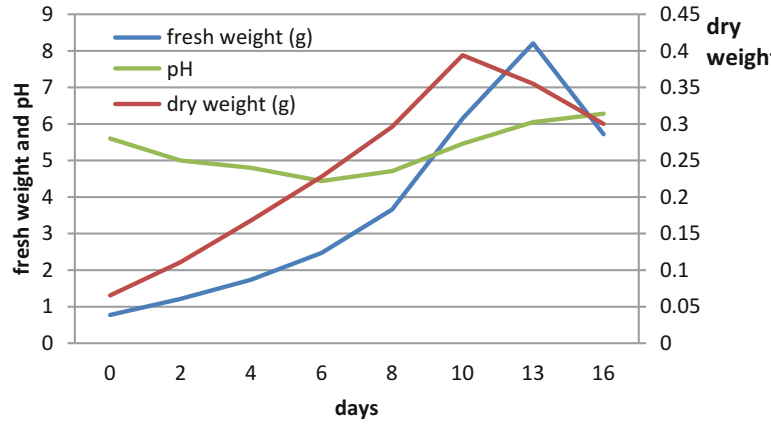

Fig. 3 Embryogenic suspension culture: growth curves of pH, fresh and dry weight during culture in batch. Starting point (T0): synchronized cells filtered at 500 μm

Table 4
Mean number of somatic embryo developed from suspension cultures grown in the light (16 h photoperiod, light intensity 35 μmol/m²/s PPFD) or in the dark and at different levels of 2,4-D after transfer in medium C (Table 1) (clone LT3; 12 replications ± standard error; mean separation by the Student-Newman–Keuls test;*, significant at $p \leq 0.05$; n.s., not significant)

	2,4-D concentration (μM)			
	4.5	9.0	18.0	*p*
Light	0.83 ± 0.30c	27.00 ± 7.00a	7.00 ± 1.60b	*
Dark	8.27 ± 2.25	10.00 ± 3.20	6.00 ± 1.70	n.s.

(Table 1); at day 2, 4, 6, 8, 11, 13, and 16, three samples were filtered, the pH was detected, and the plant material was first weighted (fresh weight) and then dried in oven at 80 °C for 36–48 h (dry weight). The growth curve (Fig. 3) shows a logarithmic increase of both fresh and dry weight from day 6 to day 13, after which the growth decreases. The pH curve shows a little variation and increasing values from day 8. A continuous growth could be obtained by adding the fresh medium at day 10 during the log phase.

7. In Table 4 data are shown about the productivity of embryogenic cell cultures related to the 2,4-D concentration, demonstrating that the concentration of 9 μM is the best to induce somatic embryos, and it also confirmed that in darkness the embryogenic process is inhibited.

Table 5
Conversion percentage of the somatic embryos developed in light conditions after 35 days of culture in several germination substrates (+ = presence; – = absence)

	Conversion (%)				
	Liquid medium		Agarized medium		
	H_2O	MS base	MS base	MS + GA_3	MS + Kin
SE converted into plant	32.0	39.0	5.6	29.5	51.5
Hyperhydration	+	–	–	–	–

8. The somatic embryo productivity of cell suspension cultures of several genotypes of Lisianthus from different origin has been described by Ruffoni and Savona [16]. Interestingly, genotypes suitable for pot plant production (from 3 to 22 somatic embryos/mL of suspension culture) showed the lowest production, while the clones suitable for cut flower production (up to 361 somatic embryos/mL of suspension) induced the highest number of somatic embryos per milliliter, thus resulting in a possible development of 180,000 somatic embryos *per* liter of culture.
9. "Conversion" is the term used for the germination of somatic embryos, the development of the cotyledonous leaves, and the elongation of the root apex, occurring better in agarized medium when Kin is added in a small amount (Table 5); the liquid medium without hormones increases somatic embryo hyperhydration, affecting the further development of the structures in the greenhouse.

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Chapter 18

Somatic Embryogenesis in Two Orchid Genera (*Cymbidium*, *Dendrobium*)

Jaime A. Teixeira da Silva and Budi Winarto

Abstract

The protocorm-like body (PLB) is the de facto somatic embryo in orchids. Here we describe detailed protocols for two orchid genera (hybrid *Cymbidium* Twilight Moon 'Day Light' and *Dendrobium* 'Jayakarta', *D.* 'Gradita 31', and *D.* 'Zahra FR 62') for generating PLBs. These protocols will most likely have to be tweaked for different cultivars as the response of orchids in vitro tends to be dependent on genotype. In addition to primary somatic embryogenesis, secondary (or repetitive) somatic embryogenesis is also described for both genera. The use of thin cell layers as a sensitive tissue assay is outlined for hybrid *Cymbidium* while the protocol outlined is suitable for bioreactor culture of *D.* 'Zahra FR 62'.

Key words Culture system, *Dendrobium*, Hybrid *Cymbidium*, In vitro propagation, Plant growth regulators, Protocorm-like body, Somatic embryo, Thin cell layer, Tissue culture

1 Introduction

Orchids are most likely the most important and interesting group of ornamentals. Their complex flowers and pollination systems, and the sheer size of the Orchidaceae, the largest family of the plant kingdom, make members of this family fascinating subjects to study. Recent reviews on the biotechnological aspects of orchids show how important this group of plants in fact is [1, 2]. In conventional seed germination, orchids form a protocorm that then develops into a plantlet [3]. In vitro, in response to various abiotic or biotic cues, orchids may also form structures, the protocorm-like body or PLB, which is the accepted de facto somatic embryo of an orchid (*see*, for example, [4]). It is a structure that resembles the true seed- and zygotic embryo-derived protocorm, but it is derived from somatic tissue, hence the "like" term in PLB. Nonetheless, the PLB forms a shoot and root, independent of any surrounding tissues, and as an independent structure, making it a

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valid case of somatic embryogenesis (SE). Thus, the term PLB will be used throughout this chapter to synonymously represent a somatic embryo. In addition, the initial development of a PLB will be referred to as primary SE and the structures as primary (1°) PLBs; then, subsequent PLBs, derived from the subculture of 1° PLBs, will be referred to as 2° PLBs, forming within a process of secondary SE, hereafter as 2° SE, so as to be consistent with previous publications that defined, and used, these terms [5, 6]. A tertiary (3°) PLB (or third-generation PLB, also derived from 2° SE) is identical to a 2° PLB (in terms of its origin), but it is strictly clonal, having an almost identical size, shape and dimensions, making it the ideal explant for controlled tissue culture experiments. Unlike 2° PLBs, which are useful starter material for establishing repeated cycles of SE, 3° PLBs could be used in commercial micropropagation.

Most likely the first orchid to have been cultured in vitro was of the genus *Cymbidium*, making this an important genus to cover within this protocol. The other genus covered by this protocol chapter, *Dendrobium*, is a commercially important orchid genus with wide-ranging medicinal properties [7]. Hybrid *Cymbidium* Twilight Moon 'Day Light' (and other cultivars) can be cultured in vitro through three main routes, one of them being through the use of thin cell layers (TCLs) [8–10]. The quantitative outcome (i.e., PLBs) from TCLs is lower than when half-PLBs—conventional explants—are used. Thus, the protocol in this chapter will use half-PLBs. For *Dendrobium*, recently published protocols for *D.* 'Jayakarta', *D.* 'Gradita 31' and *D.* 'Zahra FR 62' [11], *D.* 'Zahra FR 62' [12, 13], and *D.* 'Gradita 31' [14] serve as the basis for the protocol described herein.

2 Materials

All water used in the *Cymbidium* protocol is double-deionized, ultrapure water (18 MΩ cm at 25 °C), using a Millipore® purifier, and is prepared fresh each time. In the *Dendrobium* protocols, all water used is distilled water using a GFL Mono Water Still 2002 (Gesellschaft für Labortechnik mbH, Burgwedel, Germany) and it is prepared fresh each time. All reagents for *Cymbidium* are of tissue culture (TC) grade but the maker will differ, usually selected on the basis of the lowest price (only three choices: Sigma-Aldrich, St. Louis, USA; Wako Chemical Industries, Osaka, Japan; Nacalai Tesque, Osaka, Japan). Sigma-Aldrich products are listed below. All reagents for *Dendrobium* are TC grade from Merck, Darmstadt, Germany; Sigma-Aldrich International GmbH, St. Gallen, Switzerland; Duchefa Biochemie B.V., Haarlem, the Netherlands.

2.1 Equipment and Reagents (Cymbidium)

The following equipment and reagents are required for the *Cymbidium* protocol:

1. Petri dishes (100 mm diameter, 15 mm high) (As One, Osaka, Japan).
2. Kinetin (Kin; Sigma-Aldrich).
3. α-Naphthaleneacetic acid (NAA; Sigma-Aldrich).
4. Tryptone (Sigma-Aldrich).
5. Bacto agar (Difco Labs, Sparks, Maryland, USA).
6. Gelrite® (Duchefa-Biochemie).
7. Surgical blades (Hi stainless platinum or carbon steel; Feather Safety Razor Co., Ltd., Osaka, Japan).
8. Whatman® No. 1 filter paper (9 cm diameter; Whatman, Vienna, Austria).
9. Parafilm® (SPI Supplies/Structure Probe Inc., West Chester, PA, USA).
10. Coconut water (CW) from fresh, undamaged, green coconuts (*see* **Note 1**).
11. Cool white fluorescent tubes (CWFTs): 40 W, Panasonic or NEC, Tokyo, Japan.

2.2 Equipment and Reagents (Dendrobium)

The following equipment and reagents are required for the *Dendrobium* protocols:

1. Vertical Pressure Steam Sterilizer Model LS-B50L-I (Huanyu Pharmaceutical Equipment Co. Ltd., Zhangjiagang City, Jiangzu, China).
2. Labconco Purifier™ Clean Bench (Labconco, Kansas City, MI, USA).
3. Brand bottles (Kedaung Group Indonesia, Ungaran, Central Java, Indonesia).
4. Erlenmeyer flasks (100 mL; Pyrex, IWAKI TE-32, Asahi Glass License, PT. Anugerah Niaga Mandiri, Jakarta, Indonesia).
5. Petri dishes (9 cm in diameter; Normax, Rua Formigosa, Portugal).
6. Forceps and scalpels (stainless steel; Meiden™, Tokyo, Japan).
7. Blades (BB510, Aesculap AG & Co. KG AM, Tutlingen, Germany).
8. Mercury chloride ($HgCl_2$, Merck, Darmstadt, Germany).
9. Tween 20 (Sigma-Aldrich).
10. Thidiazuron (TDZ) (Sigma-Aldrich).
11. N^6-benzyladenine (BA) (Sigma-Aldrich).
12. NAA (Sigma-Aldrich).
13. Gelrite® (Duchefa-Biochemie).

14. CW (as for *Cymbidium*).
15. CWFTs: SL-Shinyoku, PT, Ningbo Global Lamp, Jakarta, Indonesia.

2.3 Culture Media

1. MS medium [15] (Merck).
2. Growmore (32N:10P:10K, 20N:20P:20K, 6N:30P:30K; New Century Drive, Gardena, CA, USA).
3. Rosasol medium (1.5 g/L 18N:18P:18K+1.5 g/L 25N:10P:10K+TE) (SA Engrais, Rosier, Belgium).
4. PLB induction medium (PIM): Half-strength MS medium containing 1.0 mg/L TDZ and 0.5 mg/L BA.
5. PLB proliferation medium 1 (PPM-1): Half-strength MS medium containing 0.3 mg/L TDZ and 0.1 mg/L NAA [12, 14].
6. PPM-2: Half-strength MS medium containing 0.05 mg/L BA [12].
7. PPM-3: Rosasol medium containing 150 mL/L CW [11].
8. PPM-4: Growmore medium containing 100 mL/L CW [14].
9. Somatic embryo proliferation medium (SEPM): Half-strength MS medium with 0.5 mg/L TDZ and 0.5 mg/L BA ([13]; Winarto et al. unpublished).
10. Shoot initiation medium (SIM): Half-strength MS medium containing 1.5 mg/L TDZ, 0.5 mg/L BA, and 0.02 mg/L NAA).

3 Methods

3.1 General (Cymbidium and Dendrobium)

In hybrid *Cymbidium*, PLBs can form spontaneously from the base of in vitro shoot cultures that have rooted on an organically-rich medium such as that containing banana extract. Once a single PLB has formed, it can be extracted for the induction of new PLBs, i.e., *neo*-PLB induction [5] (*see* **Note 2**). In this chapter, the term *neo-PLB* will not be used to avoid confusion, since neo-PLB can be a 2° PLB (formed from a 1° PLB) or 3° PLB (formed from a 2° PLB). In *Dendrobium*, small shoots (±0.4 cm) that are derived from greenhouse-grown plants will form the basal explants of the protocol. The reader is advised to culture donor mother plants in the greenhouse, according to the culture and environmental conditions stipulated in [12].

3.2 From Greenhouse to In Vitro: Sterilization and Preparation of Cymbidium Shoot Tips

1. Excise young shoots from 3-year-old mature plants, growing in a greenhouse, without any visible symptoms of bacterial, fungal, or viral infection.
2. Place shoots under running tap water for 30 min. Surface sterilize in 1.5 % (v/v) sodium hypochloride (NaOCl; 5 % active chlorine) for 15 min. Transfer shoots to fresh sterilizing

solution for another 15 min. Rinse shoots off three times with sterile distilled water (SDW; ×5 min each time).

3. In a sterile Petri dish, with a sharp sterilized surgical blade, isolate apical meristems (0.5–1.0 mm end of terminal tips).
4. Culture on plant growth regulator (PGR)-free half-strength MS basal salt medium to induce shoots. 1° PLBs will form spontaneously from the base about 1 % of rooted shoots in 4–6 months (*see* **Note 3**).
5. Culture these 1° PLBs in PIM, as described next.

3.3 From Greenhouse to In Vitro: Sterilization and Preparation of Dendrobium Shoot Tips

1. As described in [12] and [14], wash axillary or apical shoots (0.5–1.0 cm long) under running tap water for 30–60 min.
2. Immerse shoots in 1 % Tween-20 for 30 min and rinse with sterile distilled water (SDW) five times (×5 min each rinse). Surface sterilize shoots as follows: immerse in 0.05 % mercury chloride ($HgCl_2$) + 2–3 drops of Tween-20 for 10 min, rinse 5–6 times in SDW (×5 min each rinse). Slice off the damaged surface of rinsed explants with a tissue culture blade (BB510, Aesculap AG & Co. KG AM, Tutlingen, Germany).
3. Reduce shoot size by removing several leaves until ~0.4 cm long. Culture explants in PIM. Culture dormant lateral shoot tips (5–10 mm long) on SIM for 15–20 days. Then subculture shoot tips in half-strength, PGR-free MS medium until healthy shoots (approx. 0.5 cm long) form after 2–2.5 months of culture. After incubation, these shoots can be used as the explant source for PLB initiation (*see* **Notes 4** and **5**).

3.4 Cymbidium: 1° SE and Formation of 2° PLBs from 1° PLBs; 2° SE and Formation of 3° PLBs from 2° PLBs

There are three methods to form 2° PLBs, possible by culturing ten 1° PLBs on 40 mL/100 mL flask of PIM (*see* **Note 6**). For each method:

1. Use either Vacin and Went (VW) medium [16] or Teixeira *Cymbidium* (TC) medium [17] supplemented with Nitsch microelements [18], 2 mg/L tryptone; NAA and Kin are added, each at 0.1 mg/L.
2. Add 2 % sucrose (w/w) to PIM. Adjust pH to 5.8 ± 0.1. Add 8 g/L Bacto agar.
3. Autoclave PIM at 121 °C for 21 min.
4. Incubate all cultures at 25 ± 0.5 °C in a 16-h photoperiod provided by CWFTs with a low photosynthetic photon flux density (PPFD) of 30–40 μmol/m^2/s (*see* **Note 7**).

3.4.1 Method 1

This method involves the natural development of clusters of 2° PLBs using initial 1° PLB clusters without any cutting or processing (*see* **Note 8**).

3.4.2 Method 2

1. This method involves the use of TCLs. TCLs can be useful when the effect of some in vitro culture factors need to be examined on a very small explant such as a transverse TCL or tTCL (usually 5 mm long, 5 mm wide and 0.5–1.0 mm thick) or a longitudinal TCL or lTCL (usually 5 mm long, 1–2 mm wide and 0.5–1.0 mm thick; occasionally an epidermal strip) (*see* **Note 9**).
2. When the 1° PLB grows, 2° PLBs form on the 1° PLB, usually at the base. Select only ideal size and uniformly shaped 2° PLBs.
3. Use a new feather blade for every 6–8 PLBs. Make a 0.5–1 mm deep incision in the shape of a square, 3–5 × 3–5 mm in area. Slice this area to separate the epidermal 0.5–1.0 mm in one continuous move, thus creating an lTCL (*see* **Note 10**).
4. Using a new feather blade for every 6–8 PLBs, and only using the central 5 mm girth of the 1° PLB, make a 0.5–1 mm transverse slice throughout the whole PLB, thus creating a tTCL (*see* **Note 10**).

3.4.3 Method 3

1. Method 3 is the most recommended and can be performed in VW or TC basal medium or PIM (*see* **Note 11**). 2° PLBs form on a 45- to 60-day-old 1° PLB [19], usually at the base.
2. Separated out 1° PLBs and place them in an autoclaved glass Petri dish with a double sheet of Whatman No. 1 filter paper laid at the base (*see* **Note 12**).
3. Slice off the top 1 mm of the 1° PLB, which contains the apical meristem, with a feather blade. Also slice off the brown or yellow base in contact with medium and discard it (*see* **Notes 12** and **13**).
4. Slice the trimmed 1° PLB (i.e. without an apical meristem and base) symmetrically in half to yield two half-PLBs. Place half-PLBs cut-surface down on PIM, embedded about 1 mm into the medium (*see* **Notes 14–17**). After 45–60 days, 2° PLBs form on the outer, epidermal surface of the 1° PLB. 2° PLBs are used for 2° PLB mass production or micropropagation (*see* **Note 18**).

3.5 Dendrobium: 1° SE and Formation of 2° PLBs from 1° PLBs; 2° SE and Formation of 3° PLBs from 2° PLBs

There are two methods to form 2° and 3° PLBs.

3.5.1 Method 1

1. To form 2° PLBs, culture small shoots on PIM in the initiation stage. Culture 1° PLBs on any one of the two PPM (PPM-1 and PPM-2; *see* **Note 19**), all of which give equally successful results. 2 % sucrose (w/w) is added to all four PPM media.
2. Adjust pH to 5.8 ± 0.1. Add 2 g/L Gelrite® only to PIM-1. Autoclave for 20 min at 121 °C and at 15 kPa atm. Incubate all cultures at 24 ± 1 °C in a 12-h photoperiod provided by CWFTs with a low PPFD of ~30 μmol/m^2/s.

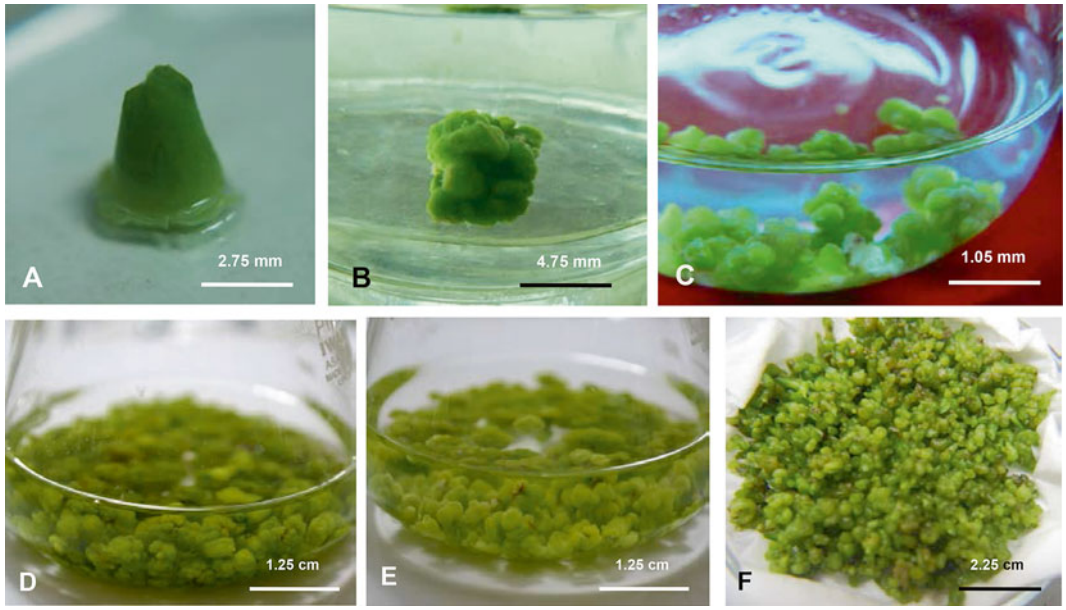

Fig. 1 Method 1: Initiation and proliferation of *Dendrobium* 'Zahra FR 62' and *D.* 'Gradita 31' PLBs (i.e., somatic embryos). (**a**) Shoot tip as the explant source. (**b**) Shoot tip with several initial PLBs in the basal part, 3.5 months after culture. (**c**) Initial proliferation of PLBs in half-strength MS medium containing 0.3 mg/L TDZ. The concentration of TDZ is reduced to 0.1 mg/L TDZ in the third subculture. (**d**) Vigorous and green PLBs form in half-strength MS medium with 0.3 mg/L TDZ and 0.1 mg/L NAA after the sixth subculture. (**e**) Light green PLBs in 1.6 g/L Growmore medium (32N:10P:10K) supplemented with 100 mL/L coconut water (CW) after the sixth subculture. (**f**) PLBs harvested from half-strength MS although they can form equally well on two alternative generic media: (1) 1.5 g/L Rosasol medium (18N:18P:18K) + 1.5 g/L Rosasol medium (25N:10P:10K + TE) containing 150 mL/L CW; (2) 1.6 g/L Growmore medium (32N:10P:10K) supplemented with 100 mL/L CW (**a**, **c**, **f** reproduced from [12] with permission from Elsevier BV; **b** reproduced from [14] with permission from TIJSAT; **d**, **e** are originals by Budi Winarto)

3. Initiate PLBs by culturing small shoots (±0.4 cm) of *D.* 'Zahra FR 62' and *D.* 'Gradita 31' on semisolid PIM. Periodically subculture shoots every 15 days for ± 2.0 months (*see* **Note 20**).
4. The method to produce 3° PLBs (Fig. 1) involves the natural development of clusters of 2° PLBs using initial 1° PLB clusters without any cutting or processing. Monthly subculture is recommended to produce vigorous PLBs (*see* **Notes 21** and **22**).

3.5.2 Method 2

1. Use PPM-2 at the initiation stage and SEPM for the proliferation stage. Add 2 % sucrose (w/w) to all media. Adjust pH to 5.8 ± 0.1. Add 2 g/L Gelrite®. Autoclave for 20 min at 121 °C and at 15 kPa atm.
2. Incubate all cultures are at 24 ± 1 °C in a 12-h photoperiod provided by CWFTs with a low PPFD of ~30 μmol/m^2/s for initiation and multiplication of somatic embryogenic callus (SEC) and ~10 μmol/m^2/s for somatic embryo proliferation.

3. Initiate SEC by culturing small shoot tips (±2.0 mm in length and 1.0–2.0 mm in diameter) on semi-solid PPM-2 medium in 9 cm Petri dishes incubated in the light for 1.5–3.0 months. This step is genotype dependent: *D.* 'Sonia Ersakul' and *D.* 'Indonesia Raya-Ina' = 1.5–2.0 months; *D.* 'Gradita 10' = 2.5–3.0 months (Winarto et al. unpublished data).
4. SEC derived from shoot tips is used as the explant for the next step in the form of somatic embryogenic callus slices (SECS; equivalent to TCLs).
5. Method 2 (Fig. 2) proper involves the use of SECS. Callus forms at the base of shoots cultured in PIM-2.
6. Culture SECS (3–10 mm in diameter; 1.0–1.5 mm thick) (*see* **Note 23**) on PPM-2 and incubate for 1 month in the light.
7. Subculture SECS three times to proliferate SEC (*see* **Note 24**).
8. In the fourth subculture, culture SECS on SEPM to regenerate 2° SEs. Cultures are placed in the light after incubation for approx. 2.0 months (*see* **Note 25**).
9. To produce a high number of 3° SEs, subculture 2–3 equally sized and healthy 2° SEs in the same medium and incubate for approx. 2 months in the light, but never exceed six subcultures (*see* **Note 26**).

4 Notes

1. Always penetrate the coconut with a sterilized borer or drill, pour out the CW into a sterilized container, and use fresh if possible. We never store CW at –20 °C for longer than 6 months.
2. 1° PLBs are not guaranteed to form on this medium. A ripe banana-based medium, rich in carbohydrates and sometimes supplemented with CW (10 %, v/v), will yield more 1° PLBs. When shoots begin to elongate (i.e., before roots elongate), cut off roots and transfer shoots to 0.5 % (w/v) Gelrite®-supplemented medium with 2 % (w/v) ripe banana and 10 % (v/v) CW. This results in more robust plantlet growth (shoots and roots).
3. This process/medium combination usually yields 100 % survival with this cultivar.

Fig. 2 (continued) callus resulting from the fourth subculture of SECS. (**k**) 2° somatic embryos regenerated from two standardized 1° somatic embryos, subcultured in half-strength MS medium supplemented with 0.5 mg/L TDZ and 0.5 mg/L BA (SEPM), 1 month after culture. (**l**) Profusion of 2° somatic embryos, regenerated from 2 to 3 standardized 1° somatic embryos subcultured on SEPM, approx. 2.0 months after culture (all photos are originals by B. Winarto, F. Rachmawat, and N.A. Wiendi)

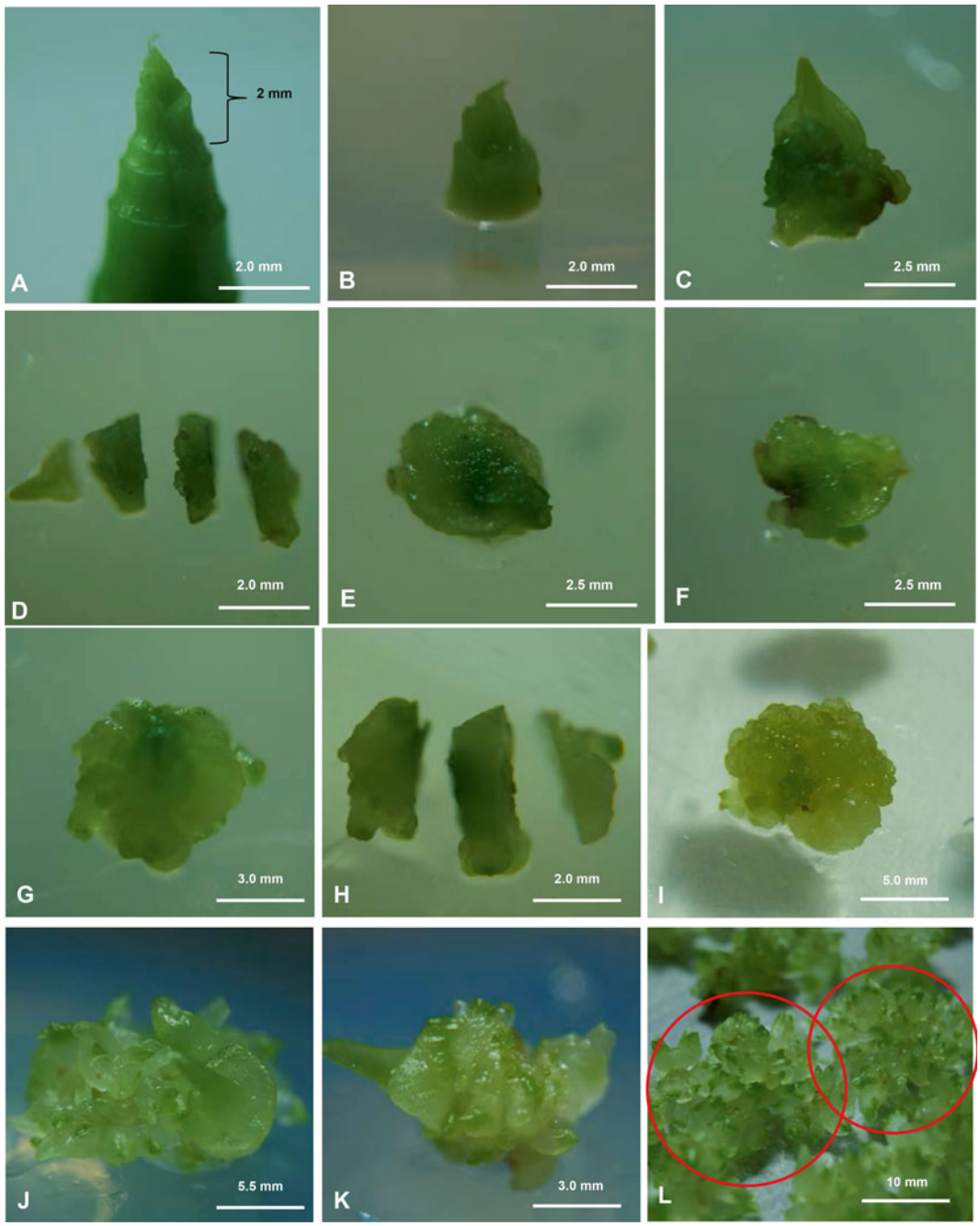

Fig. 2 Method 2: Somatic embryogenic callus slices (SECS) of several *Dendrobium* cultivars are used for initiation and multiplication of SEC and somatic embryo proliferation. (**a**) Shoot tip size and position used in initiation of somatic embryogenic callus. (**b**) Shoot tip cultured in half-strength MS medium containing 1.5 mg/L TDZ and 0.5 mg/L BA in initial culture. (**c**) SEC regenerated in the basal part of the shoot tip, 1.5 months after culture. (**d**) SECS, 1.0–1.5 mm thick. (**e**) A SECS initially cultured in medium indicated in (**b**). (**f**) Embryogenic callus growth in one SECS, 10 days after culture. (**g**) Embryogenic callus regenerated from one SECS, cultured on medium indicated in (**b**) 1 month after culture. (**h**) SECS, 1.0–1.5 mm in thickness, in the third subculture. (**i**) Embryogenic callus produced from the third subculture of one SECS, 1 month after culture. (**j**) Embryogenic

4. Initial PLB formation via callus formation or direct SE, in terms of number, quality, and speed of initiation, is genotype dependent.
5. SIM applied continually in semisolid or liquid medium for approx. 2.5 months is sometimes necessary for *Dendrobium* varieties that demonstrate a weak shoot initiation response. If using this method, PLBs form more easily in the next step.
6. Always use at least 40 replicates per treatment and repeat experiments three times for robust statistical analyses. Wherever possible, use more than one cultivar for comparison.
7. High PPFD (>80 μmol/m^2/s) or darkness can inhibit 2° PLB formation. If for the experimental treatment high PPFD or darkness are required, substitute 0.1 mg/L Kin with 1 mg/L 6-benzyladenine (BA; Sigma-Aldrich) and add 1 g/L activated charcoal (AC) [20]. With BA and AC, 2° PLBs form, but these are white and not numerous; however, once transferred to light, they regain their photosynthetic capacity. AC may mirror a darkened natural environment of the *Cymbidium* in tree tops or may absorb negative compounds, such as polyphenols, released into the medium as a consequence of wounding [21].
8. This is not a good method for testing the effect on growth of medium factors, since it is difficult to count the number of 2° PLBs that form per 1° PLB. In addition, these initial 1° PLB clusters have PLBs of different sizes, developmental stages and/or number. This method is good when one wishes to simply allow PLBs to proliferate *neo*-PLBs, without any experimental hypothesis in mind or whenever one wishes to allow shoots to form [22].
9. This method is useful for assessing the effects of several in vitro factors, such as ethylene inhibitors and aeration [23], smoke-saturated water [24], fungal elicitors [25], jasmonates and salicylic acid [26, 27], magnetic fields [28], gelling agent and medium additives [29, 30], and culture vessel [31] or use in studies related to genetic engineering and transformation [32, 33], cryopreservation and synthetic seed technology [34–37]. In this method, survival tends to be lower, and mortality is higher under extreme treatments, perhaps due to smaller size, tissue wounding, and dependence on the medium. Hence, for propagation purposes, the TCL method is not recommended.
10. Prepare the lTCL in a single stroke. If prepared in several strokes then the explant becomes excessively damaged and regeneration is low. The inner tissue (sub-epidermal layers and below) of a PLB never forms 2° PLBs [38]; thus tTCLs and lTCLs only contain epidermal tissue with 2–3 layers. tTCLs and lTCLs dry and oxidize rapidly (within the space of a few minutes) due to their size; thus any further damage to

this tissue caused during explant preparation results in rapid browning (within a few days) and, eventually, necrosis (within 1–2 weeks) of the TCL. The feather blade should thus be changed regularly and the cut lTCLs/tTCLs should be constantly submerged in sterile, double-distilled water (SDDW). Researchers that are new to TCL technology are advised to spend time practicing repeatedly the preparation of such small explants before applying them to an experimental protocol [39]. By not experimenting enough may result in very large errors in data.

11. The basal medium (abiotic factors) [40] is not as important as the explant (biotic factors) [41]. Nonetheless, the choice of medium salts and basal medium is important [42, 43]. The use of a half-PLB is the essentially important aspect of the method which allows for most stable propagation of 2° PLBs.
12. The level of macro- and micronutrients, as well as the ammonium/nitrate ratio, can have a profound impact on 2° PLB production [44].
13. Use one new autoclaved Petri dish for each 10–20 1° PLBs that need to be prepared. For a total of 1000 1° PLBs, 1000 mL of SDDW is sufficient. Pour 10–20 mL of SDDW into each Petri dish, so that the filter paper is always soaked with a thin layer of SDDW and the cut surface of PLBs are always submerged to avoid oxidation. The use of antioxidants in PIM can also help [20]. Never allow the PLBs to dry out (always almost completely cover the Petri dish so that the airflow from the clean bench does not desiccate the PLBs). Never completely submerge the PLBs in sterile SDW as an apparent hyperhydric response occurs to PLBs, which are extremely sensitive to stress caused by injury, water, light, carbon source [45], or temperature. Discard any 1° PLBs that have been left standing for more than 30 min, as an apparent hyperhydric response occurs in SDDW.
14. In the passage from the 1°–2° PLB formation, the basal part of the PLB is callus-like or opaque in appearance due to direct contact with PIM. 1° PLBs should never be used for 3° PLB production, but only 2° PLBs that form on the outer layer of 1° PLBs. Indeed, 2° PLBs are almost perfectly round, have a more consistent shape and size, and do not have a cytologically or morphologically distorted base.
15. Explants (1° half-PLBs) should never be placed with the intact surface down on the medium, but simply placed on top of the medium or slightly (0.5–1.0 mm) embedded in medium; they should never be totally embedded into the medium, as well, as in this case, PLBs will rarely form. This aspect needs to be conducted uniformly across experiments to avoid the distortion of data.

16. Usually the "mother" PLB (i.e., the 1° PLB) will gradually die away and turn brown (i.e., oxidize). This will take about 60–90 days to occur, depending on the cultivar. At that time, ideal sized 2° PLBs will have formed. Following one more subcultures, 2° PLBs form 3° PLBs, which can be used for experimental purposes, or for micropropagation. In principle, 2° PLBs of different sizes should never be used for experiments, since initial PLB size strongly affects the outcome of tissue culture experiments (Teixeira da Silva, unpublished data).
17. The sharpness of the blade is one of the most important factors that determines the success of all three methods, especially for the preparation of TCLs. Use sharp, feather, and robust blades that can be autoclaved, sterilized, boiled, sterilized in 98 % ethanol and still remain sharp for explant preparation.
18. The quantitative outcome of all three methods differs. Quantification is not easy to perform with method 1, and this method should never be used in experiments because the size, shape and developmental stages of PLBs differ so widely in PLB clusters. Very unfortunately, what is commonly observed in the literature for several orchid genera is precisely the erroneous use of method 1 rather than methods 2 or 3. As described in [6, 8], method 2 results in an average of about 14.5 2° PLBs per 1° PLB lTCL and of 6 2° PLBs per 1° PLB tTCL. The reason is the lower total surface area of tTCL than lTCL, explained by the plant growth correction factor (PGCF) [19, 46, 47]. The PGCF takes into account the size of the explant, its shape, and thus its area and thus allows hypothetical output to be calculated based on actual data for explants of a known size or area. In *Cymbidium*, two lTCLs can be prepared from an ideal-sized 1° PLB, while five tTCLs can be prepared from the same mother 1° PLB. Hypothetically, each subculture can yield a 24,280× multiplication rate after three consecutive subcultures (3 months each) for lTCLs. In other words, with two initial 1° PLB lTCLs, a total of about 351,700 3° PLBs can be obtained after a 9-month period, assuming that every single 1° and 2° PLB is used, that every single 1° and 2° PLB survives and that every single 1° and 2° PLB is able to differentiate. For tTCLs, these values are lower. Hypothetically, each subculture would yield a 4620× multiplication rate after three consecutive sub-cultures (3 months each). In other words, from five initial 1° PLB tTCLs, a total of about 28,100 3° PLBs can be obtained after a 9-month period, assuming that every single 1° and 2° PLB is used, that every single 1° and 2° PLB survives and that every single 1° and 2° PLB is able to differentiate. Method 3 results in an average of 8.21 2° PLBs per 1° half-PLB. Hypothetically, each subculture can yield a 4000× multiplication rate after four

consecutive sub-cultures (3 months each). In other words, with an initial two 1° half-PLBs, a total of about 36,350 3° PLBs can be obtained after a 12-month period, assuming that every single 1° and 2° PLB is used, that every single 1° and 2° PLB survives and that every single 1° and 2° PLB is able to differentiate.

19. Subculturing PLB clusters in PPM-1 and in PPM-2 allows for the multiplication of PLBs without PLB browning [11, 12]. However, the use of PPM-3 and PPM-4 stimulates browning in 7 % and 20 % of PLBs, respectively [11, 14].
20. A 15-day periodic subculture in the initiation stage allows shoots to remain green and vigorous. In this state, when used as the explant source, they easily produce SEC at the base of shoots. After 1 month of incubation, new PLBs formed, on average, 2.4 PLBs from one PLB in *D.* 'Gradita 31', and 2.2 in *D.* 'Zahra FR 62' [12, 14].
21. PLB multiplication by monthly subcultures is possible for a maximum of 8–9 subcultures, after which proliferation capacity decreases.
22. The productivity (i.e., number of PLBs formed) of method 1 using PPM-1 and PPM-2 is higher than when PPM-3 and PPM-4 are used. If one shoot tip (initially 0.4 mm in size) regenerates five new PLBs in the initiation stage and each 2° PLB produces five new PLBs (3° PLBs) in each subculture in the multiplication stage, then 1,953,125 PLBs of *D.* 'Jayakarta' are easily produced after nine subcultures, as well as 5504 PLBs for *D.* 'Gradita 31' and 2744 PLBs for *D.* 'Zahra FR 62'.
23. The diameter of SECS in each subculture period is 3–10 mm. In initial culture, shoot tips are about 2 mm in diameter, then they grow up to 3 mm at the end of initiation and become 5 mm in the first subculture, 7 mm in the second subculture and 10 mm in the third subculture.
24. Application of SECS can successfully produce large amounts of SEC on PPM-2 by subculturing the SECS monthly up to three times. SECS were used for *D.* 'Sonia-Ersakul', *D.* 'Indonesia Raya', and *D.* 'Gradita 10' with the highest somatic embryogenic response (i.e., formation of SEC) and subsequent somatic embryo formation exhibited by *D.* 'Indonesia Raya', followed by *D.* 'Sonia-Ersakul' and *D.* 'Gradita 10' ([13]; Winarto et al. unpublished).
25. Culturing SECS on SEPM results easily in a high number of 1° somatic embryos (15–30/slice), derived from the fourth subculture of the SECS after incubation for approx. 2.0 months ([13]; Winarto et al. unpublished).
26. Production of 2° somatic embryos (5–15/1° somatic embryo) can be continued by culturing 2–3 uniform 1° somatic embryos

in the same medium and incubation conditions up to six subcultures. Using this method, approximately one million *D.* 'Indonesia Raya-Ina' and *D.* 'Sonia-Ersakul' somatic embryos can be produced by the sixth subculture, with 10 2° somatic embryos derived from one somatic embryo produced in each subculture. In *D.* 'Gradita 10', 30–50 % fewer somatic embryos are produced in the same subculture in comparison to *D.* 'Indonesia Raya-Ina' and *D.* 'Sonia-Ersakul' ([13]; Winarto et al. unpublished). Subculturing early is essential to avoid browning, which can begin to form as early as the first month after callus induction (evidenced in closely related Pigeon orchid, *Dendrobium crumenatum* Swartz; [48]).

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Chapter 19

Somatic Embryogenesis of *Lilium* from Microbulb Transverse Thin Cell Layers

Pablo Marinangeli

Abstract

A reliable somatic embryogenesis protocol is a prerequisite for application of other plant biotechniques. Several protocols were reported for genus *Lilium*, with variable success. Between them, transverse Thin Cell Layers (tTCL) were used efficiently to induce indirect somatic embryogenesis of *Lilium*. Somatic embryogenesis potential is dependent on the genotype, explant, and culture medium composition, especially as for plant growth regulators and environmental conditions. Usually, the process comprises three phases: embryogenic callus induction, embryogenic callus proliferation and somatic embryo germination. Somatic embryo germination can be achieved in light or dark. In the first case, complete plantlets are formed, with green leaves and pseudobulb in the base. In darkness, microbulbs are formed from single somatic embryos or clusters. A last phase of microbulb enlargement allows plantlets or microbulbs to increase their biomass. These enlarged microbulbs do not need special acclimatization conditions when transferred to soil and quickly produce sturdy plants. This chapter describes a protocol for somatic embryogenesis of *Lilium* using tTCL from microbulbs.

Key words *Lilium*, Plant tissue culture, Somatic embryos, Transverse thin cell layers, tTCL

1 Introduction

Lilium is a very popular cut flower and pot plant and one of the most important ornamental bulb worldwide. According to the Flower Bulb Inspection Service of the Netherlands (Bloembollenkeuringsdienst; BKD), 2.21 billion lilybulbs were produced on 3681 ha in 2010, whereas in 2014 the planted area was 3898 ha. Other relevant lily bulb producer countries are France (401 ha), Chile (205 ha), the USA (200 ha), Japan (189 ha), New Zealand (110 ha), China (100 ha), and Israel (100 ha) [1]. Besides its ornamental attributes, the increasing popularity of lilies is due to the constant onset of novelties in terms of cultivars with superior and distinctive features. Actually, during the last 50 years, thousands of cultivars were developed and can be classified in different hybrids

Maria Antonietta Germanà and Maurizio Lambardi (eds.), *In Vitro Embryogenesis in Higher Plants*, Methods in Molecular Biology, vol. 1359, DOI 10.1007/978-1-4939-3061-6_19,

groups. This dramatic change in the assortment of lilies was possible due to the innovative new hybrid breeding strategies that include biotechnological tools, such as in vitro pollination and embryo rescue, polyploidization, molecular cytogenetics and molecular assisted breeding. Other biotechniques are not included as breeding tools, yet. This is the case of genetic transformation, but in the near future it is hypotesizable its key role in the incorporation of new traits to the *Lilium* genome [2].

Besides biotechniques directly related to breeding, there are others assisting breeders and growers to offer an assortment of quality and quantity. These are the techniques for virus eradication, propagation and conservation including, among others, somatic embryogenesis. Indeed, somatic embryos are structures that allow cloning elite material effectively in automated systems, synthetic seed production and cryopreservation, as well as the use of them or the embryogenic tissue as target for genetic transformation [3]. So, a reliable somatic embryogenesis (SE) protocol of *Lilium* is a prerequisite for application of other plant biotechniques. Several protocols were reported for genus *Lilium* (Table 1), with variable success. In all cases, somatic embryos were produced via indirect SE. Usually, the process comprises three phases: embryogenic callus induction, embryogenic callus proliferation, and somatic embryo germination [4]. SE potential is dependent on the genotype, explant, and culture medium composition, especially as for plant growth regulators (PGR), and environmental conditions [4, 5]. Most of the SE protocols for *Lilium* makes use of the MS medium [6], supplemented with sucrose as carbon source and agar for gelification (Table 2). PGRs commonly used for inducing embryogenic calli and for proliferation are auxins, as α-naphthalene acetic acid (NAA) [4, 5, 7], 2,4-dichlorophenoxyacetic acid [7], picloram [8, 9] and dicamba [8, 9], and cytokinins like thidiazuron (TDZ) [4], kinetin (Kin) [5] and N^6-benzyladenine [7, 10, 11]. Somatic embryo germination can be achieved in light or darkness. In the first case, complete plantlets are formed, with green leaves and pseudobulbs at the base. In darkness, microbulbs are formed from single somatic embryos or clusters, cultured in hormone-free MS medium [4]. A last phase of microbulb enlargement in PGR-free MS medium, containing 90 g/L sucrose, allows plantlets or microbulbs to increase their biomass. These enlarged microbulbs do not need special acclimatization conditions when transferred to soil and quickly produce sturdy plants [12].

Thin cell layers (TCL) technology consists on the in vitro culture of thin slices of tissue from different organs, and allows to induce flowers, vegetative buds, roots, and somatic embryos in a very controlled pattern of organogenesis [13]. About 1 mm thick transverse slices of tissue are termed transverse TCL (tTCL) and are efficiently used to induce somatic embryogenesis in *Lilium* [4, 5, 10, 11]. This chapter describes a protocol for somatic embryogenesis of *Lilium* using tTCL from microbulbs.

Table 1
State of the art in somatic embryogenesis of *Lilium*

Explant	*Lilium* species and hybrids	Reference
Microbulbs,bulblets, and bulb scales	*L. regale* *L. longiflorum* *L. formosanum* *L. martagon* *L. michiganense* *L. ledebourii* *L. davidii* *L. longiflorum* Longiflorum x Oriental hybrid Oriental hybrid Asiatic hybrid	[7] [14] [15] [16] [17] [10, 11] [5] [5] [5]
Leaves	*L. regale*	[7]
Seedling roots	*L. martagon* *L. x formolongi* *L. ledebourii*	[16] [18] [10, 11]
Hypocotyls	*L. martagon*	[16]
Floral pedicels	*L. longiflorum* Oriental hybrid Oriental hybrid	[8] [9]
Styles	*L. longiflorum* Oriental hybrid	[8]
Anthers	*L. longiflorum*	[19]

Table 2
Composition of media used in the different steps of *Lilium* somatic embryogenesis by tTCL

Medium	Salts and vitamins	NAA (mg/L)	TDZ (mg/L)	Kin (mg/L)	Sucrose (%)	Agar
Bulbification [20]	MS	0.03	–	–	3	0.8
Enlargement [12]	MS	0.1	–	0.1	9	0.8
Embryogenic callus induction [4]	MS	1	0.2	–	3	0.8
Embryogenic callus proliferation [4]	MS	1	0.1	–	3	0.8
Regeneration [14]	½ MS	–	–	–	3	0.8

MS Murashige and Skoog medium [6], *NAA* α-naphthaleneacetic acid, *TDZ* thidiazuron, *Kin* kinetin

2 Materials

2.1 Plant Source Material

1. Different *Lilium* hybrids can be used for SE through this protocol. Originally it was developed for *Lilium longiflorum* [4], but SE was achieved for other genotypes through slightly different protocols [5, 10, 11]. In our laboratory, this methodology has been successfully applied to *Lilium longiflorum*, cv White Heaven, Asiatic Hybrid 'Nello', Longiflorum x Asiatic Hybrid 'Royal Respect', and Longiflorum x Oriental Hybrid 'Triumphator' (*see* **Note 1**).
2. Lilybulbs can be taken from the soil after the aerial shoots die out during autumn and winter and used directly, or they can be harvested in autumn, stored for 1 year at −1.5 °C (after a cold treatment during 45–60 days) and used year round. Alternatively, bulbs can be purchased to retailers or cut flower growers and used directly (*see* **Note 2**).

2.2 Laboratory Materials and Equipment

1. Laminar flow cabinet.
2. Bunsen burner.
3. Dissecting forceps and scalpel.
4. Sterile Petri dishes 15 × 100 mm.
5. Beakers.
6. Sterile tissue paper.
7. 100–1000 mL bottles.
8. Capped test tubes (25 × 150 or 10 × 15 mm).
9. Aluminum foil.
10. Parafilm.
11. Autoclave.
12. Stereomicroscope (if necessary, depending on the skills of the operator).
13. pH meter.
14. Analytical balance.
15. Stirrer with hot plate.
16. Growth chamber with temperature control (25 ± 2 °C) and light control (dark and 16-h photoperiod, at a light intensity of 40 μmol/m^2/s provided by cool-white fluorescent tubes).
17. Greenhouse with climatic control.

2.3 Reagents, Solutions, and Culture Media

1. Sterile distilled water.
2. 70 % ethanol.
3. Sodium hypochlorite or commercial bleach (e.g., Clorox®) with 6–8 % of active chlorine.

4. Tween-20.
5. Culture media: Specific media used for all the steps, from microbulb differentiation from scale sections to shoot regeneration and bulb enlargement, are described in Table 2.

3 Methods

3.1 Preparation and Sterilization of Culture Media

Prepare media from the formulations in Table 2 (*see* **Note 1**).

1. In an appropriate sized beaker, add distilled or deionized water up to ½ the final medium volume (i.e., 500 mL for 1000 mL medium).
2. Add mineral salts from stocks, vitamins, sucrose, and growth regulators, stirring after each addition until the compound is dissolved.
3. Bring to final volume with distilled or deionized water, mix well, and adjust pH to 5.8 with 0.1 N NaOH or HCl.
4. Add agar, heat until gelling agent is fully dissolved, and dispense into autoclavable containers. Dispense 15 mL medium in each 25 × 150 mm tube or 5 mL medium in each 10 × 15 mm tube. Cap tubes and place in autoclavable racks or in high-density autoclavable polyethylene bags (*see* **Note 3**).
5. Autoclave at 121 °C for 20 min (118 kPa steam pressure).
6. Store the medium in a clean area and use within 2 weeks.

3.2 Surface Sterilization of Bulb Scales and Culture

1. Detach external and middle scales of healthy bulbs. Discard external scales showing evident signs of contamination or damage.
2. Wash carefully the scales with tap water and disinfect them by immersion in 70 % ethanol during 1 min, followed by 20 min in an aqueous solution of sodium hypochlorite (1.6 % active chlorine) plus 0.1 % Tween 20.
3. Under a laminar flow hood, rinses explants tree times with sterile water for 2 min each, and leave them in final rinse water.
4. Cut scales transversally in 2–3 mm sections on a sterile tissue paper or Petri dish. Place sections slightly embedded in the jellified bulbification medium, maintaining the polarity.
5. Cultivate explants in growth chamber at 25 °C in the dark during 30–45 days until bulblets, 3–5 mm in diameter, differentiate from the base of sections.
6. Microbulbs can be used to prepare tTCL, from which to induce SE, or they can be micropropagated in order to provide microbulbs continuously (*see* **Note 4**).

3.3 Enlargement of Microbulbs

1. In order to provide cyclic micropropagation, microbulbs developed from scale cuttings are separated and cultivated in enlargement medium for 45–60 days at 25 °C in the dark.
2. Enlarged microbulbs, about 5–8 mm in diameter, can be used to obtain tTCL for SE, or used as source of microscales for cyclic micropropagation.

3.4 Bulbification

1. Detach microscales from enlarged microbulbs and cultivate them slightly embedded in bulbification medium.
2. Cultivate in growth chamber at 25 °C in darkness during 30–45 days until bulblets, 3–5 mm in diameter, differentiate from the base of microscales.
3. Microbulbs can be used to obtain tTCL to induce SE or to continue cyclic micropropagation.

3.5 Embryogenic Callus Induction and Proliferation

1. Remove microbulbs from tubes or Petri dishes. Cut roots and etiolated leaves, while microscales should remain.
2. Excise 0.8–1.0 mm thick tTCL from the base of microbulbs and place them with the inverted polarity on embryogenic callus induction medium.
3. Cultivate in growth chamber at 25 °C in the dark with 30-day subcultures.
4. Remove embryogenic callus from the explants and cultivate them in embryogenic callus proliferation medium, in growth chamber at 25 °C in the dark with 30-day subcultures (*see* **Note 5**).

3.6 Germination of Somatic Embryos and Plant Regeneration

1. Transfer proliferating embryogenic callus to regeneration medium.
2. Cultivate in growth chamber at 25 °C with a 16-h photoperiod at a light intensity of 40 μmol/m^2/s.
3. Transfer to fresh medium every 30 days until microshoot development.

3.7 Bulbification and Soil Transfer

1. Transfer microshoots to enlargement medium and cultivate them in a growth chamber at 25 °C in darkness.
2. Cultivate during 60 days with one subculture.
3. Remove microbulbs from culture containers, wash under tap water to remove medium and plant directly in soil or substrate (*see* **Note 6**).

4 Notes

1. SE depends on the genetic background of the donor plant. The response of different species and cultivars of *Lilium* to embryogenic callus induction and proliferation is variable and it is pos-

sible that would be necessary an adjustment of the culture conditions. The main factors affecting SE are the type and concentration of PGR. So, it is recommended an adjustment of the concentrations of NAA (0.1–1 mg/L) and TDZ (0.1–0.4 mg/L) when working with novel material [4, 5, 10, 11].

2. When lilybulbs are damaged or dehydrated, high frequency of contamination appears during in vitro culture because disinfection is not efficient, reaching even 100 % of loss. In this situation, it is possible to carry out an ex vitro propagation of bulbs through scaling technique [13], during which a strong disinfection of scales is done with disinfectants, fungicides, and acaricides. Furthermore, the production of new healthy organs allows obtaining a material suitable for establishment in vitro, the explants being the microscales from bulblets differentiated at the base of the scales.
3. During the introduction in vitro it is absolutely necessary to use single culture tubes because the contamination is usually high. During the step of embryogenic callus proliferation and the embryos germination, it is possible to use Petri dishes to cultivate explants, due to the possibility to save space and culture medium. This requires preparing and sterilizing the culture medium in flasks with plastic cap, and then pouring it into sterile disposable Petri dishes while still melted.
4. Microbulbs, differentiated from scale sections of the original bulb, can be used for SE, but if year-round work is necessary, a continuous source of microbulbs is necessary. So, it is recommended following the cyclic micropropagation of *Lilium* in the dark, as mentioned in Subheadings 3.2, 3.3, and 3.4.
5. Embryogenic callus proliferation can be done both in solid and in liquid medium by cultivating embryogenic calli in either agar-solidified or liquid MS media, containing 1.0 mg/L NAA and 0.2 mg/L TDZ. However, the number of somatic embryos derived from embryogenic calli cultured in liquid medium often shows to be more than in solidified medium [14].
6. In some cultivars, microbulbs develop dormancy during enlargement. In this case, break of dormancy is possible by storing microbulbs at 4–7 °C from 45 days (*L. longiflorum*, Longiflorum x Asiatic, and Asiatic hybrids) to 60 days ('Oriental' and 'Oriental x Trumpet' hybrids). Dormancy release can be done in the same culture container or in humid peat moss within plastic bags, or in plastic containers covered with film.

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Chapter 20

Somatic Embryogenesis and Plant Regeneration of *Brachiaria brizantha*

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Abstract

The genus *Brachiaria* (Trin.) Griseb. belongs to the family Poaceae, order Poales, class Monocotyledonae. In *Brachiaria brizantha* (Hochst. ex A. Rich.) Stapf., embryogenic callus can be induced from seeds from apomictic plants, which results in high frequency somatic embryo development and plant regeneration. We report here a detailed protocol for callus induction from apomictic seed; followed by in vitro morphogenesis (somatic embryo and bud differentiation), plant regeneration, and acclimatization in the greenhouse. Important details regarding the positioning of seeds for callus induction and precautions to avoid endophytic contamination and the occurrence of albino plants are presented.

Key words Albino plants, Apomixis, Caryopses, Endophyte contamination, Forage grass, In vitro culture, Monocot

1 Introduction

The *Brachiaria* genus belongs to the family Poaceae, order Poales of the class Monocotyledonae. This genus shows a hundred species of grasses [1], and is cultivated as forage in tropical and subtropical countries. *Brachiaria brizantha* cv. Marandu is a key forage in beef cattle production in Brazil, the world's largest beef exporter. *B. brizantha* shows aposporic apomixis, an asexual mode of reproduction by seeds. The apomictic plants are thus clones of the mother plant, reducing the possibility of their use in classical breeding [2]. Furthermore, similarly to many apomicts [3], apomixis is related to polyploidy, with sexual plants being diploid and apomictic plants tetraploids. These characteristics hinder breeding of apomictic plants [4].

Alternatives to conventional breeding of *B. brizantha* would involve the introduction of genes of interest by genetic transformation, which relies on the availability of in vitro plant regeneration protocols. In monocots, in vitro plant regeneration has been

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primarily achieved through somatic embryogenesis [5, 6]. Evidences of different morphogenic responses from the same explant, under induction by different concentrations and ratios of auxin:cytokinin, have been reported in species of the Poaceae family such as sorghum (*Sorghum bicolor* (L.) Moench.), minor millet (*Paspalum scrobiculatum* L.), sugarcane (*Saccharum officinarum* L.), and baby bamboo (*Pogonatherum paniceum* Lam. Hack.) [7]. The occurrence of monopolar and bipolar embryos in sugarcane cultures, which has been earlier described as two pathways [8], was later defined as organogenesis and somatic embryogenesis [9]. In *B. brizantha* cv. Marandu, high in vitro morphogenetic efficiency was observed from seeds, with 73 % of isolated apomictic embryos forming embryogenic cultures, and 67 % of the calli regenerating plants [10]. The anatomy of somatic embryos, induced from in vitro cultivated seeds, confirmed this morphogenetic route [11]. Multiple shoot formation was also reported in basal segments obtained from micropropagated plantlets of cv. Marandu [12], a system that was used for *Brachiaria* plant recovery after colchicine treatment for in vitro chromosome duplication. Somatic embryogenesis and organogenesis in *B. brizantha* is influenced by several factors such as genotype, explant type, and culture conditions [13].

In this chapter, an efficient protocol of somatic embryogenesis induction from apomictic mature seeds is reported.

2 Materials

2.1 Plant Material and Equipment

1. Mature seeds of *Brachiaria brizantha* cv. Marandu.
2. 70 % ethanol solution (v/v) in water.
3. 2.5 % sodium hypochlorite (NaOCl) solution (v/v) in water.
4. Tween 20™.
5. Distilled water, sterilized by autoclaving at 121 °C for 20 min.
6. Conic plastic tubes, sterile, 50 mL.
7. Laminar flow hood.
8. Filter paper placed in Petri dishes, sterilized by autoclaving at 121 °C for 20 min.
9. Petri dishes, 100 × 20 mm.
10. Scalpel blades, scalpel handles, and tissue forceps.
11. Plastic film, Parafilm® M type.
12. Stock solution of 2,4-dichlorophenoxyacetic acid (2,4-D); benzylaminopurine (BAP); kinetin (KIN); naphthaleneacetic acid (NAA), and gibberellic acid (GA_3), each one at 1 mg/mL.
13. Vessels for plant tissue culture (e.g., Magenta™ or babyfood jars).

14. Incubator or growth chamber with controlled temperature and photoperiod.
15. Mixture of sand:soil:vermiculite (1:1:1, v/v), sterilized by autoclaving at 121 °C for 40 min.
16. Soil fertilized with superphosphate and organic matter.

2.2 Culture Media for Brachiaria brizantha Somatic Embryogenesis via Callus Formation

1. *Induction medium* (*M1.3*): Murashige and Skoog (MS) basal medium [14], 3 % sucrose, 300 mg/L casein hydrolysate, 3 mg/L 2,4-D, 0.2 mg/L BAP, 14 g/L agar, pH 4.2 (*see* **Note 1**), poured in Petri dishes [13].
2. *Differentiation medium* (*DM*): MS basal medium with ½ strength of major salts, 2 % sucrose, 300 mg/L casein hydrolysate, 0.5 mg/L 2,4-D, 0.05 mg/L BAP, 14 g/L agar, pH 4.2 (*see* **Note 1**), poured in Petri dishes.
3. *Regeneration medium* (*MS3*): MS basal medium with 3 % sucrose, 300 mg/L casein hydrolysate, 0.5 mg/L NAA, 1 mg/L BAP, 2.5 mg/L KIN, 14 g/L agar, pH 4.2 (*see* **Note 1**), poured in Petri dishes [13].
4. *Elongation and rooting medium* (*MMP*): MS basal medium with ½ strength major salts, 2 % sucrose, 100 mg/L casein hydrolysate, 0.5 mg/L KIN, 0.2 mg/L NAA, 0.2 mg/L GA_3, 0.7 % agar, pH 5.8, poured in Magenta™ boxes (30 mL in each box), or other vessels for plant tissue culture [13].

   The pH of media is adjusted to 5.8 with 1 N KOH or to 4.2 with 1 N HCl prior to autoclaving. Medium is autoclaved at 121 °C, for 20 min. GA_3 is filter sterilized and added to media after autoclaving.

3 Methods

1. The seeds of *B. brizantha* should be dehusked manually with the aid of a forceps (Fig. 1a) or a seed stripper. Select the well-formed and unblemished seeds with the aid of a stereomicroscope to avoid contamination. Use around 300 seeds per treatment (*see* **Note 2**).
2. Decontamination of the selected and dehusked seeds (Fig. 1b) should be carried out in a laminar flow hood, using a previously autoclaved beaker, with 100 mL of 70 % ethanol for 5 min, followed by 100 mL of a 2.5 % sodium hypochlorite solution with two drops of Tween 20 for 30 min; stir the solution with seeds repeated times.
3. Rinse the seeds thoroughly, six times, with distilled autoclaved water.
4. Dry the seeds on autoclaved filter paper.

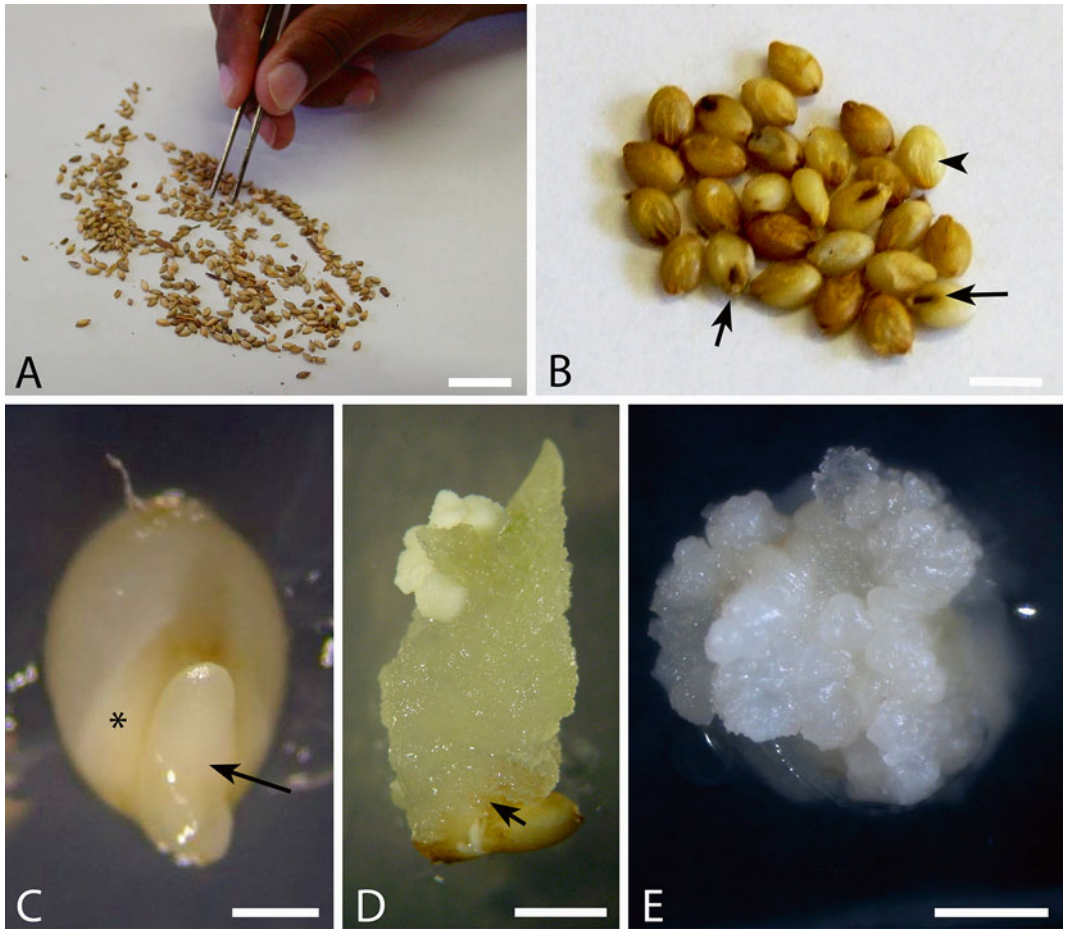

Fig. 1 Selection of *Brachiaria brizantha* cv. Marandu mature seeds and somatic embryogenesis induction: (**a**) selection of mature seeds for manually dehusking; (**b**) peeled mature seeds showing in one side the apomictic embryo (*arrow head*) and in the opposite side the hilum (*arrow*); (**c**) 5-day-old mature seed cultivated in callus induction medium, showing the swollen scutellum (*asterisk*) and embryo axis (*arrow*) of the apomictic seed embryo; (**d**) friable callus grown on the scutellum surface of the seed (*arrow*); (**e**) multiplication of embryogenic callus. Bars: **a** = 6 mm, **b** = 3 mm, **c** = 1 mm, **d** = 2 mm, **e** = 1 mm

5. Inoculate the seeds onto induction medium (M1.3) poured in Petri dishes (100 × 20 mm), 10–12 seeds per plate. To avoid condensation, leave the Petri dishes open in the hood for 15 min to remove excess of moisture. Seal the plates with Parafilm® and incubate them upside down, in the dark, at 25 ± 2 °C for 3–4 weeks (*see* **Note 3**).
6. After 5 days, mature seeds cultured in M1.3 induction medium present a swollen scutellum and embryo axis (Fig. 1c). The swelling is followed by proliferation of friable callus, after 2 weeks of induction, on the upper surface of the scutellum, and an opaque white structure is generally observed (Fig. 1d),

which is the scutellum of a somatic embryo which, in turn, repetitively produces new scutelli if the calli are subcultured to induction medium M1.3 (Fig. 1e) (*see* **Note 4**).

7. To obtain well-differentiated somatic embryos, transfer the 3- to 4-week-old induced calli from induction medium (M13) to differentiation medium (DM), following **step 7**; however, to obtain plant regeneration skip **step 7** and go directly to **step 8**. Seal the Petri dishes with Parafilm® and incubate them upside down, in the dark, at 27 ± 2 °C for 3–4 weeks. In this step visible differentiation of the somatic embryos is observed. Add this step for a better visualization of somatic embryo differentiation.
8. To obtain plant regeneration, transfer the 3- to 4-week-old induced calli from induction medium (M1.3) to regeneration medium (MS3), spreading them well on the medium, approximately 8–10 calli per Petri dish (*see* **Note 5**).
9. Incubate the Petri dishes upside down in a culture room or incubator, at 35 μmol/m²/s, 14 h photoperiod and 27 ± 2 °C for 3 days, then move the plates to a higher light intensity condition (70 μmol/m²/s) for 4 weeks (*see* **Note 6**).
10. Germinating somatic embryos, 2–3 cm long, with developing leaves, are transferred to elongation and rooting medium (MMP), in Magenta™ boxes (30 mL in each box) and kept in a culture room at 25 ± 2 °C at 70 μmol/m²/s and 14 h photoperiod, for 3–4 weeks.
11. For acclimatization, in vitro-rooted shoots are carefully washed to remove the agar, and then transferred first to plastic pots, containing vermiculite, and covered with a plastic bag to maintain high humidity (4–5 days), and afterwards to pots containing a mixture of sand:soil:vermiculite (1:1:1, v/v) in the greenhouse with natural light and temperature (*see* **Note 7**).

4 Notes

1. Endophytes are very common in *Brachiaria* spp. The acidic pH of the induction and regeneration medium (4.2) favors the reduction of endophytic bacteria contamination, enabling longer term *B. brizantha* in vitro culture, compared to culture at pH 5.8. However, at pH 4.2 agar solidification can be difficult and, to avoid this problem, we suggest the use of type A agar at twice the usual concentration (1.4 %).
2. Selection of well-formed and non-damaged seeds with the aid of a stereomicroscope is very important to prevent contamination, considering that *B. brizantha* seeds show a high unviable rate (30 %), and the viable seeds usually present fungi in the

endosperm if the storage conditions are not adequate. Seeds should be stored under refrigeration, in a desiccator.

3. Use 100 × 20 mm Petri dishes for better aeration. It is highly recommended to prepare and pour M1.3 medium in Petri dishes a day or two before inoculating the seeds, to reduce condensation and contamination. Condensation in plates should also be avoided when sowing the seeds. If there is any condensation, open the Petri dishes with medium in the hood and leave them open for 15 min. *Brachiaria* seeds should be positioned preferably with the embryo axis side up, i.e., the hilum side, which is visible (Fig. 1b), should be in contact with the culture medium. Place the seeds, one by one, applying a slight pressure in the culture medium without submerging them, so that they do not detach from the medium due to the growth of callus and/or germination of the embryo. If the seed is not in a close contact with the medium, instead of forming callus, the embryo germinates or root formation is observed; thus, the seed needs to be slightly pressed into the medium. The quality of the primary callus depends entirely on the seed quality and the induction process.
4. M1.3 medium produces a high percentage of embryogenic calli in a 2,4-D concentration ranging from 2 to 4 mg/L. We suggest avoiding a long-term maintenance of *Brachiaria brizantha* embryogenic calli in the presence of 2,4-D, due to a high probability of subsequent formation of 100 % albino plants in 4-month-old embryogenic calli [13] (Fig. 2a, b). Moreover, recently, it was shown that, in ruzigrass (*Brachiaria ruziziensis*), 4-month-old embryogenic callus generated polyploids, while all regenerants derived from 2-month-old embryogenic calli were diploid, suggesting that 2,4-D promoted not only the formation of somatic embryogenesis, but also duplication of chromosomes at early stages of embryogenic callus formation [15]. These outcomes indicate that 2-month-old or younger embryogenic calli are best suited for *Brachiaria* spp. When the supplementation of auxin decreases, there is a rapid differentiation into embryos and different patterns of distribution in the same explant may occur due to local accumulations of auxin. Auxin transport and accumulation may also have an influence in somatic embryo differentiation in *Brachiaria*. Therefore, to obtain well differentiated somatic embryos, we highly suggest transferring the induced calli to differentiation medium (DM), and after 3–4 weeks, embryos have a cream-colored embryo axis with coleoptile surrounding the shoot apical meristem of the somatic embryos, each coleoptile containing one shoot meristem (Fig. 2c). The embryo proper or embryo axis is enveloped with an opaque white-colored, isolated, well-differentiated scutellum, and in some cases show fused scutelli (Fig. 2c).

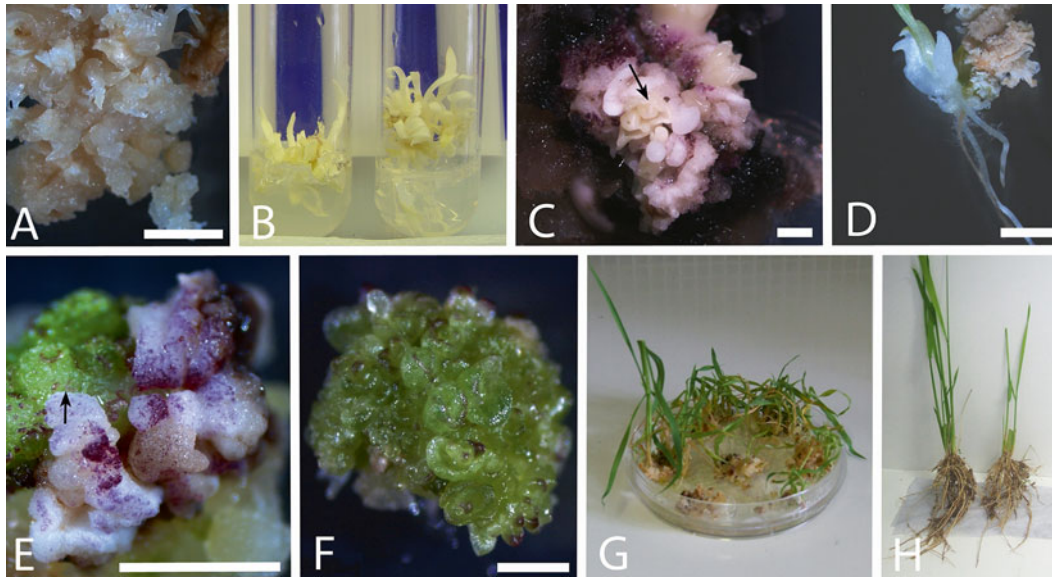

Fig. 2 Plant regeneration of *Brachiaria brizantha* cv. Marandu: (**a**) albino shoots regenerating from embryogenic callus; (**b**) elongating albino shoots; (**c**) differentiated scutellum and embryo axis of somatic embryo (*arrow*); (**d**) plantlet originated from an isolated somatic embryo; (**e**) multiple buds formed in an embryogenic callus (*arrow*); (**f**) multiple buds; (**g**) multiple shoots elongating from clumps of buds; (**h**) tiller plant (*left*) and in vitro plant after acclimatization (*right*). Bars: **a** = 1 mm, **c** = 0.5 mm, **d** = 2 mm, **e** = 2 mm, **f** = 1 mm

5. In MS3 medium plant regeneration is obtained from up to 54 % of the seeds and around 90 % of induced calli. Detailed observations of calli show that two regeneration patterns are observed after transferring the calli to regeneration medium: (1) complete plantlets originating from isolated somatic embryos (Fig. 2d), and (2) multiple buds formed from the apical meristem of somatic embryos in the presence of cytokinins in the MS3 regeneration medium (Fig. 2e, f), producing multiple shoots (Fig. 2g). The purple pigmentation observed in buds and shoots indicates a stress-induced anthocyanin production in the leaf tips of *B. brizantha* cultivated in vitro under light conditions. For reducing this stress the explant should initially be cultured for 3 days at a reduced light intensity, around 30 μmol/m^2/s, returning to a higher light intensity (70 μmol/m^2/s), for plantlet development.
6. If two light intensity conditions are not available, as an alternative pile up the Petri dishes at 70 μmol/m^2/s^1 for the initial period of 3 days at reduced light intensity, then spread the plates side by side at the same light intensity of the climatic chamber. The initial lower light intensity helps to prevent anthocyanin accumulation.
7. Regenerated plantlets from this protocol show a morphological pattern of growth, flowering, and seed production, similar to naturally propagated plants (Fig. 2h).

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Part III

Protocols of Somatic Embryogenesis in Selected Forest Trees

Chapter 21

Somatic Embryogenesis in *Pinus* spp.

Itziar Aurora Montalbán, Olatz García-Mendiguren, and Paloma Moncaleán

Abstract

Somatic embryogenesis (SE) has been the most important development for plant tissue culture, not only for mass propagation but also for enabling the implementation of biotechnological tools that can be used to increase the productivity and wood quality of plantation forestry. Development of SE in forest trees started in 1985 and nowadays many studies are focused on the optimization of conifer SE system. However, these advances for many *Pinus* spp. are not sufficiently refined to be implemented commercially. In this chapter, a summary of the main systems used to achieve SE in *Pinus* spp. is reported.

Key words Conifer, Forest biotechnology, In vitro, Plant tissue culture, Propagation, Somatic embryos

1 Introduction

During the last years, one of the main objectives of conifer genetic improvement programs has been the development and application of biotechnological tools, able to achieve production systems of elite plants adapted to different environmental conditions in the future scenery of climate change. In vitro propagation systems have been one of the most studied aspects worldwide in programs of genetic improvement. In this sense, although the utility of in vitro organogenesis using juvenile material has been developed and optimized in some *Pinus* species [1–5], the high cost of the process is still a limitation for mass production on a commercial scale. Other systems to achieve in vitro propagation of *Pinus* spp. adult trees have been developed [6–10], but changes in the attributes of resulting plants have sometimes been observed and rejuvenation of the material has been transitory in in vitro conditions. For the abovementioned reasons, propagation via somatic embryogenesis (SE) has many advantages:

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- It captures all specific gene combinations of selected individuals, giving high genetic gain [11];
- Combined with cryopreservation of the embryonal masses (EM) and the selection of elite clones in field tests, it enables implementing multivarietal forestry [11];
- It is an ideal system for genetic transformation, through initiation of somatic embryos from single cells [12];
- It offers the capability to produce unlimited numbers of plantlets from somatic embryos [13, 14] and artificial seeds [15].

Although EM initiation protocols are now fairly well established, maturation of EM into cotyledonary normal somatic embryos is not always successful in *Pinus* spp. Problems such as low or asynchronous embryo production [16, 17], abnormal morphology, or poor root development have also been reported for *P. pinea* [18] and *P. kesiya* [19]. In conifer SE, physical and chemical conditions are factors that should be studied carefully. In this sense, culture medium composition takes on special importance in SE, being a substitute of the megagametophyte, supplying adequate amounts of nitrogen and carbon [20]. For this reason, studies were carried out to analyse the effect of culture conditions in SE of *Pinus* spp. [21–24]. Thanks to these studies, a considerable increase of the number and quality of the produced *Pinus* somatic plantlets has been achieved [23].

In this chapter, recent studies focused on the development and optimization of successful protocols of SE in various *Pinus* species are described.

2 Materials

2.1 Plant Material

One-year-old green female cones, enclosing immature zygotic embryos of *Pinus* spp. at the precotyledonary stage [21], are collected from open or control pollinated trees (*see* **Note 1**). The cones are stored at 4 °C until processing (*see* **Note 2**). Cones are usually processed within a week, although they can be stored for more than one month with no detriment in SE initiation rates [13]. Recently, it has also been possible to initiate EM from differentiated cells in epicotyledonary region of post-cotyledonary zygotic embryos [25].

2.2 Media

1. Initiation, proliferation, and maturation phases of SE are usually carried out in the same basal medium. As for macroelements, microelements and vitamins, different media formulation and their modifications are used depending on the species [24, 28], i.e.:

- DCR [26] with *P. nigra* [27], *P. sylvestris* [28], *P. palustris* [29], *P. patula* [30], and *P. brutia* [16];
- EDM [31], LP [32] and Glitz [33] for *P. radiata* [15, 23, 33];
- 505 [34] for *P. taeda* [35];
- LV [36] for *P. pinaster* [37], *P. monticola* [38], *P. pinea* [18], *P. strobus* [39], and the hybrid *P. rigida x P. taeda* [40]. Moreover, different modifications of culture media described are used, depending on the species [41].

2. As a carbon source, sucrose in *P. halepensis* [22], *P. pinaster* [37], *P. pinea* [18] and *P. strobus* [39], and/or maltose in *P. densiflora* [42], *P. kesiya* [43], *P. patula* [30] and *P. taeda* [34] are used in concentrations ranging: (i) from 10 g/L in *P. armandii* [44] and *P. luchuensis* [45] to 30 g/L in *P. bungeana* [12], *P. pinaster* [37], and *P. radiata* [21, 33] for initiation and proliferation, and (ii) from 30 g/L in *P. monticola*,[38] and *P. luchuensis* [45] to 60 g/L in *P. pinea* [18], *P. radiata* [23], and *P. strobus* [39] for maturation (*see* **Note 3**).
3. Gellan gum (Gelrite® or Phytagel®) is added to the medium. The concentration of gellan gum varies, depending on the specific phase of SE process from 2 g/L in *P. brutia* [16] to 4 g/L in *P. pinea* [18] and *P. monticola* [38] for the initiation stage, from 3 g/L in *P. oocarpa* [46] to 5.5 g/L in *P. radiata* [21] for the proliferation stage, and from 4 g/L in *P. nigra* [47] to 10 g/L in *P. pinaster* [37], *P. rigida* x *P. taeda* [40], and *P. sylvestris* [48] for the maturation stage (*see* **Note 3**). Moreover, polyethylene glycol (PEG) is used in the maturation stage of species such as *P. armandii* [44], *P. brutia* [16], *P. densiflora* [42] and *P. patula* [30] to increase the osmolarity of culture media and ensure the success of the process (*see* **Note 4**). Germination stage (i.e., the conversion of somatic embryos to emblings) can be carried out at a broader range of concentrations of Gelrite® or other gellan gum brands.
4. Once medium is sterilized, it is supplemented with a source of organic nitrogen that varies among *Pinus* species, being casein hydrolysate plus l-glutamine in *P. nigra* [47], *P. strobus* [39], and *P. sylvestris* [48], or EDM amino acid mixture [31] in *P. armandii* [44], *P. densiflora* [42], *P. radiata* [21], the most commonly used (*see* **Note 5**).
5. Plant growth regulators (PGR) added to medium are as follows: (i) at initiation and proliferation stages, a cytokinin (benzyladenine, BA) and an auxin (2,4-dichlorophenoxyacetic, 2,4-D) both at the concentration of 2.2 μM for *P. strobus* [39], 2.7 and 4.5 μM, respectively, for *P. radiata* [21], 4.4 and 13.6 μM for *P. pinaster* [49] or the hybrid *P. rigida* x *P. taeda* [50], 2.2 and 9 μM for *P. nigra* [27] (*see* **Note 6**). Other hormones

can also be used for initiation and proliferation of EM, i.e., kinetin instead of BA (at 2.7 μM in *P. halepensis* [22]) or in combination with BA (both at 2.0 μM in *P. taeda* [34]), and auxins such as 1-naphthaleneacetic acid (NAA) instead of 2,4-D (at 10.7 μM in *P. taeda* [34]) or in combination with 2,4-D (both at 4.5 μM in *P. halepensis* [22]); (ii) at maturation stage I (initial procedure of tissue resuspension), liquid medium lacks PGR and organic nitrogen [23]. For improving *P. pinaster* [37] and *P. densiflora* [42] maturation process, activated charcoal (5 to 10 g/L) can be added to the liquid medium used to resuspend EM (*see* **Note 7**); at maturation stage II (tissue culture on the filter paper), abscisic acid (ABA) at a concentration ranging from 40 μM (in *P. oocarpa* [46]) to 120 μM (in *P. strobus* [39]) is used.

6. The basal medium for germination phase is the same used for the previous stages of the process, except in some species such as *P. radiata* in which somatic embryos are germinated in LP medium [51]. Germination medium lacks PGR and is usually supplemented with sucrose. Sucrose concentration varies depending on the species, e.g., at 10 g/L in *P. densiflora* [42], 15 g/L in *P. taeda* [52] and 30 g/L in *P. radiata* [23] and *P. halepensis* [22]. *P. nigra* germination culture medium contains maltose (20 g/L), instead of sucrose [47]. In species such as *P. armandii* [44], *P. halepensis* [22], *P. nigra* [47], or *P. radiata* [23], culture medium is supplemented with activated charcoal to germinate the somatic embryos (*see* **Note 8**).
7. The pH of culture media for all *Pinus* spp. is adjusted to 5.7–5.8.
8. For initiation, proliferation and germination, explants can be cultured into 90 × 15 mm Petri dishes (20–25 mL of semisolid medium), while for maturation the use of 90 × 20 mm Petri dishes is recommended (40 mL of semisolid medium) (*see* **Note 9**).

3 Methods

3.1 Initiation and Proliferation

1. Spray intact cones with 70 % (v/v) ethanol, split into quarters and remove immature seeds. Use 10 % (v/v) H_2O_2 plus two drops of Tween 20® for 8 min for sterilizing immature seeds, then rinse three times with sterile distilled H_2O under the sterile conditions of a laminar airflow cabinet.
2. Excise out aseptically (*see* **Note 10**) whole megagametophytes containing immature embryos and place them horizontally onto initiation medium (*see* **Note 11**). Then, lay out cultures in the growth chamber (Fig. 1a) (*see* **Note 12**).

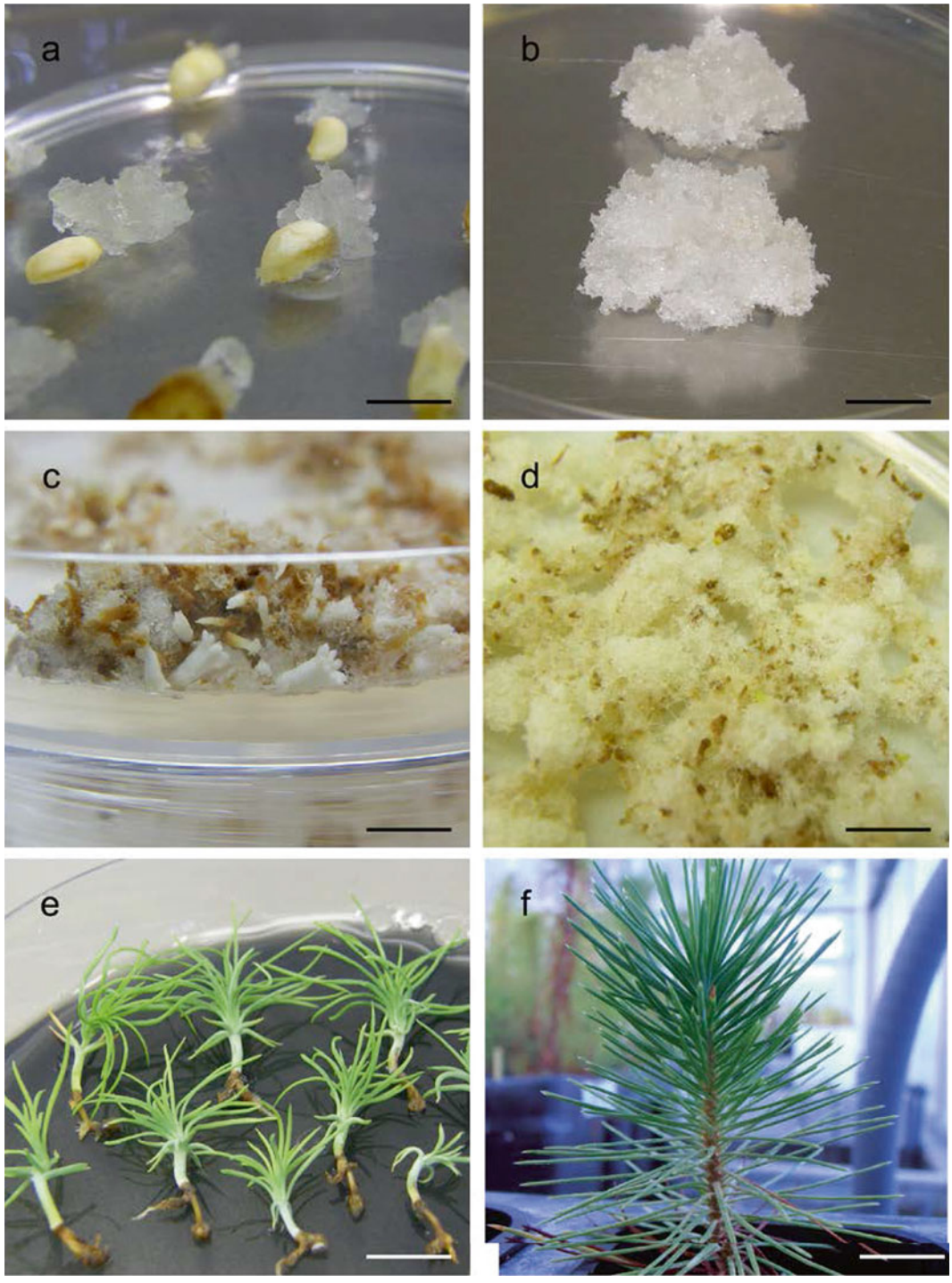

Fig. 1 (**a**) Initiation of EM in *P. radiata* megagametophytes cultured on EDM medium, bar 10 mm. (**b**) Proliferation of EM of *P. halepensis* cultured on DCR medium, bar 6 mm. (**c**) *P. radiata* somatic embryos obtained from 100 mg of EM cultured on EDM supplemented with 60 g/L sucrose and 60 μM ABA, bar 6 mm. (**d**) Tissue overgrowth obtained from 150 mg of EM cultured on EDM supplemented with 60 g/L sucrose and 60 μM ABA, bar 10 mm. (**e**) *P. radiata* somatic plantlets after 14 weeks germinating on half strength LP supplemented with 2 g/L activated charcoal, bar 12 mm. (**f**) Somatic *P. halepensis* plant growing in the greenhouse, bar 25 mm.

3. In most of the *Pinus* species tested, after 4–10 weeks on initiation medium, proliferating EM with a size around 3–5 mm in diameter is separated from megagametophytes. EM is subcultured to proliferation medium every 2 weeks (Fig. 1b). In *P. sylvestris* [28] and *P. pinaster* [37], in order to attain a high amount of EM in a short period of time, weight 300 mg of EM and resuspend it in liquid medium. Then pour it onto filter-paper disc and drain it using a Büchner funnel. Thereafter, transfer the filter paper with attached EM to proliferation medium and subculture it to fresh medium each 2 weeks (*see* **Note 13**).
4. During initiation and proliferation, keep cultures in darkness [21] or under low light intensity (5 μmol/m^2/s [33]) at 21–24 °C [41].

3.2 Maturation

1. Maturation process is divided in two stages. For maturation stage I, resuspend EM in liquid medium in 50 mL centrifuge tubes (*see* **Note 14**). Then, shake EM suspension vigorously by hand for a few seconds.
2. 4–5 mL aliquot of the suspension, containing 300–500 mg of EM, is used in *P. brutia* [16] and *P. strobus* [53] and is poured onto a filter paper disc (Whatman no. 2, 70 mm) in a Büchner funnel. For *P. sylvestris* [48], EM amount can be decreased to 200 mg and, in species such as *P. halepensis* [22], *P. pinaster* [37], or *P. radiata* [23], a low amount of EM (60–100 mg fresh weight) is used to obtain the best results (Fig. 1c) and avoid overgrowth (Fig. 1d) (*see* **Note 15**).
3. Apply a vacuum pulse for 10 s. For maturation stage II, transfer the filter paper disc with attached EM to maturation medium (*see* **Note 16**), such as in *P. monticola* [38], *P. nigra* [47], or *P. taeda* [54]. On the contrary, in *P radiata* [23], *P. pinaster* [37], and *P. sylvestris* [48], the filter paper discs with attached EM are not subcultured through all maturation process.
4. During maturation, cultures are kept in darkness or under low light intensity (5 μmol/m^2/s), at 16-h photoperiod and 21–24 °C.

3.3 Germination and Acclimatization

1. After 6–15 weeks, collect mature somatic embryos, i.e., white to yellowish, non-germinating somatic embryos with a distinct hypocotyl region and at least three cotyledons (Fig. 1c). A partial desiccation pre-germination treatment has been described in *P. thunberghii*, *P. densiflora*, *P. armandii* [55], *P. patula* [30], *P. nigra* [47], and *P. oocarpa* [46]. Partial desiccation can be carried out at 25 °C in a laminar airflow cabinet for 0–4 h (fast method) or at high relative humidity, placing embryos over 30 mm diameter filter paper disks into a multiplate in

which some wells are filled with 5–6 mL of sterile water, sealed tightly and placed in darkness at 25 °C for 0–3 weeks (slow method) [55]. *P. elliotii* [56] somatic embryos can also be stratified in order to increase somatic embryo conversion to emblings (*see* **Note 17**).

2. Culture somatic embryos on Petri dishes with embryonal root caps pointing downwards and tilt the Petri dishes vertically at an angle of approximately 45–60°. In *P. radiata* [23], *P. halepensis* [22] and *P. nigra* [47], the cultures are maintained at 21–24 °C under a 16-h photoperiod at 40 μmol/m^2/s for 1–2 weeks, and then at 120 μmol/m^2/s provided by cool white fluorescent tubes. In *P. densiflora* [55], *P. taeda*, *P. elliottii*, and *P. palustris* [57], red wavelengths provided by light-emitting diode (LED) improve somatic embryo germination.
3. After 6–8 weeks on germination medium, subculture the plantlets once onto fresh germination medium. After another 6–8 weeks on germination medium, transfer the somatic plants to trays with a sterile potting mix (Fig. 1e). As potting mix, use a peat:perlite, ratio 3:1 and 7:3 in *P. radiata* [23] and *P. halepensis* [22], respectively, pine bark in *P. patula* [30], vermiculite in *P. kesiya* [19], peat:vermiculite (3:1) in *P. pinaster* [37], and perlite:peat:vermiculite (1:1:1) in *P. taeda* [52] and *P. rigida* x *taeda* [50] (*see* **Note 18**).
4. Acclimatize the plantlets in a greenhouse under controlled conditions, decreasing humidity progressively [8] (*see* **Note 19**) (Fig. 1f).

4 Notes

1. Ten megagametophytes per seed family or control cross should be destructively sampled; the megagametophyte is carefully cut longitudinally under an inverted microscope. Sometimes the use of acetocarmine can help to see the zygotic embryo, especially at early stages of development [21]. If the stage of most zygotic embryos is not between early cleavage polyembryony and first "bullet" stages with a dominant embryo [33], initiation rates of SE will be very low or zero.
2. To minimize high humidity and contamination, cones are wrapped in filter paper and stored in expanded polystyrene boxes.
3. For the development of somatic embryos, it is necessary to restrict water availability by physical or chemical means, such as increasing osmotic agents (e.g., gellan gum or sugars) concentration.
4. In this sense, some authors also add polyethylene glycol to maturation medium [16, 30, 42, 44, 46].

5. It is important to adjust the pH of thermolabile organic nitrogen solution (i.e., l-glutamine) to 5.7–5.8.
6. The most commonly used PGR sources and concentrations are those presented in Subheading 2.2, but some authors use different plant growth regulators for initiation such as *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU) in *P. pinaster* [58], or PGR concentration is reduced to improve proliferation (in *P. densiflora* [42]).
7. When using a high concentration of activated charcoal in liquid medium, it is important to adjust carefully pH of the medium to 5.7–5.8. It is also important to shake liquid medium before resuspending the EM, in order to avoid sedimentation of activated charcoal.
8. Activated charcoal can be a critic factor for conversion of somatic embryos into plantlets, as proved in *P. radiata* [23]. Thus, when trying to achieve conversion into plantlets of somatic embryos in a specific *Pinus* species for the first time, it is suggested to test germination media with and without activated charcoal.
9. Semisolid media are prepared at least one week before being used, while liquid medium is prepared the day before and totally used within a week.
10. For this purpose we use Gerald forceps and scalpels (scalpel blades number 11 or 20, depending on the size of the seeds).
11. Although some authors [33] have increased initiation rates by excising out immature zygotic embryos from megagametophytes (using a dissecting microscope), this procedure is time consuming and requires sophisticated technical skills to avoid damages or contamination of zygotic embryos.
12. It is advisable not to put more than ten explants per Petri dish, in order to avoid later overlapping of extruding EM. Petri dishes in all stages of the process are sealed with cling film.
13. Only peripheral parts of EM must be taken for proliferation, and particularly for maturation of EM (it is recommended the use of forceps). It is also important to be careful with the amount of EM per aliquot; otherwise overgrowth of tissue on the filter paper would hinder development of somatic embryos (Fig. 1d). For this purpose, if SE has not been previously studied in a given species, it is convenient to test different amounts of EM per aliquot.
14. Maturation is carried out once a sufficient amount of tissue is achieved, usually after 4–8 subculture periods.
15. Sterilize filter papers prior to use them for maturation process. In order to avoid cross contaminations, use a different Büchner funnel for each embryogenic cell line maturated; the use of autoclavable plastic funnels makes this procedure easier.

16. In some Pinus spp., such as *P. sylvestris* [48] and *P. pinaster* [37], the filter paper with attached EM is subcultured fortnightly to fresh proliferation medium.
17. In *P. radiata* [23], *P. monticola* [38], or *P. halepensis* [22] it is not necessary to perform any pre-germination treatment if somatic embryos show a normal morphology.
18. These mixes can be supplemented with slow release osmocote (at 750 g m^3 [38]). As suggested by several authors [41], fertilization and pesticide treatments are the same as used for seedlings, except that somatic seedlings are fertilized immediately after transplanting [41].
19. The first 2 weeks after transplanting the plantlets, an acclimatization tunnel is recommendable to maintain the humidity at 90–95 %.

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Chapter 22

Somatic Embryogenesis of *Abies cephalonica* Loud.

Jana Krajňáková and Hely Häggman

Abstract

Greek fir (*Abies cephalonica* Loudon) belongs to the Mediterranean fir species and is widely distributed in the mountains of Central and Southern Greece. Considering a climatic scenario, infestation by pathogens or insects and fire episodes, it has been proposed that Mediterranean firs could be in danger in some parts of their present range but, on the other hand, could also replace other species in more northern zones with temperate humid climates (e.g., silver fir, *Abies alba* Mill.). As fir species are generally highly productive and therefore important for commercial forestry, they have traditionally been involved in conventional tree improvement programs. A lot of effort has been put into the development of vegetative propagation methods for firs, in order to rapidly gain the benefits of traditional breeding to be utilized in reforestation. The present paper provides up to date information on protocols for somatic embryogenesis (i.e., the most promising in vitro method for vegetative propagation) of Greek fir. Moreover, the protocols for cryopreservation and long-term storage of embryogenic material are described as well.

Key words Cryopreservation, Ectomycorrhizal fungi, Fulvic acids, Greek fir, Initiation, Maturation, Proliferation

1 Introduction

Greek fir (*Abies cephalonica* Loudon) is a medium-sized tree, widely distributed in mountains of Central and Southern Greece, mainly at altitudes of 800–1700 m, covering an area of 200,000 ha of productive and conservation forests [1]. Most stands are rather degraded and the present distribution is just a fraction of its potential natural area [2]. The decrease has been attributed to various reasons such as air pollution [3], drought-related extreme periods, infestation by mistletoe, pathogens or insects, root damage [1, 4, 5], fire episodes spreading over high altitudes [6], as well as the effects of climate change [7]. Considering a climatic scenario, it has been proposed that Mediterranean firs could be in danger in some parts of their present range, but, on the other hand, could also replace other species in more northern zones with temperate humid climates (e.g. silver fir, *Abies alba* Mill) [8]. As fir species are generally highly productive and therefore important for

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commercial forestry, they have traditionally been involved in conventional tree improvement programs. A lot of effort has been put into the development of vegetative propagation methods for firs, in order to rapidly gain the benefits of traditional breeding to be utilized in reforestation [9]. Somatic embryogenesis (SE), i.e., the development of embryos from somatic cells, with its potential for mass multiplication has become a useful technique for large-scale propagation of many coniferous species [10]. In combination with cryopreservation somatic embryogenesis makes it possible to preserve important genotypes during field tests (reviewed in [10]).

In Greek fir, like in other conifers, the multi-step regeneration process of SE starts with induction of pro-embryogenic masses, followed by somatic embryo formation, maturation, desiccation and plant regeneration (Fig. 1). *Abies* species were among the first coniferous species where the induction of SE was reported [11, 12]. However, a standard protocol for propagation by SE on a large scale is still lacking, the only exception is SE of *A. nordmanniana* in which case the technology has already been tested in large scale (Jens Find, personal communication). *A. cephalonica* was regenerated by Krajňáková et al. [13] and hybrid *A. alba*×*A. cephalonica* was regenerated by Salajová et al. [14]. Embryogenic cultures of *A. cephalonica* and hybrid *A. alba*×*A. cephalonica* [13, 14] have been derived from immature zygotic embryos. In case of hybrid (*A. alba*×*A. cephalonica*), initiation of embryogenic cultures was achieved when using also mature embryos [15] and cotyledons derived either from seedlings or somatic embryos (secondary or repetitive SE) [16].

Somatic embryogenesis of several *Abies* species, including *A. cephalonica*, differs from most of the other genera of the *Pinaceae*, because only cytokinin is needed for induction and proliferation [13, 17]. Maturation of Greek fir and hybrid *A. alba*×*A. cephalonica* somatic embryos is promoted by abscisic acid and maltose is the preferable carbohydrate. The addition of polyethylene glycol promoted the development of somatic embryos [15, 18]. For germination, well-developed cotyledonary somatic embryos are selected and subjected to a partial desiccation treatment for 3 weeks [13, 18]. Despite positive achievements, the bottlenecks in *A. cephalonica*, like in most conifers, are the low initiation rate, uneven maturation of embryos, problems in rooting and germination phases. This is due to poor understanding of embryo development and therefore inability to develop proper SE methods for

Fig. 1 (continued) embryogenic cell mass and detail of proembryogenic cell masses after staining with acetocarmine and Evan's blue (**f**). (**g**) Option for cryopreservation of the germplasm. (**h**) Maturation of somatic embryos with embryogenic cell masses spread on filter paper or (**i**) as clumps over solid medium (a cotyledonary somatic embryo is showed in a *small box*). (**j**) Plants prepared for experimental field trail

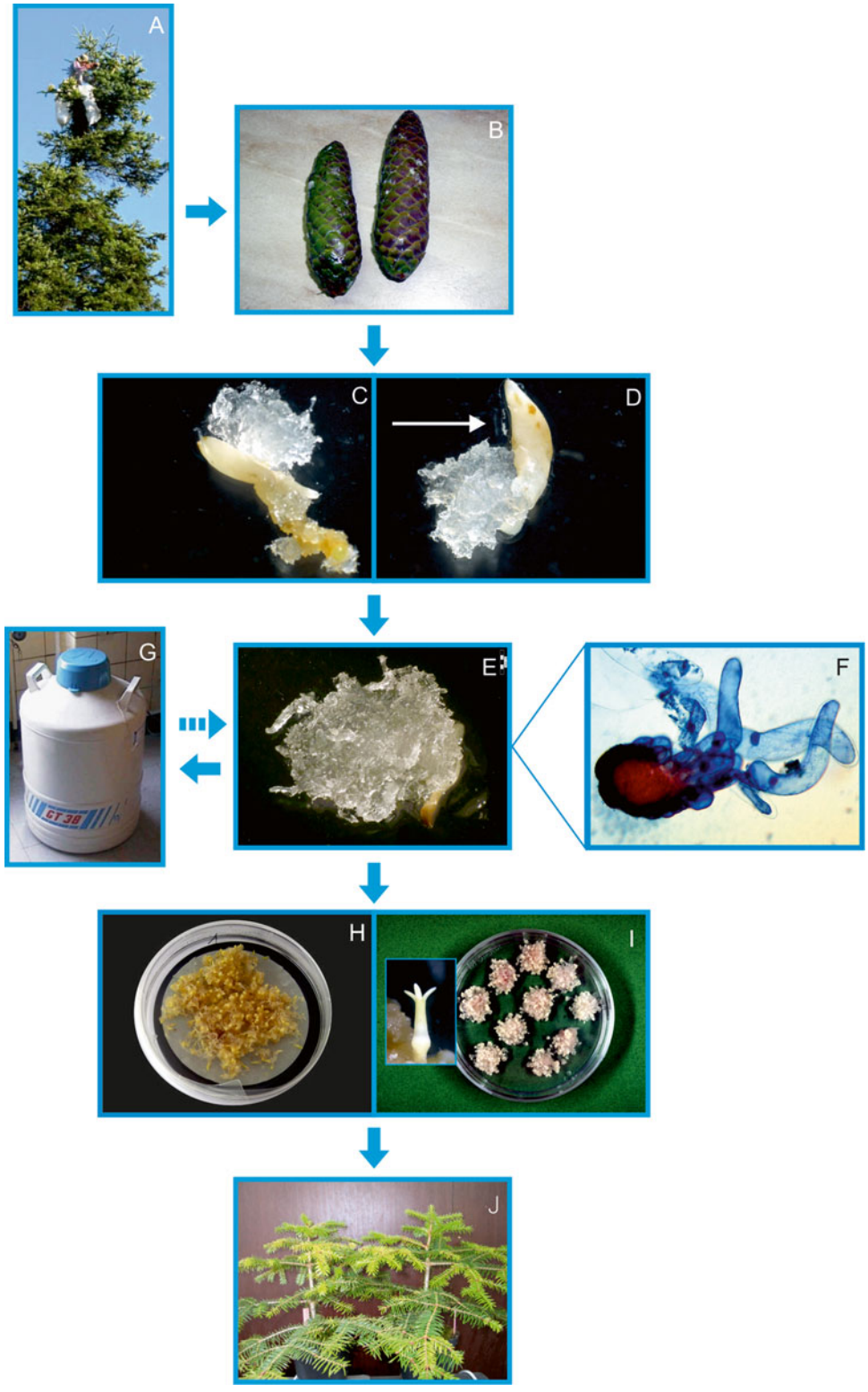

Fig. 1 Somatic embryogenesis of *Abies cephalonica*. (**a**) Elite tree of *A. cephalonica*. (**b**) Developing green cone, shortly after meiosis. (**c**) Initiation of somatic embryogenesis using immature embryos and proliferation of embryogenic cell mass, protruding from both sides of megagametophyte. (**d**) Initiation of somatic embryogenesis and proliferation of embryogenic cell masses (*arrow* is pointing on the resin residuals). (**e**) Proliferating

practical purposes. Recently, the use of fulvic acids, for improving the proliferation abilities of Greek fir was studied [19], as well as the technique of cocultivation of ectomycorrhizal fungi with embryogenic cell masses which have led to improvements during maturation [20].

The aim of present paper is to provide up-to-date information on protocols for Greek firsomatic embryogenesis, cryopreservation of embryogenic cell masses, and their long-term storage.

2 Materials

General equipment for tissue culture:

1. Laminar flow hood.
2. Scalpels.
3. Forceps.
4. Growth chamber or cultivation room.
5. Autoclave.
6. pH-meter.

2.1 In Vitro Protocols for Somatic Embryogenesis

1. Seed cones containing immature seeds.
2. 70 % ethanol.
3. 4 % (w/v) CaOCl (Ca-hypochlorite).

2.1.1 Initiation, Induction, and Proliferation

4. Sterile distilled water.
5. 9 cm sterile Petri dishes.
6. 100 mL sterile beaker.
7. Initiation medium (Tables 1 and 2): Initiate either on solid MS-based medium [21] or SH [22] medium. Media are modified as follows: Half-strength macroelement MS medium supplemented with 20 g/L (58 mmol/L) sucrose, 1 mg/L (4.44 μmol/L) benzyl adenine (BA), 500 mg/L (3.4 mM) L-GLUTAMINE, and solidified with 0.3 % (w/v) gellan gum Phytagel™ (Sigma) [13]; SH medium, containing 20 g/L (58 mmol/L) sucrose, 1 mg/L (4.44 μmol/L) BA, 500 mg/L (3.4 mM) L-glutamine, and solidified with 0.3 % (w/v) gellan gum Phytagel™ (Sigma) [13].
8. Proliferation medium: MS-based initiation medium with addition of 0.1 % (w/v) casein hydrolysate [13].

2.1.2 Maturation

1. Sterile filter paper discs.
2. Falcon tubes.
3. 9 cm Petri dishes.

Table 1

Concentrations of basic ingredients in half-strength macroelement MS medium [21], SH medium [22], and DCR medium [23] used for somatic embryogenesis of Greek fir

Component	Half-strength macroelement MS		SH		DCR	
Ingredient	**[mg/L]**	**mM**	**[mg/L]**	**mM**	**[mg/L]**	**mM**
Inorganic macro						
NH_4NO_3	825	10.3	–	–	400	5
KNO_3	800	9.4	2500	25	334	3.3
$Ca(NO_3)_2{\cdot}4H_2O$	–	–	–	–	543	2.3
$CaCl_2{\cdot}2H_2O$	220	1.5	200	1.4	84	0.57
$MgSO_4{\cdot}7H_2O$	185	0.75	400	1.6	370	1.5
KH_2PO_4	85	0.625	–	–	163	1.2
$NH_4H_2PO_4$	–	–	300	2.6	–	–
Inorganic micro						
KI	0.83	0.005	1.0	0.006	0.8	0.005
H_3BO_3	6.2	0.1	5.0	0.08	6.2	0.1
$MnSO_4{\cdot}4H_2O$	22.3	0.1	–	–		
$MnSO_4{\cdot}H_2O$	–	–	10	0.06	22	0.13
$ZnSO_4{\cdot}7H_2O$	8.6	0.030	1.0	0.0035	8.6	0.03
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.001	0.1	0.0004	0.24	0.001
$CuSO_4{\cdot}5H_2O$	0.025	0.0001	0.2	0.0008	0.25	0.001
$CoCl_2{\cdot}6H_2O$	0.025	0.0001	0.1	0.0004	0.024	0.0001
$NiCl_2{\cdot}6H2O$	–	–	–	–	0.024	0.0001
Na_2-EDTA	37.25	0.1	20	0.055	34	0.1
$FeSO_4{\cdot}7H_2O$	27.85	0.1	15	0.055	27.8	0.1
Organics						
Myoinositol	100	0.555	1000	5.55	198	1.1
Nicotinic acid	1.0	0.0812	5.0	0.41	0.5	0.0041
Pyridoxine-HCl	1.0	0.0048	0.5	0.0024	0.5	0.0024
Thiamine-HCl	1.0	0.003	5.0	0.015	1	0.003
Glycine	2.0	0.0266	–	–	2.0	0.0266
L-glutamine	500	3.42	500	3.42	248	1.7

Table 2

Composition of the initiation, proliferation, maturation, and conversion media during cultivation of Greek fir embryogenic cultures [13, 18, 24]

Medium composition	Initiation [13]		Proliferation [13, 24]	Liquid medium for suspension [24]	Maturation			Conversion
Inorganics and organics	Half- strength macroelement MS	SH	Half-strength macroelement MS	Half-strength macroelement MS	DCR [13]	Half-strength macroelement MS [24]	Half-strength macroelement MS [24]	Half-strength DCR [13, 18]
Casein hydrolysate (g/L)			1	1	0.5	0.5	0.5	
L-Glutamine			0.5	0.5	0.25	0.25	0.25	
Sucrose (g/L)	20	20	20	20				
Maltose (g/L)					30	30	30	20
BA (mg/L)	1	1						
ABA (mg/L)					8.5	17	8.5	
Phytagel (g/L)	3	3	3		2.5	2.5	2.5	
Agar (g/L)								10
PEG 4000 (g/L)						10		

4. Liquid proliferation media without plant growth regulators (Table 2) for making suspension with embryogenic cell masses (ECMs) [24].
5. Perform maturation either on solid DCR medium: DCR with 8.5 mg/L (32 μM) abscisic acid (ABA), 10 % (w/v) polyethylene glycol (PEG) 4000, 0.05 % (w/v) casein hydrolysate, 250 mg/L, 1.7 nmol/L L-glutamine, 30 g/L (83.3 mM) maltose [13] (*see* **Note 1**) or solid MS media: (a) half-strength macronutrient MS medium with 17 mg/L (64 μM) ABA, 10 % (w/v) polyethylene glycol (PEG) 4000, 0.05 % (w/v) casein hydrolysate, 250 mg/L (1.7 nmol/L) L-glutamine, 30 g/L (83.3 mM maltose) [24]; (b) half-strength macronutrient MS medium with 8.5 mg/L (32 μM) ABA, 0.05 % (w/v) casein hydrolysate, 250 mg/L (1.7 nmol/L) L-glutamine, 30 g/L (87.6 mM) maltose [24] (Tables 1 and 2).

2.1.3 Conversion and Acclimatization to Ex Vitro

1. 150 mL tissue culture jars.
2. Solid DCR medium for conversion: Half-strength DCR hormone-free medium with 20 g/L (58 nmol/L) maltose [13, 18] (Tables 1 and 2).
3. Non-fertilized horticultural peat and perlite.
4. Plastic containers, commercial fertilized peat (VAPO, Finland) with 1 kg/L basic fertilizer: 9.7 % N, 7.5 % P, 14.4 % K, 5.0 % Ca, 6.6 % S, 3.8 % Mg, 0.27 % Fe, 0.13 % Mn, 0.04 % B, 0.05 % Zn, 0.25 % Cu, and 0.09 % Mo and 3 kg/L limestone dust with Mg, commercial 0.2 % 5-Superex fertilizer (Kekkilä, Finland).

Table 3

Composition of proliferation and pretreatment media for cryopreservation of Greek fir embryogenic cell masses [13, 24, 25]

Medium composition	Proliferation	Pretreatment		Cryo-treatment
Inorganics and organics	**Half-strength macroelement MS**	**Half-strength macroelement MS**	**Half-strength macroelement MS**	**Half-strength macroelement MS**
Casein hydrolysate (g/L)	1	0.5	0.5	1
L-Glutamine	0.5	0.25	0.25	0.5
Sucrose (g/L)	20	68.5	137	137
BA (mg/L)	1	1	1	
Phytagel (g/L)	3	3	3	

2.2 Cryopreservation

1. Cryovials and markers.
2. Cryobox or cryocanes for immersion of the cryovials in liquid nitrogen (LN).
3. Sterile tips for pipets of different volumes (0.2 μL to 1 mL).
4. Programmable controlled-temperature chamber or Nalgene™ freezing container and isopropanol.
5. Ice.
6. Dewar for the conservation of samples in LN.
7. Actively proliferating embryogenic cell masses (10- to 12-day old, after the last regular transfer).
8. Solid MS based medium for cryopreservation: (a) half-strength macroelement MS medium, hormone-free, containing 68.5 g/L (0.2 M) sucrose; (b) half-strength macroelement MS medium, hormone-free, containing 137 g/L (0.4 M sucrose) [24, 25] (Tables 1 and 3).
9. Liquid MS-based medium for cryopreservation: Half-strength macroelement MS medium, hormone-free, containing 137 g/L (0.4 M) sucrose [24, 25] (Tables 1 and 3).
10. Solid MS-based proliferation medium: Half-strength macroelement MS medium with 20 g/L (58.4 mM) sucrose, 1 mg/L (4.44 μmol/L) BA, 500 mg/L (3.4 mM) L-glutamine, 0.1 % (W/v) casein hydrolysate [13, 24, 25] (Tables 1 and 3).
11. PGD cryoprotectant solution: 10 % PEG 6000, 10 % glucose, 10 % dimethyl sulfoxide (DMSO) in H_2O, filter sterilized.

3 Methods

3.1 Somatic Embryogenesis

3.1.1 Culture Media Preparation, Explant Excision and Sterilization, and Culture Initiation

1. Solid MS, SH, and DCR culture media (Tables 1 and 2) for initiation, proliferation, and maturation are prepared in 9 cm Petri dishes and liquid media to arrest proliferation in 250 mL Erlenmeyer flasks. The pH of medium is adjusted to 5.7 prior adding the solidifying agent. Media for conversion are prepared in tissue culture jars (Magenta vessels). Aqueous stock solutions of L-glutamine and ABA are filter sterilized and added to the medium after autoclaving. Separately autoclaved polyethylene glycol is mixed with the rest of the maturation medium in laminar flow hood to get the final volume.
2. Immature zygotic embryos surrounded by the megagametophyte (called immature zygotic embryos) and isolated from immature seed cone are most favorable material for initiating SE of Greek fir. The optimum developmental stage of immature zygotic embryos for initiation is the precotyledonary stage (i.e., 1 month after fertilization but before the formation of cotyledons) [13] (*see* **Note 2**) (Fig. 1a, b). However, in case of

hybrid *A. alba* × *A. cephalonica*, also mature zygotic embryos are used [15] as well as cotyledons from seedlings and emblings [16] (*see* **Notes 3** and **4**).

3. Immature seed cones are rinsed with 70 % ethanol for 2 min, after which immature seeds are removed from the cones using scalpels and forceps and placed in sterile beaker with sterile distilled water.
4. Seeds are surfaced sterilized for 20 min in 4 % (w/v) CaOCl, and rinsed three times for 5 min with sterile distilled water (*see* **Note 5**).
5. Seed coats are opened and removed with forceps and immature zygotic embryos surrounded by megagametophytes are excised and placed onto MS or SH medium for initiation (*see* **Note 6**) (Fig. 1c).
6. Immature zygotic embryos are first cultured for 4 weeks, and thereafter transferred onto new media for an additional 4 weeks. However, it is recommended to control the contamination problems within the first week of cultivation. The contaminated immature embryos should be discarded and not contaminated transferred to a fresh medium (*see* **Note 7**) (Fig. 1d).
7. Initiation and induction is performed in the dark at 22 ± 2 °C.

3.1.2 Proliferation of Embryogenic Cultures

1. Embryogenic cell masses start to protrude from different parts (micropylar end being the most frequent) of the responsive explants (immature zygotic embryos, surrounded by megagametophytes) 4–6 weeks after initiation (Fig. 1c). Embryogenic tissues are excised from each explant separately (each one representing one genotype) and transferred to a new Petri dish with proliferation medium (MS or SH) to form a new cell line.
2. To maintain the proliferation of ECMs, they are transferred to fresh medium every 3 weeks. ECMs can be subcultured for several months (*see* **Note 8**) (Fig. 1e–g) in the dark at 22 ± 2 °C.
3. ECMs can be used as such for maturation.

3.1.3 Maturation of Embryogenic Cultures

1. Clumps of ECMs (or filter paper covered by a thin layer of ECM suspension) are transferred to maturation medium 1 (Table 2; *see* **Notes 9** and **10**) for the first 6 weeks, followed by regular transfers to fresh media at 2-week intervals (Fig. 1h, i). For further development of somatic embryos, ABA concentration is decreased and PEG-4000 is omitted from the medium (Table 2).
2. For preparing the suspension, 4 g of fresh ECM is transferred to sterile Falcon flasks with 20 mL of liquid hormone-free proliferation medium (Table 2). Suspension is gently mixed by

vortex and allowed to settle. After the removal of supernatant, 1 mL of suspension, containing approximately 250 mg ECM (fresh weight), is plated onto sterile Whatman filter paper placed on maturation medium [24].

3. Maturation is performed in the dark at 22 ± 2 °C.

3.1.4 Desiccation-Conversion of Somatic Embryos and Acclimatization to Ex Vitro

1. Mature healthy cotyledonary somatic embryos are carefully detached from the embryogenic cell masses (Fig. 1I, small box) and transferred on empty Petri plates (diameter ca. 4 cm) which are placed into bigger Petri plates (diameter ca. 9 cm) with sterile distilled water for 3 weeks desiccation period.
2. During the desiccation period, somatic embryos are stored in the dark, at the temperature of 4 °C.
3. Afterwards, desiccated embryos are placed onto the hormone-free half-strength DCR medium with 20 g/L (58 mM) maltose, solidified with 1 % (w/v) agar [13, 18] (Tables 1 and 2).
4. The base of somatic embryos is gently inserted into the medium.
5. Somatic embryos are germinated at the temperature of 22 ± 2 °C and the light intensity is kept for the first 2 weeks at 30 μE/m^2/s (16 h photoperiod), and then gradually augmented up to 75 μE/m^2/s.
6. Somatic embryo-derived plantlets are carefully detached from the medium and roots are washed. Thereafter plantlets are planted into small plastic greenhouses containing non-fertilized horticultural peat and perlite (*v:v*) (2:1). For the first 2 weeks the plantlets are kept under mist in order to keep relative humidity at approximately 90 %, after which the humidity is gradually decreased.
7. After 1 month the plantlets are transferred into bigger containers (diameter ca. 5–6 cm) containing commercial fertilized peat (VAPO, Finland) in a greenhouse. During the growing season, plantlets are fertilized monthly with commercial 0.2 % 5-Superex fertilizer (Kekkilä, Finland) (Fig. 1j).

3.2 Cryopreservation by Controlled-Rate Cooling

3.2.1 Cold Hardening, Pretreatments, and Cryostorage

1. Transfer actively proliferating ECMs, size 300 ± 50 mg, on MS-based proliferation medium and cultivate at 5 °C in the dark for 14 days (*see* **Note 11**).
2. After cold hardening, transfer the culture onto proliferation medium supplemented with 0.2 M sucrose for 24 h, and afterwards onto 0.4 M sucrose medium for another 24 h.
3. Transfer about 3–4 ECM clumps into 400 μL of hormone-free proliferation medium (**item 9**, Subheading 2.2) which is added into 2 mL cryotubes on ice.
4. Add PGD cryoprotective solution dropwise over a period of 30 min to give a final concentration of 5 %.

5. Leave the cryotubes to stand for 2 h on ice before freezing.
6. After finishing the cryoprotection phase, freeze the samples at a rate of 10 °C/h (0.17 °C/min) to the prefreezing temperature of −38 °C, using a programmable controlled-rate freezer (*see* **Note 12**).
7. After reaching the terminal temperature, immerse the cryotubes containing samples in LN and store.

3.2.2 Thawing and Recovery

1. Thaw the cryovials in a 37 °C water bath and then transfer them on ice.
2. Rinse the surfaces of cryovials with 70 % ethanol. Pay attention to labeling.
3. Plate (dispense) the contents of the cryovials on an autoclaved filter paper disc, placed on proliferation medium in a 90 mm Petri dish with 0.4 M sucrose. Incubate cultures for 1 h.
4. After 1 h, transfer filter papers with suspensions onto fresh proliferation medium with 0.2 M sucrose and incubate for 24 h in the dark at 22 ± 2 °C.
5. After 24 h, transfer filter papers with suspensions on the proliferation medium (Tables 1 and 2).
6. Examine the viability of cells by staining the suspension culture with 0.5 % FDA (fluorescein diacetate) and observe at the microscope under UV light.
7. Monitor cultures regularly and transfer them onto fresh proliferation medium at 2-week intervals (*see* **Note 13**).
8. After observing the recovery (i.e., new proliferation growth), transfer the embryogenic cell masses on fresh proliferation medium without filter paper disc.
9. Embryo maturation is established when proliferation of cryopreserved ECMs is comparable to non-cryopreserved cultures (*see* **Note 14**).

4 Notes

1. DCR medium was originally used for tissue cultures of Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] [23] and have been used for cultivation of other coniferous species, such as *Pinus* [26] and *Abies* species [13, 14].
2. The cones with immature zygotic embryos can be collected and stored at 4 °C for at least 2 months without losing the ability to induce somatic embryogenesis [13, 27].
3. Mature embryos, used for initiation of SE, were excised from hybrid seeds of *A. alba* × *A. cephalonica*, stored from 6 months

to 4 years [15]. Embryos isolated from seeds stored for 6 months showed 27 % initiation frequencies, and those isolated from 1-year stored seeds 29 %. Embryos from seeds stored for 4 years did not response.

4. Embryogenic cultures have been initiated on cotyledon explants dissected from seedlings or emblings of the hybrid *A. alba* × *A. cephalonica* [16]. Cotyledons of seedling origin gave relatively low initiation frequencies (about 2 %). In embling-derived cotyledons, the initiation was cell-line dependent and reached values between 1 and 24 %.
5. Due to the toxic nature of $HgCl_2$ (0.1 %, w/v, solution used), this sterilizing agent was omitted from tissue culture protocols. However, it was successfully used when applied to immature and mature seeds of *A. cephalonica* [13] and *A. alba* × *A. cephalonica* hybrid [15]. Positive results were also obtained with 15 % H_2O_2 [14].
6. There were no significant differences in initiation frequencies when half-strength MS and SH media were compared [13]. DCR and LM media have also been used for induction of somatic embryogenesis from hybrid immature zygotic embryos of *A. alba* × *A. cephalonica*. However, these media turned out to be impropriate for initiation of embryogenic cell masses [14]. On the other hand, ECMs were induced on cotyledon explants isolated from emblings and seedlings of *A. alba* × *A. cephalonica* hybrid on DCR-based medium [16].
7. Seeds of *A. cephalonica*, like other *Abies* species, are full of resins. Sometimes these resins are transferred during isolation of embryos onto initiation medium and they create white plaques similar to bacterial contamination. When observing this phenomenon it is recommended to cultivate "suspicious" Petri plates overnight at higher temperature (37 °C), as well as to transfer the culture on a bacterial cultivation medium. If the plaques remain of the same size, they are resins; if they grow, then it is bacterial contamination.
8. During prolonged proliferation, the regeneration ability of ECMs decreases. It is therefore very important to start with cryopreservation when stable proliferation is achieved.
9. A suspension made with embryogenic cell masses can also be used for maturation [19, 24, 27, 28].
10. Arrest of proliferation can be achieved either on solid or liquid hormone-free half-strength macroelement MS medium [24, 27, 29].
11. Step of cold-hardening has been omitted from cryopreservation protocol without noticing the decrease in viability of cultures after thawing, however only two cell lines were tested [30].

12. Nalgene Freezing Container, Mr. Frosty, filled with isopropanol alcohol, was successfully used for cooling down the cryovials, instead of using a programmable controlled-rate freezer [30].
13. The most precise way of monitoring the new proliferation is determination of proliferation ratio (w_0/w_i) in which w_i is the initial fresh weight of sample after thawing and w_0 is the weight at the time of subculturing, generally 2, 4, or 6 weeks after thawing [24, 30].
14. Successful cryopreservation was published also for the ECMs of fir hybrids (*A. alba* × *A. cephalonica*, *A. alba* × *A. numidica*) with pre-culturing on media with 0.4 or 0.8 M sorbitol for 24, 48, or 72 h and addition of 5 % (v/v) DMSO as a cryoprotectant [31].

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Chapter 23

Somatic Embryogenesis in Horse Chestnut (*Aesculus hippocastanum* L.)

Maurizio Capuana

Abstract

Embryogenic cultures of horse chestnut (*Aesculus hippocastanum* L.) can be obtained from different organs and tissues. We describe here the induction from stamen filaments and the procedures applied for the successive phases of somatic embryo development and maturation. Embryogenic tissues are obtained on Murashige and Skoog medium containing 9.0 μM 2,4-dichlorophenoxyacetic acid. Somatic embryos develop after transfer to hormone-free medium enriched with glutamine. Maturation and germination of isolated embryos are achieved by transfer to medium containing polyethylene glycol 4000 and activated charcoal, successive desiccation treatment, and cold storage at 4 °C for 8 weeks.

Key words Anther filament, Conversion, Desiccation, Maturation, Plant growth regulators

1 Introduction

Common horse chestnut (*Aesculus hippocastanum* L.) is one of the 12 species of the genus *Aesculus*, family Hippocastanaceae, that comprises deciduous trees and shrubs distributed in the Northern Hemisphere. There are two Eurasian species, *A. hippocastanum* and *A. chinensis*, var. *chinensis*, both commonly used in medicine. From *A. hippocastanum*, bark, leaves, and seed extract (HCSE) have been used for several medical treatments [1]; among its natural compounds, aescin, a saponin mixture extracted from this species, displays diverse activities, including anti-inflammatory, antiviral, and antioxidative properties [2]. Since the beginning of this century, the number of horse chestnuts with bleeding cankers has increased in Europe [3], highlighting the need to accelerate the release of tolerant genotypes. Vegetative propagation of selected superior trees is, thus, important for both environmental and industrial purposes [4]. Ornamental forms of horse chestnut are generally multiplied by grafting or cuttings [5]. In vitro propagation methods have the advantage of speeding up the multiplication

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process and embryogenesis, in particular, has a huge productive potential to be exploited.

Somatic and gametic embryogenesis have been obtained from different primary explants of horse chestnut, such as microspores [6], anther filaments [7], zygotic embryos [8], leaf segments [9], and stem explants [10]. Embryogenic tissues may also be used for long-term conservation by cryopreservation [11]. In this chapter, a protocol for the induction and development of somatic embryos from flower filaments is described. Compared to other kinds of explants, this material offers the advantage of a lower presence of contaminants and, consequently, an easier in vitro establishment of culture.

2 Materials

2.1 Plant Material

1. Collect flower buds before their opening and preferably from the outer part of the crown of the selected plant(s), where the lower humidity conditions promote the collection of healthier explants; store them at 4 °C until use (*see* **Note 1**).

2.2 Preparation of Culture Media

1. Murashige and Skoog salts and vitamins (MS) [12]; Woody Plant Medium salts and vitamins (WPM) [13] (Table 1).
2. D-Sucrose pure.
3. Agar (B&V, Italy).
4. 2,4-Dichlorophenoxyacetic acid (2,4-D).
5. N_6-benzyladenine (BA).
6. Indole-butyric acid (IBA).
7. 0.1 and 1.0 M KOH solutions.
8. 0.1 and 1.0 M HCl solutions.
9. Glutamine.
10. Polyethylene glycol 4000 (PEG).
11. Activated charcoal (AC).
12. 125, 500 mL glass flasks.
13. 500, 1000 mL beakers.
14. 500, 1000 mL cylinders.
15. Sterile Petri dishes (90 mm in diameter).
16. Tissue culture facilities: Precision balance, magnetic stirrer, magnetic bars, microwave cooker, pH meter, autoclave for sterilization, forceps, scalpels, sterilizer, laminar flow bench, growth chamber, refrigerator.

2.3 Explant Sterilization

1. Tap water.
2. Ethanol (70 %).

Table 1
Plant culture media: formulations of Murashige and Skoog (MS, [12]) and Lloyd and McCown (WPM, [13])

	MS (mg/L)	WPM (mg/L)
KNO_3	1900	–
NH_4NO_3	1650	400
$MgSO_4 \cdot 7H_2O$	370	370
KH_2PO_4	170	170
$CaCl_2 \cdot 2H_2O$	440	96
$Ca(NO_3)_2 \cdot 4H_2O$	–	556
K_2SO_4	–	990
H_2BO_3	6.2	6.2
$MnSO_4 \cdot 4H_2O$	22.3	22.3
$ZnSO_4 \cdot 7H_2O$	8.6	8.6
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.25
$CoCl_2 \cdot 6H_2O$	0.025	–
KI	0.83	–
$FeSO_4 \cdot 7H_2O$	27.8	27.8
$Na_2EDTA \cdot 2H_2O$	37.3	37.3
Sucrose	20,000	20,000
Glycine	2.0	2.0
Pyridoxine·HCl	0.5	0.5
Nicotinic acid	0.5	0.5
Thiamine·HCl	0.1	1.0
Myo-inositol	100	100

3. Sodium hypochlorite solution (bleach solution at 7 g/L active chlorine).
4. Distilled water (autoclaved reverse-osmosis water).
5. 125, 250 mL sterilized glass flasks.
6. 50, 100, 250 mL cylinders.
7. Tissue culture facilities: Forceps, sterilizer, laminar flow bench, growth chamber, refrigerator.

2.4 Laboratory Equipment

1. Precision balance.
2. Magnetic stirrers, magnetic bars.
3. Microwave cooker.
4. pH meter.
5. Autoclave for sterilization.
6. Laminar flow bench.
7. Refrigerator.

2.5 Acclimatization of Plantlets

1. Plastic trays (with 3.5 cm diameter holes).
2. 6–8 cm diameter plastic pots.
3. Potting medium (garden soil, peat, sand, 3:1:1 by volume).
4. Greenhouse equipped with "mist" system.

3 Methods

It is possible to induce somatic embryogenesis on different kinds of explants, such as mature or immature zygotic embryos, portions of leaves, and flower parts. In this chapter we describe the induction of somatic embryogenesis from anther filaments. Using these explants we can start a clonal propagation cycle from a material of identifiable genetic origin, allowing the mass propagation of plants selected for superior traits (shape, vigor, pest and insect resistance, stress adaptability, etc.).

The following protocol, based on the experiences of different authors [14–17], comprises the following stages: (1) culture media preparation; (2) plant material collection and sterilization; (3) somatic embryogenesis induction; (4) somatic embryo development; (5) somatic embryo maturation; (6) somatic embryo conversion; and (7) plantlet acclimatization.

3.1 Culture Media and Conditions

1. Prepare MS and WPM media (Table 1) in double-distilled water, supplemented with 2 % sucrose. Store at 4 °C.
2. Prepare 2,4-D, BA, and IBA stock solution: 2,4-D must be dissolved in a few drops of absolute ethanol. For BA, use 1.0 M KOH. Store at 4 °C.
3. Induction medium: Use MS supplemented with 9 μM 2,4-D.
4. Embryo development medium: Use plant growth regulator (PGR)-free MS medium, containing 400 mg/L glutamine (filter-sterilized).
5. Maturation medium: Use PGR-free MS medium, containing 50 mg/L PEG and 1 g/L AC.

6. For conversion, apply a slow-desiccation procedure by placing the mature somatic embryos, contained in empty and non-sealed Petri dishes, on the laminar flow bench and leave the material under the air flow for 48 h. Then, transfer somatic embryos to conversion medium: WPM supplemented with 2 % sucrose, 0.7 % agar, 0.2 mg/L BA and 0.02 mg/L IBA.
7. Adjust the pH of the media to 5.6 using HCl or KOH (1.0 and 0.1 M).
8. Add agar (0.7 %).
9. Sterilize the media by autoclaving at 121 °C and 108 kPa for 20 min.
10. Store the autoclaved media at 4 °C for a maximum of 60 days.

3.2 Explant Surface Sterilization

1. Cut the filaments and rinse them under slow running tap water for 1 h.
2. Sterilize the laminar flow surface by 70 % ethanol before use.
3. Disinfect the filaments by soaking in 70 % ethanol solution for 2 min, followed by two 2-min rinses in sterile distilled water; disinfect again by soaking in 20 % sodium hypochlorite (1.4 % active chlorine) solution for 20 min, with three final rinses in sterile distilled water, under the laminar air flow and using sterilized glass flasks.

3.3 Somatic Embryogenesis Induction

1. Under the laminar flow bench, pick up the filaments and place them horizontally on the induction medium (20–25 filaments per Petri dish).
2. Incubate the cultures in the growth room (or cabinet) in darkness.
3. After 1 month, transfer the explants onto fresh induction medium.

3.4 Somatic Embryo Development and Embryogenic Tissue Proliferation

1. After 1 month, transfer explants with emerging embryogenic tissues from the induction medium to a PGR-free MS medium containing 400 mg/L of filter-sterilized glutamine (embryo development medium).
2. Incubate cultures at 16-h photoperiod light condition, 60.0 μmol/m^2/s photosynthetically active radiation, for 1 month.
3. Subculture the material at 4-week interval (*see* **Note 2**).

3.5 Somatic Embryo Maturation and Conversion

1. From clusters of maturing somatic embryos (at stage from globular to torpedo, before the developmental phase showed in Fig. 1b), isolate globular embryos ("singularization") and culture them for 4 weeks on MS medium containing 50 g/L PEG and 1 g/LAC (maturation medium) (*see* **Note 3**).

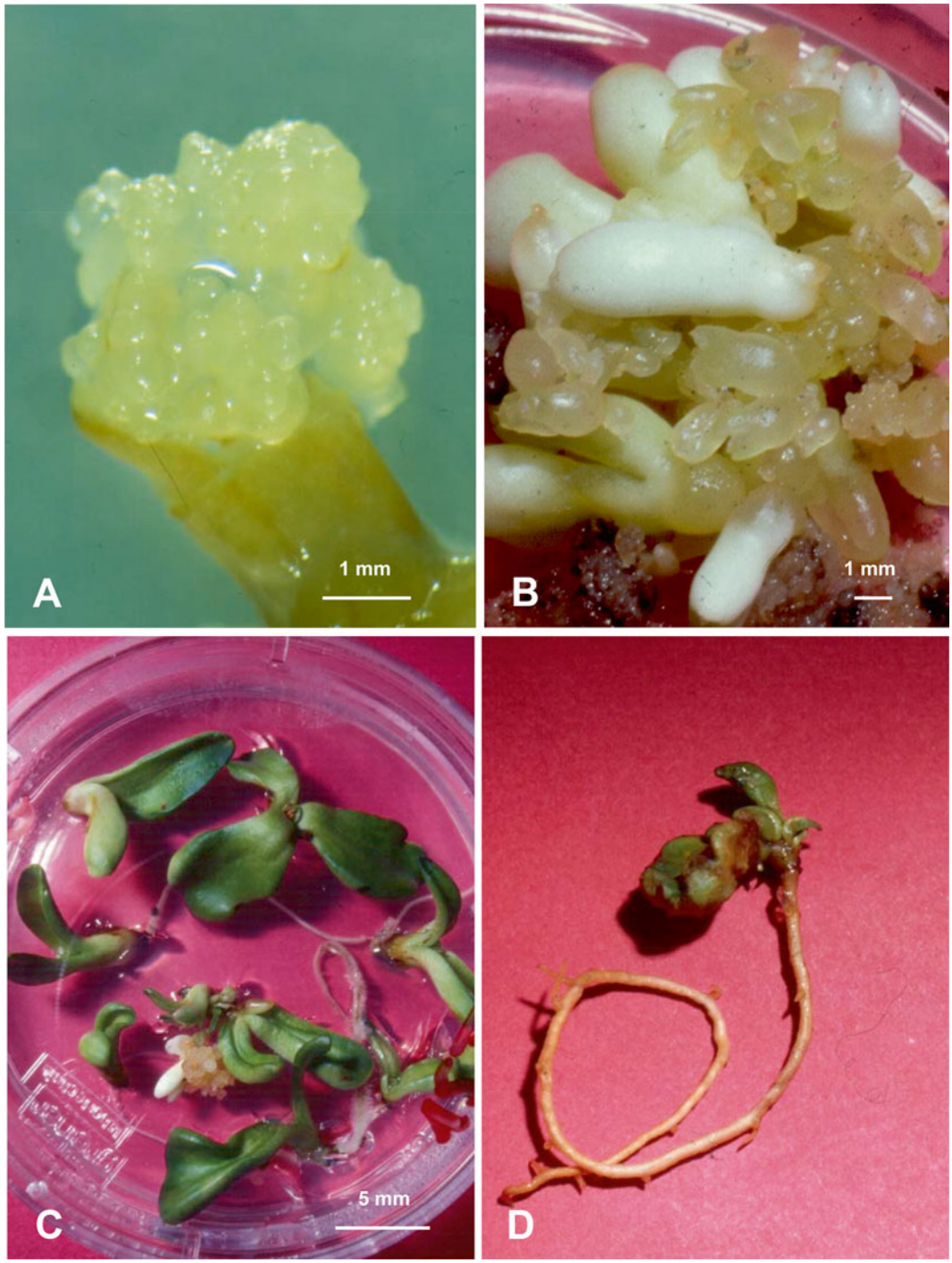

Fig. 1 (**a**) Embryogenic tissues protruding from the anther filament of horse chestnut. (**b**) Cluster of maturing somatic embryos: most embryos are at cotyledonal stage, many others are at stages from globular to torpedo. (**c**) Converting somatic embryos. (**d**) Plantlet ready for transplant to pot and acclimatization

2. For conversion, apply a slow-desiccation procedure, by placing the mature somatic embryos, contained in empty and non-sealed Petri dishes, on the laminar flow bench and leave the material under the airflow for 48 h (Fig. 1c; *see* **Note 4**).
3. Transfer somatic embryo to PGR-free MS medium and store cultures at 4 °C in darkness for 8 weeks.
4. Transfer somatic embryos to conversion medium for a 4-week period.

3.6 Acclimatization

1. Select the converted somatic embryos (i.e., with developing apical pole and roots) and wash the roots under running tap water to remove the adhering solidified culture medium.
2. Insert the plantlets in 35 mm diameter trays, filled with potting mixture and place the trays on a greenhouse bench equipped with a mist system for 3–4 weeks.
3. Move the trays to a non-misted bench under a tunnel covered with plastic foil, where they remain for about 3 weeks to allow a gradual transition to ambient atmosphere.
4. Transplant the plantlets to larger pots (60–80 mm diameter) and place the pots in a shaded area of the nursery fur further growth.

4 Notes

1. Following the indication of Radojevic [5], flower buds must be collected at stage 3–4, when the buds (2–3 mm in length) are completely closed.
2. Generally, embryogenic tissues continue to proliferate after transfer to PGR-free medium This material can be sub-cultured for years showing a very slow decline of proliferation capacity; it is advisable, however, to transfer the cultures onto a BA-containing medium (4.4 μM) after some months of sub-culturing on PGR-free medium.
3. An asynchronous development of somatic embryos may be observed in every phase of maturation (Fig. 1b). It is frequent the development of irregular embryos showing hypertrophy, absence of a well-organized shoot meristem, abnormal cotyledon shapes, or more than two cotyledons.
4. Somatic embryo conversion in horse chestnut can be problematic. Better results can be achieved if, before applying the desiccation procedure, somatic embryos, as illustrated above, are cultured for 4 weeks on medium containing PEG (50 g/L) in combination with AC (1 g/L).

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Chapter 24

Somatic Embryogenesis in *Araucaria angustifolia* (Bertol.) Kuntze (Araucariaceae)

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Abstract

This chapter deals with the features of somatic embryogenesis (SE) in *Araucaria angustifolia*, an endangered and native conifer from south Brazil. In this species SE includes the induction and proliferation of embryogenic cultures composed of pro-embryogenic masses (PEMs), which precede somatic embryos development. *A. angustifolia* SE model encompasses induction, proliferation, pre-maturation, and maturation steps. Double-staining with acetocarmine and Evan's blue is useful to evaluate the embryonic somatic structures. In this chapter we describe *A. angustifolia* SE protocols and analyzes morphological features in the different SE developmental stages.

Key words Conifers, Forest biotechnology, Germplasm conservation, Plant cell culture, Plant physiology, Somatic embryogenesis

1 Introduction

The Brazilian pine *Araucaria angustifolia* (Bertol.) Kuntze (Araucariaceae) is a native conifer with relevant economic importance in Brazil, representing the most exploited timber source until the 1970s [1]. Uncontrolled exploitation of the high-quality wood has led to the species classification as critically endangered in the International Union for the Conservation of Nature and Natural Resources Red Book [2]. In the last years, it has been suggested for *A. angustifolia* conservation integrated ex situ and in situ strategies to conserve genetic resources [3]. In addition, the maintenance of ex situ seed banks is not feasible for recalcitrant seeds, such as *A. angustifolia* requiring the use of in vitro techniques to germplasm conservation [4].

Biotechnological tools have a large potential in breeding and biodiversity conservation programs for woody species [5].

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In this sense, somatic embryogenesis (SE) has been successfully applied for somatic cells and viable embryos obtaining, in a morphogenetic process closely related to the natural process of zygotic embryogenesis (ZE) [6]. SE in *A. angustifolia* is a complex and multifactorial pathway that includes induction and proliferation of embryogenic cultures (EC), composed of proembryogenic masses (PEMs) preceding somatic embryo formation [1, 5, 7]. *A. angustifolia* SE model encompasses two cycles. The cycle A consists in induction, proliferation and pre-maturation steps. Induction is characterized by EC formation in zygotic embryo apex (Fig. 1a), which is retrieved and in vitro cultivated in both auxin and cytokinin presence (Fig. 1b) or in plant growth regulator (PGR)-free culture medium [8–10]. Through double-staining analysis with acetocarmine and Evan's blue, it is possible to identify in the PEMs the presence of two typical conifer cells: embryogenic cells and suspensor-like cells (SCs) [11–13]. During proliferation step, PEMs evolve through three specific developmental stages, PEM I, II, and III, evaluated by the abundance of embryogenic cells and SCs [10–12].

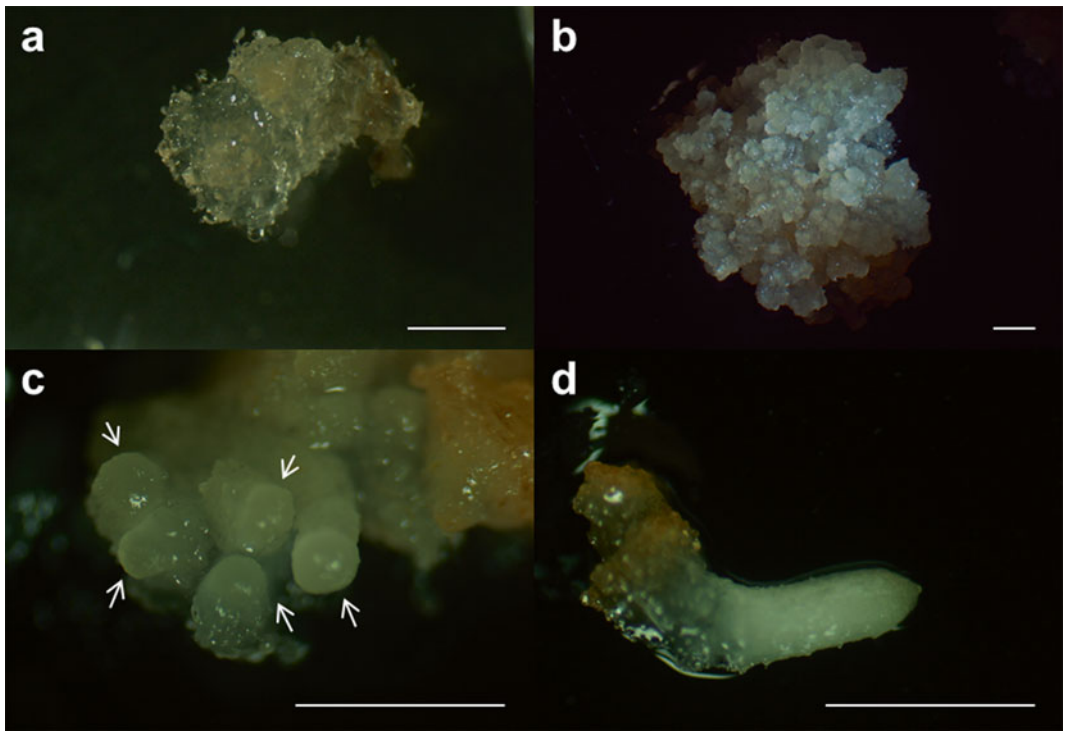

Fig. 1 Morphological aspects of *Araucaria angustifolia* embryogenic cultures. (**a**) Embryogenic callus 30 days after somatic embryogenesis induction. (**b**) Embryogenic callus during multiplication cycles in gelled culture medium. (**c**) Callus with globular-staged somatic embryos during maturation cycle. (**d**) Torpedo-staged somatic embryo after 90 days in maturation culture medium (*arrows* indicate globular-staged somatic embryos). Bar, 2 mm

PEMs-to-early somatic embryo transition is a central event in conifers SE [6]. In *A. angustifolia* SE, pre-maturation step is the starting point of the early SE polarization and individualization from PEM III [7]. The trigger for this process is the PGR removal of culture medium, followed by maltose and PEG supplementation [11, 14]. Early somatic embryos arise when compact clusters of embryogenic cells grow from PEM III with two regions, the dense globular embryonal mass (EM) in the apical part, and suspensor (S) in the basal part [5]. After pre-maturation step, in the cycle B, starts the maturation phase, where early somatic embryos (Fig. 1c) are able to develop in late somatic embryos (Fig. 1d). Late somatic embryos formation can be achieved when the early embryos are capable to respond to the new specific signals with osmotic and hormonal adjustment during maturation step [3, 7, 11]. The initiation of early somatic embryo formation can be observed with the embryonic cell group increase, while the elongated suspensor cells undergo programmed cell death [9, 13–15]. The early somatic embryo development marks the beginning of structural differentiation with the protoderm formation around the early somatic embryo followed by the meristem determination (root and shoot apical meristems). After that, the somatic embryos obtained can be converted into plantlets. Thus, the approach of this chapter is to describe SE protocols and describe morphological features of SE developmental stages in *A. angustifolia*.

2 Materials

Prepare all solutions using distilled water and analytical grade reagents. Prepare all stock solutions at room temperature. All stock solutions can be autoclaved excepting solutions containing vitamins and amino acids.

2.1 Plant Material and Surface Sterilization

1. Immature zygotic embryos of *A. angustifolia* excised of seeds collected from female cones in December.
2. 70 % (v/v) ethanol.
3. 2 % sodium hypochlorite.
4. Sterile distilled water.
5. Glass flasks.

2.2 Stock Solutions of the Induction and Proliferation Culture Medium

1. BM-macrosalt solution [16], 20×: Add about 500 mL of distilled water to a 1000 mL glass beaker. Weigh 12.07 g NH_4NO_3, 18.20 g KNO_3, 2.72 g KH_2PO_4, 4.93 g $MgSO_4{\cdot}7H_2O$, 5.13 g $Mg(NO_3)_2{\cdot}4H_2O$, 1 g $MgCl_2{\cdot}6H_2O$, and 4.72 g $Ca(NO_3)_2{\cdot}4H_2O$, transfer to the beaker, and solubilize. Make up to 1000 mL with water. Store at 4 °C.

2. BM-microsalt solution 200× [16]: Add about 500 mL of distilled water to a 1000 mL glass beaker. Weigh 1.59 g $MnSO_4 \cdot H_2O$, 2.82 g $ZnSO_4 \cdot 7H_2O$, 3.1 g H_3BO_3, 0.83 g Kl, 25 mg $CuSO_4 \cdot H_2O$, 25 mg Na2MoO4·5H2O, and 25 mg $CoCl_2 \cdot 6H_2O$. Transfer to glass beaker, and solubilize. Make up to 1000 mL with water. Store at 4 °C.
3. BM-amino acid solution 100×: Add 5 mL of distilled water to a 10 mL glass beaker. Weigh 1 g L-glutamine, 0.5 g casein, 1 g myoinositol, transfer to beaker and solubilize. Make up to 10 mL with water. Prepare just before use, do not stock.
4. Fe-EDTA solution 20×: Add about 500 mL of distilled water to a 1000 mL glass beaker. Weigh 187.2 mg $Na_2EDTA \times 2H_2O$ and 139 mg $FeSO_4 \times 7H_2O$, transfer to beaker and solubilize. Make up to 1000 mL with water. Store at 4 °C.
5. Vitamins and glycine solution 500×: Add about 500 mL of distilled water to a 1000 mL glass beaker. Weigh 500 mg thiamine HCl, 250 mg pyridoxine HCl, 250 mg nicotinic acid, 1 g glycine, add to the beaker and solubilize. Make up to 1000 mL with water. Store aliquots of 2 mL microtubes at −20 °C.

2.3 Stock Solutions of the Pre-maturation and Maturation Culture Medium

1. MSG-macrosalt solution [17], 20×: Add about 500 mL of distilled water to a 1000 mL glass beaker. Weigh 29 g NH_4NO_3, 38 g KNO_3, 8.8 g $CaCl_2 \cdot 2H_2O$, 3.4 g KH_2PO_4, 7.4 g $MgSO_4 \cdot 7H_2O$, and 14.9 g KCl. Transfer to the beaker and solubilize. Make up to 1000 mL with water. Store at 4 °C.
2. MSG-microsalt solution [17], 200×: Add about 500 mL of distilled water to a 1000 mL glass beaker. Weigh 3.38 g $MnSO_4 \cdot H_2O$, 1.72 g $ZnSO_4 \cdot 7H_2O$, 1.24 g H_3BO_3, 0.16 g Kl, 5 mg $CuSO_4 \cdot H_2O$, 50 mg $Na_2MoO_4 \cdot 5H_2O$, and 5 mg $CoCl_2 \times 6H_2O$, transfer to the beaker, and solubilize. Make up to 1000 mL with water. Store at 4 °C.
3. MSG-amino acid solution 100×: Add about 5 mL of distilled water to a 10 mL glass beaker. Weigh 1.46 g L-glutamine, 0.1 g myoinositol, and transfer to the beaker and solubilize. Make up to 10 mL with water. Prepare just before use, do not stock.
4. Fe-EDTA solution 20×: Use the same solution described in Subheading 2.2.
5. Vitamins and glycine solution 500×: Use the same solution described in Subheading 2.2.

2.4 Other Stock Solutions

1. 1000 μM 2,4-Dichlorophenoxyacetic acid (2,4-D): Weigh 22.10 mg of 2,4-D and transfer to a 100 mL glass beaker. Add 1 mL of NaOH 1 M to dissolve 2,4-D. Make up to 100 mL with water. Store at 4 °C (*see* **Note 1**).

2. 1000 μM 6-benzylaminopurine (BAP): Weigh 22.50 mg of BAP and transfer to a 100 mL glass beaker. Add 1 mL of NaOH 1 M to dissolve BAP. Make up to 100 mL with water. Store at 4 °C (*see* **Note 1**).
3. 1000 μM kinetin (KIN): Weigh 21.50 mg of KIN and transfer to a 100 mL glass beaker. Add 1 mL of NaOH 1 M to dissolve KIN. Make up to 100 mL with water. Store at 4 °C (*see* **Note 1**).

2.5 Culture Medium Supplements

1. Sucrose.
2. Maltose.
3. Phytagel®.
4. Gelrite®.
5. Polyethylene glycol 3350.
6. Polyethylene glycol 4000.
7. Reduced L-glutathione.
8. Abscisic acid (ABA).
9. Activated charcoal.

2.6 Culture Medium Preparation (See Note 2)

2.6.1 Induction Culture Medium (BMi)

1. To prepare 1 L of BMi add 30 g of sucrose to 400 mL of water in a 1000 mL glass beaker and stir on a magnetic stirrer. Add 50 mL of BM-macrosalt stock solution, 5 mL of BM-microsalt stock solution, 5 mL of Fe-EDTA stock solution, 5 mL of 2,4-D stock solution, 2 mL of BAP stock solution, and 2 mL of KIN stock solution.
2. At this step, PGR-free culture medium is also used for SE induction.
3. Add water to just under the final volume of 988 mL. While stirring, adjust the pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 and add 2 g of Phytagel®. Autoclave for 15 min at 121 °C.
4. Wait the autoclaved mixture temperature to reach 40 °C. In the laminar air flow cabinet, add the filter-sterilized (*see* **Note 3**) solution containing 10 mL of BM-amino acid stock solution and 2 mL of vitamins and glycine stock solution. Adjust pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 before filter-sterilization.

2.6.2 Proliferation Culture Medium (BMp)

1. To prepare 1 L of BMp add 30 g of sucrose to 400 mL of water in a 1000 mL glass beaker and stir on a magnetic stirrer. Add 50 mL of BM-macrosalt stock solution, 5 mL of BM-microsalt stock solution, 5 mL of Fe-EDTA stock solution, 2 mL of 2,4-D stock solution, 0.5 mL of BAP stock solution, and 0.5 mL of KIN stock solution.

2. Cultures induced in PGR-free culture medium can be multiplied either in the culture medium described in 1, or in PGR-free culture medium.
3. Add water to just under the final volume of 988 mL. While stirring, adjust the pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 and add 2 g of Phytagel®. Alternatively the EC can be multiplied in liquid medium. Autoclave for 15 min at 121 °C.
4. Wait the autoclaved mixture temperature to reach 40 °C. In the laminar airflow cabinet, add the filter-sterilized (*see* **Note 3**) solution containing 10 mL of BM-amino acid stock solution and 2 mL of vitamin and glycine stock solution. Adjust pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 before filter-sterilization.
5. For gelled culture medium, shake the solution to homogenize the mixture while warming. Distribute the mixture by pouring into sterile 15 mm × 90 mm Petri dishes (1 L of culture medium provides ~40 dishes). Leave the dishes to cool and solidify. Close and seal the dishes with Parafilm®.
6. For liquid culture medium, shake the solution to homogenize the mixture. Distribute 50 mL of the mixture into a sterile 250 mL Erlenmeyer flask. Close and seal with Parafilm®.

2.6.3 Pre-maturation Culture Medium 1 [14]

Recently, two pre-maturation protocols have been described for *A. angustifolia* SE and both of them can be successfully applied [14, 18].

1. To prepare 500 mL of pre-maturation culture medium (MSGpm1), add 45 g of maltose and 35 g of PEG 3350–200 mL of water in a 500 mL glass beaker and stir on a magnetic stirrer. Add 25 mL of MSG-macrosalt stock solution, 2.5 mL of MSG-microsalt stock solution, 2.5 mL of Fe-EDTA stock solution.
2. Add water to just under the final volume of 494 mL. While stirring, adjust the pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8. Autoclave for 15 min at 121 °C in a 750–1000 mL Erlenmeyer flask.
3. Wait the temperature to reach 40 °C and add the filter-sterilized (*see* **Note 3**) solution containing 5 mL of MSG-amino acid stock solution, 1 mL of vitamin and glycine stock solution, and 1.53 g of reduced L-glutathione in the laminar airflow cabinet. Adjust pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 before filter-sterilization. This procedure should be done preferably in the dark to prevent reduced L-glutathione degradation.

4. Shake the solution to homogenize the mixture. Distribute 2 mL of the mixture by pipetting into sterile 12-well culture plate (500 mL of culture medium provides ~20 multiwell culture plates). Close and seal with Parafilm®.

2.6.4 Pre-maturation Culture Medium 2 [18]

1. To prepare 500 mL of pre-maturation culture medium (MSGpm2), add 15 g of sucrose, 35 g of maltose, 45 g of PEG 4000, and 1.5 g of activated charcoal to 200 mL of water in a 500 mL glass beaker and stir on a magnetic stirrer. Add 25 mL of MSG-macrosalt stock solution, 2.5 mL of MSG-microsalt stock solution, and 2.5 mL of Fe-EDTA stock solution.
2. Add water to just under the final volume of 494 mL. While stirring, adjust the pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.7. Add 1.5 g of Gelrite® and autoclave for 15 min at 121 °C in a 750–1000 mL Erlenmeyer flask.
3. In the laminar airflow cabinet, wait the temperature to reach 40 °C and add the filter–sterilized (*see* **Note 3**) solution containing 0.73 g of L-glutamine, and 1 mL of vitamin and glycine stock solution. Adjust pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.7 before filter-sterilization.
4. Shake the solution to homogenize the mixture while warm. Distribute the mixture by pouring into sterile 15 mm × 90 mm Petri dishes (500 mL of culture medium provides ~20 dishes). Leave the dishes to cool and solidify. Close and seal the dishes with Parafilm®.

2.6.5 Maturation Culture Medium

1. To prepare 1 L of maturation culture medium (BMm), add 90 g of maltose, 70 g of PEG 3350 and 1.5 g of activated charcoal to 400 mL of water in a 1000 mL glass beaker and stir on a magnetic stirrer. Add 50 mL of BM-macrosalt stock solution, 5 mL of BM-microsalt stock solution, and 5 mL of Fe-EDTA stock solution.
2. Add water to just under the final volume of 988 mL. While stirring, adjust the pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 and add 2 g of Phytagel®. Autoclave for 15 min at 121 °C.
3. Wait the temperature to reach 40 °C and add the filter-sterilized (*see* **Note 3**) solution containing 31.7 mg of ABA (*see* **Note 4**), 10 mL of BM-amino acid stock solution, and 2 mL of vitamin and glycine stock solution in the laminar flow cabinet. Adjust the solution pH by adding 0.5 M NaOH or 0.5 M HCl solution to reach a pH of 5.8 before filter-sterilization.
4. Shake the solution to homogenize the mixture while warm. Distribute the mixture by pouring into sterile 15 mm × 90 mm Petri dishes (1 L of culture medium provides ~40 dishes). Leave the dishes to cool and solidify. Close and seal the dishes with Parafilm®.

2.7 Cytochemical Analysis

1. 2 % carmine: Dissolve 2 g carmine in 100 mL acetic acid 45 % (v/v). Boil in reflux condenser for 3 h. Cool at room temperature and filter with filter paper.
2. 0.05 % (w/v) Evan's blue: Dissolve 1 g Evan's blue in 100 mL distilled water.
3. Slides and cover glass.
4. Light microscope.

2.8 Other Useful Materials

1. Scalpel, forceps.
2. Spirit burner.
3. Magnetic stirrer.
4. pH meter, autoclave.
5. Bottles, Petri dishes, Erlenmeyer flasks, glass beaker, 12-well culture plate.
6. Parafilm®.
7. Syringe, sterile syringe filters Chromafil®, filter paper.
8. Analytical balance.
9. 0.5 N sodium hydroxide (NaOH), 0.5 N hydrochloric acid (HCl).
10. Incubator chamber, laminar flow cabinet, stereomicroscope.

3 Methods

All the procedures described below must be performed in laminar flow cabinet, with sterilized instruments.

3.1 SE Induction

1. Only immature seeds of *A. angustifolia* with globular-staged zygotic embryos are used, in order to induce SE. Surface sterilize seeds in a glass beaker with 70 % ethanol for 5 min. Remove ethanol and add 2 % sodium hypochlorite for 20 min. Remove sodium hypochlorite and wash seeds three times with autoclaved distilled water. All solutions must be added in enough volume to cover the seeds into the beaker.
2. With the aid of a stereomicroscope, scalpel, and forceps on a sterilized Petri dish, excise the immature zygotic embryo and inoculate into the induction culture medium. Cultures are maintained in BOD incubator chamber at 24 ± 2 °C.

3.2 EC Proliferation

After 30-day culture in BMi culture medium, EC is generally obtained. During proliferation step, EC are composed by PEMs, maintained in repetitive multiplication cycles for an undetermined period of time. At this point, EC proliferation can be achieved with or without PGR supplementation. Proliferation can also be performed in gelled or liquid culture medium.

1. To perform the subculture for gelled BMp culture medium, friable and translucent EC should be removed from the BMi medium with the aid of a forceps and transferred to fresh gelled BMp culture medium. Colonies of cells should be mixed during the process of subculture to promote uniform distribution of nutrients contained in culture medium. The subculture procedure must be performed every 21 days to fresh gelled BMp culture medium and can be done indefinitely. Cultures are maintained in BOD incubator chamber at 24 ± 2 °C.
2. For liquid BMp culture medium, about 500 mg of friable and translucent EC should be removed from the BMi medium with the aid of a forceps and transferred to a fresh liquid BMp medium. The subculture procedure must be performed every 15 days to a fresh liquid BMp culture medium and can be done indefinitely. This procedure is realized with the aid of "Cell Dissociation Sieve" (Sigma-Aldrich), 80 mesh screens. Capture the EC by pouring the culture medium with EC in proliferation in the "Cell Dissociation Sieve." With the aid of a forceps, take 500 mg of EC and transfer to a new flask. Cultures are maintained in an orbital shaker at 90 rpm, at 24 ± 2 °C in the dark.

3.3 EC Pre-maturation 1

Pre-maturation is an important step in conifers SE, and it was recently applied to *A. angustifolia* protocol [14, 18]. In this step, the transition of PEMs to early somatic embryos is observed.

1. After proliferation step, repeat the same procedure described above (*see* Subheading 3.2) to capture the EC. Transfer about 50 mg of EC with the aid of a forceps to a 12-well culture plate containing 2 mL MSGpm1 per well.
2. The plates should be incubated in an orbital shaker at 90 rpm in the dark. Cultures are maintained in a growth room at 24 ± 2 °C for 15 days.

3.4 EC Pre-maturation 2 [18]

1. After proliferation step, repeat the same procedure described above (Subheading 3.2, **step 2**) to capture the EC. Transfer 100 mg of EC with the aid of a forceps to a sterile filter paper disc (Ø 80 mm). Transfer the filter paper with the cultures to Petri dish containing MSGpm2 culture medium.
2. Cultures are maintained in a growth room at 24 ± 2 °C for 30 days.

3.5 Early Somatic Embryo Maturation

1. For somatic embryos maturation, about 500 mg of EC containing early somatic embryos is transferred with the aid of a forceps to BMm culture medium.
2. Petri dishes are maintained in BOD incubator chamber at 24 ± 2 °C for 60 days. One subculture should be performed at day 30 in culture to a fresh BMm culture medium.

3.6 Morphological and Cytochemical Analysis Procedure

The quality of cultures is evaluated by double staining under light microscope based on acetocarmine and Evan's blue staining [19]. This double-staining analysis reveals the presence of the two typical embryonic conifer structures: the embryogenic cells, which are isodiametric and densely cytoplasmic, reacting in red with acetocarmine, and the suspensor-like cells, which are vacuolated and reacts in blue to Evan's blue [20].

1. Take an aliquot of 50 mg of EC and transfer to a watch glass.
2. Add a drop of 1 % acetocarmine (w/v) to the sample, gently mix, and wait for 1 min.
3. Carefully remove the acetocarmine with the aid of toilet paper.
4. Drop 0.05 % Evan's blue (w/v) to the sample, gently mix and wait 1 min.
5. Carefully remove the Evan's blue with the aid of toilet paper.
6. Drop 1 mL of sterile distilled water.

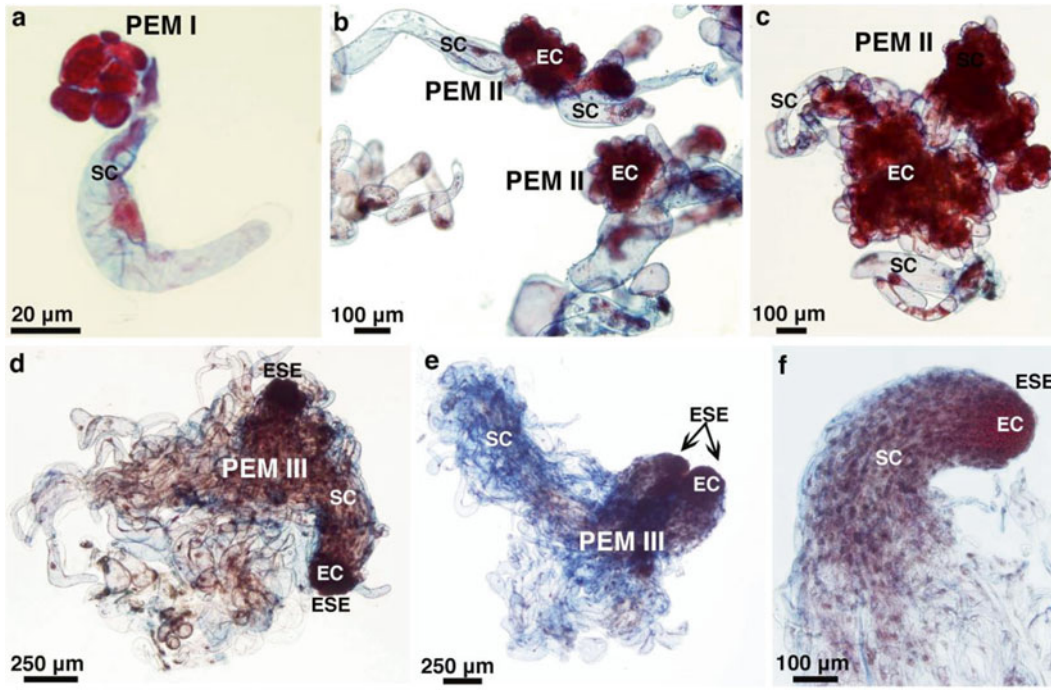

Fig. 2 *Araucaria angustifolia* embryogenic cultures morphological and cytochemical analysis with acetocarmine and Evan's blue. (**a**–**c**) Proembryogenic masses at PEM I stage (**a**), PEM II stage (**b**) and PEM III stage (**c**). (**d**) PEM III-staged embryogenic cells starting polarization and individualization process. (**e**) Early somatic embryos (ESE) individualized and polarized. (**f**) Globular-staged ESE. EC, embryogenic cells stained with acetocarmine; SC, suspensor-like cells stained with Evan's blue

7. Drop with a pipette an aliquot on a slide glass, and then visualize in the light microscope.
8. Analyze and quantify the presence of PEM I (Fig. 2a), PEM II (Fig. 2b), PEM III (Fig. 2c), and early somatic embryos (Fig. 2e, f) as well as the presence of SCs (Fig. 2a) and embryogenic cells (Fig. 2a, b).

4 Notes

1. The PGR stock solutions can be autoclaved for 15 min to decrease bacterial and fungal contamination, and improve the solubilization.
2. Culture medium should be prepared at least 3 days before the inoculation procedure. This is the required period to ensure that there was no fungal or bacterial contamination during the culture medium preparation.
3. Filter-sterilization is made with the aid of a syringe and sterile Syringe filters Chromafil® (Macherey-Nagel), with PTFE membrane, 0.20 μm pore size into the laminar flow cabinet.
4. Abscisic acid cannot be maintained in stock solution. Weigh the abscisic acid with the aid of a analytical balance, add 200 μL of NaOH 1 M to dissolve ABA, and then add the vitamins, amino acids, or other stock solutions you need to filter-sterilize.

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Part IV

Protocols of Gametic Embryogenesis in Selected Higher Plants

Chapter 25

Anther Culture in Eggplant (*Solanum melongena* L.)

Giuseppe Leonardo Rotino

Abstract

The technique of in vitro anther culture is the most favorite to incite the production of plants from microspore through direct embryogenesis or regeneration from callus. Anther culture has been employed since 1980s in eggplant to obtain double-haploid plants from microspore derived embryos. From that time it has been refined and widely applied both at commercial level for a fast generation double-haploid parental lines of F1 hybrids, as well as for experimental studies as the complete homozygosis of the microspore-derived plants make more simply the genetic analysis. In this chapter, a step-by-step procedure is reported, taking into consideration all the aspects of the technique, including the growth condition of the anther donor plant, the in vitro regeneration of the androgenetic plantlets, their ploidy analysis, and the colchicine treatment to double the chromosome number of the haploids.

Key words Androgenesis, Double-haploid, Haploid, Tissue culture, Microspore

1 Introduction

Haploidy through natural parthenogenesis has never been observed in eggplant. The first haploids in eggplant were obtained by the "Chinese Research Group of Haploid Breeding" [1] and by Isouard et al. [2]. Then, Dumas de Vaulx and Chambonnet [3] and Chambonnet [4] greatly improved the yield of in vitro anther derived plantlets by using a method similar to the one applied to pepper [5]. This protocol is based on a high temperature (35 °C) treatment during the first period of anther culture. Studies on eggplant isolated-microspore culture have been carried out and plantlets were regenerated from microspore-derived callus following either anther pre-culture [6] or direct culture of microspores [7, 8]. However, no consistent technique has been so far published about a direct regeneration of plantlets at a satisfactory rate through embryogenesis from isolated microspore culture.

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Dumas de Vaulx and Chambonnet method [3] resulted in a reliable protocol to produce pollen-derived plants, enabling a successful integration of doubled haploid lines in eggplant breeding programs. Subsequent minor modifications of this method allowed to further enlarge the usefulness of in vitro androgenesis for the production of modern eggplant varieties [9]. According to this method, excised anthers are cultured in the induction medium (C), supplemented with appropriate plant growth regulators (PGR), and placed in the darkness at 35 °C for 8 days of culture. In the following days, Petri dishes are kept in the growth chamber at 25 °C under a 16-h illumination (50 μmol/m^2/s, fluorescent light). On the 13th day, anthers are transferred to the differentiation medium (R). Generally, embryos become visible from the anthers after 1 month from the beginning of culture and the embryo production lasts for 3–4 months. Well-formed embryos of 4–6 mm are transferred to the PGR-free medium (V3) for further development. Complete plantlets can easily be propagated in vitro by cuttings, using the apical bud with 3–4 nodes, and transferred to soil. Hereafter, a slightly modified Dumas de Vaulx protocol [3], regarding the PGR composition of the culture media, is described, together with advices on the various steps of the procedure. Pluriannual observations about the response of different genotypes evidenced that some of them were able to regenerate androgenetic plants only in a medium containing specific PGR combinations. For this reason, it is suggested to try simultaneously alternative induction and regeneration media, especially for novel donor material or segregating progenies (Table 1).

Table 1
Sucrose content and PGR composition of the induction (C3, C6, C9, and C13) and regeneration media (R1K and R1Z) media for anther culture of eggplant (plus 8 g/L agar; pH 5.9 before autoclaving. KIN, kinetin; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; NAA, 1-naphthaleneacetic acid; ZEA, zeatin)

	Induction (C)				Regeneration (R)	
Compounds	**C3**	**C6**	**C9**	**C13**	**R1K**	**R1Z**
Sucrose (g/L)	30	30	30	120	30	30
KIN (mg/L)	3	5	–	5	0.1	–
2,4-D (mg/L)	–	–	–	5	–	–
IAA (mg/L)	1	–	–	–	–	–
NAA (mg/L)	–	5	3	–	–	–
ZEA (mg/L)	–	–	1	–	–	0.1

2 Materials

2.1 Plant Materials

The highly responsive cv Dourga can be employed to start practicing the protocol, with plants preferably grown in greenhouse. However, also donor plants from the open field can be used.

2.2 Equipment

1. Laminar flow hood.
2. Sterile Whatman or blotting paper.
3. Forceps, scalpels and spatulas.
4. Petri dishes, 60 mm diameter.
5. Glass or plastic jars (e.g., 250 and 500 mL, Magenta box).
6. Autoclave.
7. Plastic wrap film.
8. Stereo and optical fluorescence microscopes.
9. Microscope slides and cover slips.
10. Incubator at 35 °C.
11. Stainless steel tea mesh infuser spoon.
12. Growth chambers.
13. Flow cytometer.

2.3 Solutions and Media

1. Tween 20 and dish soap.
2. 80 % ethanol.
3. Sodium hypochlorite.
4. Sterile deionized water.
5. TRIS.
6. Triton X-100.
7. Induction (C), regeneration (R), and multiplication (V3) media (Tables 1 and 2).
8. Acetocarmine [1 g carmine, 100 mL glacial acetic acid (45 %), 5 mL $FeCl \cdot 6H_2O$].
9. Lysis solution for leaf mesophyll nuclei extraction.
10. Staining solution for nuclear DNA (Partec).
11. FDA stock solution: Fluorescein-diacetate dissolved into acetone (5 mg/mL).
12. Colchicine.
13. Lanoline.

Table 2
Macronutrients, micronutrients, and vitamins of the three basal media utilized for eggplant anther culture (mg/L)

Macroelements[a]	Media		
	C	R	V3
KNO_3	2150	2150	1900
NH_4NO_3	1238	1238	1650
$MgSO_4 \cdot 7H_2O$	412	412	370
$CaCl_2 \cdot 2H_2O$	313	313	440
KH_2PO_4	142	142	170
$Ca(NO_3)_2 \cdot 4H_2O$	50	50	–
$NaH_2PO_4 \cdot H_2O$	38	38	–
$(NH_4)_2SO_4$	34	34	–
KCl	7	7	–
Microelements[a]			
$MnSO_4 \cdot H_2O$	22.130	20.130	0.076
$ZnSO_4 \cdot 7H_2O$	3.625	3.225	1.000
H_3BO_3	3.150	1.550	1.000
KI	0.695	0.330	0.010
$Na_2MoO_4 \cdot 2H_2O$	0.188	0.138	–
$CuSO_4 \cdot 5H_2O$	0.016	0.011	0.030
$CoCl_2 \cdot 6H_2O$	0.016	0.011	–
$AlCl_3 \cdot 6H_2O$	–	–	0.050
$NiCl_2 \cdot 6H_2O$	–	–	0.030
Vitamins and amino acids			
Myoinositol	100.00	100.00	100.00
Pyridoxine HCl	5.500	5.500	5.500
Nicotinic acid	0.700	0.700	0.700
Thiamine HCl	0.600	0.600	0.600
Calcium pantothenate	0.500	0.500	0.500
Vitamin B12	0.030	–	–
Biotin	0.005	0.005	0.005
Glycin	0.100	0.100	0.200
Chelated iron			
Na_2 EDTA	18.65	18.65	37.30
$FeSO_4 \cdot 7H_2O$	13.90	13.90	27.80

[a]As reported in [4]

3 Methods

3.1 Anther Donor Plants and Choice of Floral Buds

1. Generally, healthy and vigorous eggplants provide anthers with the highest androgenetic potential. It is important to prevent seed-setting and plant aging by removing open flowers and small fruits. It is important to control insect and mite attacks; however anthers should be collected several days after spraying pesticide (*see* **Note 1**).
2. Detach from the donor plants the flower buds with, roughly, the upper fused edge of the sepal, almost of the same height of the petals (Fig. 1, *see* **Note 2**). This stage of bud development ensures that a large part of the microspores is at the uninucleate or the very early binucleate stage of development which, generally, gives better results. Such evidences have been recently confirmed by Salas et al. [10], which demonstrated that vacuolate microspores and young bicellular pollen are the most responsive stages when microspores are directly cultured in liquid medium, whereas cultured anthers, mostly containing microspores at these stages, displayed a reduced or even null androgenetic response. The authors ascribed such discrepancy to a delayed contact of the media components with the microspores which became too old and lose their androgenic inducible state because of the time needed by the active substances to reach the anther locule.

3.2 Sterilization and In Vitro Culture

1. Collected flower buds are briefly immersed and gently stirred into a solution of demineralised water with a few drops of Tween 20 or dish soap, followed by 30 s in a solution of 80 % ethanol.

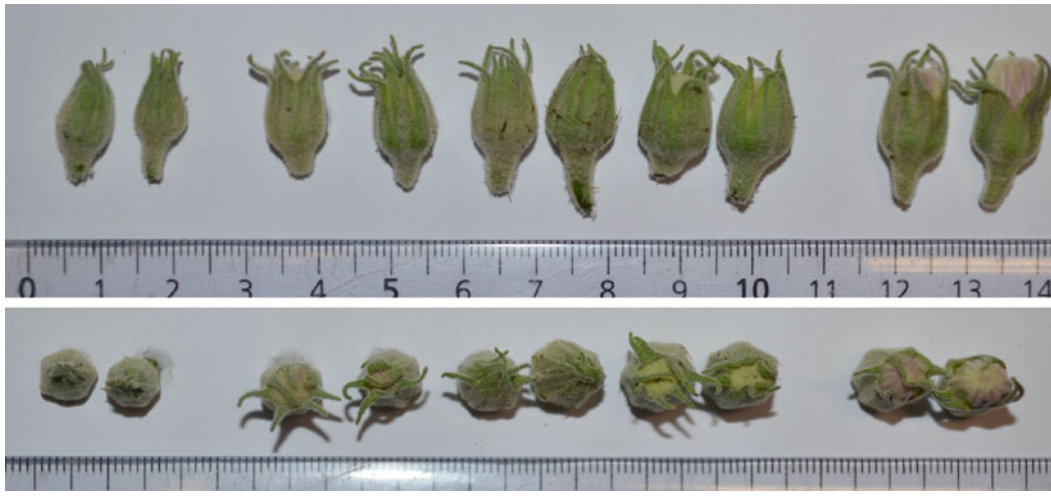

Fig. 1 Flower buds at different developmental stages. The six buds in the *middle* are suitable for anther culture, whereas the two buds on the *right* and the two buds on the *left* are, respectively, too young and too old

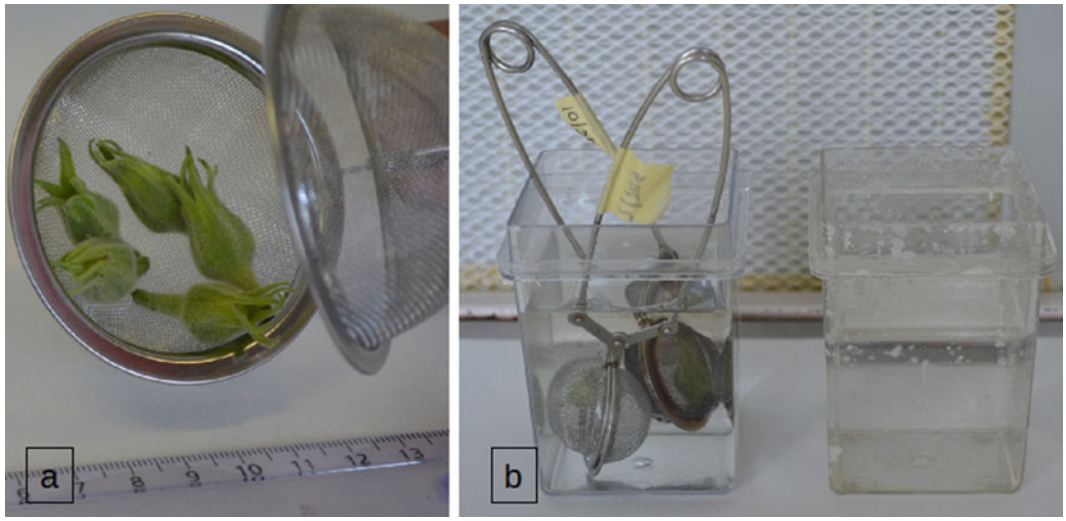

Fig. 2 Buds sterilization. (**a**) Buds inside the steel tea mesh infusers. (**b**) Tea mesh containing the buds immersed in the chlorine sterilization solution next to a Magenta box filled with water for the first washing

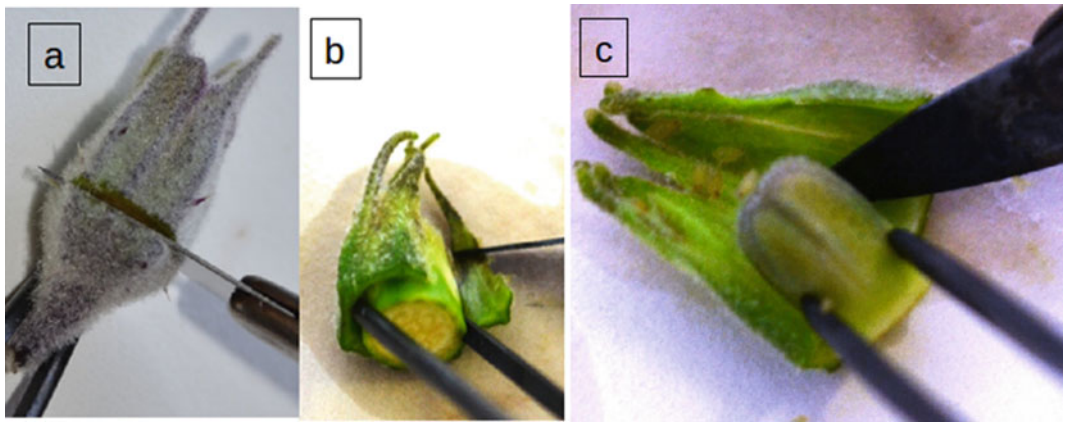

Fig. 3 Extraction of the petal cone containing the anthers from the buds. (**a**) Transversal cut of bud below the anther. (**b**) Longitudinal cut of the sepal. (**c**) Extraction of the petal cone containing the anthers

Then, in the laminar flow hood, the floral buds are immersed for 20 min in a solution of 30 % commercial bleach (1 % active chlorine), and finally rinsed three times with sterile demineralized water (3–4 min for each washing) (Fig. 2, *see* **Note 3**).

2. The flower buds are placed over a sterile paper to excise the anthers by using a scalpel and a forceps (Figs. 3 and 4). Plate, in a 60 mm Petri dish, 10–12 anthers with their concave (external) zone onto medium C (Fig. 4d). The Petri dishes are sealed using household plastic wrap. Keep plated anthers at 35 °C in the darkness for 8 days (*see* **Note 4**).

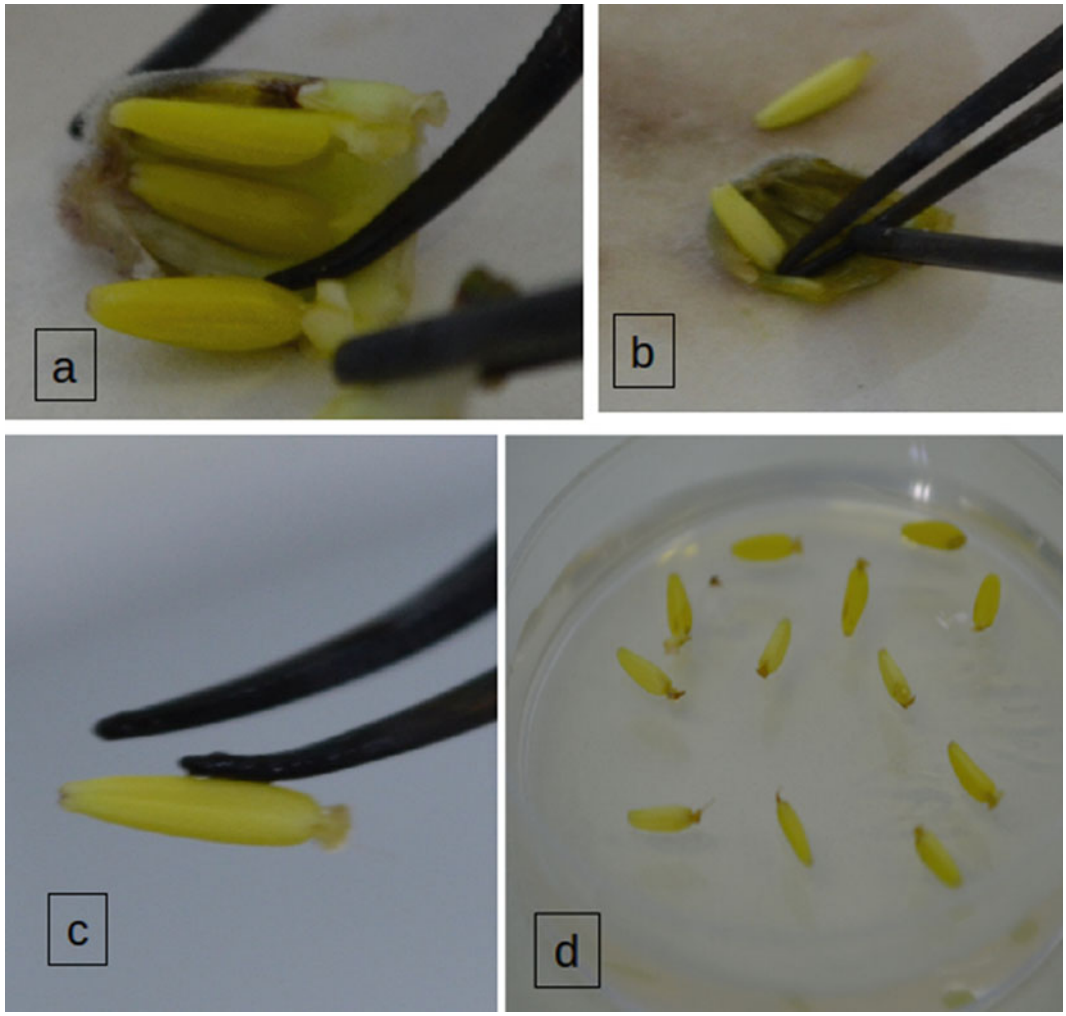

Fig. 4 Extraction and plating of the anthers. (**a**) Petal cone cut and opened. (**b**) Excision of anther by pushing to its filament. (**c**) Anther stuck with forceps. (**d**) Anthers plated in the C medium

3. Transport and keep the anthers at 25 °C, 16-h photoperiod (50 μmol/m^2/s).
4. After 4 days transfer the anthers to R medium (*see* **Note 5**).
5. Every 5–6 weeks transfer anthers to fresh R medium (*see* **Note 5**).
6. Move the embryos sprouting out from the anthers to a 60 mm Petri dish, containing either the V3 medium, if they have a well formed principal root, or the R medium in the case the embryos are younger (3–4 mm) (*see* **Note 6**).
7. Well formed in vitro plantlets, with good root system and foliage, are acclimatized under growth chamber condition (16-h light at 22 °C, 8-h dark at 18 °C, light intensity ~200 μmol/m^2/s) by transplanting into pots (Fig. 5, *see* **Note 7**).

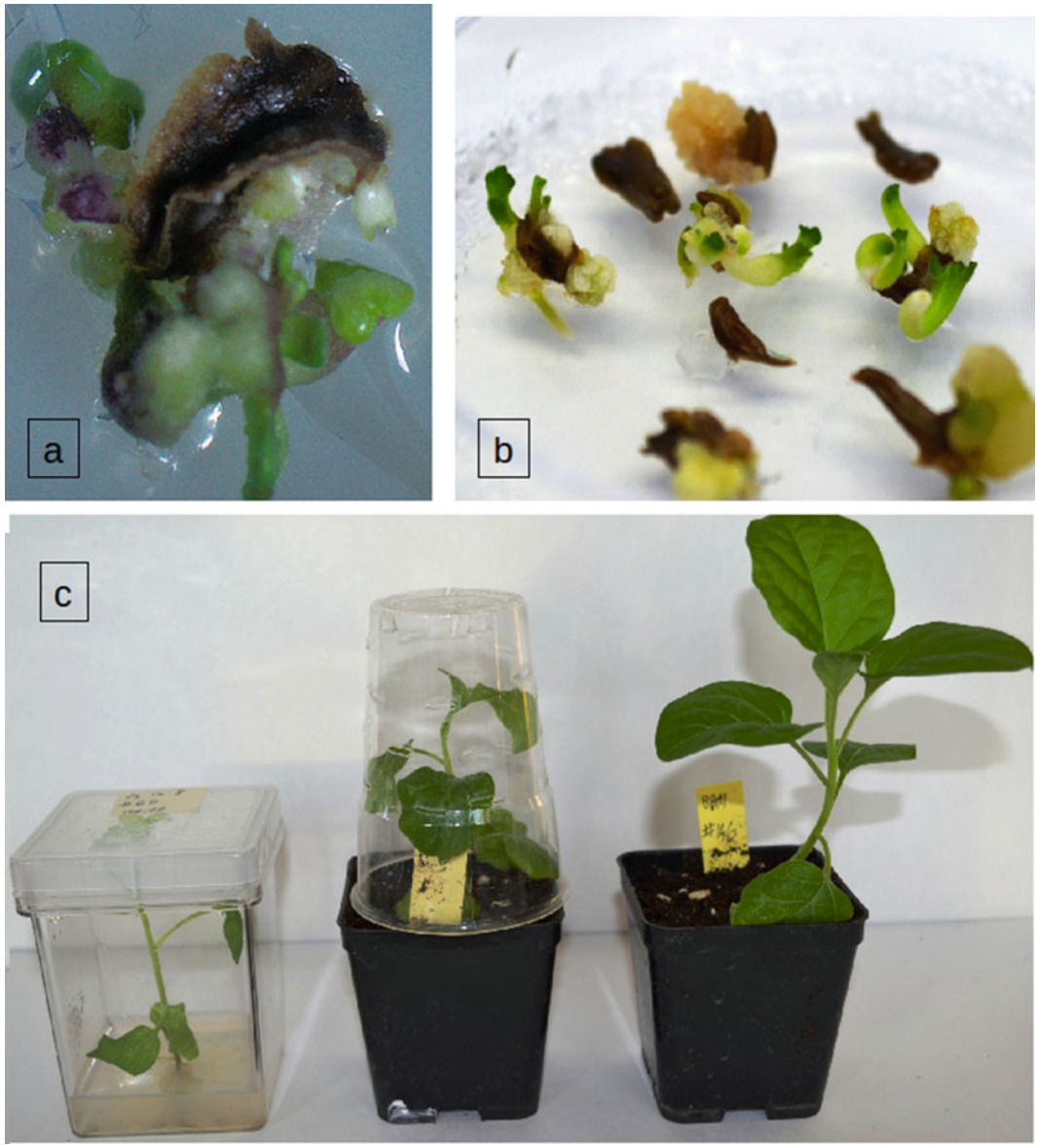

Fig. 5 From androgenetic embryos to plantlets. (**a**) An anther cultured in the R medium giving rise to several microspore-derived embryos at different developmental stages. (**b**) Cultured anthers producing embryos or callus. (**c**) From *left* to *right*: rooted androgenetic plants in the V3 medium ready to be transferred to soil, freshly plantlet in ex vitro condition, and acclimatized plantlets, ready to be transferred to the greenhouse

8. After 1–2 weeks, acclimatized plants can be moved to the greenhouse, keeping them under shadow if the temperature and light intensity are high.

3.3 Ploidy Determination

Analysis of ploidy determination can be performed through direct quantification of nuclear DNA content by flow cytometer, or indi-

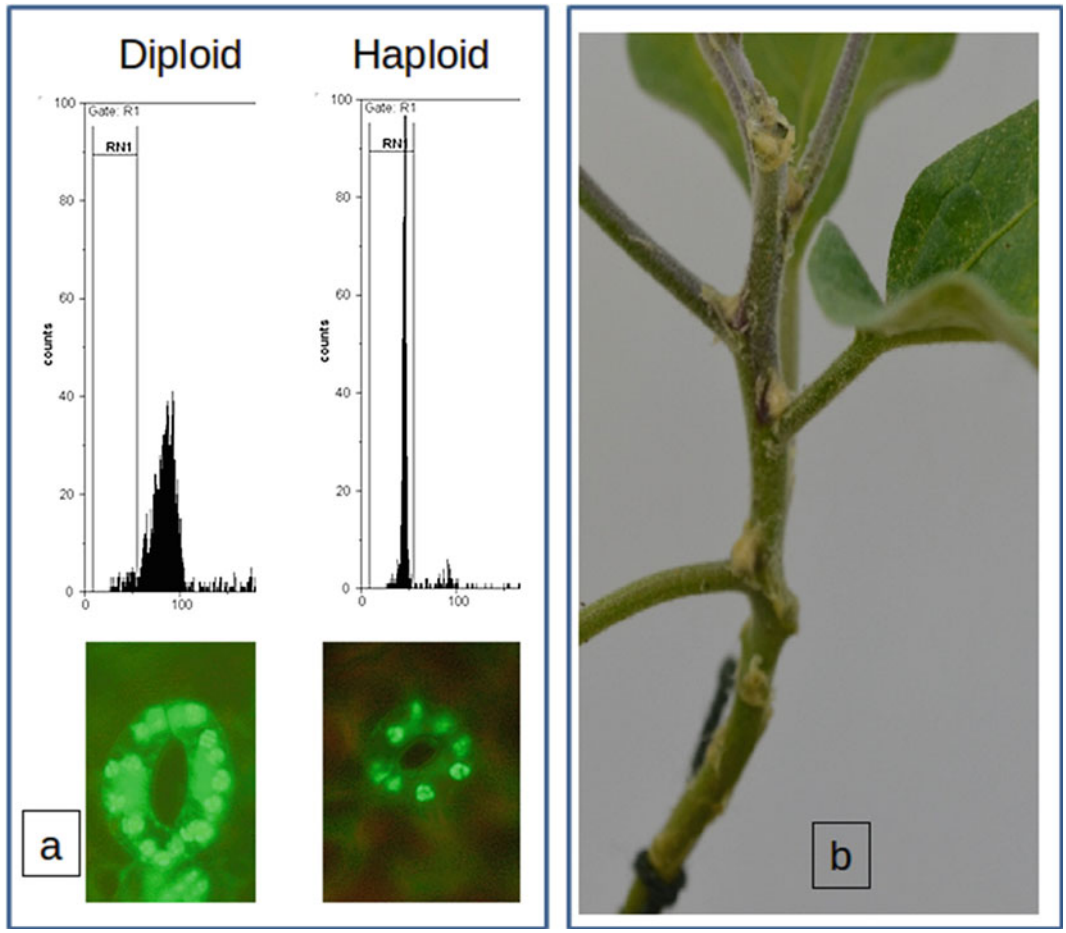

Fig. 6 Ploidy determination and colchicine treatment. (**a**) Flow cytometric determination of DNA content in the nuclei and number of chloroplasts in the stomata guard cells of a diploid and haploid androgenetic plant, obtained from the same anther donor. (**b**) Lanoline paste containing 0.5 % colchicine, applied to the secondary axillary buds of a haploid plant

rectly by counting the number of chloroplasts in the stomata guard cells (Fig. 6a). Leaves from in vitro rooted plantlets are the best material for both the analyses (*see* **Note 8**).

3.3.1 Flow Cytometry Analysis According to the Manufacturer Protocol (E.g., Partec CyFlow, SL) (see ***Note 9****)*

1. About 0.5 cm^2 of a young leaf of an in vitro grown plant is chopped in the extraction buffer in a small Petri dish.
2. Filter (30 μm mesh) to enrich the solution with the nuclei; add the staining buffer containing the fluorophore (DAPI, Fluorescein, etc.) and keep at 4 °C for 1 h.
3. Start the analysis.

*3.3.2 Chloroplast Counting in Stomata Guard Cells (See **Note 10**)*

1. Cut a piece of 0.3–0.5 cm^2 of leaf and put it on the microscope slide with the lower leaf lamina upward.
2. Wet the lower epidermal with 1–2 drops of FDA stock solution diluted in water, add the cover slip, and wait for a maximum of 20–30 s.
3. Observe under a fluorescent light. The chloroplast will appear green-colored in the stomata cells (*see* **Note 10**).

3.4 Diploidization of the Haploid Plants

Haploid plants, apart specific employment (e.g., basic research), need to be treated to restore their diploid status, so that selfed seeds can be obtained and the double-haploid lines established. Chromosome doubling may be performed in several ways. Here it is described the treatment of the secondary axillary buds with colchicine in the already acclimatized plantlets which, generally, ensure that 50–70 % of the treated plants will produce seeds after selfing.

1. Use preferably fast growing plantlets with 4–8 leaves. Trim the apical bud (to suppress apical dominance) and remove the axillary buds with a scalpel.
2. Dissolve colchicine (0.5 %) in lanoline paste.
3. Apply with the aid of a spatula the lanoline-containing colchicine to the secondary axillary buds.
4. Keep the treated plantlets in the dark for 48 h and then transfer them back to greenhouse conditions.
5. Remove the shoots produced by the untreated buds (e.g., those below the soil level) (Fig. 6b, *see* **Note 11**).

4 Notes

1. Avoid excessive fruit setting, especially the presence of mature and overripe fruits. Unfortunately, it is not known the environmental conditions that allow to maximize the androgenetic response of the anther donor eggplant. Seasonal variation has been observed in the yield of eggplant androgenetic embryos. It has been reported [11] that during the period July–October (in the Northern Hemisphere), the highest number of responding anthers were found from the middle of September until the middle of October. These results are in accordance with our observations that, in the Mediterranean climate, higher androgenetic frequencies are obtained during cooler months, and the best periods are spring and autumn (unpublished). Most likely, the photoperiod, as well as the day/night temperature, affects anther response. More precise information could be obtained by growing donor plants in a phytotron.
2. For beginners, it is suggested to check the exact stage of microspore development during the various step of the flower bud

growth. This is important because the sepal coverage of the petal strongly varies among the different eggplant genotypes; it is influenced by plant ageing and is also affected by the environment. Anyway, due to this appreciable variation, it is suggested to collect also flower buds either slightly larger, or slightly smaller than the ones considered of optimal size. For the cytological analysis of the microspore stage: squash one anther on freshly prepared DAPI-TRIS buffer (TRIS buffer: 0.05 M Tris–HCl, 0.5 % Triton X-100, 5 % sucrose, pH 7) and observe under UV light. Alternatively, the anther can be squashed in acetocarmine and observed under optical microscope.

3. The use of stainless steel spoons (like the ones used as tea mesh infuser) makes very easy the sterilization of flower buds, as it is only necessary to move the spoon from one jar to the next (Fig. 2). This ensures that the whole flowers are completely immersed in the sterilization and washing solutions. After washing, the buds are kept into a Petri dish, or left in the in tea mesh infuser within the empty jar.
4. Extract the anthers by cutting transversally the flower bud at its base, in correspondence of the anther filament or slightly below (Fig. 3a). Then, cut longitudinally only the sepal and extract the petal cone with inside the anthers still joined together (Fig. 3b, c). Cut and open the petal cone and excise the anthers by pushing with the tip of the forceps to the anther filament (Fig. 4a, b), so avoiding to touch the anther. To place the anthers onto the medium C, it is advisable to immerse one tip of the forceps into the medium as the small amount of agar-medium remaining on the tip allows to stick the anther (Fig. 4c). The anther is placed in the 60 mm Petri dish containing medium C (Fig. 4d). It is extremely important to manipulate very gently the floral buds and, especially, the anther, avoiding squeezing or excessive pressure, because the wounds stimulate proliferation of somatic callus which, in turn, reduces the androgenetic response. Detaching the whole filament it is also important; however, it is better to leave the filament rather than to risk damaging the anther, as the filament can be easily removed when the anther will be subcultured from medium C to medium R.
5. Use the R medium which contains the same cytokinin of the C medium (i.e., use R1Z for the anthers plated onto C9 medium and R1K for C3, C6, and C13 media; *see* Table 1). Remove the portion of the filament which remains attached to the anther, as well as the somatic callus developed from the anther tissue.
6. The well-formed embryos obtained in the R medium are then transferred to V3 medium in Petri dishes as soon as they are germinated. Plantlets are moved to a bigger container (jars or

Magenta box). It is also advisable to make a backup (cuttings) of each androgenetic plantlet by subculturing the apical shoot with 2–3 nodes in V3 medium.

7. Gently wash out the roots from the agar and transplant the plantlets in pots (maximum 6 cm diameter) filled with a mixture of peat (65 %), perlite (25 %), and sand (10 %). To ensure the acclimatization of plantlets to the ex vitro conditions, the freshly potted plantlets are maintained under high humidity condition by covering each potted plantlet with a plastic cup (Fig. 5c), having 3–4 holes; the cup is then progressively removed from the pot to reduce gradually the humidity to the one of the growth chamber. Otherwise the freshly potted plantlets are put in a case sealed with a transparent plastic sheet, with holes in the top, which will be progressively removed in 10–14 days.
8. A certain percentage of plantlets will be diploid, and they can be promptly employed in the breeding program, without the need to double their ploidy. Diploidization is generally caused by spontaneous chromosome doubling during the first microspore division. Molecular analyses, using polymorphic heterozygous loci markers (SSR, SNP) in the anther donor, may be performed to confirm their gametophytic origin (i.e., having all the loci at the homozygous state, *see* Fig. 7). It is advisable to make this analysis if you are not completely sure that the plantlets originated from a well-formed embryo. In fact, the media employed for anther culture are not suitable to trigger embryogenesis from the somatic anther tissue.
9. Use preferably young leaf for cytofluorimetric analysis.
10. Use mature and healthy leaves, cut a piece of leaf missing of primary, secondary and tertiary veins that ensures a better adherence of the cover slip. Observe quickly under fluorescent microscope, after keeping the leaf in the FDA solution for a maximum of 3–5 min. In fact, the stomata cells are the first reacting to FDA, as a strong green background coloration will hamper the chloroplast counting as soon as the FDA will penetrate into the other leaf cells. In diploid and haploid plants, the average number of chloroplasts in the stomata is about 12 and 7, respectively [9].
11. Cloning of haploids by in vitro cutting increases the probability of their conversion into the diploid status. Dissolve colchicine in the lanoline by manual stirring with the aid of a spatula. Check daily the plantlets to eliminate the young shoots coming from the untreated buds, as this will further stimulate the development of shoots from the secondary/adventitious buds whose meristems have been exposed to the colchicine.

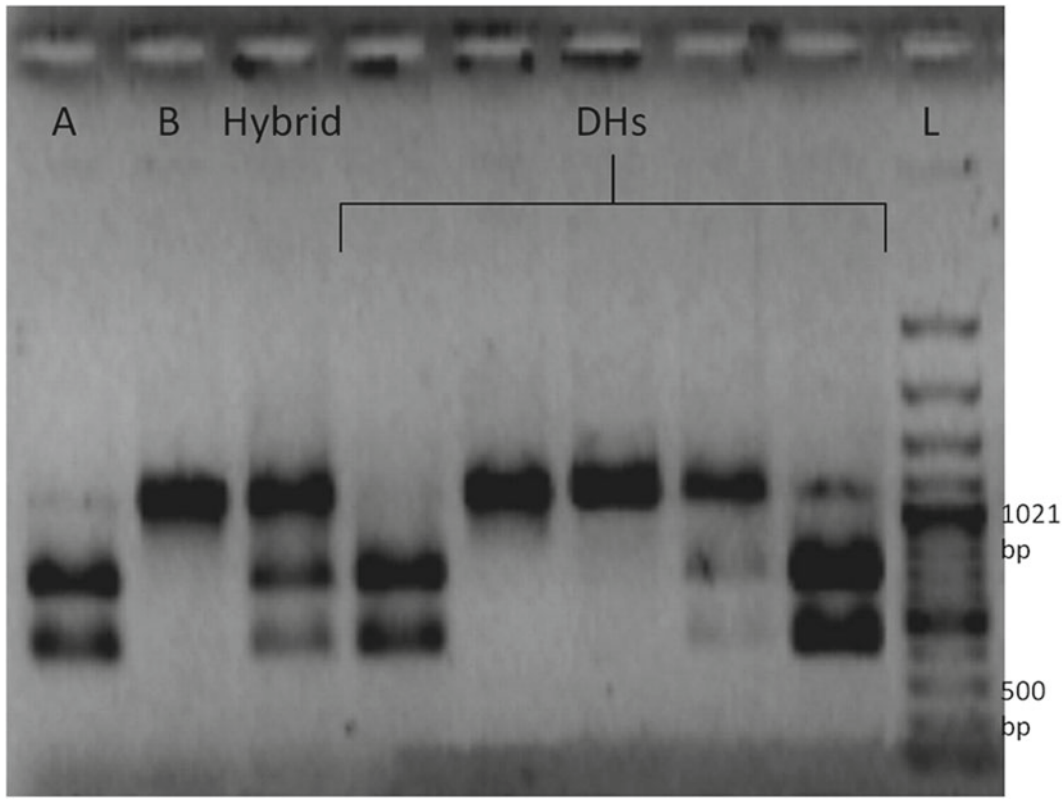

Fig. 7 Molecular analysis confirming the pollen origin of double-haploid plants (DHs), as they resulted homozygous using a bi-allelic polymorphic SSR marker which is heterozygous in the anther donor (Hybrid); the DH plants displayed the one or the other of the single marker present in the parental lines (A, B)

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Chapter 26

Anther Culture in Pepper (*Capsicum annuum* L.)

Verónica Parra-Vega and Jose M. Seguí-Simarro

Abstract

Anther culture is the most popular of the techniques used to induce microspore embryogenesis. This technique is well set up in a wide range of crops, including pepper. In this chapter, a protocol for anther culture in pepper is described. The protocol presented hereby includes the steps from the selection of buds from donor plants to the regeneration and acclimatization of doubled haploid plants derived from the embryos, as well as a description of how to analyze the ploidy level of the regenerated plants.

Key words Androgenesis, Doubled haploid, Embryogenesis, Haploid, Microspores, Pollen, Tissue culture

1 Introduction

Androgenesis can be defined as the generation of an individual derived from a nucleus of male origin, usually a haploid microspore or young pollen grain [1]. Haploid embryos or calli are produced through the deviation of the microspore from its original gametophytic pathway towards a new sporophytic pathway. Haploid embryos may become doubled haploid individuals by themselves or through the application of treatments for genome doubling [2]. Doubled haploid individuals can be used as pure lines to produce hybrid seeds, which reduces considerably the time and resources needed to obtain pure lines when compared with conventional breeding methods [3].

For most of the studied species the optimal stage of male gametophyte development to induce embryogenesis is the transition between vacuolate microspores and young bicellular pollen [1, 4]. Technically, microspore embryogenesis can be induced through anther culture or isolated microspore culture. Isolated microspore culture is based on the isolation of the microspores in liquid medium. Since the maternal tissue is removed, microspores are directly in contact with the medium components. Therefore, the possible formation of somatic embryos coming from the anther

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walls is avoided. Despite these advantages, isolated microspore culture is more complex than anther culture and therefore it is well set up just in a few species. Just tobacco (*Nicotiana tabacum*), rapeseed (*Brassica napus*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*) can be considered as model systems for microspore culture [5]. For most crops of agronomic interest, the most used technique is still anther culture. Anther culture consists on the cultivation of the anthers in a solid or semisolid medium. It can be applied to a wide range of crops and it is the preferred technique used to produce doubled haploids due to its simplicity [6], and to the possibility of culturing large amounts of anthers per isolation. In some species, the presence of the anther walls in the culture medium seems to provide a proper environment for microspore development, allowing for the induction of the microspores towards embryogenesis [7]. Anther culture in pepper (*Capsicum annuum* L.) has been used to produce doubled haploid plants for breeding programs since the mid-1980s (*see* Chapter 9, this volume).

In this chapter, a protocol for anther culture of sweet pepper is explained according to Dumas de Vaulx et al. [8] with some modifications. The protocol was adapted for commercial F1 hybrids of sweet pepper [9] and the selection of donor flower buds was made according to Parra-Vega et al. [10]. In this protocol, the combination of two morphological markers (calix-bud length ratio and anther pigmentation) is used to select the optimal flower buds.

2 Materials

2.1 Plant Material

This stepwise protocol was developed with the following commercial F1 hybrids of pepper (*C. annuum* L.): 'Herminio' (Lamuyo type, from Syngenta Seeds), 'Coyote', 'Quito' (California type, both from Syngenta Seeds), and 'Vélez' (California type, from Enza Zaden).

2.2 Equipment

1. Plastic tubes of 50 mL.
2. Box with melting ice.
3. Laminar flow hood.
4. Sterile Whatman paper.
5. Sterile forceps and scalpel.
6. Sterile Petri dishes 90 × 25 mm (Ø × height).
7. Parafilm.
8. Inverted or light microscope.

9. Microscope slides and cover slips.
10. Aluminum paper.
11. Incubator at 35 and 25 °C.
12. Sterile baby food jars with plastic caps.
13. Plastic plant pots 90 × 100 mm (width × height).
14. Composite soil.
15. Transparent plastic glass.
16. Growth chamber at 25 °C.
17. Pasteur pipettes, 3 mL.
18. Razor blades.
19. Filters of 30 μm pore (CellTricks, Partec).
20. Plastic tubes 3.5 mL, 55 × 12 mm (Ø × height).
21. Flow cytometer Partec Ploidy Analyzer I.

2.3 Solutions and Culture Media

1. 70 % ethanol (v/v).
2. 4 g/L sodium hypochlorite with 0.05 % Tween (v/v).
3. Sterile distilled water (three glass jars) autoclaved at 121 °C for 20 min.
4. Induction medium: C medium (Table 1) supplemented with 0.01 mg/L kinetin and 0.01 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D).
5. Regeneration medium: R medium (Table 1), supplemented with 0.1 mg/L kinetin. Adjust the pH of media C and R to 5.9. Autoclave media at 121 °C for 20 min, and then pour it in 90 × 25 mm sterile Petri dishes.
6. Rooting medium: V3 medium (Table 1). Adjust the pH to 5.9. Autoclave medium at 121 °C for 20 min and pour it in 90 × 25 mm sterile Petri dishes and sterile baby food jars (200 mL).
7. Lysis buffer (LB01) [11]: 5 mM Tris(hydroxymethyl)aminomethane, 2 mM Na_2EDTA, 0.5 mM espermine, 80 mM KCl, 20 mM NaCl, 15 mM β-mercaptoethanol, and 0.1 % (v/v) Triton X-100. The pH is adjusted at 7.5.
8. Staining buffer: 4,6-Diamidino-2-phenylindole (DAPI) (Partec CyStain UV precise P, PARTEC GmbH).

3 Methods

3.1 Donor Plant Growth Conditions

Donor plants are grown in a growth chamber at 25 °C, light intensity of 200 μmol/m^2/s with a 16 h photoperiod and 60–65 % relative humidity.

Table 1
Macroelements, microelements, and vitamins used in the three basal media for pepper anther culture (mg/L). C and R media from Dumas de Vaulx et al. [9]. V3 medium from Chambonnet [12]

	Medium C	Medium R	Medium V3
Macroelements			
KNO_3	2150	2150	1900
NH_4NO_3	1238	1238	1650
$MgSO_4 \cdot 7H_2O$	412	412	370
$CaCl_2 \cdot 2H_2O$	313	313	440
KH_2PO_4	142	142	170
$Ca(NO_3)_2 \cdot 4H_2O$	50	50	–
$NaH_2PO_4 \cdot H_2O$	38	38	–
$(NH_4)_2SO_4$	34	34	–
KCl	7	7	–
Microelements			
$MnSO_4 \cdot H_2O$	22.130	20.130	0.076
$ZnSO_4 \cdot 7H_2O$	3.625	3.225	1.000
H_3BO_3	3.150	1.550	1.000
KI	0.695	0.330	0.010
$Na_2MoO_4 \cdot 2H_2O$	0.188	0.138	–
$CuSO_4 \cdot 5H_2O$	0.016	0.011	0.030
$CoCl_2 \cdot 6H_2O$	0.016	0.011	–
$AlCl_3 \cdot 6H_2O$	–	–	0.050
$NiCl_2 \cdot 6H_2O$	–	–	0.030
Vitamins and amino acids			
Myo-Inositol	100.00	100.00	100.00
Pyridoxine HCl	5.500	5.500	5.500
Nicotinic acid	0.700	0.700	0.700
Thiamine HCl	0.600	0.600	0.600
Calcium pantothenate	0.500	0.500	0.500
Vitamin B12	0.030	–	–
Biotin	0.005	0.005	0.005
Glycine	0.100	0.100	0.200
Chelated iron			
Na_2 EDTA	18.65	18.65	37.30
$FeSO_4 \cdot 7H_2O$	13.90	13.90	27.28
Sucrose	30,000	30,000	30,000
Bacto-agar	8000	8000	8000

3.2 In Vitro Culture of Anthers

1. Select by eye the optimal buds for anther culture. In our genotypes, they are covered approximately the 80 % of them by the sepals (Fig. 1a), according to Parra-Vega et al. [10] (*see* **Note 1**). Excise the buds from the plant. Bring them to the lab in plastic tubes immersed on melting ice (*see* **Note 2**).
2. Take the buds to the laminar flow hood.

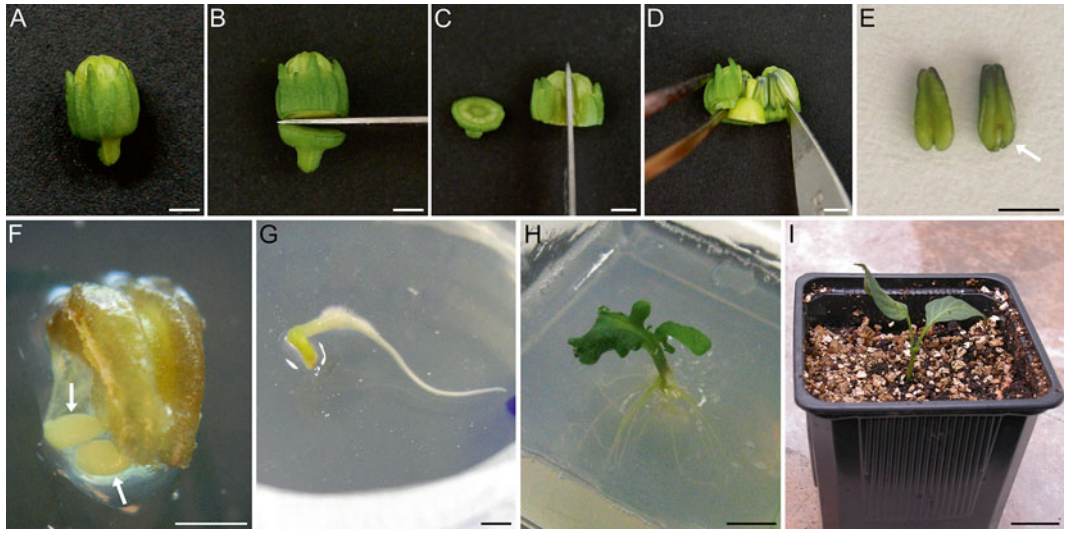

Fig. 1 Process of anther culture in pepper. (**a**) Flower bud at the right stage for anther isolation. (**b**–**d**) Anther extraction out of the bud: transversal cut of the flower bud (**b**), longitudinal cut of the flower bud surface (**c**), and opening of the flower bud with scalpel and forceps to extract the anthers (**d**). (**e**) Anthers at the right stage for isolation. *White arrow* points the right position to culture the anthers in medium (*concave part*). (**f**) Anther cultured in vitro producing two microspore-derived embryos (*white arrows*) in **c** medium. (**g**) Microspore-derived embryo germinated in V3 medium. (**h**) Microspore-derived seedling cultured in vitro in V3 medium. (**i**) Acclimated seedling cultured ex vitro in a plastic plant pot. Bars: **a**–**e**, 2 mm; **f** and **g**, 5 mm; **h**, 1 cm; **i**, 2 cm

3. Surface sterilize the buds with 70 % ethanol for 30 s, and then with sodium hypochlorite 4 g/L for 5 min, and finally three washes of 4 min each with sterile distilled water (*see* **Note 3**).
4. Place the buds over sterile Whatman paper and excise them to extract the anthers (*see* **Note 4**). At this step, make a second selection of the buds. Culture only buds containing anthers with purple distal tips (Fig. 1e), according to Parra-Vega et al. [10]. In case the optimal stage of anther development has not been well set up in advance for the genotype used, it is highly recommended, at this point, to check the microspores/pollen stage of every bud before culturing them (*see* **Note 5**).
5. Place the selected anthers in Petri dishes with C medium. Place them with their concave part in contact with the medium. Seal the dishes with Parafilm and introduce them in the incubator at 35 °C in darkness for 4 days (*see* **Note 6**).
6. At day 4, place the dishes in the incubator at 25 °C with a 12-h photoperiod for 4 days more.
7. At day 8, transfer the anthers to R medium and incubate them at 25 °C, light intensity of 32 μmol/m^2/s and a 12 h photoperiod. Every 2 months, change the anthers to fresh R medium.
8. As soon as the embryos pop out of the anthers, pick them with forceps and transfer them to V3 medium in 90×25 mm Petri

dishes, incubate them at 25 °C, light intensity of 32 μmol/m^2/s and a 12 h photoperiod. Transfer the embryos that germinate correctly to sterile baby food jars with V3 medium (*see* **Note 7**).

9. When seedlings develop a proper root system (one or two primary roots and some secondary roots), transfer them to plastic plant pots with wet soil.
10. Acclimate the seedlings in the growth chamber at 25 °C and a 16 h photoperiod (*see* **Note 8**).

3.3 Analysis of the Ploidy Level

1. Analyze the nuclear DNA content with a flow cytometer (Partec Ploidy Analyzer I) according to its commercial specifications. Use DAPI as the fluorescent stain.
2. Use donor plants as control for 2C DNA content. Plants derived from embryos will be analyzed in order to know the ploidy level (*see* **Note 9**).
3. Excise young leafs from the plant and place them in a box with ice (*see* **Note 10**).
4. Chop with a razor blade a piece of 1 cm^2 of a young leaf in a plastic Petri dish containing 0.5 mL of lysis buffer (*see* **Note 11**).
5. Filter the extracted nuclei with a 30 μm pore filter into a 3.5 mL plastic tube.
6. Add 1.5 mL of DAPI staining buffer with a 3 mL Pasteur pipette.
7. Keep the tubes on ice for 2 min prior to analyze the samples using the flow cytometer. Count a minimum of 10,000 cells per sample.

4 Notes

1. The selection of anthers is one of the critical steps of anther culture. The anthers must contain vacuolate microspores and young bicellular pollen grains to efficiently induce embryogenesis. As this parameter determination is highly genotype dependent, it is recommended to study previously, in each genotype, the right size and appearance of anthers containing the appropriate stage of microspore/pollen development to be induced towards embryogenesis.
2. Once the buds are excised from the plant, keep them on ice in order to slow down the development of the microspores/pollen. Also, keep the sterilized solutions at 4 °C before using them to reduce the degradation process of anthers.

3. Pour the sterilized solutions into the plastic tube, close the lid and shake the solutions during the corresponding time for each solution. After that, open the lid and remove the liquid keeping the buds. Pour the next solution into the tube and repeat the process. An alternative to the plastic tubes is to use tea filter sieves.
4. Excise the anthers with a scalpel avoiding breaking them. First, make a transversal cut at the basal part of the bud (near to the pedicel), removing the basal part of the floral bud (Fig. 1b). Second, make a longitudinal cut, only at the surface of the bud (Fig. 1c), to open the sepals. Later, take away the sepals and petals with forceps, and extract the anthers (Fig. 1d). It is important to remove the anther filament as much as possible, just to avoid callus formation from this tissue, which is especially prone to proliferate.
5. After extracting the anthers from the bud, take one anther to observe it under the microscope and keep the remaining anthers waiting in the laminar flow hood. Place the anther onto a microscope slide with a drop of water, chop the anther with a razor blade in order to extract the microspores/pollen and cover it with a standard cover slip. Observe the preparation under a light or inverted microscope checking the stages of microspores contained. If the anther contains mostly vacuolate microspores and young bicellular pollen, the rest of anthers from the same bud will be used for anther culture.
6. Cover the Petri dishes with aluminum paper to create a darkness environment inside the incubator.
7. Transfer the germinated embryos to baby jars in order to increase the space to develop the roots and aerial parts of the new plant.
8. In order to avoid drastic change in humidity conditions, use a transparent plastic cup to protect the seedlings. Pinch holes in the cup every 2 days, to gradually reduce the humidity inside the cup down to the levels of the growth chamber. Then remove the glass.
9. The flow cytometer is used to analyze the ploidy level, but when a 2C individual appears, molecular analysis marker (preferentially SSRs) has to be performed in order to clarify whether this individual has a somatic or an embryogenic origin. For donor plants polymorphic for the SSR used, if the regenerated samples analyzed are homozygous for the used molecular markers, the origin of these plants will be gametophytic. However, if the samples are heterozygous for the SSRs used, their origin will likely be somatic (most likely coming from anther wall tissues).

10. Young tissues are used to analyze the ploidy level because these tissues present more cells in G2 phase; therefore the second peak of the histogram appears clearer.
11. The nucleic extraction buffer from Partec (CyStain UV precise P, PARTEC GmbH) may be used at this step. However, with pepper is recommended to use the lysis buffer in order to slow down the oxidizing process of pepper samples.

Acknowledgments

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Chapter 27

Microspore Embryogenesis Through Anther Culture in *Citrus clementina* Hort. ex Tan.

Benedetta Chiancone and Maria Antonietta Germanà

Abstract

Anther culture is a biotechnological method that allows to obtain, in one step, homozygous plants, very important to plant breeding, due to their numerous applications in mutation research, selection, genome sequencing, genetic analysis, and transformation. To induce the microspores, i.e., the immature male gametes, to switch from the normal gametophytic pathway to the sporophytic one, it is necessary to submit them to a type of stress, such as high or low temperature, starvation, or magnetic field. Stress can be applied to the donor plants and/or the floral buds or the anthers or the isolated microspores, before or during the culture. In this chapter, the protocol to induce gametic embryogenesis from anther culture of several cultivars of *Citrus clementina* Hort. ex Tan. is reported.

Key words Anther culture, Citrus, Clementine, Doubled haploids, Gametic embryogenesis, Isolated microspore culture, Microspore embryogenesis, Somatic embryogenesis

1 Introduction

The conventional methods applied to *Citrus* breeding are time-consuming and limited by many factors. Biotechnological methods, and, among them, haploidy technology, are a valuable support to increase the efficiency and to speed up the breeding programs. The interest of breeders in haploids and doubled haploids relies mainly in the possibility of obtaining homozygosity in one step, particularly in woody plants, generally characterized by a long reproductive cycle, a high degree of heterozygosity, large size, and, sometimes, by self-incompatibility [1–3]. Haploid technology is important for its potential use in mutation research, selection, genetic analysis, transformation, and the production of homozygous cultivars. Moreover, in *Citrus*, where somatic hybridization is a well-established protocol, haploid protoplasts can be fused with diploid ones in order to obtain triploids, which are particularly important since they are seedless [2–4].

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In *Citrus*, the first haploid seedlings were obtained by the application of gamma rays in *Citrus natsudaidai* [5]. After that, many studies have been carried out on gametic embryogenesis to obtain haploid and doubled haploid plants, through anther culture, but not much of them have been successful. For example, only heterozygous plantlets have been obtained by anther culture in *C. aurantium* [6, 7], *C. aurantifolia* [8], *C. madurensis* [9], *C. reticulata* [10], *Poncirus trifoliata* [11, 12], and *C. sinensis* [13, 14]. Haploid plantlets have been recovered from *Poncirus trifoliata* [15] and *C. madurensis* [16]; one doubled haploid plantlet has been obtained from the hybrid No. 14 of *C. ichangensis* × *C. reticulata* [12]; haploid but albino embryoids of Mapo tangelo *C. deliciosa* × *C. paradisi* [17], haploid and diploid calli, embryoids and leafy structures but no green plants of *C. limon* [18], and haploid embryoids of *Clausena excavata* [19] have been also achieved. Furthermore, haploid, doubled haploid and triploid plantlets, and highly embryogenic calli of *C. clementina* Hort. ex Tan. were recovered [10, 14, 20–23].

To induce gametic embryogenesis, it is necessary to switch microspore development from the gametophytic to the sporophytic pathway, usually subjecting microspores to a stress treatment [2, 6, 24, 25, 26]. Stress can be provided through the growing conditions of the donor plants and/or as treatments applied to the floral buds or to the anther or to the isolated microspores, before or during the culture. Actually, all aspects of the in vitro culture protocol could be classified as stresses [27]. The stress seems to act by altering the polarity of the division at the first haploid mitosis, involving reorganization of the cytoskeleton [28], delaying and modifying pollen mitosis, blocking starch production, or dissolving microtubules [29].

Also in *Citrus*, numerous studies were conducted to obtain regeneration through anther and isolated microspore culture techniques, testing the microspore response to different stress treatments applied before and after the culture [4, 10, 17, 18, 23, 24, 30, 31]. In particular, in *Citrus clementina* Hort. ex Tan., several stress treatments have been tested to induce microspore embryogenesis, both by isolated microspore and by anther culture, particularly low- and high-temperature pretreatments and magnetic field treatments (10, 13, 14, 20–23, 32, 33, and unpublished results).

In this chapter, the protocol successfully used to induce microspore embryogenesis through anther culture in several *Citrus clementina* Hort. ex Tan. cultivars is reported [13, 14, 22, 23].

2 Anther Culture

2.1 Materials

2.1.1 Plant Material

Immature flower buds of *Citrus clementina* Hort. ex Tan., cvs. Nules, SRA 63, Monreal, Corsica, and Hernandina, with anthers containing microspores at the vacuolated stage of development, collected from field growing trees.

2.1.2 Equipment

1. Stereo microscope, light microscope, fluorescent microscope, slides.
2. Laminar flow hood, forceps, scalpels, glass bead sterilizer or burners.
3. Plastic/glass 1000 mL beakers, 1000 mL graduated cylinders, Petri dishes (60 mm diameter tissue culture Petri dishes), Magenta boxes (Sigma V8505), test tubes, 1000 mL screw-capped Pyrex bottles, Parafilm, magnetic stirrers, spin bars, 100 and 1000 μL micropipettes, and micropipette tips.
4. pH meter.
5. Autoclave.
6. Jiffypots, peat moss, sand, soil, polythene bags.

2.1.3 Solutions and Culture Media

1. Sterile distilled water, 70 % (v/v) ethyl alcohol, 20 % (v/v) commercial bleach.
2. 1 mg/mL of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI).
3. In Table 1, media used for inducing gametic embryogenesis (IM), for embryogenic calli proliferation (PM), and for embryo germination (GM) are reported (*see* **Note 1**).

2.2 Methods

2.2.1 Flower Bud Collection

1. Collect unopened flowers 3.5–5.0 mm long, from plants in February to April, depending on the season and on the genotype (Fig. 1a).
2. Store flower buds at the immature stage at 4 °C for about 1 week (*see* **Note 2**).

2.2.2 DAPI Staining and Developmental Stage Determination

1. Determine the pollen development stage, staining one or more anthers per floral bud size with DAPI.
2. Squash anthers in few drops of DAPI solution (1 mg/mL) and observe slides under a fluorescent microscope to identify the pollen development stage (*see* **Note 3**).

2.2.3 Culture Medium Preparation and Sterilization

1. To prepare a finale volume of 1 L, start with 500 mL of distilled water containing a magnetic stirrer (*see* **Note 4**).
2. Start adding salts and vitamin mixture, then the carbon source and the growth regulators, mixing properly.

Table 1
Media composition used for *Citrus clementina* Hort. ex Tan. anther culture

Components	IM	PM	GM
	Per liter		
N6 Chu basal salts	1×		
MS basal salts	–	1×	1×
N&N vitamins	1×		
MS vitamins		1×	1×
Galactose	18 g		
Lactose	36 g		
Sucrose		50 g	30 g
Ascorbic acid	500 mg	500 mg	500 mg
Myo-Inositol	5 g		
Biotin	500 mg		
Thiamine	5 mg		
Pyridoxine	5 mg		
Casein	500 mg		
Glycine	2 mg		
Glutamine	800 mg		
Serine	100 mg		
Malt extract	500 mg	500 mg	500 mg
Coconut water	100 mL		
6-Benzylaminopurine	0.5 mg		
2,4-Dichlorophenoxyacetic acid	0.5 mg		
Gibberellic acid	0.5 mg		1 mg
Kinetin	0.5 mg		
1-Naphthaleneacetic acid	–	0.02 mg	0.01 mg
Thidiazuron	0.5 mg		
Zeatin	0.5 mg		
Agar	8.5 g	8.0 g	7.5 g
pH	5.8	5.8	5.8

Abbreviations: MS = [41]; N&N = [42]; N6 = [43]; IM = induction medium; PM = proliferation medium; GM = germination medium

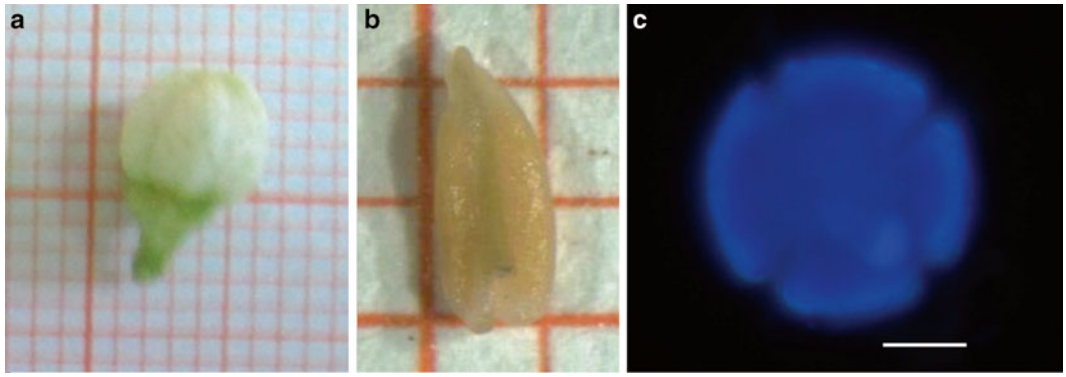

Fig. 1 Plant material. (**a**) *Citrus clementina* Hort. ex Tan., cv SRA 63 flower bud (5 mm) at the suitable developmental stage. (**b**) Anther of *Citrus clementina* Hort. ex Tan., cv Nules containing microspores at the vacuolate developmental stage (**c**) Bar = 10 μm

3. Adjust the pH of media to 5.8, with 1 N KOH or 1 N HCl, and then bring to volume adding distilled water till 1 L.
4. Add agar directly in the bottle before the medium, without mixing (*see* **Note 5**).
5. Put the bottle, without closing completely the cap, in the autoclave and sterilize it at 110 kPa, 121 °C for 20 min.
6. Under the laminar flow hood, pour in 60 mm Petri dishes the medium, only when its temperature is lower than 60 °C.

2.2.4 Flower Bud Sterilization, Anther Isolation, and Culture

1. Under the laminar flow hood, to sterilize flower buds, immerse them, firstly for 3 min in 70 % (v/v) ethyl alcohol and then in 25 % commercial bleach solution (about 1.5 % active chlorine in water) with few drops of Tween 20, for 15–20 min. Finally, rinse them three times with sterile distilled water.
2. Isolate anthers, first by removing the petals and then separating them from stamens (Fig. 1b).
3. Put 60–80 anthers per each Petri dish containing 10 mL of induction medium (IM) (Table 1).
4. Use Parafilm to seal Petri dishes, before the incubation at 27 ± 1 °C, in the dark, for the first month and then under cool white fluorescent lamps (Philips TLM 30W/84) with a photosynthetic photon flux density of 35 μmol m^{-2} s^{-1} and a 16 h light photoperiod.
5. Observe the cultures for 10 months, every 2 weeks.

2.2.5 Embryogenic Callus Maintenance

1. Once embryos and embryogenic calli start to appear (after 2–3 months), transfer them to proliferation medium (PM) (*see* **Note 6**) (Fig. 2a, b).
2. Subculture the stock culture lines every 45 days, keeping them at the same light and temperature conditions.

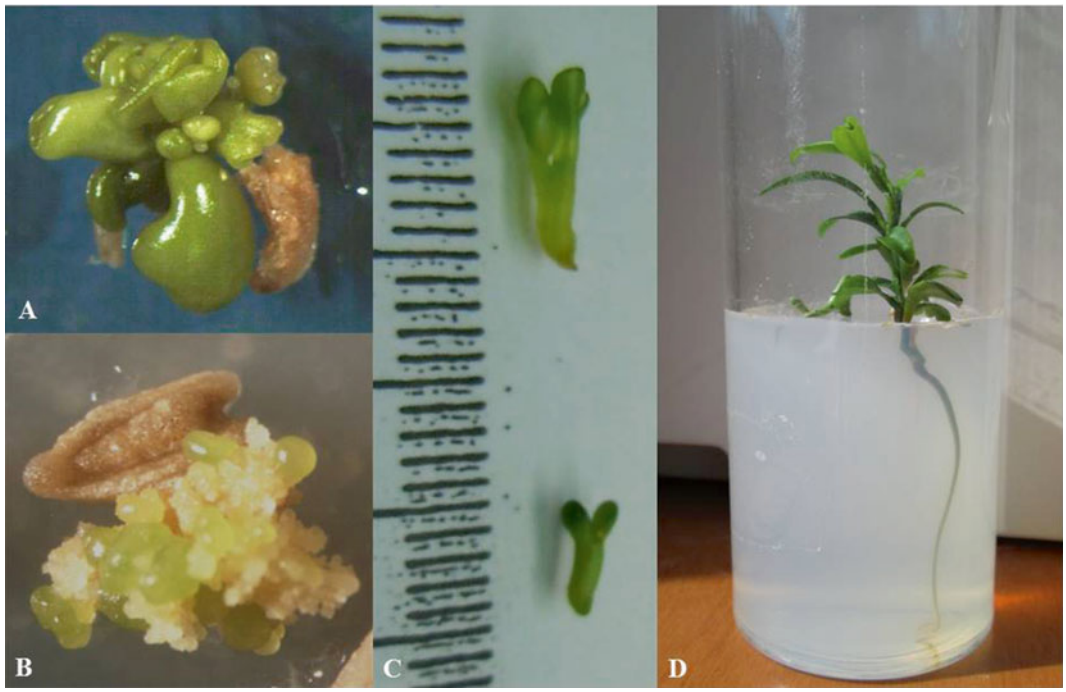

Fig. 2 Microspore embryogenesis through anther culture. (**a**) Direct embryogenesis from an anther of *Citrus clementina* Hort. ex Tan., cv Monreal. (**b**) Embryogenic callus production from an anther of *Citrus clementina* Hort. ex Tan., cv Nules, after 3 months of culture. (**c**) Microspore-derived embryos of *Citrus clementina* Hort. ex Tan., cv Hernandina. (**d**) Plantlet of *Citrus clementina* Hort. ex Tan., cv Corsica, regenerated from anther culture and transferred to test tube

2.2.6 Embryo Germination

1. Isolate the well-developed embryos and culture in 100 mm Petri dishes containing the germination medium (GM) (Fig. 2c) (Table 1) (*see* **Note 7**).
2. Keep the culture in the light at 27 ± 1 °C (with a 16 h photoperiod).
3. Move germinated embryos in Magenta boxes or in test tubes containing the same medium, with 5–6 week subcultures (*see* **Note 8**) (Fig. 2d).

2.2.7 Plant Development and Acclimatization

1. Wash the rooting apparatus of well-developed plantlets with sterile distilled water to remove the medium residues.
2. Transplant plantlets, 4–5 cm high, in Jiffypots or in pots containing sterile peat moss, sand, and soil, in the ratio of 1:1:1, and grow them in the greenhouse.
3. To avoid dehydration, cover the plantlets with polythene bags for the first 40–50 days (*see* **Note 9**).

3 Regenerant Characterization

3.1 Ploidy Analysis of Regenerants by Flow Cytometer: Materials and Method

3.1.1 Materials

1. A portion of 0.5 cm^2 leaf tissue collected from a regenerated plantlet (or the equivalent part of a regenerated embryo) and the same amount of a mother plant young leaf.
2. Razor blade, nylon gauze filter (Partec CellTrics®).
3. Extraction buffer (Partec CyStain® UV Precise); staining buffer (Partec CyStain® UV Precise).
4. Flow cytometer (Partec, Münster, Germany).

3.1.2 Methods

1. Cut by a razor blade a portion of 0.5 cm^2 leaf tissue collected from a regenerated plantlet (or the equivalent part of a regenerated embryo) and the same amount of a mother plant young leaf. Chop them together in 1 mL of extraction buffer (Partec CyStain® UV Precise), to release the nuclei from the cells.
2. Use 30 μm nylon gauze filter (Partec CellTrics®) to remove debris.
3. Add the staining buffer (Partec CyStain® UV Precise) to the suspension.
4. Inject the suspension in the flow cytometer (Partec, Münster, Germany) to determine relative DNA content of the samples (Fig. 3a) (*see* **Note 10**).

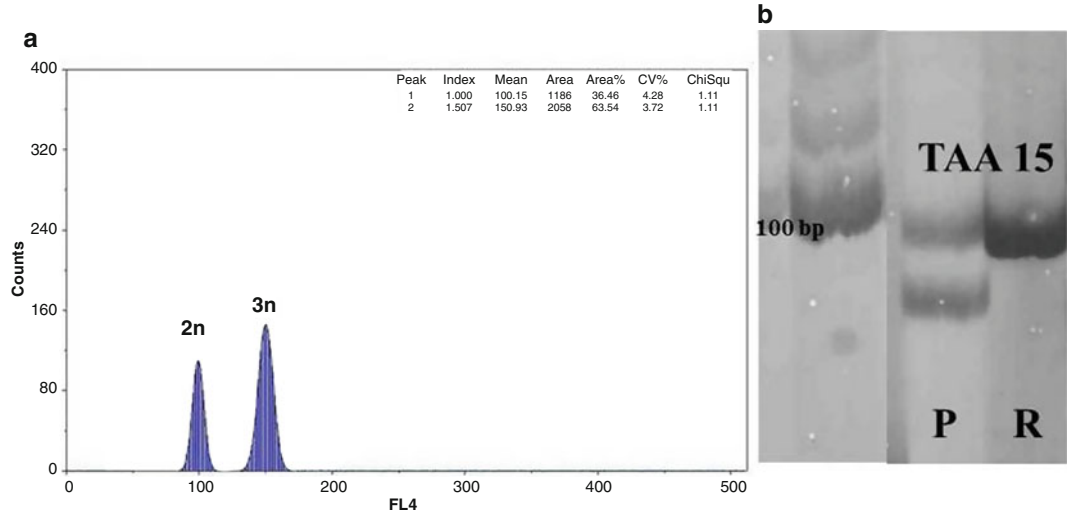

Fig. 3 Characterization of anther culture regenerants. (**a**) Cytofluorimetric analysis: histograms of fluorescence intensity of a diploid control (*C. clementina* Hort. ex Tan. mother plant) and of a triploid regenerant of *C. clementina* Hort. ex Tan., cv Corsica, obtained by anther culture. (**b**) Microsatellite analysis: polyacrylamide gel electrophoresis of microsatellites TAA15 showing the homozygosity of a regenerant from anther culture of *C. clementina* Hort. ex Tan., cv Nules. DNA was extracted from leaves of mother plants (P) and of one anther culture regenerant (R)

3.2 Molecular Characterization: Microsatellite Analysis

Microsatellite analysis has several applications in *Citrus* breeding. For example, in the case of anther culture, because it is possible to regenerate from the somatic anther tissue, as well as from the microspores, microsatellites, being codominant markers, allow to discriminate heterozygous and homozygous regenerants (Fig. 3b) (*see* **Note 11**).

3.2.1 Materials

DNA extraction

1. Sterile Eppendorf tubes, sterile 100 and 1000 μL tips, 100 and 1000 μL micropipettes, centrifuge, vortex, timer, liquid nitrogen container, gloves, sterile pestles, biosafety cabinet, water bath.
2. Ethyl alcohol, liquid nitrogen, phenol, ammonium acetate, isopropanol.
3. Extraction buffer (EB) (stocks 100 mM Tris–HCl pH 8, 50 mM Na_2EDTA pH 8, 500 mM NaCl, 10 mM β-mercaptoethanol, 3 % sodium dodecyl sulfate, SDS).
4. TE buffer [10 mL of 1 M Tris–HCl (pH 8.0), 2 mL EDTA (0.5 M), Milli-Q water to 1000 mL].

DNA amplification

1. PT 100 thermal cycler (MJ Research, USA).
2. 0.5 mL Eppendorf tubes, micropipettes (1–20 and 20–200 μL), gloves, crushed ice.
3. Primers (such as TAA1, TAA15, TAA41, TAA 45, [34]), dNTPs, template DNA, Taq DNA polymerase.
4. 10× PCR buffer (500 mM KCl, 15 mM $mgCl_2$, 100 mM Tris–HCl, pH 8.3).

Polyacrylamide (PA) gel electrophoresis

1. Gloves and polyacrylamide gel electrophoresis system.
2. PA mixture: 6 % acrylamide solution, 50 μL *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), 600 μL 10 % ammonium persulphate (APS).

Silver staining

1. Polyacrylamide gel, tray.
2. Fixer (10 % acetic acid): 50 mL glacial acetic acid in 450 mL distilled water.
3. Silver stain: 3 mL 1 N silver nitrate solution; 500 mL distilled water; sodium thiosulphate solution (0.1 N), formamide.
4. Developer: 15 g sodium carbonate; 500 mL distilled water and put it at 4 °C, 75 mL of sodium thiosulphate solution (0.1 N), and 0.75 mL of formamide.

3.2.2 Methods

DNA extraction

1. Isolate a young leaflet or 150 mg of callus from each regenerant and from the mother plant; process each sample separately.
2. Warm up the EB on the 37 °C water bath, under sterile biosafety cabinet.
3. Cool the centrifuge to 4 °C.
4. Put the sample in the Eppendorf tube and reduce it in powder, adding liquid nitrogen.
5. Add 700 μL of EB, vortex, incubate at 65 °C for 10 min, and centrifuge for 5 min at 13,000 rpm (16,060 RCF).
6. Transfer the supernatant in a clean Eppendorf tube, add 700 μL of phenol, and vortex for few seconds.
7. After centrifuging for 5 min at 13,000 rpm, transfer the supernatant in a clean Eppendorf tube.
8. Add 65 μL of ammonium acetate (NH_4^+ Ac) and 450 μL of cold isopropanol and mix lightly.
9. Centrifuge for 10 min at 13,000 rpm, eliminate the supernatant, and add 700 μL of 70–75 % of cold ethyl alcohol.
10. Centrifuge for 10 min at 13,000 rpm and eliminate the ethyl alcohol, leaving uncovered the Eppendorf tubes.
11. Resuspend DNA in 100 μL of TE buffer and store at 4 °C for one night.
12. Quantify or store at −20 °C.

DNA amplification

1. In a 0.5 mL Eppendorf tube on ice, add all the reagents in the following order: 30 μL sterile distilled H_2O, 5 μL 10× PCR buffer, 4 μL dNTP Mix (1.25 mM), 2.5 μL per each primer, 4 μL $MgCl_2$ (25 mM), and just before the reaction starts, add the Taq DNA polymerase (Amersham Biosciences, USA) (*see* **Note 12**).
2. Add 15 μL of cocktail to the genomic DNA.
3. Place the Eppendorf tubes in the thermocycler. Use the PCR thermal profile: 94 °C for 5 min for 1 cycle; 94 °C for 60 s, 55 °C for 30 s, 72 °C for 60 s for 32 cycles, 72 °C for 5 min [21].

Polyacrylamide (PA) gel electrophoresis

1. Place 0.4 mm spacers on glass plates and pour the acrylamide mixture between the plates using a syringe, until solution fills the space between the plates (*see* **Note 13**), and then lay the plates flat.
2. Insert comb teeth up and clamp, and then leave it to polymerize for 30–45 min.

3. Put the plates in the apparatus. Add in the chambers warm 0.5× TBE (±1 cm over the shorter glass), and pre-run the gel at low wattage for 10 min (40 °C).
4. Load the samples and connect the apparatus: 40 °C, 40 W constant ± 2 h.
5. Stop the running, open the circuit, and eliminate TBE from upper chamber.
6. Open the glass plates, remove the spacers, and stain gel with silver staining.

Silver staining

1. Leave the gel in the fixer for 30 min, and then wash the gel two times, 10–15 min each time.
2. Immerse the gel in the silver stain for 30 min.
3. Just before the gel developing, add the cold developer solution.
4. Agitate the silver stain for 10 s, before eliminating it, and then add the developer.
5. Wait the band development, and then add the fixer.
6. Wash the gel with water for 20 min, and then put it vertically to dry.
7. Photograph or scan the gel for observations.

4 Notes

1. Culture medium composition is one of the crucial factors affecting the gametic embryogenesis induction. In the last 20 years, several culture media have been used in *Citrus clementina* Hort. ex Tan. in vitro anther culture. In particular, experiments were carried out testing the effect of the addition of different carbon sources [10] and gelifying agents (data not published) or of different growth regulator combinations, including thidiazuron [21] and polyamines [36].
2. Storage at 4 °C has the double function of preserving the flower buds from senescence and of stressing the microspores before the culture.
3. Several studies report that in *Citrus clementina* Hort. ex Tan., flower buds of 3.5–5.0 mm size contain the highest ratio of uninucleated/vacuolated microspores (Fig. 1c) [1–4].
4. Starting with a lower volume guarantees to not overcome the final 1 L volume.
5. Agar is not dissolvable at room temperature.

6. Anthers require 2–3 months to initiate callus and embryo production. Many investigations report that most of the calli obtained in *Citrus* anther culture are non-morphogenic, but some of them appear friable and white and differentiate into a clump of embryos. This type of callus is highly embryogenic, and its embryogenic potential is maintained for several years. From only one anther, it is possible to obtain a high amount of embryogenic callus and more than 100 embryos after several subcultures [22].
7. In the germination medium (GM), the microspore-derived embryos follow the same developmental steps of the zygotic ones: globular, heart, torpedo, and cotyledonary stages. Furthermore, secondary embryogenesis can be observed, more frequently in the root region of the embryos. Sometimes, teratomatal structures and morphological anomalies, cotyledonary-fused, pluricotyledonary, or fascinated and thickened embryos, are observed [35].
8. It is expected that 80–89 % of the cultured embryos will germinate in vitro [36].
9. In order to reduce the humidity level, it is recommended to make some holes in the plastic bag and gradually increase their sizes.
10. In *Citrus clementina* Hort. ex Tan., through anther culture, regeneration of calli and plantlets of different ploidy levels, haploid, doubled haploid, triploid, tetraploid, aneuploid, and mixoploid, have been reported, with a preponderance of triploids (around 80 %) [22]. The obtaining of non-haploids may be due to the regeneration from the anther walls to the fusion of several nuclei, to the endomitosis within the pollen grain, and to meiotic irregularities in the microspores [37–40].
11. In *Citrus*, several microsatellites, such as TAA 1, TAA 15, TAA27, TAA 33, TAA 41, TAA 45, TAA 52, CAGG 9, and CAC23, were used [34]. It is needed to individuate which microsatellites are heterozygous in the mother plant. The presence of one band in the regenerant and two bands in the mother plant is considered the confirmation of the gametic origin of the regenerant.
12. To have enough solution, the dose of each component has to be multiplied for the number of samples plus 1; Taq DNA polymerase has to be kept on ice.
13. It is important to avoid the bubble formation in the gel; for this reason, it is worth to invert the syringe to expel any trapped air.

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Part V

Stepwise Protocols on Pivotal Topics

Chapter 28

Detection of Epigenetic Modifications During Microspore Embryogenesis: Analysis of DNA Methylation Patterns Dynamics

Pilar S. Testillano and María Carmen Risueño

Abstract

Methylation of 5-deoxy-cytidines of DNA constitutes a prominent epigenetic modification of the chromatin fiber which is locked in a transcriptionally inactive conformation. Changes in global DNA methylation are involved in many plant developmental processes during proliferation and differentiation events. The analysis of the changes of global DNA methylation distribution patterns during microspore embryogenesis induction and progression will inform on the regulatory mechanisms of the process, helping in the design of protocols to improve its efficiency in different species. To investigate the DNA methylation dynamics during microspore embryogenesis in the different cell types present in the cultures, the analysis of spatial and temporal pattern of nuclear distribution of 5-methyl-deoxy-cytidine (5mdC) constitutes a potent approach. The immunolocalization of 5mdC on sections and subsequent confocal laser microscopy analysis have been developed for in situ cellular analysis of a variety of plant samples, including embryogenic microspore and anther cultures. Quantification of 5mdC immunofluorescence intensity by image analysis software also permits to estimate differences in global DNA methylation levels among different cell types during development.

Key words Anther culture, Confocal laser scanning microscopy, Embryo, Epigenetics, Immunofluorescence, 5-Methyl-deoxy-cytidine, Microspore culture, Pollen

1 Introduction

Plant developmental processes, as differentiation and proliferation, are accompanied by chromatin remodeling and epigenetic reprogramming. DNA methylation constitutes a prominent epigenetic modification of the chromatin fiber which is locked in a transcriptionally inactive conformation [1]. Microspore embryogenesis can be induced either in anther or isolated microspore cultures [2, 3]; in these in vitro systems, after a stress treatment, microspores are reprogrammed and change their gametophytic developmental pathway toward embryogenesis. Nevertheless, this process presents a low efficiency in many species because its regulatory mechanisms

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are not well known. Stress-induced plant cell reprogramming involves changes in global genome organization, being the epigenetic modifications, DNA methylation and histone modifications, key factors in the regulation of genome flexibility. Therefore, the analysis of the epigenetics modifications involved in microspore embryogenesis induction will inform on its regulatory mechanisms and open the door to exploit the process more efficiently for plant breeding and biotechnology purposes in agriculture, selection and propagation of forest resources, and environment control.

Research in the past years has revealed exciting findings with regard to epigenetic mechanisms controlling plant developmental processes [4]. However, the knowledge of the DNA methylation and histone modification regulation during relevant developmental programs in flowering plants, such as gametogenesis or embryogenesis, is limited [4–8]. Difficultly in accessing specific cell types inside the very young embryo or endosperm inside the maternal tissues or the developing microspores inside the anthers has made biochemical and molecular analysis sometime problematic. Although partially overcome by the use of in vitro systems, in situ localization approaches using modern bioimaging technology have become essential tools [9–11]. To investigate the global DNA methylation dynamics during plant embryogenesis, the analysis of spatial and temporal pattern of nuclear distribution of 5-methyl-deoxy-cytidine (5mdC) constitutes a potent approach, which permitted to distinguish among cell types in the same embryo, in comparison with the electrophoretic and ELISA assays used to quantify the percentage of methylated cytidines in genomic DNA.

Immunolocalization of 5mdC and confocal analysis have been developed to several plant cell types, tissues, and organs [5–7, 12, 13], and the results demonstrate the versatility and feasibility of the approach for different plant samples, revealing defined DNA methylation nuclear patterns associated with differentiation and proliferation events of various plant cell types and developmental programs. Quantification of 5mdC immunofluorescence intensity by appropriate confocal image software also permits to estimate differences in global DNA methylation levels among different cell types of the same organ during development and under different physiological conditions. During microspore embryogenesis, the analysis of the dynamics of DNA methylation distribution patterns by the 5mdC immunolocalization approach presented here revealed that a decrease in the DNA methylation and its nuclear redistribution is associated with microspore reprogramming and embryogenesis initiation, whereas a progressive increase in DNA methylation accompanies the progression of microspore embryogenesis and embryo differentiation [5, 7, 13].

The processing of the plant culture sample previous to the 5mdC immunolocalization constitutes a key step which should fit with the compromise of preserving the antigenicity together with the good structural preservation. The processing methods are

different for different culture samples due to the different characteristics for in situ cellular analysis on section of the samples, e.g., hardness, heterogeneity, cell accessibility, tissue compactness, etc. At advanced stages of microspore embryogenesis, the individual developing embryos that can be separated from the microspore or anther cultures constitute samples with low/mild hardness and relatively homogeneous structure. Therefore, it could be sectioned without embedding media by the cryostat, providing thick sections with good structural preservation. At early stages of the anther culture, the embryogenic anther is a heterogeneous organ, composed by very different cell types with different wall hardness and vacuoles. Moreover, the anthers contain microspores and very early embryos inside the pollen sac which would be lost in non-embedded sections. Therefore, anthers have to be processed and embedded in acrylic resins at low temperature, to maintain their structural integrity and to preserve their antigenic properties as much as possible. The resins of choice are Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany) and Lowicryl K4M (Polysciences Inc, Eppelheim, Germany). In isolated microspore cultures, the samples of the first stages containing isolated microspores and small embryos are previously embedded in gelatin in order to manipulate them as tissue pieces. After that, they are processed like anthers and embedded in acrylic resins.

The 5mdC immunofluorescence protocol involves several permeabilization steps for thick cryostat sections, including freezing-thawing, dehydration-rehydration, and mild cell wall enzymatic digestion. After permeabilization, cryostat sections are treated with the same protocol than resin sections. Denaturation of the DNA in sections with HCl is essential to expose the 5mdC antigen to the antibodies. Further steps included the blocking and the incubations with the first (anti-5mdC) and secondary (fluorochrome Alexa-conjugated) antibodies. The microscopical analysis of the immunofluorescence preparations is performed in a confocal laser scanning microscope (CLSM) which permitted to obtain optical sections and avoided the out-of-focus fluorescence of the thick (30–50 μm) cryostat sections. 1–2 μm semithin resin sections can be analyzed by both CLSM and epifluorescence microscopes, even though the CLSM provided fluorescent images of higher resolution and quality (Figs. 1 and 2). Controls are performed by eliminating the DNA denaturation by HCl and by immunodepletion assays in which the antibody is pre-blocked with the antigen (5mdC) in vitro, and this pre-blocked antibody is used for immunofluorescence experiments. Negative results of the first control indicate that the antibody does not cross-react with double-stranded DNA or other nuclear antigens. Absence of signal in the immunodepletion experiments indicate that the antibody only recognized the 5mdC as antigen and did not cross-react with other antigens, since it was completely blocked in vitro with the 5mdC (Fig. 1b, c).

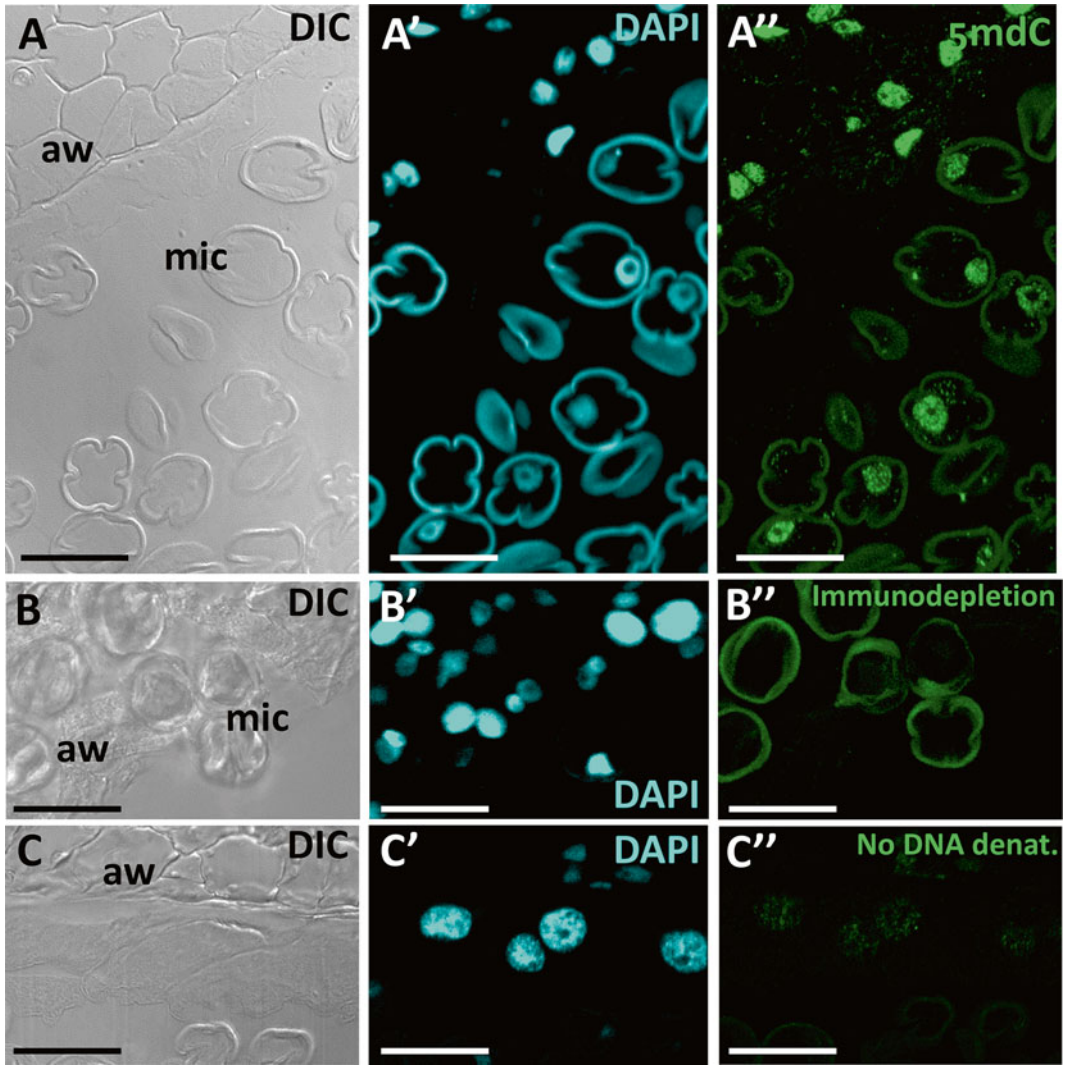

Fig. 1 5mdC immunofluorescence in anthers developed in vivo. Tobacco anthers at the developmental stage of vacuolated microspore-young bicellular pollen, the most responsive for embryogenesis in vitro induction. Confocal images of 1 μm semithin Technovit 8100 sections. Same sections of anthers showing microspores (mic) and anther wall (aw) observed with differential interference contrast (**A, B, C**), DAPI staining for nuclei, cyan fluorescence (**A′, B′, C′**), and 5mdC immunofluorescence, green fluorescence (**A″, B″,C″**). (**A, A′, A″**) 5mdC immunofluorescence. (**B, B′, B″**) Control by immunodepletion of the antibody by in vitro pre-blocking with 5mdC. (**C, C′, C″**) Control by eliminating the DNA denaturation step. The microspore wall, the exine, showed unspecific autofluorescence of different intensities in *cyan* and *green* channels. Bars, 30 μm

The method presented here provides unique information on the DNA methylation nuclear patterns of different plant cell types, like microspores, pollen grains, anther and embryo cells, and their dynamics in relation to chromatin organization during proliferation and differentiation processes that occur during microspore embryogenesis in different in vitro systems (isolated microspore

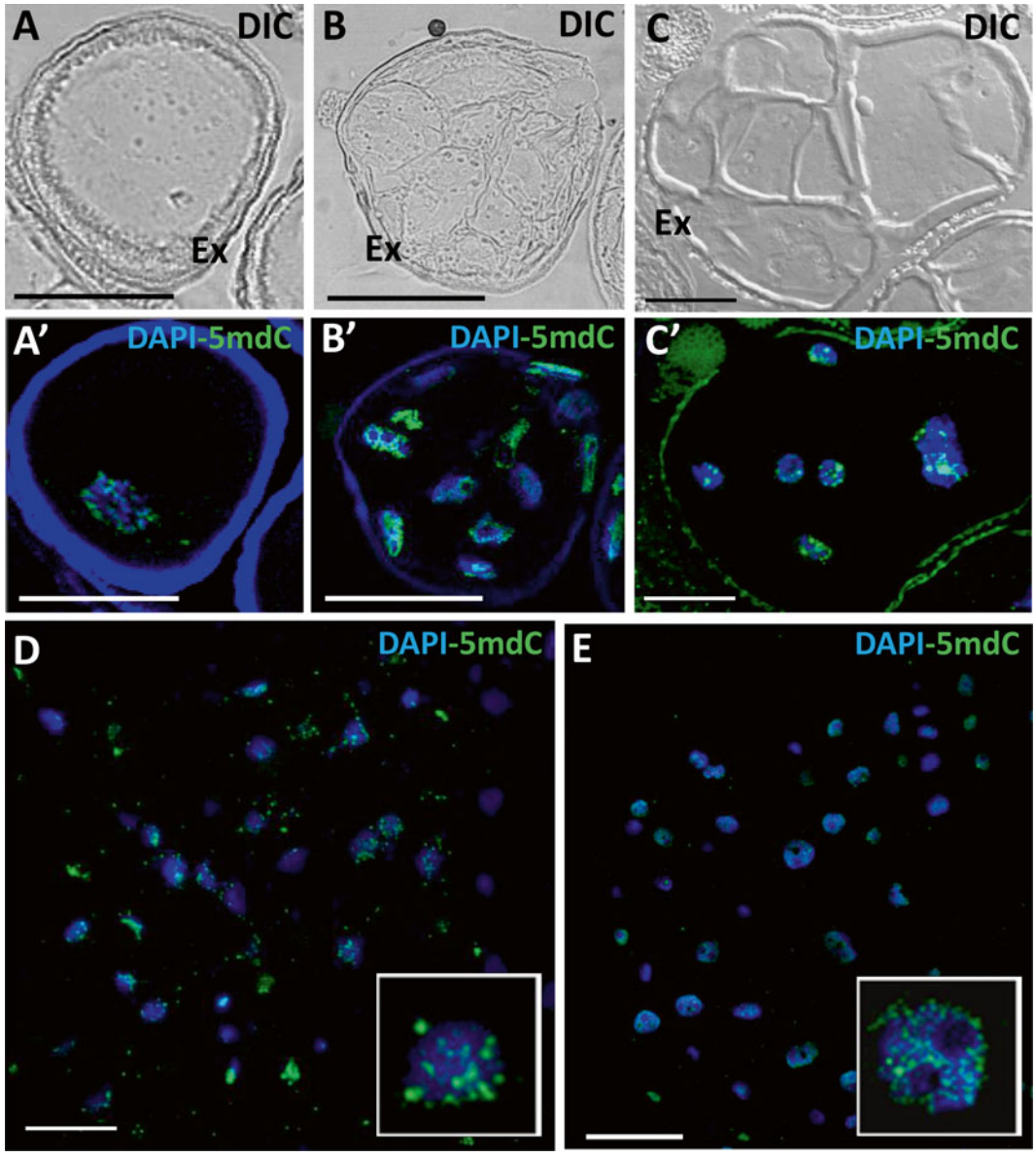

Fig. 2 5mdC immunofluorescence during different stages of microspore embryogenesis in different systems. (**A, A′**) Barley vacuolated microspore before embryogenesis induction. (**B, B′**) Multicellular embryo still surrounded by the exine (Ex) from an isolated microspore culture of barley. (**C, C′**) Multicellular embryo at the exine breakdown from an isolated microspore culture of rapeseed. (**D**) Advanced microspore-derived embryo developed from anther culture of cork oak. (**E**) Advanced microspore-derived embryo developed from isolated microspore culture of barley. Confocal images of 1 μm semithin Technovit 8100 sections. (**A, B, C**) Differential interference contrast (DIC) images of the same sections than in **A′, B′** and **C′**. (**A′, B′, C′, D**, **E**) Merged images of DAPI staining for nuclei (*blue*) and 5mdC immunofluorescence (*green*). (*Insets* in **D**, **E**) Individual nuclei of advanced microspore embryos of cork oak and barley, respectively. In **A′**, **B′**, **C′** the microspore wall, the exine (Ex), showed unspecific autofluorescence of different intensities in *blue* and *green* channels. Bars in **A**, **A′**, 10 μm; in **B**, **B′**, **C**, **C′**, 20 μm; in **D**, **E**, 25 μm

cultures and anther cultures) and in various plant species, based on the versatility of the immunolocalization protocol and the good resolution and quality provided by the CLSM analysis. The information raised will give new insights into the mechanisms regulating epigenetic patterns and chromatin remodeling during in vitro microspore embryogenesis.

2 Materials

2.1 Fixative, Dehydration, and Resin Solutions

1. Fixative: 4 % paraformaldehyde in PBS. Prepare a solution of 4 % formaldehyde (from paraformaldehyde powder) in PBS, pH 7.0. Heat in a hot bath (no more than 80 °C) until the solution is transparent. If necessary, a small drop of sodium hydroxide can be added, but pH should be checked afterward. Then put it on melting ice. Aliquots of freshly prepared 4 % formaldehyde solution can be stored at −20 °C and thawed just before use.
2. Dehydration solutions: Prepare an acetone series of 30, 50, 70, 90, and 100 % in volume of acetone in water, and keep at 4 °C until use.
3. Resin: Commercial acrylic resin Technovit 8100 (Heraeus Kulzer, Germany). Prepare the infiltration solution and embedding solution following the manufacturer instructions, just before use, and keep them at 4 °C (*see* **Note 1**).

2.2 Components for Processing in Cryostat

1. Cryoprotectant: Prepare a series of sucrose in increasing concentrations in PBS, 0.1, 0.5, 1, 1.5, 2, and 2.3 M. Aliquots of them can be stored at −20 °C and thawed just before use.
2. Commercial OCT (optimal cutting temperature) compound is kept in liquid-viscous form at room temperature and used to embed samples during freezing over carbonic ice.

2.3 Solutions for Immunofluorescence

1. Phosphate-buffered saline (PBS).
2. Blocking agents: 5 % BSA and bovine serum albumin (w/v), in PBS. Dilute 0.5 g BSA in 10 mL PBS with a magnetic stirrer (without heating or the BSA will coagulate), and prepare aliquots of 1 mL that can be stored at −20 °C and thawed just before use.
3. Permeabilization agents: Prepare a methanol series of 30, 50, 70, 90, and 100 % in volume of methanol in water and keep at room temperature until use. Prepare a mixture of enzymes for partial cell wall degradation with the following composition: 2.5 % pectinase, 2.5 % cellulase, and 2.5 % pectoliase in PBS. Prepare aliquots which can be stored at −20 °C and thawed just before use.

4. DNA denaturation agent: Prepare a solution of 2 N HCl (chlorhydric acid) in water, and keep it at room temperature until use.
5. Commercial mouse monoclonal anti-5-methyl-deoxy-cytidine (anti-5mdC) antibody (Eurogentec, Belgium, Cat. Number: BI-MECY-0100).
6. Commercial goat anti-mouse IgG conjugated to Alexa Fluor 466 secondary antibody (Molecular Probes, Leiden, The Netherlands).
7. DNA staining agent: Prepare a 1 mg/mL DAPI (4′,6-diamidino-2-phenylindole) solution in PBS and keep at 4 °C until use.

3 Methods

3.1 Sample Processing, Section Preparation, and Storage

Two types of sections can be used for in situ analysis of DNA methylation patterns by 5mdC immunofluorescence: cryostat sections and resin sections.

1. Anther and microspore culture samples collected at different culture times are fixed overnight with 4 % paraformaldehyde in PBS at 4 °C. Samples immersed in the fixative are subjected to a short vacuum step (1–5 min) for proper penetration of the fixative into the cells.
2. After fixation, samples are washed three times in PBS for 5 min each washing step.
3. Culture samples of the first developmental stages containing isolated vacuolated microspores and early multicellular embryos have to be previously embedded in 15 % gelatin in PBS and gel solidified on ice for further manipulation, like embryo or anther samples.
4. Fixed samples can be either dehydrated and resin embedded, or processed for freezing and cryostat sectioning. The samples of early stages, which were embedded in gelatin, are dehydrated and resin embedded. Larger samples that were not embedded in gelatin, like anthers, globular, torpedo, and cotyledonary embryos, can be processed either for cryostat or resin embedding.

3.1.1 Cryostat Sections

1. To obtain cryostat sections, fixed samples are washed in PBS, and cryoprotected through a gradual infiltration in sucrose solutions: 0.1, 0.5, 1, 1.5, and 2 M for 1 h each and 2.3 M overnight, at 4 °C, embedded in Tissue-Tek optimal cutting temperature (OCT) compound and frozen on dry ice forming small pieces of solidified frozen OCT containing the samples at their interior that should be kept at –20 °C until use.

2. Frozen samples are placed in the cryostat and sectioned at 20–30 μM thickness under −20 °C/−30 °C working temperature.
3. Cryostat sections are collected on glass slides, washed with water to eliminate the OCT, and transferred to a water drop over silanized slides, air-dried and stored at −20 °C until use for immunofluorescence (*see* **Note 2**).
4. Cryostat sections are then subjected to permeabilization before their use for immunofluorescence assays.

3.1.2 Resin Sections

1. Fixed samples are dehydrated in an acetone series of 30, 50, 70, 90, and 100 % and then immersed in the Technovit 8100 resin infiltration solution overnight at 4 °C.
2. After infiltration, individual samples are embedded in resin embedding solution (*see* **Note 3**) in gelatin capsules which are covered by a gelatin cap to avoid oxygen that interferes with the polymerization.
3. Resin capsules are polymerized at 4 °C overnight, and sections of 1–2 μm thickness are obtained in an ultramicrotome, placed in a water drop on silanized slides, dried, and stored at 4 °C until use for immunofluorescence.
4. Semithin resin sections do not require permeabilization and are subjected directly to the immunodetection, after incubation in PBS for a few minutes.

3.2 Permeabilization of Cryostat Sections

For cryostat sections, permeabilization is required prior to immunofluorescence.

1. After thawing the sections at room temperature, they are dehydrated and rehydrated in a methanol series (30, 50, 70, 90, 100, 90, 70, 50, 30 %, 5 min each) and PBS.
2. Sections are subsequently subjected to enzymatic digestion of cell walls for additional permeabilization by treatment with an enzymatic mixture (2.5 % pectinase, 2.5 % cellulase, and 2.5 % pectoliase) in PBS for 45 min (*see* **Note 4**), then washed in PBS, and subjected to immunofluorescence (IF) procedure without drying of the section in any step.

3.3 5mdC Immunofluorescence on Cryostat and Resin Sections

At this step, both section types, cryostat and resin sections, follow the same protocol.

1. Sections are denatured with 2 N HCl for 45 min, washed in PB*S* two times, 5 min each, and then blocked with 5 % (w/v) bovine serum albumin (BSA) in PBS for 10 min.
2. Sections are then directly incubated for 1 h with the mouse monoclonal anti-5mdC antibody diluted 1:50 in 1 % BSA in PBS. After three rinsing steps in PBS, 5 min each, sections are

incubated for 45 min in darkness with the secondary antibody, an anti-mouse IgG conjugated to Alexa Fluor 488 diluted 1:25 in 1 % BSA.

3. After washing in PBS three times, 5 min each, nuclei are stained with DAPI (4′,6--diamidino-2-phenylindole) staining solution for 5 min (*see* **Note 5**), washed in sterile water, and mounted in Mowiol.
4. Immunofluorescence preparations are then examined under either an epifluorescence or a confocal laser scanning microscope (CLSM). CLSM permits to obtain optical sections and avoid the out-of-focus fluorescence of the thick (20–30 μm) cryostat sections. 1–2 μm semithin resin sections can be analyzed by both CLSM and epifluorescence microscopes, even though the CLSM provides fluorescent images of higher resolution and quality. The results obtained are similar in both cryostat and resin sections, intense immunofluorescence signals on defined regions of the nuclei, which are clearly identified by DAPI staining. Confocal optical sections are collected either at 0.5 or 0.1 μm length intervals in the *z* axis (section thickness) for cryostat or resin sections, respectively, and images of maximum projections can be obtained with software running in conjunction with the confocal microscope.

3.4 Controls for 5mdC Immunofluorescence Experiments

Apart from the general control experiments in immunofluorescence assays by eliminating the first and secondary antibodies, two main controls should be performed to assess the specificity of the 5mdC immunofluorescence signal, a control by the elimination of the DNA denaturation step and another by the immunodepletion of the 5mdC antibody with the antigen.

3.4.1 Control by Eliminating the DNA Denaturation Step

This control is performed in samples by applying the whole immunofluorescence protocol and eliminating the DNA denaturation step by avoiding the HCl treatment before the antibody incubation. Instead of it, a washing step with PBS during the same time than the HCl treatment is carried out. The results of this control should be negative, showing a complete absence of signal which indicates that the antibody do not cross-react with double-stranded DNA or other nucleic acid antigens.

3.4.2 Control by Immunodepletion of the 5mdC Antibody with the Antigen

1. The anti-5mdC antibodies are pre-blocked with its corresponding immunogen, the 5mdC, by incubating the antibody in an Eppendorf tube with a 5mdC solution (5 μg/μL in water) in a proportion of 1:2, v/v, at 4 °C, overnight. During this time, the immunoglobulins contained in the antibody solution that specifically bind to 5mdC are blocked by the excess of immunogen in the immunodepletion solution and cannot bind to any antigen present in the section, providing negative immunofluorescence results.

2. After the above reaction, the pre-blocked antibody solution is used as primary antibody for immunofluorescence on the sections, following the same protocol and conditions as described above. Negative results of the immunodepletion control experiment indicate that the antibody only recognizes the 5mdC as antigen and does not cross-react with other antigens in the sections, since it was completely blocked in vitro with the 5mdC molecules.

3.5 Quantification of Fluorescence Intensity in 5mdC Immunolocalization Confocal Images

The analysis of the immunofluorescence assays by confocal microscopy using the same laser excitation and sample emission capture settings for image acquisition in all immunofluorescence preparations allows the accurate comparison among signals from cells at different developmental stages and the further quantification of the signal intensities.

1. For each immunofluorescence microscopy preparation, confocal optical sections are collected at the same z-intervals, e.g., 0.5 μm for cryostat sections and 0.1 μm for resin sections, with the same total number of optical sections (15–20). Then, images of maximum projections are obtained and used for relative fluorescence intensity quantification with software running in conjunction with the confocal microscope.
2. Fluorescence intensity quantification is performed on random nuclei of each sample, in a minimum number of nuclei statistically significant, around 30–50 nuclei per sample (e.g., the minimum sample size can be estimated by the progressive mean method).
3. Significant differences among the mean values of relative 5mdC fluorescence intensities of microspore embryogenesis developmental stages are compared by appropriate statistical tests like Student's *t* test or one-way variance analysis.

4 Notes

1. Once the infiltration solution is freshly prepared by mixing the components of the commercial kit and stored at 4 °C, it can be used for 1 month either for infiltration of new samples or to prepare the embedding solution.
2. In general, for immunofluorescence of sections, the use of Teflon-printed multi-well slides (Immuno-Cell Int. Mechelen Belgium) is very convenient; on one hand, they help to minimize the volumes of antibodies required since the drops of solutions are confined by the well (10 μL, even less in critical cases, for 7 mm well is enough to cover the section, when placed in a humid chamber to avoid drying during antibody

incubation). Secondly, they permit to perform individual experiments in each section/well and therefore several assays with different antibodies, dilutions, or samples in one unique slide.

3. The embedding solution mixture starts its polymerization rapidly after its preparation; therefore, it should be prepared in small quantities to avoid the risk of its polymerization along the procedure of embedding in capsules. When there are numerous samples to embed, they can be infiltrated together, but the embedding should be performed in consecutive time steps. For example, prepare only 3 mL of embedding solution in a tube, proceed with the transfer of the samples to capsules and filling with embedding solution until finishing the embedding solution, then prepare another tube with 3 mL, and repeat with other infiltrated samples. With this sequential procedure, the time of manipulation of the embedding solution is short and it will keep liquid.
4. In case of samples with thick or differentiated cell walls, or in the case of thicker sections, the permeabilization should be more efficient. In this case, the activity of the enzymes can be optimized by performing the incubation at higher temperature (e.g., 37 °C) in a humid chamber or prolonging the time.
5. For thick sections, to facilitate DAPI to penetrate into the nuclei, add to the staining solution 0.1 % Triton X-100.

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Chapter 29

Embryogenesis and Plant Regeneration from Isolated Wheat Zygotes

Jochen Kumlehn

Abstract

Wheat zygotes can be mechanically isolated and cultivated to continue their development in vitro. Since each zygote needs to be individually isolated, only relatively few of these cells are available per experiment. To facilitate embryonic growth despite of this limitation, the zygotes are kept within a culture insert placed in a larger dish which itself contains embryogenic pollen cocultivated for continuous medium conditioning. This setup ensures that the two cultures, while being physically separated from one another, can exchange essential intercellular signal molecules passing through the bottom of the insert which is made of a permeable membrane. Thanks to the natural fate of zygotes, which is to form an embryo followed by the generation of a plant, embryogenesis and plant regeneration are achieved at much higher efficiency as compared to other single-cell systems. While the method is largely independent of the genotype, it allows for the nondestructive observation, manipulation, and individual analysis of zygotes and very young embryos.

Key words Cocultivation, Embryonic development, Fertilized egg cell, Single-cell culture

1 Introduction

The commencement of ontogenesis is a fundamental process in plant development. However, zygotes and young embryos are hardly accessible to observation over time, manipulation, and cell-specific analyses, because they are hidden under several layers of tissue within the pistil. In vitro embryogenesis and plant regeneration via culture of isolated zygotes have been preferentially achieved in Poaceae species such as barley, maize, wheat, and rice [1–4]. Historically, however, zygotes of maize and wheat were first cultivated following isolation of gametes (egg and sperm cells) and in vitro fertilization [5, 6], which was later achieved in rice as well [7].

The isolation of zygotes and their further embryonic development in vitro have been an important technical advance toward the elucidation of structural patterns and molecular mechanisms in the

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context of fertilization and early embryogenesis [8, 9]. Live-cell imaging of cultivated wheat and rice zygotes has resulted in valuable descriptive information on early embryonic development [8, 10]. Isolated zygotes or bicellular proembryos also proved to be very useful for precise transcriptomic analyses as was shown in maize [11], wheat [9, 12], tobacco, and rice [13, 14]. In addition, isolated barley zygotes were used for stable transgenesis by means of microinjection of plasmid DNA [15].

Survival and development of plant cells are dependent upon intercellular exchange of signals, which is typically provided in cell and tissue culture systems by a suitable cell population density or sufficient explant size. To cope with the limited cell number available per experiment, Kumlehn and coworkers [16] transplanted isolated wheat zygotes into cultivated wheat or barley ovules, which succeeded in efficient embryonic development and plant regeneration. The cocultivation of heterologous cell types proved to be a viable alternative approach to effective medium conditioning. In the method described here, barley microspores previously treated to undergo pollen embryogenesis were used for cocultivation to facilitate embryonic growth of isolated wheat zygotes. To prevent the zygotes and zygotic embryos from getting lost in the comparatively huge population of pollen-derived embryogenic structures, culture inserts featuring a permeable membrane instead of a solid bottom are used. Such insert harboring some zygotes is placed in a larger dish that itself contains the embryogenic pollen culture so that extracellular signal molecules are allowed to diffuse from the outer medium portion through the membrane into the insert, while the zygote- and pollen-derived structures are kept separated from one another.

2 Materials

All solutions and media are prepared using doubled-distilled water or equivalent quality and analytical grade chemicals, unless specified otherwise.

2.1 Plant Material

1. The German wheat (*Triticum aestivum* L.) cultivars Florida (winter type), Ralle and Remus (spring type) as well as the Mexican breeding line Veery #5 (spring type) were used to isolate and cultivate zygotes.
2. Embryogenic pollen cultures used for co-culture with isolated zygotes were produced in the German barley (*Hordeum vulgare* L.) cv Igri (winter type).

2.2 Specific Laboratory Equipment

2.2.1 For the Production of Barley Embryogenic Pollen Cultures

1. Filter paper disks, 7 cm diameter, ash-free, autoclaved.
2. Refrigerated centrifuge equipped with swing-out baskets.
3. Waring blender (Eberbach, MI, USA), sterilizable by heat, with drive unit.
4. Sterile screw-cap polypropylene centrifuge tubes, 50 mL.
5. Sterile screw-cap round-bottomed polycarbonate cell culture tubes, 12 mL.
6. Clear-transparent (e.g., Magenta) boxes, ca. 250 mL, autoclavable.
7. Nylon mesh, 100 μm grid, autoclavable.
8. Hemocytometer, type Rosenthal.

2.2.2 For Isolation and Culture of Wheat Zygotes

1. Inverted microscope equipped with long-distance condenser lens, allowing to conduct preparations in culture dishes placed on the microscope stage.
2. Fine-tipped glass needles, custom- or self-made by a pulling device.
3. Glass capillary, 100 μM interior diameter, custom- or self-made by a pulling device.
4. Cell Tram Vario (Eppendorf, Germany) equipped with polypropylene tubing.
5. Millicell inserts, 0.4 μm pore size membrane (Millipore, Germany).
6. 4-well plates, 1.9 cm^2 culture area per well (Nunc, Denmark).

2.3 Stock Solutions

1. K macro minerals [17] (×20): 40.4 g/L KNO_3, 1.6 g/L NH_4NO_3, 6.8 g/L KH_2PO_4, 8.8 g/L $CaCl_2{\cdot}2H_2O$, 4.9 g/L $MgSO_4{\cdot}7H_2O$ (*see* **Note 1**); filter-sterilized, stored at room temperature.
2. K micro minerals [17] (×1000): 8.4 g/L $MnSO_4{\cdot}H_2O$, 7.2 g/L $ZnSO_4{\cdot}7H_2O$, 3.1 g/L H_3BO_3, 120 mg/L $Na_2MoO_4{\cdot}2H_2O$, 24 mg/L $CoCl_2{\cdot}6H_2O$, 25 mg/L $CuSO_4{\cdot}5H_2O$, 170 mg/L KI (*see* **Note 1**); filter-sterilized, stored at 4 °C.
3. Chu N6 macro minerals [18] (×10): 28.3 g/L KNO_3, 4.62 g/L $(NH_4)_2SO_4$, 4 g/L KH_2PO_4, 1.86 g/L $MgSO_4{\cdot}7H_2O$, 1.66 g/L $CaCl_2{\cdot}2H_2O$ (*see* **Note 1**); filter-sterilized, stored at room temperature.
4. Chu N6 micro minerals (×1000): 4 g/L $MnSO_4{\cdot}4H_2O$, 500 mg/L H_3BO_3, 500 mg/L $ZnSO_4{\cdot}7H_2O$, 25 mg/L $Na_2MoO_4{\cdot}2H_2O$, 25 mg/L $CuSO_4{\cdot}5H_2O$, 25 mg/L $CoCl_2{\cdot}6H_2O$ (*see* **Note 1**); filter-sterilized, stored at room temperature.

5. NaFeEDTA (Ferric sodium ethylenediaminetetraacetate; 75 mM): 2.75 g dissolved in 100 mL; filter-sterilized, stored at 4 °C.
6. $CaCl_2$ (1 M): 14.7 g $CaCl_2 \cdot 2H_2O$ dissolved in 100 mL; filter-sterilized, stored at room temperature.
7. KM organics [19] (×100, Sigma K-3129): 2 mg/L *p-aminobenzoic* acid, 200 mg/L L-ascorbic acid, 1 mg/L D-BIOTIN, 100 mg/L D-calcium pantothenate, 2 mg/L cyanocobalamin, 40 mg/L folic acid, 10 g/L myo-inositol, 100 mg/L nicotinamide, 100 mg/L pyridoxine·HCl, 1 mg/L retinol, 20 mg/L riboflavin, 100 mg/L thiamine·HCl; stored at −20 °C.
8. Gamborg B5 organics [20] (×1000): 100 mg/L myo-inositol, 1 mg/L nicotinic acid, 1 mg/L pyridoxine·HCl, 10 mg/L thiamine·HCl; filter-sterilized, stored at −20 °C.
9. L-glutamine (0.25 M): 1.83 g dissolved in 50 mL with a few drops of 0.1 M KOH by heating in a water bath; filter-sterilized, stored at −20 °C.
10. Casein hydrolysate (0.1 g/mL, Sigma A-2427): 1 g dissolved in 10 mL; filter-sterilized, stored at −20 °C.
11. Maltose (1 M, ≥99 %): 360 g maltose·H_2O dissolved in 1 L; filter-sterilized, stored at room temperature.
12. Maltose (1 M, ≥95 %): 360 g maltose·H_2O dissolved in 1 L; filter-sterilized, stored at room temperature.
13. Maltose (0.55 M, ≥95 %): 198 g maltose·H_2O dissolved in 1 L; filter-sterilized, stored at 4 °C.
14. Glucose (1 M): 180 g dissolved in 1 L; autoclaved, stored at 4 °C.
15. Mannitol (0.4 M): 72.9 g dissolved in 1 L; autoclaved, stored at 4 °C.
16. Mannitol (0.55 M): 100.2 g dissolved in 1 L; autoclaved, stored at 4 °C.
17. Xylose (×1000): 1.5 g dissolved in 10 mL; filter-sterilized, stored at 4 °C.
18. IBA (3-indolbutyric acid, 1 mM): 2 mg dissolved in a few drops of 50 % ethanol, made up to final volume of 10 mL with hot water (*see* **Note 2**); filter-sterilized, stored at 4 °C.
19. 2,4-D (2,4-dicholophenoxyacetic acid, 1 mM): 2.2 mg dissolved in a few drops of 50 % ethanol, made up to final volume of 10 mL with hot water (*see* **Note 2**); filter-sterilized, stored at 4 °C.
20. BAP (6-benzylaminopurine, 1 mM): 224 mg/L dissolved in a few drops of 1 M NaOH, made up to final volume of 50 mL with hot water (*see* **Note 2**); filter-sterilized, stored at 4 °C.

21. Kinetin (1 mM): 10.8 mg/L diluted in a few drops of 1 M NaOH, made up to final volume of 50 mL with hot water (*see* **Note 2**); filter-sterilized, stored at 4 °C.
22. Phytagel (×2): 3.5 g suspended in 250 mL cold water; autoclaved, stored at room temperature.
23. NaOCl (sodium hypochlorite, 2.5 %): 10 mL concentrated NaOCl (25 %, containing 12 % Cl) diluted in 90 mL water with three drops of Tween 20; freshly prepared before use.
24. Double distilled water: autoclaved, stored at room temperature.
25. Tap water: autoclaved, stored at room temperature.

2.4 Nutrient Media

1. Barley pollen culture (KBP, 17) medium: 50 mL/L K macro minerals, 1 mL/L K micro minerals, 1 mL/L NaFeEDTA, 12 mL/L L-glutamine stock, 10 mL/L KM organics, 4 mL/L BAP stock, 250 mL/L maltose (1 M, ≥99 %), pH adjusted to 5.9; stored at 4 °C.
2. Zygote culture (N6Z) medium [3]: 50 mL/L Chu N6 macro minerals (*see* **Note 3**), 1 mL/L Chu N6 micro minerals, 10 mL/L KM organics, 27 mL/L L-glutamine stock, 2.5 mL/L casein hydrolysate stock, 472 mL/L glucose (1 M; *see* **Note 4**), 1 mL/L xylose stock, 0.9 mL/L 2,4-D stock, pH adjusted to 5.7; stored at 4 °C (*see* **Note 5**).
3. Regeneration (N6D) medium [3]: 100 mL/L Chu N6 macro minerals, 2.44 mL/L $CaCl_2$ stock (*see* **Note 6**), 1 mL/L Chu N6 micro minerals, 10 mL/L KM organics, 20.3 mL/L L-glutamine stock, 2.5 mL/L casein hydrolysate stock, 1 mL/L xylose stock, 150 mL/L maltose (1 M, ≥95 %), 2.5 mL/L IBA stock, 2.3 mL/L kinetin stock, all components mixed in half of the final medium volume, pH adjusted to 5.7, heated to about 40 °C, then mixed 1:1 with Phytagel stock previously melted by heating (*see* **Note 5**).

3 Methods

All procedures are carried out at room temperature unless specified otherwise.

3.1 Growth of Donor Plants

1. Barley or wheat grain is germinated in trays filled with 3:1:2 substrate of garden mulch, sand and peat (Substrate 2, Klasmann, Germany) and placed for 2 weeks in a chamber providing a 12 h photoperiod (136 μmol/m^2/s photon flux density) and 14/12 °C (day/night).
2. Seedlings of cvs. Igri (barley) and Florida (wheat) need to be vernalized at 4 °C under an 8 h photoperiod for 8 weeks (*see* **Note 7**).

3. Seedlings are transferred to 18 cm diameter pots, filled with 2:2:1 substrate formulation of compost, substrate 2 (Klasmann, Germany), and sand, fertilized by providing 15 g Osmocote (Scotts Celaflor, Germany; 19 % N, 6 % P, and 12 % K) per pot, and further held in a chamber providing a 12 h photoperiod (136 µmol/m²/s photon flux density) and 14/12 °C (day/night).
4. As of the tiller elongation stage, the plants are held in a glasshouse at 18/14 °C (day/night) with a minimum of 16 h photoperiod (170 µmol/m²/s photon flux density) provided by SON-T-Agro lamps (Philips, Netherlands, ca. 200 W/m²) used in addition to natural daylight if required.

3.2 Production of Embryogenic Pollen Cultures of Barley Used for Cocultivation

1. Barley spikes are harvested when the tips of the awns have emerged from the boot. The anthers of these spikes predominantly contain highly vacuolated, pre-mitotic microspores.
2. The boots are cut and surface-sterilized by spraying with 70 % ethanol (*see* **Note 8**). The flag leaf sheath is removed and five dissected spikes placed onto a moistened, 7 cm filter paper disk per 10 cm Petri dish. After sealing, the plates are held in the dark at 4 °C for 3–5 weeks.
3. Fifteen pretreated spikes are chopped into ca. 1 cm fragments and macerated in a Waring blender in the presence of 20 mL 0.4 M mannitol (*see* **Note 9**). The blender drive unit is set on "low" speed and run twice for 15 s.
4. The macerate is filtered through a 100 µm mesh into a transparent box. The blender is flushed with 10 mL of 0.4 M mannitol, which is then also passed through the mesh.
5. The debris remaining on the mesh is squeezed gently to release further suspension into the box, then returned to the blender for re-maceration (twice for 10 s) in another 10 mL of 0.4 M mannitol and the macerate passed through the mesh, which is again followed by flushing the blender.
6. The suspension collected in the box is transferred into a 50 mL tube, and the box flushed with 5 mL 0.4 M mannitol, which is then added to the tube. The suspension is centrifuged (100 × *g*, 10 min, 4 °C).
7. The pellet is re-suspended in 3 mL 0.55 M maltose in a round-bottomed 12 mL tube with a screw cap. The centrifuge tube is flushed with 2 mL 0.4 M mannitol, which is poured carefully over the top of the 0.55 M maltose suspension, thereby forming two distinct liquid layers with different density.
8. The suspension is subjected to density gradient centrifugation in swing-out baskets (100 × *g*, 10 min, 4 °C) with the centrifuge set to give slow acceleration and deceleration to prevent the two established layers with different density from becoming

mixed. The interphase, where viable immature, highly vacuolated pollen have accumulated, is withdrawn by pipetting, transferred to a fresh 50 mL tube to which 10 mL 0.4 M mannitol is added.

9. The pollen is gently suspended evenly, and a representative 100 μL aliquot is removed to a hemocytometer cell in order to estimate the population density. Meanwhile the remaining microspores are pelleted by centrifugation (100 × *g*, 10 min, 4 °C). Before the supernatant is withdrawn, the tube is left stand for ca. 5 min to allow still floating pollen to settle down.
10. The pellet is re-suspended in an appropriate volume of KBP medium to deliver a density of 100,000 immature pollen per 1-mL aliquots that are transferred to 35-cm Petri dishes which are then sealed and incubated until use for cocultivation at 24 °C in the dark (*see* **Note 10**).

3.3 Emasculation and Manual Pollination of Florets Used for Zygote Isolation

1. Spikes are manually emasculated 1–3 days before anthesis, using only the spikelets of the central third of the rachis and the two major (outer) florets per spikelet. All other spikelets and florets are removed from the rachis before detaching the anthers from the florets using fine-tipped forceps. To avoid any unwanted pollination, the spikes are covered by polyethylene bags.
2. During the period of pistil receptivity, one or two freshly dehisced anthers taken from non-emasculated spikes are transferred into each floret, so that fresh pollen is released onto the stigmas.

3.4 Isolation of Zygotes

1. 1–9 h after manual pollination, the spikes are cut, and, after removal of bracts and lemmas, surface-sterilized in 2.5 % NaOCl solution for 10 min, then rinsed four times using autoclaved tap water. All following steps are conducted under aseptic conditions using surface-sterile materials.
2. The preparation of tissue is conducted using sterile, fine-tipped forceps and a scalpel (*see* **Note 11**). The pistils are carefully detached from the florets and collected in a 35-cm Petri dish, containing 2 mL of 0.55 M mannitol.
3. Using a binocular, the lodicules and filaments are removed from the pistils, the basal tips are cut without squashing the tissue (Fig. 1a) and transferred to another dish, containing 2 mL of 0.55 M mannitol.
4. After having collected some pistil tips with their cut side facing the liquid surface, the explants are submerged into the solution to allow them to settle at the bottom of the dish.
5. Using an inverted microscope, the ovule tips are isolated from the pistil tips, and the remaining outer integument and pericarp tissue is discarded (Fig. 1b, *see* **Note 12**).

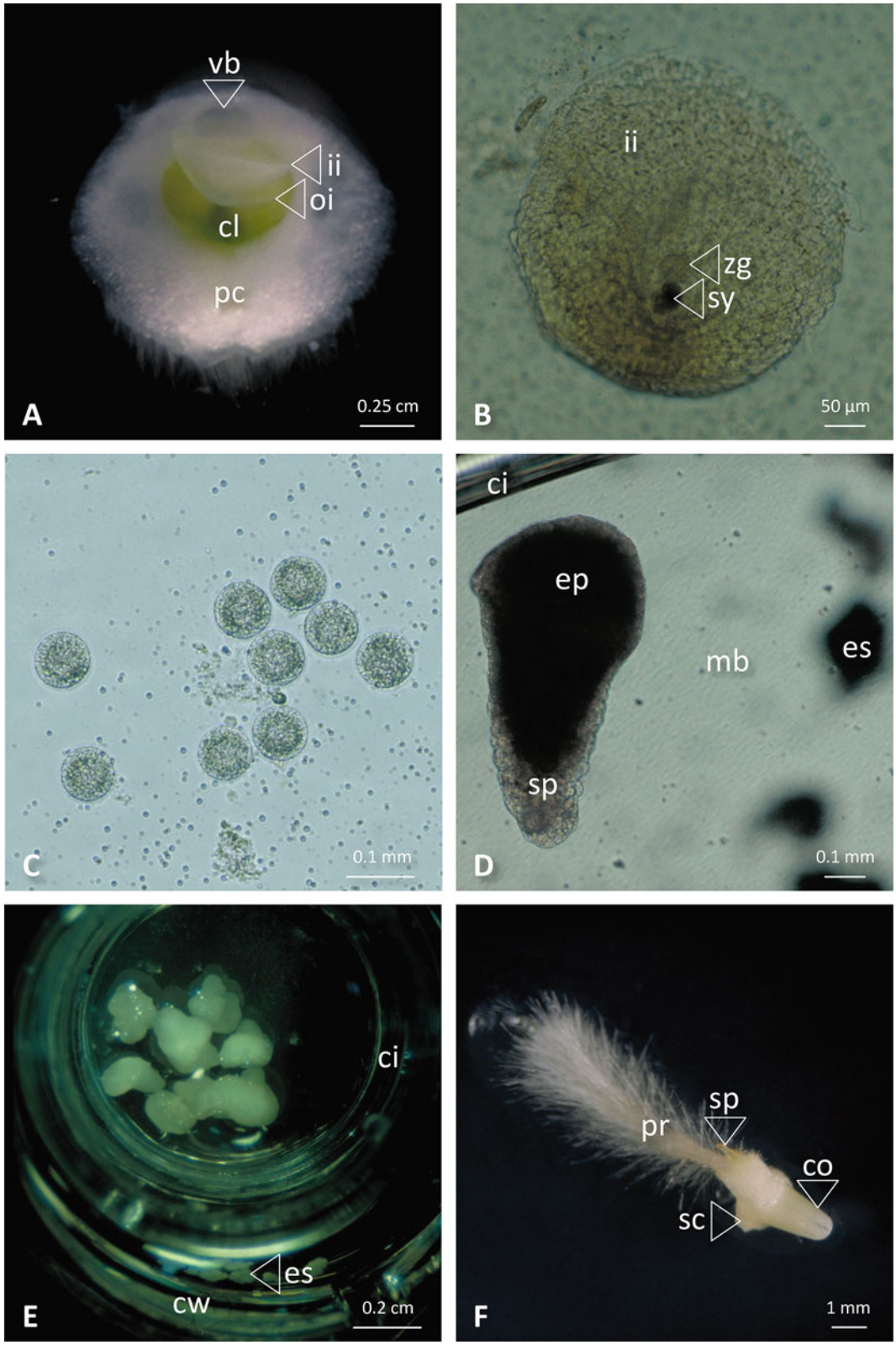

Fig. 1 Isolation and culture of wheat zygotes. (**a**) Cut side of a pistil tip showing pericarp (pc), vascular bundle (vb), chlorophyll layer (cl), outer integument (oi), and inner integument (ii) (binocular); (**b**) ovule tip consisting of

6. Using two fine-tipped glass needles, the zygotes can be gently pushed to be released from the ovule tips (Fig. 1b, *see* **Note 13**).
7. Isolated zygotes are collected close to one another at the bottom of the dish (Fig. 1c) using a glass capillary connected by polypropylene tubing, filled with 0.55 M mannitol solution, to a manually controlled cell tram. This equipment facilitates to take up and release single cells in a few nanoliters of liquid. Before filling the system with mannitol solution, the interior of cell tram, tubing, and glass capillary is to be surface-sterilized using 70 % ethanol, followed by flushing with water.

3.5 Zygotic Embryogenesis and In Vitro Plant Regeneration

1. Using the glass capillary, as many as ten zygotes are transferred onto the membrane of a culture insert containing 100 µL of N6Z medium and placed in a well of a 4-well plate with the well containing another 0.35 mL of the same medium.
2. Per well, 0.15 mL of 1–2 weeks old embryogenic pollen culture is added to the medium outside the culture inserts (Fig. 1d, e). After being sealed, the 4-well plates are incubated in a larger plastic box at 26 °C in the dark for 4 weeks (*see* **Note 14**).
3. Macroscopically visible zygote-derived embryos are transferred to plates containing N6D medium and grown until plantlet formation (Fig. 1e, f). Embryos and small plantlets are subcultivated to plates or containers with fresh N6D medium after 3 weeks (*see* **Note 15**).

3.6 Establishment of Plantlets in Soil

1. Regenerants are transferred to 6 cm diameter pots, filled with Petuniensubstrat (Klasmann, Germany), and placed in a tray covered by a transparent hood to maintain a high humidity environment. The tray is placed in a chamber providing a 12 h photoperiod (136 µmol/m^2/s photon flux density) and 14/12 °C (day/night).
2. After 2 weeks, the hood is removed and the tray left uncovered for another week.
3. Plantlets are further grown as described above for the donor plants.

Fig. 1 (continued) inner integument (ii) and displaying degenerated synergid (sy) and zygote (zg) (inverted microscope); (**c**) Freshly isolated zygotes collected at the bottom of the Petri dish used for dissection (inverted microscope); (**d**) globular zygotic embryo with suspensor (sp) and embryo proper (ep) residing on the membrane (mb) of a culture insert (ci) after 2 weeks of culture; embryogenic structures (es) derived from cocultivated barleypollen are visible behind the membrane (inverted microscope); (**e**) zygotic embryos in a culture insert (ci) after 4 weeks of culture, embryogenic structures (es) derived from cocultivated barley pollen are visible in the outer medium portion between insert and culture well (cw) (binocular); (**f**) zygotic embryo on regeneration medium with primary root (pr), degenerated suspensor (sp), scutellum (sc), and coleoptile (co) (binocular)

4 Notes

1. Components of mineral stocks are dissolved separately before mixing, and then the whole solution is made up to the required volume.
2. The use of hot water is to prevent the dissolved molecules from re-precipitating when being exposed to reduced solvent concentration. As soon as the final concentration of the stock is established, precipitation will no longer appear to happen even after the solution is cooled down.
3. N6Z contains only half the concentration of macro minerals as compared to the original N6 medium according to Chu and coworkers [18].
4. The reason for the use of glucose as major osmoticum and carbohydrate source in N6Z medium is that a disaccharide, such as sucrose or maltose, would effect a higher specific density which entails freshly isolated zygotes to float to the medium surface where most of them would burst.
5. Since the addition of some organic acids formerly used in N6Z and N6D media [3] proved to be unnecessary, these can be omitted.
6. N6 minerals [18] need to be supplemented with additional $CaCl_2$ to obtain a final concentration of 3 mM, so making sure that the medium will solidify using Phytagel.
7. The vernalization treatment is not required in the spring-type accessions.
8. All steps following the surface-sterilization of the boots are to be conducted under aseptic conditions, using surface-sterile equipment and solutions.
9. All equipment and solutions used to process the pretreated spikes and immature pollen need to be precooled to 4 °C and should be kept on ice.
10. 1–2 week-old embryogenic pollen cultures are used for cocultivation with isolated zygotes, whereas cultures older than 2 weeks showed a reduced capability of supporting zygotic embryogenesis in vitro.
11. To keep the scalpel blade and forceps clean, it is advisable to remove tissue debris after each preparation step using a piece of household viscose foam, autoclaved and moistened with distilled water.
12. While the zygote is visible through the inner integument that forms the isolated ovule tip, the removal of the outer integument is essential to facilitate zygote isolation.

13. Wheat zygotes are shaped pear-like as long as being embedded in ovular tissue. Owing to the step-wise release from the stabilizing embryo sac and the use of hypotonic solution for the isolation procedure, the zygotes take on a spherical form before they can be recognized within the ovule tips (Fig. 1b). Since the zygote is protoplast-like and not interconnected with neighbor cells via plasmodesmata, it can be mechanically isolated without the use of cell wall-degrading enzymes. The diameter of isolated wheat zygotes is 60–80 μm.
14. In vitro zygotic embryogenesis follows fairly the same pattern formation as the one of embryos growing *in planta*. While the first zygotic cell division appears to be symmetrical with regard to the volume of daughter cells and is generally completed within 24 h after pollination, the following period of 1–2 weeks of bisymmetric proembryo development includes the differentiation of suspensor and embryo proper (Fig. 1d). This is followed by dorsoventral embryo development, characterized by the formation of scutellum, shoot apical meristem, coleoptile, primary root primordium, and coleorhiza.
15. The efficiency of embryo formation and plant regeneration proved to be genotype independent. However, a drop in isolation efficiency was observed when exotic accessions with smaller grains were used [21]. Using the described procedure, 80–90 % of the isolated zygotes form embryos, most of which are capable of plant regeneration. The plants obtained show phenotypically normal development and grain set.

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Chapter 30

From Somatic Embryo to Synthetic Seed in *Citrus* spp. Through the Encapsulation Technology

Maurizio Micheli and Alvaro Standardi

Abstract

In vitro propagation by somatic embryogenesis represents an efficient alternative method to produce high-quality and healthy plants in *Citrus* species. The regenerated somatic embryos need protection from mechanical damages during manipulation and transport, as well as nutritive support for their evolution in plantlets after sowing. The encapsulation technology allows to obtain synthetic seeds by covering somatic embryos with a gel of calcium alginate enriched by nutrients. This chapter describes the procedure for producing synthetic seeds containing somatic embryos from different *Citrus* genotypes.

Key words Artificial seed, Calcium alginate matrix, Plant tissue culture, Somatic embryogenesis, Synseed

1 Introduction

The increasing world's demand for new and promising *Citrus* genotypes requires effective and innovative technologies for high-quality plant production. Consequently, research is looking for an innovative procedure able to join the advantages of micropropagation (high productive efficiency, sanitary plant conditions, and reduced space requirements) with the technologic characteristics of the zygotic seed, as handling, storability, and transportability [1], actually represented by the synthetic seed technology. The original concept of synthetic seed (artificial seed or synseed) was applied to desiccated or hydrated somatic embryos (SEs) and did not involve the encapsulation [2, 3]. Later Murashige [4] gave the first definition of synthetic seed as "an encapsulated single SE inside a covering matrix."

The large use of sodium alginate as encapsulating agent is due to its moderate viscosity, low spin ability of solution, low toxicity, quick gellation, low cost, and biocompatibility characteristics [5–7]. The encapsulation technology was proposed to safeguard the SEs from mechanical damages during handling in the nursery and

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transportation in the farms, as well as to provide nutrients (*artificial endosperm*) during their evolution in plantlets under in vivo or in vitro conditions (*conversion*). In fact, SEs are structurally similar to gamic or zygotic embryos, but lack nutritive and tegument structures [5]. Nevertheless, the first experiments on the encapsulation were conducted employing SEs, as their bipolar nature, able to convert in plantlets in a single step, made them suitable for synthetic seed production [2, 8]. SEs develop from somatic cells, and this regenerative pathway allows the clonal propagation. Their use as encapsulated explants for synthetic seed preparation is however limited because of the involved difficulties, due to asynchronism during SEs formation and development, somaclonal variation, recurrent embryogenesis [8], and embryo dormancy [9]. Moreover in vitro SE production requires expensive manual labor, even though they could be obtained by bioreactors [10]. Therefore, different propagules were tested to produce synthetic seeds. New perspectives emerged with the use of non-embryogenic unipolar plant propagules. In fact, the most recent concept involves every meristematic tissues (in vitro or in vivo derived), as long as able to convert in a whole plantlet after encapsulation and possible storage [5, 7, 8, 11–14]. However, the abovementioned limitations of SEs for synthetic seeds production seem to be infrequent in *Citrus* spp., and several studies are focused on the application of the encapsulation technology to citrus [7, 15–21].

2 Materials

2.1 Plant Material

Since some researchers found that the SE size affects the conversion in different plant species [22–25], we carried out preliminary experiments using different sized SEs of *Citrus* genotypes for encapsulation (unpublished data). The results indicated that the largest SEs (5–6 mm) showed the highest values in terms of *viability* (green appearance of explants, with no necrosis or yellowing), *regrowth* (increasing in size of the explants with consequent breakage of the involucre and extrusion of at least one visible shoot or root after the sowing), and conversion [16, 17]. Nevertheless their encapsulation involves the formation of an irregular alginate layer around the propagule, reducing the protective and nutritive functions. So, in our experiments, we used only medium-sized SEs (3–4 mm) discarding the larger and the smaller ones (Fig. 1), hence limiting the negative effects of asynchronism (*see* **Note 1**) and recurrent embryogenesis (*see* **Note 2**).

Usually our experiments were carried out using hydrated SEs of *Citrus reticulata* Blanco cv Mandarino Tardivo di Ciaculli, *Citrus limonimedica* Lushington, and *Citrus clementina* Hort. ex Tan. cvs Nules and Monreal, obtained according the procedures described

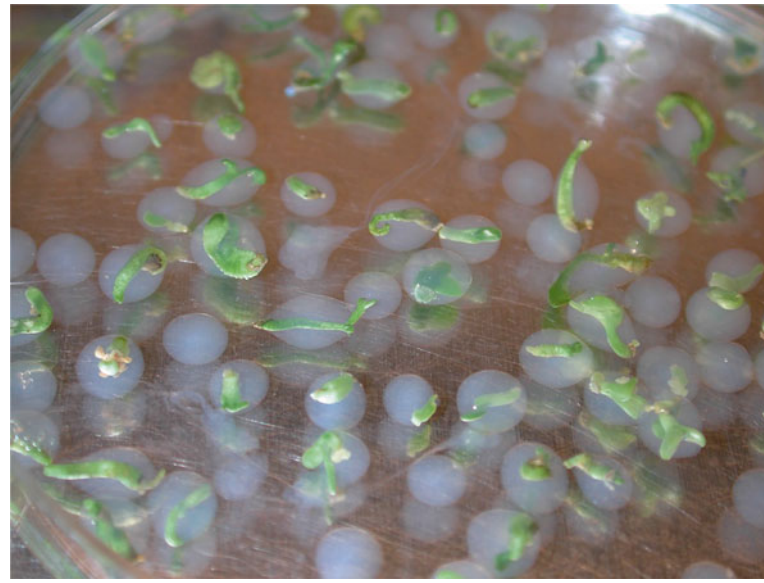

Fig. 1 Synthetic seeds obtained from different sized SEs of *Citrus*

by Germanà and co-workers [26–30]. The synthetic seeds of these genotypes were sown and maintained in aseptic conditions. In addition, synthetic seeds of *Citrus reticulata* Blanco cv Mandarino Tardivo di Ciaculli were sown also in non sterile conditions.

2.2 Encapsulation Solutions

1. Tissue culture facilities: Graduate cylinders, pipettes, lab pipettor, glass beakers, magnetic stirrer, spin bar, analytical balance, lab spoons, weighing boats, pH meter, NaOH and HCl solution (0.1 N), 100 mL screw capped Pyrex glass jars, autoclave, horizontal flow cabinet, forceps, scalpels, blades, and electric incinerator.

2a. Aseptic conditions: Distilled water, half strength MS basal medium [31], 0.25 g/L malt extract, 0.25 g/L ascorbic acid, 1 mg/L gibberellic acid, (GA_3), 0.02 mg/L a-naphthalene acetic acid (NAA), and 68 g/L sucrose (artificial endosperm).

b. Nonsterile conditions: Artificial endosperm and 100 mg/L Thiophanate-methyl TM® (Pestanal, Riedel-de-Haen).

3. Alginate sodium salt, medium viscosity (2.5 % w/v).

4. Calcium chloride anhydrous (1.1 % w/v).

2.3 Sowing Media and Culture Conditions

1. Tissue culture facilities: Graduate cylinders, pipettes, lab pipettor, glass beakers, magnetic stirrer, spin bar, analytical balance, lab spoons, weighing boats, pH meter, NaOH and HCl solution (0.1 N), Magenta® jars (7 × 7 × 7 cm), autoclave, horizontal flow cabinet, forceps, scalpels, blades, electric

incinerator, and growth chamber (temperature of 21 ± 2 °C, photosynthetic photon flux density of 40 μmol/m²/s, and photoperiod 16 h).

2a. Aseptic conditions: Distilled water, full strength MS basal medium [31], 0.5 g/L malt extract, 0.5 g/L ascorbic acid, 68 g/L sucrose and 7 g/L agar, and filter paper bridges.

2b. Nonsterile conditions: Filter paper bridges, perlite, soil (Compo-Cactea®), or Jiffy-7 Pellets (J7).

3 Methods

Three solutions are required to encapsulate SEs: *coating*, *complexing*, and *rinsing* solutions (Fig. 2). The common component is represented by the artificial endosperm (*see* Subheading 2.2) added of 2.5 g/L sodium alginate (coating matrix) and 1.1 g/L calcium chloride (complexing solution). The rinsing solution is composed only by the artificial endosperm. All solutions and media are adjusted to pH 5.5 and autoclaved at 115 °C for 20 min just after their transferring into the containers. During the autoclaving cycle, the sodium alginate is completely dissolved forming a dense dark yellow solution. The artificial endosperm of the synthetic seeds sown in nonsterile conditions is enriched by Thiophanate-methyl TM® (*see* **Note 3**).

Fig. 2 Coating, complexing, and rinsing solutions employed for encapsulation of *Citrus* SEs (*from left to right*)

3.1 Encapsulation

1. Single SEs are immersed in alginate solution for a few seconds (*see* **Note 4**).
2. The alginate-coated SEs are then dropped into the complexing solution for 25–30 min (*see* **Note 5**).
3. The encapsulated SEs are washed 2–3 times in the rinsing solution for 10–15 min in order to remove the toxic residual ions of chloride and sodium (*see* **Note 6**). The whole procedure is carried out in aseptic conditions under a horizontal flow cabinet.

3.2 Sowing and Evaluation

1a. Aseptic conditions: After washing, the synthetic seeds are aseptically transferred into closed Magenta® jars, containing sterilized agar sowing medium or filter paper bridge, moistened with 10 mL of artificial endosperm (*see* **Note 7**).

b. Non-sterile conditions: After washing the synthetic seeds are aseptically transferred into Magenta® jars containing sterilized filter paper bridge, perlite, soil (Compo-Cactea®), or "Jiffy-7 Pellets" (J7) moistened with appropriate amount of artificial endosperm (*see* **Note 7**).

2a. Aseptic conditions: The Magenta® jars are hermetically closed, and the cultures are transferred into the growth chamber.

b. Nonsterile conditions: The cultures are then transferred into the growth chamber, and the Magenta® jars are not hermetically closed, allowing the gas exchanges and the water evaporation. To prevent the synthetic seeds dehydration, the substrates moisture is periodically monitored and restored with distilled water.

3. After 1 week, fungal or bacterial contamination is monitored.
4. After 45 days, viability, regrowth and conversion (Fig. 3) are evaluated.

4 Notes

1. The asynchronism involves the simultaneous presence of different sized SEs at the end of regenerative cultures. Their encapsulation determines the formation of heterogenous synthetic seeds with different ability and energy of conversion. So, the synchronism is a crucial step in taking advantage of somatic embryogenesis for the commercial production of plants by synthetic seeds.
2. Recurrent or secondary somatic embryogenesis in the production of new SEs from the mature ones.
3. The application of synthetic seeds in the nurseries should imply their conversion in non-sterile conditions using substrates as perlite, sand, paper, or peat. In this case, the protection of the

Fig. 3 Extrusion of shoot and root apex (*black arrows*) from the alginate matrix at the beginning of conversion

synthetic seeds from fungal and bacterial contaminations during conversion is essential. The beneficial effect of Thiophanate-methyl fungicide on the *Citrus* synthetic seeds conversion has been showed [17].

4. In substitution to sodium alginate, several substances were tested, like mixture of sodium alginate with gelatin, potassium alginate, polyco 2133, carboxymethyl cellulose, carrageenan, Gelrite, guar gum, sodium pectate, and tragacanth gum [5–7].
5. During the complexation step, ion exchange occurs through the replacement of Na^+ by Ca^{2+}, forming calcium alginate by ionic cross-linking among the carboxylic acid groups and the polysaccharide molecules and producing a polymeric structure called "egg box" [13, 32, 33]. Hardening of calcium alginate bead is affected by the concentration of sodium alginate and calcium chloride, as well as the complexing time. Usually, at higher consistence corresponds good protection during transport and manipulation but higher difficulty of explants in breaking the alginate coat [8].

6. Automation systems have been proposed, as somatic embryogenesis and encapsulation are expensive techniques due to the high manual labor requirement. The use of bioreactors for temporary immersion system has shown to be effective for the production of *Citrus deliciosa* SEs [34]. Concerning automation, several devices are available for the encapsulation of SEs or other in vitro-derived vegetative propagules, using systems based on concentric tube nozzle, multiple wire loops, rotating disks, perforated plates, or precision dripping [10, 35, 36].
7. Before sowing, the synthetic seeds can be stored at 4–6 °C in darkness, using closed sterile dishes or vials containing some drops of artificial endosperm solution to avoid dehydration.

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Chapter 31

From Stress to Embryos: Some of the Problems for Induction and Maturation of Somatic Embryos

Sergio J. Ochatt and Maria Angeles Revilla

Abstract

Although somatic embryogenesis has been successfully achieved in numerous plant species, little is known about the mechanism(s) underlying this process. Changes in the balance of growth regulators of the culture medium, osmolarity, or amino acids as well as the genotype and developmental stage of the tissue used as initial explant may have a pivotal influence on the induction of somatic embryogenic cultures. Moreover, different stress agents (ethylene, activated charcoal, cold or heat or electrical shocks), as well as abscisic acid, can also foster the induction or further development of somatic embryos. In the process, cells first return to a stem cell-like status and then either enter their new program or dye when the stress level exceeds cell tolerance. Recalcitrance to differentiation of somatic cells into embryos is frequently observed, and problems such as secondary or recurrent embryogenesis, embryo growth arrest (at the globular stage or during the transition from torpedo to cotyledonary stage), and development of only the aerial part of somatic embryos can appear, interfering with normal germination and conversion of embryos to plants. Some solutions to solve these problems associated to embryogenesis are proposed and two very efficient somatic embryogenesis protocols for two model plant species are detailed.

Key words *Arabidopsis thaliana*, Embryogenesis recalcitrance, *Medicago truncatula*, Stress agents

1 Introduction

Somatic embryogenesis is in many species the predominant plant regeneration pathway, during which dedifferentiated somatic plant cells become totipotent and develop into embryos which, subsequently, convert into plants [1–3]. Surprisingly, although somatic embryogenesis is widely used for propagation and has in recent years become one of the preferred regeneration methods for commercially cultivated biotech crops, little is known about the mechanisms underlying this process. One of the main interesting features of somatic embryogenesis is that it may sidestep aging limitations of cultures to yield large numbers of embryos from elite genotypes including for woody species [4], where somatic embryogenesis is likely the only way of producing juvenile tissues of recalcitrant

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species [5]. In addition, as somatic embryo development resembles that of zygotic embryos [2, 6], they are also an interesting model system to understand the physiological, biochemical, and molecular mechanisms taking place during embryo development [6–9]. The use of somatic embryogenesis has been reviewed in a large number of species [10–12]. The ability of cells to retain totipotency and developmental plasticity in a differentiated stage makes them unique and renders them capable to dedifferentiate, proliferate, and subsequently regenerate into mature plants, provided that optimum culture conditions are developed [3, 13, 14].

Embryogenic cultures are initiated from primary explants on a medium containing mainly an auxin alone but often also a cytokinin [1, 6, 15, 16] and, sometimes, even only a cytokinin [16]. The most commonly used auxin is 2,4-dichlorophenoxyacetic acid (2,4-D) [1, 12, 15, 17], which has been suggested to downregulate gene expression through changes in the level of DNA methylation [14, 18]. In addition, various culture conditions and treatments have an impact on the induction of somatic embryogenesis, including balance of plant growth regulators [15, 17], medium osmolarity [19, 20], pH [1], amino acid, or salt concentration [21], while the most influential traits identified so far remain the particular genotype studied and the developmental stage of the tissue used as initial explant [22].

Somatic embryogenesis may be initiated either directly by inducing embryos to develop on the surface of the initial explant, or indirectly, via an intermediary step of callus formation from which the embryos subsequently regenerate [6, 21]. Once embryogenic cells are initiated, they undergo a continuous, unlimited cycle producing further pro-embryogenic or embryogenic masses [23], resulting in multiplication of the original plant. A special case of somatic embryogenesis is secondary or recurrent embryogenesis, which occurs when the first somatic embryo formed fails to germinate and, instead, gives rise to new successive cycles of embryogenesis [17]. In some species, this has been sought as a means of cloning embryogenic lines as the process can be maintained indefinitely [5, 24, 25]. However, secondary embryos develop directly from epidermal and subepidermal cells of embryos [26], mostly at their root pole or on the main axis and cotyledons [27–29], whereby they interfere with normal germination of the original embryo and, *in fine* being repetitive, with the conversion to plants. For somatic embryos, to reach the cotyledonary stage and then accumulate the storage products needed for conversion to plants [6], the medium and culture conditions have to be changed. One of the main growth regulators in embryo maturation in vivo is abscisic acid (ABA), and treating embryogenic cultures in vitro with this hormone has been beneficial in some species [6, 23, 29, 30], particularly because of its involvement in the acquisition of partial desiccation [5, 25] or cold tolerance of mature

somatic embryos that precedes their competence to germinate [17, 23]. Likewise, ethylene, activated charcoal [31], pH [1], cold [20, 32] or heat [1] shocks, osmotic stress [19, 20, 33, 34], electricity [35], and even centrifugation [36, 37], and sonication [37–39] have been reported to foster somatic embryo induction but also maturation in different species. No such clear effect can be ascertained for light conditions. Dark culture has been beneficial for embryogenesis, reducing the activity of enzymes responsible for release of phenols that induce callus and early embryo browning in species prone to suffering from such phenomena [3, 4, 15, 17]. On the other hand, photoperiodic light regimes have been preferred and even required for other species [37, 40]. Other stress agents such as heavy metals, starvation, and wounding have also been reported to promote responses in several models [41, 42], and they are moreover an integrating part of dedifferentiation. In this respect, several studies have shown that, prior to redifferentiating, cells first return to a stem cell-like status and then either enter their new program or dye when the stress level exceeds cell tolerance or when a mediation of cell responses to stress is hampered by their physiological status [3, 6, 43, 44]. Indeed, stress-induced morphogenetic response has been ascribed to the redirection of growth to better acclimate to an exposure to stress [13, 42, 45].

Only those mature somatic embryos with a normal morphology having accumulated enough storage products will be able to convert into normal plants [6, 7, 29]. Following transfer to a germination medium, somatic embryos develop similarly to zygotic embryos, yielding plants that should be true to type [6, 16, 33, 46]. The hormonal composition needed for germination of the somatic embryos will mostly depend on the species (and sometimes genotype) studied and there is no generally applicable rule. Hormone-free media have been reported for both herbaceous [1, 29] and woody [47] species, but media with various auxin/cytokinin contents have been employed with species as wide apart as legumes [16] and forest trees [17]. Besides a number of publications referred the need to add extra miscellaneous compounds to the medium such as glutamine, casein hydrolysate, etc. [1, 17], conversely, there is consensus in the literature on the conditions required to acclimatize the somatic embryo-derived plantlets to in vivo conditions which is similar to that usually employed for micropropagated plants [1].

The most frequent applications of somatic embryogenesis are the mass propagation of selected material, obtained after in vitro selection or genetic transformation [5, 6, 17, 48, 49], and there are several examples of its commercial exploitation, in particular for gymnosperms [23], while this is generally still to be done for most angiosperms with the exception of several ornamentals. It is also employed for a better understanding of various fundamental mechanisms and processes, including those dealing with the acquisition

and eventual loss of regeneration competence, as well as for the recovery of novel genotypes following in vitro selection for stress tolerance, somatic hybridization, or gene transfer. These aspects have already been reviewed and discussed in the past. Here, we shall focus on the problems that may arise during somatic embryogenesis and we shall also discuss some possible solutions for them.

2 Development of Somatic Embryos Formed In Vitro

Somatic embryogenesis is one of the two major pathways for plant regeneration in vitro, and it may take place from undifferentiated tissues (protoplasts, cell suspensions, callus) but also from highly differentiated cells (immature gametes), leading respectively to regeneration of normal plants (that should resemble the mother plants) or to haploids that will thereafter have to undergo chromosome doubling for genome fixation. Since somatic embryos can arise from a single cell, it is a way of choice to regenerate transgenic plants. The process includes a sequence of developmental stages, the first of which is often the induction of callus from explants (Fig. 1a), followed by the induction of somatic embryos from such callus tissues that will thereafter follow a common developmental path from globular- to heart-shaped embryos (Fig. 1b), then torpedo-shaped embryos (Fig. 1c), and finally mature cotyledonary embryos (Fig. 1d) which are capable of "germinating" (Fig. 1e), i.e., of converting into whole viable plantlets. To date, these general

Fig. 1 A typical sequence of somatic embryogenesis from root- or leaf-derived callus of *Arabidopsis*. (**a**) Leaf explant starting to produce callus. (**b**) A highly embryogenic callus with many somatic embryos at early stages of development, i.e., from globular to heart. (**c**) A cluster of torpedo stage embryos showing new globular embryos developing on one of the torpedo embryos (*arrow*). (**d**) A highly embryogenic cluster with mostly torpedo to cotyledonary stage embryos. (**e**) Liquid culture of embryogenic clusters whereby, with time, only embryos and plantlets proliferate in culture

steps have been successfully applied to many species [50]. In this context, auxin is required to induce and maintain a high rate of proliferation of unorganized plant cells, but low-auxin or simply a hormone-free medium is needed to induce those developmental responses that are normally dependent on endogenous hormonal factors [43, 51]. Embryos of a unicellular origin are similar to globular zygotic embryos and they are sometimes connected to the maternal tissue by a suspensor like structure, while those derived from multiple cells initially look like a smooth and bright nodule where the embryos at its base are usually connected to the maternal tissue through their epidermis [52]

2.1 Some of the Problems Arising During Somatic Embryogenesis and Possible Solutions

Not all plant cells are capable of expressing totipotency in vitro, a process that strongly depends on the genetic background, the physiological status of the donor plant, the type of explant, and its physiological/developmental status, the culture medium and conditions, and any possible interactions among all these factors [15, 53]. Activation of key regulators of somatic embryogenesis is preceded by a reprogramming of cellular metabolism which is often induced by some kind of physiological stress and will not be expressed by somatic cells although it already potentially exists in the plant genome [13, 41, 42, 51]. Thus, the developmental switch from a somatic to an embryogenic status in cells occurs under the influence of both physical and chemical inductors but also requires a major and dynamic reprogramming in gene expression [7, 41, 42, 54], entailing the activation of a number of signal cascades leading to a differential (released) gene expression which, in turn, renders the undifferentiated cells capable to acquire an embryogenic capacity [6, 13, 43].

As opposed to the highly efficient embryogenesis sequence shown in Fig. 1, there are many species where attaining this is still difficult and sometimes even impossible. Indeed, there are three key stages in this process where a blockage may occur, two of them

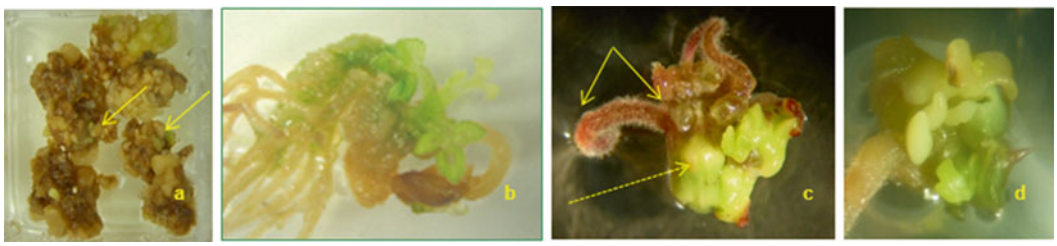

Fig. 2 Some problems that may be found during early stages of somatic embryogenesis in pea (*Pisum sativum* L.). (**a**) Browning of globular and heart somatic embryos (*arrows*) and of the callus supporting them. (**b**) Callus overgrowth and secondary somatic embryogenesis on developing embryos that will fail to convert into plants. (**c**) A cluster of abnormal somatic embryos showing browned roots (*solid arrows*) and fused cotyledons (*dotted arrow*). (**d**) Pale embryos that have not accumulated storage products are blocked at the late cotyledonary stage and are unable to germinate

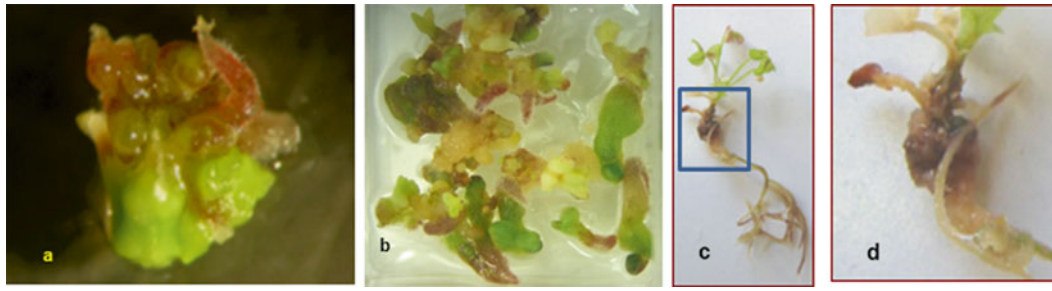

Fig. 3 Problems with rhizogenesis from somatic embryos of *Medicago truncatula*. (**a**) By 9 days on an unsuitable medium with root primordia browned and recallusing. (**b**) Embryos of the same age on the right medium. (**c**) Senescent seedling due to the lack of vascular connection between the roots and the aerial part. (**d**) Close-up of hypocotyl/root section of the plantlet in (**c**) showing the interfering callus proliferation

during early development of embryos (Fig. 2) and the third one at the latest stages leading to rooting (Fig. 3), as follows:

1. The earlier stages of somatic embryo development, with embryo growth arrested at the globular stage, whereby mitoses stop and embryos start to brown or are covered by de novo callus overgrowth (Fig. 2a), which may also sometimes be associated with secondary embryogenesis (repetitive or not) (Fig. 2b).
2. During the transition from torpedo to cotyledonary embryos, where abnormal (i.e., "trumpet" (Fig. 2c)) fused cotyledons are formed and/or embryos become pale in color (Fig. 2d), due to a lack of accumulation of the storage compounds needed for the somatic embryo to mature and eventually germinate.
3. At the end of embryo development where, as may happen also with organogenesis-derived regenerants, only the aerial part of the somatic embryos develops (Fig. 3a) and they have to be transferred to a different medium for rooting. Then, while the root pole should grow fast, in an unsuitable medium callus starts to proliferate instead (Fig. 3b), mostly at the junction between the aerial part and the root of the somatic embryo-derived plantlet (Fig. 3c). Such plantlets often lack vascular connection between the root and shoot portions and die upon transfer in vivo.

Species with a tendency to undergo any one or all of these processes are regarded as recalcitrant to somatic embryogenesis and thus to biotechnology approaches based on it for the recovery of novel genotypes, as in haplo-diploidization and various genetic transformation protocols, e.g., with legumes [16, 55] and cereals [13].

As the developmental blockages above tend to concern different processes, they require different solutions. It is difficult to suggest general strategies that will successfully resolve these problems

but some simple measures can often be applied to at least palliate or delay their occurrence and thereby permit the recovery of somatic embryo-derived normal and fertile plants. Thus, for blockages occurring at the earliest stages above, shortening the periodicity between subcultures restrains callus development and allows very immature embryos to develop better. This operation, however, is not without risks as early globular somatic embryos must be excised and transferred individually for subsequent development on a medium that, generally, will have to be enriched in cytokinins and, sometimes, also in gibberellic acid. This problem is frequently observed among protoplast-derived callus of pea [29], and several early cytological predictors of the acquisition of somatic embryogenesis competence [19] may help to monitor the evolution of callus tissues during their culture, in order to act before the shift from embryogenic back to a sporophytic path is onset. Also at this stage, increasing the agar concentration of the medium or, likewise, increasing its osmolarity either by replacing, at least partially, the sucrose or glucose with a polyalcohol such as mannitol, or by adding polyethylene glycol (MW 6000), may also be helpful to slow down the callus proliferation while having little deleterious effect on the developing somatic embryos [15, 20, 34]. On the other hand, when the problem encountered is secondary embryogenesis, a distinction should be made between situations where this process is repetitive and those where the first secondary embryos formed will normally convert into plants. Thus, in the latter case the best solution is probably to simply disregard the problem and try to enhance secondary embryogenesis instead, so as to increase the potential for regeneration. Conversely, when the process is constant and no embryos ever germinate, little information about possible solutions is available in the literature. One possibility that has worked to date with several genotypes of legumes [15] is to add abscisic acid to the medium for at least one passage and in combination with an auxin.

For situations when embryo development stops at the transition from torpedo to cotyledonary stage, modifications to the nutrient composition of the medium may prove appropriate to warrant a sufficient accumulation of storage compounds as needed for embryo maturation. In this respect, it has been shown that nitrogen, sulfur [6], and also sugar (type and concentration, 7) in the medium play a major role in embryo maturation. Likewise, some studies have shown that introducing a mild ionic stress (Na^+ or K^+) at this transition stage might favor embryo maturation [34]. Finally, when the main blockage found is for the rooting of shoots derived from somatic embryos (whose root pole would not develop easily on the embryogenic media), this is likely due to the use of an unsuitable combination of medium salt-strength and hormone (auxin) content. Such cultures should be treated as for conventionally propagated difficult-to-root shoots, i.e., by testing a reduction

of the salt-strength to half (or even less in very recalcitrant species such as some cereals and various neglected crops), by replacing a strong auxin by a weaker one (i.e., if NAA was used, replacing it with IBA or IAA), or by totally deleting auxin from the medium. In extremely difficult species, a last resort would be the micrografting of the somatic embryo-derived shoots on suitable in vitro germinated seedlings of the same species (and ideally also genotype).

3 Two Example Protocols of Somatic Embryogenesis

As an example, optimized protocols for the induction of somatic embryogenesis in *Arabidopsis thaliana* and *Medicago truncatula* are reported here.

3.1 Materials

All stock solutions are prepared using ultrapure water (Milli-Q, prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and, unless stated otherwise, analytical grade reagents. All reagents were purchased from Kalys (fr.kalys.com) or Sigma-Aldrich (www.sigmaaldrich.com) and disposable plastic ware (culture dishes, multiwall plates, pipettes, etc.) from Dutscher (www.dutscher.com). When dealing with GMO material, all needed precautions in terms of biosecurity are respected. Reagents and stock solutions are generally stored in the fridge or frozen until use, while media are kept at room temperature in the dark until use.

3.1.1 Plant Materials

For studies with *Arabidopsis thaliana*, seeds of wild-types C24 and Col but also of the cytokinin-overproducing mutants *hoc* [56] and *amp1* [57] are used. These seeds are stored in a cold chamber (4 °C, in darkness) until used. For experiments with *Medicago truncatula*, R108-1 genotype seeds are generally used as the source of explants.

3.1.2 Composition of Media Used

Table 1 details the composition of basal media and stock solutions used to prepare them. For *Arabidopsis thaliana*, MPic medium is based on MS medium [58] and contains 0.2 mg/L picloram and benzylaminopurine (BAP) at 0.5 mg/L (for leaves) [29] or 1.0 mg/L (for roots). The sequence of SH-based media used for *Medicago truncatula* is based on N6 major salt formula [61], SH microelements and vitamins [62], 0.38 mM FeEDTA, 0.55 mM myoinositol, and having the pH adjusted to 5.8 prior to autoclaving. They differ in their sucrose content, mineral strength, and hormonal composition. SH3 contains 4 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) plus 0.5 mg/L BAP and 30 g/L sucrose, while media SH9 and ½ SH9 are both hormone-free and only differ in the salt strength which is reduced by half

Table 1
Composition of media and stock solutions

Stock solutions for SH Medium (*59*)

N6 Macro-salts (*58*)
(for 1 L of 10× stock solution)

$MgSO_4 \cdot 7H_2O$	1.85 g
KNO_3	28.30 g
$(NH_4)_2SO_4$	4.63 g
$CaCl_2 \cdot 2H_2O$	1.66 g
KH_2PO_4	4.0 g

Macro-salts
(for 500 mL of 20× solution)

KNO_3	25 g
$MgSO_4 \cdot 7H_2O$	4 g
$CaCl_2 \cdot 2H_2O$	2 g
$NH_4H_2PO_4$	3 g

Micro-salts
(for 100 mL of 1000× solution)

$MnSO_4 \cdot H_2O$	1 g
H_3BO_3	500 mg
$ZnSO_4 \cdot 7H_2O$	100 mg
KI	100 mg
$Na_2MoO_4 \cdot 2H_2O$	10 mg
$CuSO_4 \cdot 5H_2O$	20 mg
$CoCl_2 \cdot 6H_2O$	10 mg

Vitamins
(for 100 mL of 1000× solution)

Nicotinic acid	500 mg
Thiamine HCl (Vitamin B1)	500 mg
Pyridoxine HCl (Vitamin B6)	500 mg

Medium SH3 (for 1 L); if solidified add 3 g/L Phytagel

N6 Macro	100 mL
SH Micro	1 mL
SH vitamins	1 mL
Fe EDTA	5 mL
Myo-inositol	100 mg
Sucrose	30 g
2-4 D	4 mg
BAP	0.5 mg

Medium SH9 (for 1 L); if solidified add 7 g/L agar

N6 Macro	100 mL
SH Micro	1 mL
SH vitamins	1 mL
Fe EDTA	5 mL
Myo-inositol	100 mg
Sucrose	20 g

Medium 0.5× SH9 (for 1 L); if solidified add 7 g/L agar

N6 Macro	100 mL
SH Micro	1 mL
SH vitamins	1 mL
Fe EDTA	5 mL
Myo-inositol	100 mg
Sucrose	20 g

Medium SHb10 (for 1 L); if solidified add 6 g/L agar

SH Macro	50 mL
SH Micro	5 mL
SH vitamins	5 mL
Fe EDTA	5.5 mL
Myo-inositol	1 g
Sucrose	10 g

for ½ SH9. For preparation of semisolid media, SH3 medium is further supplemented with 3 g/L Phytagel, while media SH9 and ½ SH9 are gelled with 7 g/L HP696 agar (Kalys).

3.2 Methods

3.2.1 *Arabidopsis thaliana*

A very straightforward protocol will permit the establishment of the highly embryogenic culture in *Arabidopsis*, detailed in Fig. 1, as follows:

1. Seeds of *Arabidopsis* wild-types C24 and Col but also the cytokinin-overproducing mutants *hoc* [56] and *amp1* [57] are sterilized during 10 min with a calcium hypochlorite solution (2.5 %, w/v) and imbibed at 4 °C for 3 days before germination in constant light at 22 °C.
2. Germinated plants are placed in MS [58] and grown in a growth chamber at 22 °C with a 16/8 h day/night photoperiod of a light intensity of 90 μmol/m^2/s.
3. Plant age is calculated from the first day at 22 °C; 27 days after germination, plants are harvested and individual leaves are wounded with a scalpel, and roots cut to 1–2 cm length.
4. Leaf/root explants are kept stationary in liquid MS and MPic media (pH 5.6), respectively, to regenerate embryos.

5. Cultures are observed weekly during 6 weeks, and the medium is not renovated until the end of the experiments, i.e., until 63 days.
6. By 6 weeks from culture initiation, cultures are transferred for 3 weeks onto hormone-free MS medium for expression of embryogenesis.
7. After 9 weeks from culture initiation, cultures exhibit large numbers of globular somatic embryos and are transferred to liquid medium of the same composition as used to induce embryogenesis, cultured with shaking (80 rpm) under the same conditions as above, and subcultured every 4 weeks thereafter.
8. By the first passage with shaken conditions, the tissues from both explant sources are completely covered with somatic embryos at different stages of development. The somatic embryos start to detach from the explants, and 1 month later the flasks will mostly contain germinating embryos only.

3.2.2 Medicago truncatula (Table 1)

The barrel medic, *Medicago truncatula*, is considered as a model legume species in terms of biotechnology approaches, and R108 is one of the genotypes that can be regenerated by somatic embryogenesis and genetically transformed [59], even if gene transfer is efficient only for a few genotypes.

1. Use seeds of the R108-1 genotype of *M. truncatula* that have been stratified (48–72 h in the dark at 4 °C), as described in Trinh et al. [60] and Ochatt et al. [16].
2. Scarify seeds (1 M H_2SO_4 for 2 min or grated with sand paper) for efficient germination.
3. Germinate seeds on humid filter paper at 24 °C in the dark for 48–72 h.
4. Transfer germinated seeds to hormone-free MS medium [16] or SHb10 medium (Table 1; 60), at 22 ± 2 °C under a 16 h light photoperiod at 90 μmol/m^2/s from warm white fluorescent tubes, for 4–6 weeks.
5. Use individual folioles from trifoliate leaves (i.e., the cotyledonary leaves were discarded), harvested from the 4–6-week-old plantlets grown in vitro (*see* **step 4**). Alternatively, folioles from trifoliate leaves on in vivo seedlings of the same age, but grown in the glasshouse (19–22 °C, 60–70 % relative humidity, 16/8 h light/dark photoperiod at 200 μmol/m^2/s) may also be used.
6. For the initiation of callus followed by somatic embryo induction, folioles are cut 3–4 times with the blade of a scalpel (perpendicularly to the main vein) and transferred to SH3 medium, which contains 3 % (w/v) sucrose (commercial sugar can be

used) and is also supplemented with 16 μM (4 mg/L) 2,4-D and 2 μM (0.5 mg/L) BAP. Cultures are kept at 25 °C in the dark, and explants transferred to fresh medium every 2 weeks.

7. During 8–10 weeks of culture, callus develops and some early globular pro-embryonic structures appear on the callus surface.
8. After 8–10 weeks, callusing explants are transferred to hormone-free medium SH9, which contains only 2 % (w/v) sucrose, and cultures are transferred to the photoperiodic light and temperature regime as reported above for expression of somatic embryogenesis. On this medium, somatic embryos develop up to the cotyledonary stage and start to germinate.
9. Transfer germinating somatic embryos to half-strength SH9 medium for complete rooting of plantlets, which are thereafter acclimatized and transferred to the glasshouse until maturity and seed set.

It is important to note that if this protocol is used for gene transfer or in vitro selection for stress tolerance, once a given callus produces a shoot from embryos, this is harvested and the callus is discarded to prevent multiplication of several plants derived from the same transformation or selection event.

4 Conclusions

The FAO [63] has been ringing the alert about the ever-increasing demand for food and feed resulting from the constant demographic increase, while most quality arable land is already under exploitation, which pushes cultivation to more marginal areas and soils so that crops are confronted with novel or increased stress agents [64]. The need for developing new more stress-resistant genotypes to ensure food supply is becoming urgent, and one of the ways to producing them is by exploiting biotechnology approaches in vitro, such as somatic embryogenesis. Against this background, integration of conventional breeding programs and molecular and cell biology approaches based on somatic embryogenesis proves invaluable to fasten the generation of genetically improved commercial crop species [10, 13, 49, 64].

Despite all the knowledge about the requirements for in vitro regeneration accumulated in the literature over the last decades, it is still a matter of controversy why certain genotypes, cells, or explants are embryogenic, while others are not [13, 15, 19]. Thus, the optimization of culture conditions remains mainly an empirical exercise driven by experience and intuition of researchers to assess a range of combinations of potentially effective parameters. However, various recent advances would permit to better define

and measure the totipotent status and hence the degree of cell specificity toward regeneration competence. In this context, it is most likely that the reason for this gap in knowledge comes from the complex interactions in place between the environmental conditions (including the composition of the used culture medium) and the physiological status of the cells at the time of culture which are under the tight control of genes whose expression also depends on many factors and conditions [6, 13, 19, 45].

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Chapter 32

Cryotechniques for the Long-Term Conservation of Embryogenic Cultures from Woody Plants

Elif Aylin Ozudogru and Maurizio Lambardi

Abstract

Since its development in the 1960s, plant cryopreservation is considered an extraordinary method of safe long-term conservation of biological material, as it does not induce genetic alterations and preserve the regeneration potential of the stored material. It is based on the storage of explants at cryogenic temperatures, such as the one of liquid nitrogen (−196 °C), where the metabolism within the cells is suspended; thus, the time for these cells is theoretically "stopped". Cryopreservation is particularly important for embryogenic cultures, as they require periodic subculturing for their maintenance, and this, in turn, increases the risk of losing the material, as well as its embryogenic potential. Periodic re-initiation of embryogenic cultures is possible; however, it is labor intensive, expensive, and particularly difficult when working with species for which embryogenic explants are available only during a limited period of the year. Among various methods of cryopreservation available for embryogenic cultures, slow cooling is still the most common approach, especially in callus cultures from softwood species. This chapter briefly reviews the cryopreservation of embryogenic cultures in conifers and broadleaf trees, and describes as well a complete protocol of embryogenic callus cryopreservation from common ash tree (*Fraxinus excelsior* L.) by slow cooling.

Key words Cryopreservation, Embryogenic cultures, *Fraxinus excelsior* L., Slow cooling, Somatic embryos, Two-step freezing, Woody trees

1 Introduction

Cryopreservation refers to the storage of biological material (such as seeds, somatic/zygotic embryos, embryogenic and organogenic callus cultures, shoot tips, axillary buds) in liquid gases, at ultra-low temperatures. Among various gases available, liquid nitrogen (LN), which is in such a physical state at −196 °C, is usually preferred due to its numerous advantages, such as low cost, ease in handling, ease in delivery, and no toxicity for preserved plant specimens. When a biological material is subjected to such a low temperature, almost all of the biological reactions within its cells are hampered. Suspension of the metabolism in such way ensures the storage of the material for theoretically unlimited time, without inducing any genetic alteration. Hence, the technique is considered

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the only valuable approach to the ex situ long-term conservation of plant biodiversity that can be regarded as complementary to the traditional storage in seed banks and in in-field collections [1]. Water content of the cells during the immersion in LN is the key factor affecting the success of cryopreservation. It should be low enough to prevent the formation of lethal intracellular ice crystals and yet high enough to enable recovery of viable explants after storage in LN. This is induced by triggering the "vitrification" of cytosol, which is a physical state of water solutions, in which the solidification of water molecules at ultra-low temperature is obtained through their transition to an amorphous ("glassy") state, instead of crystallization [2]. Cell vitrification can be induced in several ways: (1) by imposing "cryodehydration" of explants, i.e., by a gradual decrease of their temperature (usually at a rate of −0.5/−1 °C/min) up to −40 °C before the immersion in LN ("slow cooling"), (2) by the use of highly concentrated vitrification solutions ("chemical dehydration"), or (3) by exposing the explants to sterile air flow or silica gel ("physical dehydration"). The latter two techniques allow the direct immersion of the explants in LN and thus are referred as "one-step freezing" [3].

Cryopreservation is particularly important for embryogenic cultures because, once a cell culture is established, it requires periodic subculturing for the maintenance, and this is not only labor intensive but also increases the risk of losing either the material through contamination, human errors or technical failures, or its embryogenic potential through the frequent long-term subculturing [4, 5]. Periodic re-initiation of embryogenic cultures can provide a solution to this drawback. However, this is labor intensive and expensive, too, and is particularly difficult when working with species for which suitable explants for embryogenic callus induction are available only during a limited period of the year [6–8]. Nevertheless, development and optimization of efficient cryopreservation protocols for embryogenic cultures allow the safe, low-cost, and long-term conservation of this unique material [9]. Cryopreservation of embryogenic cultures is a relatively recent application of the cryogenic technology, first examples of detailed and successful protocols being available only in the early 1990s [10, 11]. Sakai's work on *Citrus sinensis* also reported the development of Plant Vitrification Solution no. 2 (PVS2), a mixture of cryoprotectants which instantly became a milestone to induce cell vitrification. It was soon evident that the possibility of storing valuable embryogenic culture lines in LN could allow the long-term maintenance of their embryogenic potential, making them available only when necessary and avoiding the above-mentioned drawbacks induced by repeated subculturing [9].

Slow cooling is the most common approach for embryogenic callus cultures. In recent years, this approach allowed the development of effective protocols for various conifer (Table 1) and broadleaf (Table 2) trees. One disadvantage of slow cooling is the

Table 1
Cryopreservation of embryogenic cultures of conifers (*LN* liquid nitrogen, *NR* not reported, *RT* room temperature)

Species	Explant[a]	Preculture	Pre-freezing treatments	Cooling rate	Thawing	Maximum recovery (%)	Reference
Slow cooling							
Abies cephalonica	CC	5 °C (14 days)+, darkness + sucrose, 0.2 M (1 day) + 0.4 M (1 day)	10 % PEG6000+ 10 % glucose + 10 % DMSO 30 min, 0 °C	−10 °C/h to −38 °C	37 °C	75	[16]
Abies hybrids	CC	Sorbitol, 0.4 or 0.8 M (2 or 3 days)	0.4 or 0.8 M sorbitol + 5 % DMSO, 1 h, 0 °C	−80 °C (~100 min) to −40 °C[c]	40 °C, 3 min	37–100	[17]
Picea abies	SC	Sorbitol, 0.4 M (2 days)	5 % DMSO, 30 min	−0.3 °C/min to −35 °C	37 °C, 2–3 min	NR	[18]
Picea abies	SC	Sorbitol, 0.2 M (1 day) + 0.4 M (1 day), darkness	5 % DMSO, 0 °C	−0.5 °C/min to −40 °C	45 °C, 90 s	NR	[19]
Picea abies	CC	Sorbitol, 0.4 M (2 days)	5 % DMSO, 30 min	−0.3 °C/min to −35 °C	37 °C, 2–3 min	NR	[20]
Picea glauca engelmannii	SC	–	0.4 M sorbitol + 5 % DMSO	Multistep cooling[b]	37 °C, 1–2 s	100	[21]
Picea glauca	SC	Sorbitol, 0.4 M (1 day)	0.4 M sorbitol + 5 % DMSO, 30 min	−0.3 °C/min to −35 °C	37 °C, 90 s	94	[22]
Picea sitchensis	SC	Sorbitol, 0.2 M (1 day) + 0.4 M (1 day)	5 % DMSO, 0 °C	−0.5 °C/min to −40 °C	45 °C	NR	[23]
Picea sitchensis	SC	Sorbitol, 0.2 M (1 day) + 0.4 M (1 day), darkness	5 % DMSO, 0 °C	−0.5 °C/min to −40 °C	45 °C, 90 s	NR	[19]
Pinus caribaea 'Hondurensis'	SC	Sucrose, 0.4 M	0.4 M sucrose + 5 % DMSO	−0.5 °C/min to −35 °C	40 °C, 2 min	100	[24]
Pinus nigra	CC	Sucrose or maltose, 0.5 M (1 day)	0.5 M sucrose or maltose + 7.5 % DMSO, 1 h	−80 °C (~100 min) to -40 °C	40 °C, 3–4 min	87	[25]
Pinus patula	SC	Sorbitol, 0.3 M (1 day)	0.3 M sorbitol + 5 % DMSO, 20 min, 0 °C	−70 °C, 2 h[c]	42 °C, 2–3 min	60	[26]
Pinus pinaster	SC	Maltose, 0.2 M (1 day) + 0.4 M (1 day), darkness	10 % PEG4000 + 10 % sucrose + 10 % DMSO 0 °C	−80 °C, 1 day[c]	45 °C	97	[27]

(continued)

Table 1
(continued)

Species	Explant[a]	Preculture	Pre-freezing treatments	Cooling rate	Thawing	Maximum recovery (%)	Reference
Pinus pinaster	SC	Sucrose, 0.22 M (1 day) + 0.4 M (2 h)	5 % PSD solution (20 g/L PEG4000 + 20 g/L sucrose + 20 % (v/v) DMSO), 1 h at 0 °C	−70 °C, 80 min[c]	40 °C	100	[28]
Pinus radiata	SC	Sorbitol, 0.4 M (1 day) 20 °C	0.4 M sorbitol +10 % DMSO	−80 °C, 75–90 min[c]	RT, 30 s + 40–45 °C, 2 min	100	[29]
Pinus roxburghii	SC	Sorbitol, 0.3 M (1 day)	0.3 M sorbitol +5 % DMSO	Multistepcooling[d]	45 °C, 2–3 min	70	[30]
Pinus sylvestris	CC	5 °C (14 days) + sucrose, 0.2 M (1 day) + 0.4 M (1 day)	10 % PEG6000+ 10 % glucose + 10 % DMSO, 30 min, 0 °C	−10 °C/h to −38 °C	37 °C	78	[31]
Pinus sylvestris	CC	Sucrose, 0.2 M (1 day) + 0.4 M (1 day)	10 % PEG6000 + 10 % glucose + 10 % DMSO, 1 h, 0 °C	−10 °C/h to −38 °C	37 °C, 1–3 min	80–93	[32]
Direct immersion in LN							
Picea mariana	CC	Sorbitol, 0.8 M (2 days)	PVS2, 30 min, 0 °C	–	40 °C	67	[33]

Updated from [9]

[a] *CC* clumps of embryogenic callus (developmental stage not reported), *SC* cells from suspension cultures

[b] Slow cooling was achieved as follows: −0.3 °C/min to −3 °C → −15 °C/min to −8 °C → −25 °C/min to −32 °C → −0.3 °C/min to −35 °C

[c] Slow cooling was achieved by incubating the samples in a freezer at −70 or −80 °C before plunging them into LN

[d] Slow cooling was achieved as follows: −0.3 °C/min to −35 °C → −25 °C/min to −50 °C

Table 2
Cryopreservation of embryogenic cultures of broadleaf trees (*LF* laminar flow, *LN* liquid nitrogen, *MC* moisture content, *NR* not reported, *RT* room temperature, *SG* silica gel)

Species	Explant[a]	Preculture	Pre-freezing treatments	Cooling rate	Thawing	Maximum recovery (%)	Reference
Slow cooling							
Citrus deliciosa	SC	–	10 % DMSO, 30 min, 4 °C	−0.5 °C/min	37 °C, 5 min	NR	[34]
Citrus sinensis	SE	–	–	−0.5 °C/min to −42 °C	RT, 15 min	5	[35]
Citrus sinensis	HSE	–	10 % DMSO	−0.5 °C/min to −42 °C	37 °C, 5 min	31	[36]
Citrus ssp.	CC	–	10 % DMSO, 30 min, 0 °C	−0.5 °C/min to −40 °C	37 °C, 5 min	100	[37]
Fraxinus excelsior	CC	–	0.61 M sucrose +7.5 % DMSO, 1 h, 0 °C	−1 °C/min to −40 °C	40 °C, 2-3 min	100	[12]
Hevea brasiliensis	CC	–	1 M sucrose + 10 % DMSO, 1 h, 0 °C	−0.2 °C/min to −40 °C	40 °C	49	[38]
Hevea brasiliensis	CC	–	1 M sucrose + 10 % DMSO, 1 h, 0 °C	−0.2 °C/min to −40 °C	40 °C	70	[39]
Persea americana	CC	–	5 % DMSO + 5 % glycerol + 0.13 or 1 M sucrose, 30 min, 0 °C	−1 °C/min to −80 °C	40 °C, 5 min	NR	[40]

(continued)

Table 2
(continued)

Species	Explant[a]	Preculture	Pre-freezing treatments	Thawing	Maximum recovery (%)	Reference
Direct immersion in LN						
Aesculus hippocastanum	TSE	4 °C (5 days), darkness	2 M glycerol + 0.4 M sucrose, 30 min, 25 °C+ PVS2, 90 min, 0 °C	45 °C, 50 s	94	[41]
Citrus sinensis	SC	–	60 % PVS2, 5 min, 25 °C+ PVS2, 3 min, 0 °C	25 °C	84	[11]
Citrus ssp.	SE	Sucrose, 0.7 M (1 day)	Encapsulation + dehydration, 5 h, LF	RT, 2–3 min	100	[42]
Fraxinus angustifolia	CSE	4 °C (10 h) → sucrose, 0.5 M (1 day)	Encapsulation + dehydration, 1 h, SG	40 °C, 3 min	31	[43]
Litchi chinensis	CC	–	PVS2, 30 min, 0 °C	38–40 °C, 2 min	100	[44]
Mangifera indica 'Zihua'	SC	Sucrose, 0.5 M (1 day) 25 °C	PVS3[b], 20 min, 25 °C	25 °C, 2–3 min	94	[45]
Olea europaea 'Canino'	CC	4 °C (4 days), darkness	2 M glycerol + 0.4 M sucrose, 30 min, 25 °C+ PVS2, 90 min, 0 °C	RT, 10 s + 40 °C, 50 s	38	[46]
Olea europea 'Nabali'	CC	30 °C (1 day)	Encapsulation + 0.4 M sucrose + 2 M glycerol, 60 min + PVS2, 3 h, 0 °C	38 °C, 2 min	64	[47]
Olea europea 'Picual'	CC	Sucrose, 0.4 M (7–8 weeks)	PVS2, 60 min, 0 °C, droplet vitrification	1.2 M sucrose, RT	100	[48]
Persea americana	CC	–	PVS2, 60 min	40 °C, 5 min	NR	[40]
Prunus avium	CC	Multistep sucrose preculture[c]	Dehydration, LF (until 20 % MC)	1.2 M sucrose at 40 °C	89	[49]
Quercus robur	GSE-HSE	Sucrose, 0.3 M (3 days)	PVS2, 60 min, 25 °C	40 °C, 2 min	70	[50]
Quercus robur	CC	Multistep sucrose preculture[c]	Dehydration, 4–5 h, LF	1.2 M sucrose at 40 °C	NR	[51]
Quercus suber	GSE	Sucrose, 0.3 M (3 days)	PVS2, 60 min, 0 °C	40 °C, 2 min	93	[52]
Quercus suber	GSE	Sucrose, 0.7 M (3 days)	Dehydration, LF (until 25–35 % MC)	38 °C, 2 min	90	[53]
Theobroma cacao	SSE	Sucrose, 0.5 M (5 days)	PVS2, 60 min, 0 °C	42 °C, 3 min	74.5	[54]

Updated from [9]

[a]*CC* clumps of embryogenic callus (developmental stage not reported), *CSE* clumps or isolated somatic embryos at the cotyledonary stage, *GSE* clumps or isolated somatic embryos at the globular stage, *HSE* clumps or isolated somatic embryos at the heart stage, *SC* cells from suspension cultures, *SSE* secondary somatic embryos, *SE* isolated somatic embryos, *TSE* clumps or isolated somatic embryos at the torpedo stage

[b]PVS3: 50 % sucrose (w/v) + 50 % glycerol (w/v) in standard culture medium

[c]Multistep sucrose preculture was performed as follows: sucrose, 0.25 M (1 day) + 0.5 M (1 day) + 0.75 M (2 days) + 1.0 M (3 days)

requirement of an expensive equipment, the controlled-rate freezer. The Nalgene freezing container "Mr. Frosty"® (Sigma-Aldrich) is a cheaper alternative approach to slow cooling; however, it should be noted that "Mr. Frosty"® provides only a rate of −1 °C/min gradual temperature decrease and thus is useful only when this cooling rate is suitable for the plant material [9]. Vitrification-based protocols have also been developed for embryogenic cultures from various important plant species, such as *Citrus* spp., *Olea europaea*, *Fraxinus* spp., and *Quercus* spp. (Tables 1 and 2). A sample protocol on cryopreservation of *Fraxinus excelsior* embryogenic callus cultures by slow cooling approach is included to the chapter, providing a detailed information of the procedure to the reader.

2 Materials

2.1 Plant Material

Slow cooling of *Fraxinus excelsior* is presented as a sample protocol [12], where embryogenic callus cultures at the proliferation phase (Fig. 1a) are used as a plant material (*see* **Notes 1** and **2**). For embryogenic callus induction, following seed collection and their decontamination, zygotic embryos are isolated, and embryonic axes are excised aseptically and cultured on ½-strength MS [13] medium supplemented with 8.8 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.4 μM benzyladenine (BA) (*see* **Note 3**) for 2 months in the dark, followed by an additional 1-month incubation period on plant growth regulator (PGR)-free medium (MS0) under standard culture conditions, i.e., 23 ± 1 °C under a 16-h photoperiod and low light intensity (20 μmol/m²/s). The de novo formed embryogenic callus is then transferred to Woody Plant Medium (WPM, [14]) (*see* **Note 4**), supplemented with 4.4 μM BA, and maintained by subculturing at 2-week intervals (see also [15]).

2.2 Cryoprotective Solutions and Semi-solid Media

1. Sucrose-rich liquid medium: Liquid WPM, containing 0.61 M (210 g/L) sucrose (*see* **Note 5**).
2. Dimethyl sulfoxide (DMSO) and sucrose-rich liquid medium: Liquid WPM, containing 0.62 M (210 g/L) sucrose and 15 % DMSO (w/v) (*see* **Note 6**).
3. Post-thaw recovery and plantlet development medium: Semi-solid WPM supplemented with 4.4 μM BA.
4. Somatic embryomaturation medium: PGR-free semi-solid WPM.

2.3 Laboratory Facilities

1. Graduate cylinders (100, 500, 1000 mL).
2. Glass beakers (250, 500, 1000 mL).

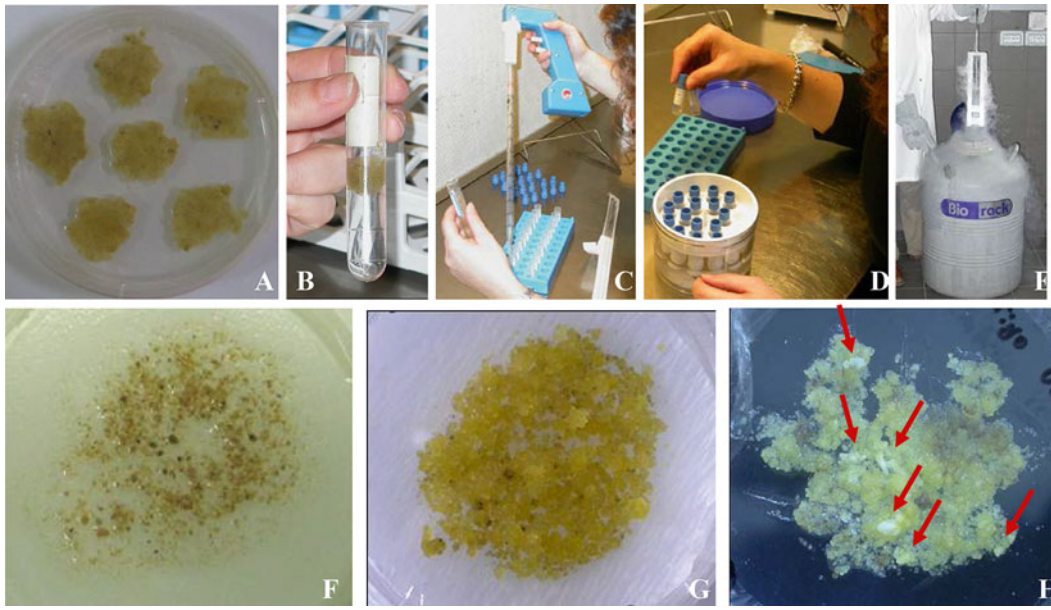

Fig. 1 Slow cooling of embryogenic callus cultures of *Fraxinus excelsior*. (**a**) Embryogenic callus cultures at the proliferation phase used in the cryopreservation trials. (**b**) Callus samples (~2 g) transferred to sterile 10-mL glass tubes for the treatment with cryoprotective solutions. (**c**) Distribution of the mixture into sterile 2-mL cryovials. (**d**) "Mr. Frosty"® containing cryovials to be cooled in a −80 °C freezer. (**e**) Immersion of the samples in LN. (**f**) Cryopreserved callus samples immediately after thawing and placing on post-thaw recovery medium. (**g**) Proliferation of the callus samples 42 days after thawing and recovery. (**h**) Somatic embryomaturation on the cryopreserved callus samples (*arrows*) (Figg. **g** and **h** reproduced from [12] with permission from CryoLetters)

3. Magnetic stirrer and spin bar.
4. Analytical balance, weighting containers, and lab spoons.
5. pH meter.
6. Autoclave.
7. Horizontal flow cabinet.
8. Automatic pipettor.
9. Nalgene benchtop cooler.
10. Nalgene freezing container ("Mr. Frosty"®, Sigma-Aldrich).
11. Nalgene box.
12. LN dewar.
13. Appropriate gloves and masks for protection from LN.
14. Growth chamber (temperature of 23 ± 1 °C, photosynthetic photon flux density of 20 μmol/m²/s and 16-h photoperiod).

2.4 Consumables

1. Distilled water.
2. LN.
3. NaOH and HCl solution (1.0 and 0.1 N).

4. Stock solutions of macro- and microelements, organics, and iron of MS and WPM media.
5. Stock solutions of growth regulators (2,4-D and BA).
6. Sucrose.
7. Gelrite.
8. Schott bottles (250, 500, 1000 mL).
9. Petri dishes (Ø 90 mm).
10. 50-mm Whatman filter paper.
11. Forceps, scalpels, and blades.
12. Sterile medical gloves.
13. Sterile tubes (10 mL).
14. Sterile cryovials (2 mL).
15. Pipettes.
16. Trays (35 mm Ø).

3 Methods

Cryopreservation of embryogenic callus cultures (as it is for cryopreservation of almost all kinds of plant material) is a multistep process. It involves several consecutive preconditioning (to enhance the cold tolerance of plant material before the immersion in LN) and promotive steps (to help the plant material recovering after storage). Although some of these steps can be skipped, depending on the cryopreservation approach applied, a complete cryopreservation protocol is composed of (1) cold hardening, (2) preculture, (3) osmoprotection, (4) cryoprotection, (5) immersion and storage in LN, (6) thawing, (7) rinsing, and (8) plating on regeneration medium.

Cryopreservation approach proved to be suitable for the embryogenic callus cultures of *Fraxinus excelsior* is based on slow cooling, inducing "cryodehydration" (*see* **Note 7**) of the samples. Here, the main step is the gradual decrease of the temperature at a rate of –0.5/–1 °C/min to an intermediate temperature of –40 °C before the immersion in LN, while some of the steps preceeding immersion in LN (i.e., cold hardening, preculture, and osmoprotection) can be skipped. Gradual decrease of the temperature of the samples, inducing cryodehydration, can be achieved by using a controlled-rate freezer or the Nalgene freezing container "Mr Frosty"®, a specially designed plastic box containing 250 mL isopropyl alcohol, which cools the samples at a rate of about –1 °C/min [3, 9].

3.1 Cryopreservation of Fraxinus excelsior

1. Embryogenic callus samples (~2 g) are placed in sterile 10-mL glass tubes (Fig. 1b) and incubated with 5 mL sucrose-rich liquid medium, i.e., liquid WPM, containing 210 g/L (0.61 M) sucrose.
2. Five mL DMSO and sucrose-rich liquid medium, added of 15 % DMSO, is then gradually added in three steps (1 mL, 2 mL, and 2 mL, respectively) over a total period of 60 min (15 min, 15 min, and 30 min, respectively), to reach a final DMSO concentration of 7.5 % in a final volume of 10 mL.
3. Following DMSO treatment, suspension cultures are mixed thoroughly and transferred into sterile 2-mL cryovials (each cryovial containing 1 mL of the mixture, Fig. 1c) (*see* **Note 8**).
4. Slow cooling of the samples is achieved by placing the cryovials in the cells of "Mr. Frosty"® (Fig. 1d, *see* **Note 9**) and transferring the device in a −80 °C freezer, where it is kept (for about 1 h) until the temperature reaches −40 °C.
5. Afterward, the cryovials are rapidly transferred to Nalgene boxes that are then plunged into LN (Fig. 1e) where they are stored for at least 1 h.
6. Callus samples that are treated with DMSO (control 1), or treated with DMSO and cooled to −40 °C, but not frozen in LN (control 2) serve as control.

3.2 Thawing and Post-Thaw Recovery

1. The embryogenic callus samples are thawed in a 40 °C water bath until the DMSO solution is totally melted.
2. They are then poured onto a 50-mm Whatman filter paper, placed on post-thaw recovery medium, i.e., WPM supplemented with 4.4 μM BA (Fig. 1f), and cultured at 23 ± 1 °C in the dark for 2 days.
3. Subsequently, embryogenic callus samples are subcultured under standard culture conditions (i.e., 23 ± 1 °C, 16-h photoperiod, with a light intensity of 20 μmol/m^2/s) in every 2 weeks by transferring the filter paper onto semi-solid fresh post-thaw recovery medium until the 42nd day (Fig. 1g).
4. The callus clumps are then put in direct contact with fresh semi-solid medium.

3.3 Embryo Maturation

1. In order to stimulate somatic embryomaturation and conversion into plantlets, cryopreserved callus samples are transferred onto PGR-free, semi-solid WPM (embryo maturation medium) and subcultured at 2-week intervals (Fig. 1h).
2. Somatic embryos at the cotyledonary stage are then isolated and subcultured on the same medium at 4 °C, in darkness for 4 weeks, followed by transfer to WPM containing 4.4 μM BA under the above-mentioned standard culture conditions.
3. The plantlets developed are then transferred to trays (35 mm Ø) and acclimatized under greenhouse conditions.

4 Notes

1. For cryopreservation trials, only established embryogenic callus cultures (i.e., coming from cultures maintained for at least 1 year) are used.
2. Cultures were initiated from immature zygotic embryos of selected ash trees in Florence, Italy [15].
3. In our laboratory, stock solutions of PGR are prepared in 10^{-3} M concentration and are stored at 4 °C. 2,4-D and BA are sterilized by autoclaving; thus, they are included in the medium before sterilization.
4. MS and WPM media, supplemented or not with PGR, contained 20 g/L sucrose and 3.6 g/L Gelrite (pH 5.8).
5. During the course of the study, sucrose concentration, as well as the application time of the solution, should be optimized carefully for each species. Accordingly, depending on the plant species used in the study, the solution can be prepared using different basal medium formulations.
6. As stated in **Note 5**, also here sucrose concentration, as well as DMSO concentration, should be optimized carefully according to the sensitivity of the specific embryogenic callus line. The solution can be prepared in any kind of basal medium formulation. However, what is crucial is that the sucrose concentrations and basal medium formulations of these two solutions should be identical.
7. "Cryodehydration" refers to a state of losing moderate amount of potentially freezing water molecules from the cell cytosol, in response to gradual decrease of the temperature. If temperature decrease is performed too fast, cells do not lose sufficient amount of water, and thus they risk the cryo-damages induced by ice crystals formed in the intra cellular spaces during immersion in LN. On the contrary, if temperature decrease is performed too slowly, cells lose extreme amount of water, which results in dehydration injuries due to cell plasmolysis. Moderate water loss is proved to be induced by decreasing the temperature at a rate of −0.5/−1 °C/min.
8. This application is rather "tricky" while working with compact callus samples, as it is difficult to have homogeneous distribution of callus samples in the solution and thus is difficult to transfer equal amount of callus sample from the 10-mL glass tubes into each 2-mL cryovial. Alternatively, callus samples of equal amount can be directly transferred to cryovials and can be treated with the cryoprotective solutions in that container. If this is the case, it should be recalled that the final volume of the solutions will be 1 mL, in total.

9. "Mr. Frosty"® has a plate of 18 cells. Thus, it should be considered that, using one "Mr. Frosty"® container, only 18 cryovials can be managed for each trial.

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INDEX

A

B

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C

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E

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N

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P

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R

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