

## 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

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### 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.

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 rupestris* [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).

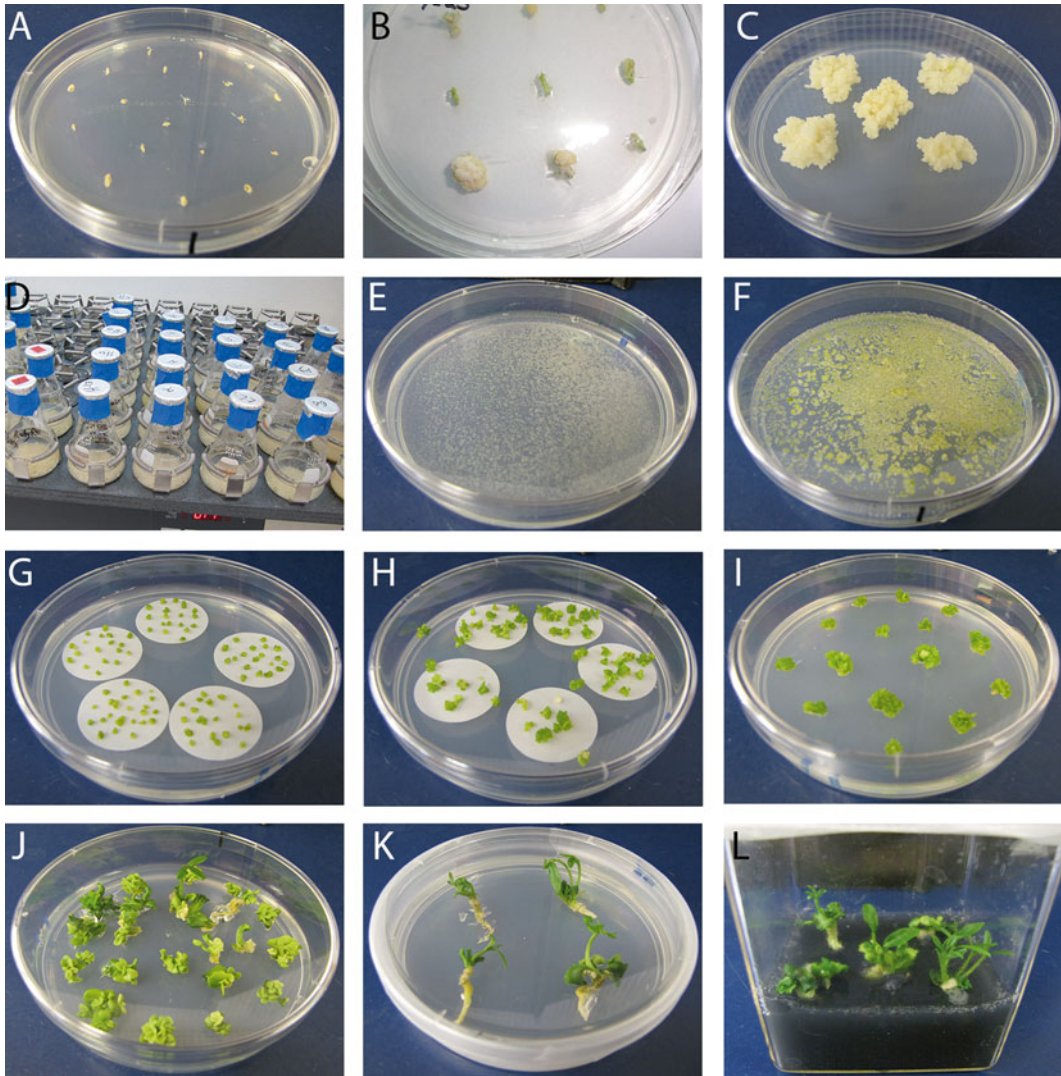
The application of *in vitro* systems based on SE for plant regeneration is determined not only by a high efficiency of somatic embryo formation, but frequently depends on capacity of the embryos for complete plant development. The process of developmental changes, which a somatic embryo undergoes, is 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<sub>3</sub>) 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  $\text{NH}_4\text{NO}_3$  that can often be toxic to protoplasts. Glutamine or  $\text{Ca}(\text{NO}_3)_2$  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 cancer challenge assay to determine if the transfer of the kumquat mt genome can indeed improve their resistance to citrus canker.

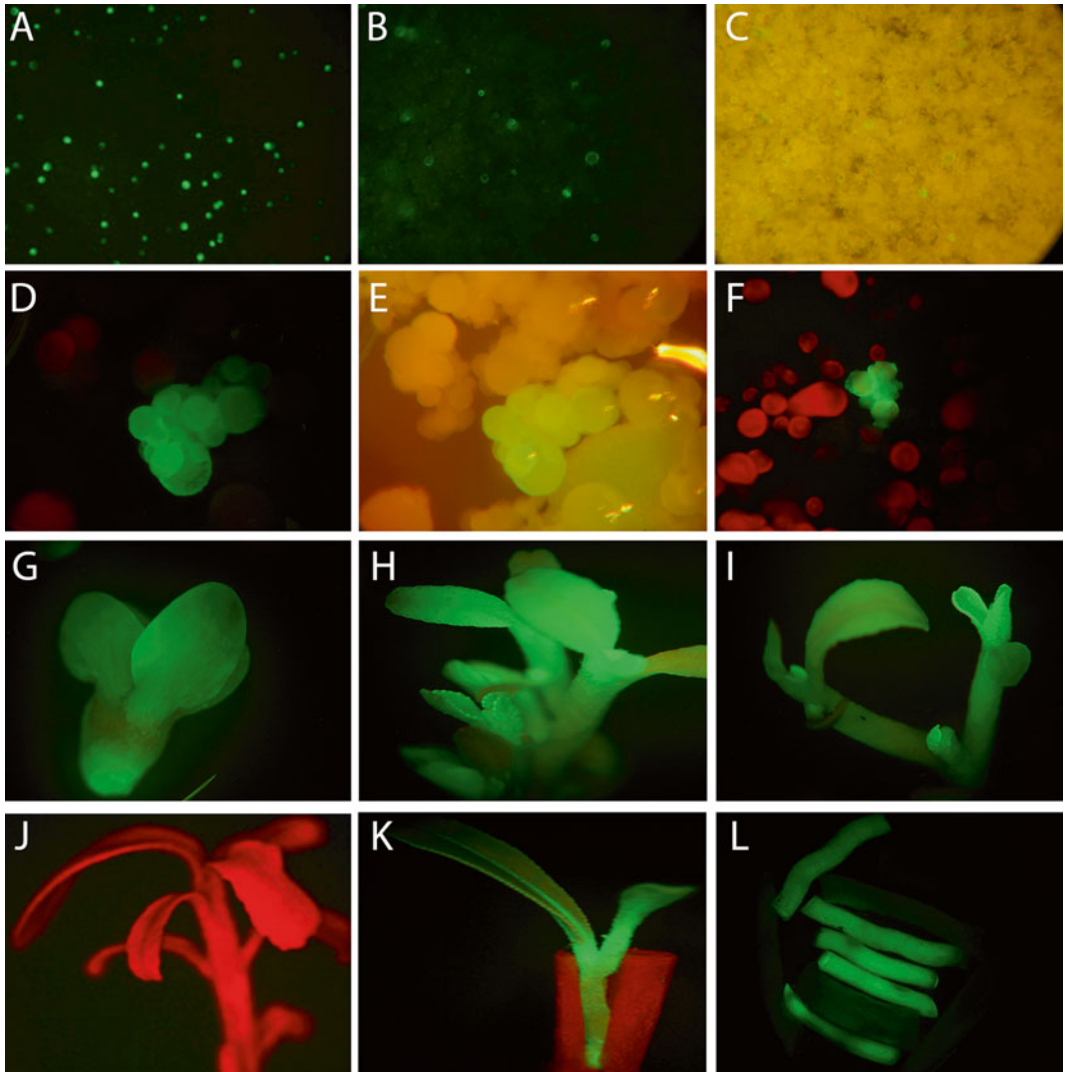
### **1.3 Citrus Transformation Involving Somatic Embryogenesis**

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-calls (transformed and non-transformed) under *blue light*, (c) protoplast derived micro-calls (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].

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## 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  $28 \pm 2$  °C.
3. Laminar flow cabinet.
4. pH meter.
5. Autoclave.
6. Sterilized paper plates.
7. Syringe filter units, 0.2  $\mu$ m pore size.
8. Centrifuge with 100–400  $\times g$  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<sub>4</sub>·7H<sub>2</sub>O, 15 g/L KH<sub>2</sub>PO<sub>4</sub>, 2 g/L K<sub>2</sub>HPO<sub>4</sub>; dissolve in H<sub>2</sub>O and store at 4 °C.
3. Murashige and Tucker (MT) macronutrient stock [101]: 95 g/L KNO<sub>3</sub>, 82.5 g/L NH<sub>4</sub>NO<sub>3</sub>, 18.5 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 7.5 g/L KH<sub>2</sub>PO<sub>4</sub>, 1 g/L K<sub>2</sub>HPO<sub>4</sub>; dissolve in H<sub>2</sub>O and store at 4 °C.
4. MT micronutrient stock: 0.62 g/L H<sub>3</sub>BO<sub>3</sub>, 1.68 g/L MnSO<sub>4</sub>·H<sub>2</sub>O, 0.86 g/L ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.083 g/L KI, 0.025 g/L Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.0025 g/L CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.0025 g/L CoCl<sub>2</sub>·6H<sub>2</sub>O; dissolve in H<sub>2</sub>O 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<sub>2</sub>O and store at 4 °C.
6. MT calcium stock: 29.33 g/L CaCl<sub>2</sub>·2H<sub>2</sub>O; dissolve in H<sub>2</sub>O and store at 4 °C.
7. MT iron stock: 7.45 g/L Na<sub>2</sub>EDTA, 5.57 g/L FeSO<sub>4</sub>·7H<sub>2</sub>O; dissolve in H<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O and store at -20 °C.
11. BH3 KI stock: 0.83 g/L KI; dissolve in H<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O; store at 4 °C.
2.  $\alpha$ -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<sub>2</sub>O 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<sub>2</sub>O; 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<sub>2</sub>O; store at 4 °C.
5. Gibberellic acid (GA<sub>3</sub>; stock solution, 1 mg/mL): Dissolve the powder in a few drops of 95 % (v/v) ethanol, bring to final volume with H<sub>2</sub>O, 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<sub>2</sub>·2H<sub>2</sub>O stock solution, 0.98 M): Dissolve 14.4 g in 100 mL H<sub>2</sub>O and store at -20 °C.
2. Monosodium phosphate (NaH<sub>2</sub>PO<sub>4</sub> stock solution, 37 mM): Dissolve 0.44 g in 100 mL H<sub>2</sub>O 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<sub>2</sub>O and store at -20 °C.
4. Enzyme solution: 0.7 M mannitol, 24 mM CaCl<sub>2</sub>, 6.15 mM MES buffer, 0.92 mM NaH<sub>2</sub> PO<sub>4</sub>, 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<sub>2</sub>O and add 1 mL of CaCl<sub>2</sub>·2H<sub>2</sub>O, NaH<sub>2</sub>PO<sub>4</sub> and MES stock solutions; bring volume to 40 mL with H<sub>2</sub>O, 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<sub>4</sub>·7H<sub>2</sub>O, 10 g/L KNO<sub>3</sub>, 2.72 g/L KH<sub>2</sub>PO<sub>4</sub>, 0.016 g/L KI, 0.025 ng/L CuSO<sub>4</sub>·5H<sub>2</sub>O; dissolve in H<sub>2</sub>O and store at -20 °C.
2. CPW salts stock solution 2: 15 g/L CaCl<sub>2</sub>·2H<sub>2</sub>O; dissolve in H<sub>2</sub>O and store at -20 °C.
3. 13 % CPW (13 %, w/v, mannitol solution with CPW salts): Dissolve 13 g mannitol in 80 mL H<sub>2</sub>O, add 1 mL each of CPW salts stock solutions 1 and 2; bring volume to 100 mL with H<sub>2</sub>O, 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<sub>2</sub>O, add 1 mL each of CPW salts stock solutions 1 and 2; bring to 100 mL with H<sub>2</sub>O, 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<sub>2</sub>O, 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<sub>2</sub>·2H<sub>2</sub>O, pH 6.0. To prepare 100 mL of PEG solution, dissolve 0.97 g CaCl<sub>2</sub>·2H<sub>2</sub>O and 5.41 g glucose in 10 mL H<sub>2</sub>O, add 80 mL of PEG stock solution (50 %) and adjust the volume to 100 mL with H<sub>2</sub>O, 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<sub>2</sub>·2H<sub>2</sub>O, 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](http://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  $\mu\text{m}$  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<sub>3</sub> (add 1 mL GA<sub>3</sub> 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<sub>3</sub> (add 1 mL GA<sub>3</sub> 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.

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### 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  $28 \pm 2$  °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 ( $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) at  $28 \pm 2$  °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  $4 \times 10^6$  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 ( $2 \times 10^6$  protoplasts/mL).
2. Add 30–40  $\mu$ g 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  $\times$  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  $28 \pm 2$  °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  $28 \pm 2$  °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 ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  intensity) with a 16 h photoperiod at  $28 \pm 2 \text{ }^\circ\text{C}$ .
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 ( $70 \mu\text{mol m}^{-2} \text{s}^{-1}$  intensity) at  $28 \pm 2 \text{ }^\circ\text{C}$  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 semisolid 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  $-80 \text{ }^\circ\text{C}$  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  $28 \text{ }^\circ\text{C}$  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  $\mu\text{mol m}^{-2} \text{s}^{-1}$  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.

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## 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 ( $5\text{--}10 \times 10^6$  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  $4 \times 10^6$  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  $\mu\text{g}/\mu\text{l}$ , 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.  $1\text{--}1.5 \times 10^6$  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  $1 \times 10^6$  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.



## References

1. Soost RW, Cameron JW (1975) Citrus. In: Janick J, Moore JN (eds) *Advances in fruit breeding*. Purdue University Press, West Lafayette, IN, pp 507–540
2. Soost RW, Cameron JW (1969) Tree and fruit characters of Citrus triploids from tetraploid by diploid crosses. *Hilgardia* 39: 569–579
3. Frost HB, Soost RK (1969) Seed reproduction: development of gametes and embryos. In: Reuther W, Batchelor HJ, Datchelar LD (eds) *The citrus industry, vol 1*. University of California, Berkeley, CA
4. Gmitter FG Jr, Grosser JW, Moore GA (1992) Citrus. In: Hammerschlag FA, Litz RE (eds) *Biotechnology of perennial fruit crops*. CAB International, Wallingford, pp 335–369
5. Barrett HC, Rhodes AM (1976) A numerical taxonomic study of affinity relationships in cultivated citrus and its close relative. *Syst Bot* 1:105–136
6. Scora RK (1975) On the history and origin of citrus. *Bull Torrey Bot Club* 102:369–375
7. Hodgson RW (1969) Horticultural varieties of citrus. In: Reuther W, Batchelor HJ, Datchelar LD (eds) *The citrus industry, vol 1*. University of California, Berkeley, CA
8. Hearn CJ (1989) Yield and fruit quality of ‘Ambersweet’ orange hybrid on different rootstocks. *Proc Fla State Hort Soc* 102: 75–78
9. Grosser JW, Gmitter FG Jr (1990) Protoplast fusion and citrus improvement. *Plant Breed Rev* 8:339–374
10. Grosser JW, Gmitter FG Jr (2011) Protoplast fusion for production of tetraploids and triploids: applications for scion and rootstock breeding in citrus. *Plant Cell Tiss Org Cult* 104(3):343–357
11. Litz RE, Gray DJ (1992) Organogenesis and somatic embryogenesis. In: Hammerschlag FA, Litz RE (eds) *Biotechnology of perennial fruit crops*. CAB International/University Press, UK/Cambridge, pp 3–34
12. Bajaj YPS (1995) Somatic embryogenesis and its applications for crop improvement. In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry. Somatic embryogenesis and synthetic seed I*, vol 30. Springer, Berlin, pp 105–125
13. Germanà MA (2005) Protocol of somatic embryogenesis from *Citrus* spp. anther culture. In: Jain SM, Gupta PK (eds) *Protocols of somatic embryogenesis-woody plants*. Springer, Netherlands, pp 191–207
14. Carman JG (1990) Embryogenic cells in plant tissue cultures: occurrence and behaviour. *In Vitro Cell Dev Biol Plant* 26: 746–753
15. Emons AMC (1994) Somatic embryogenesis: cell biological aspects. *Acta Bot Neerl* 43: 1–14
16. Turgut K, Barghchi M, Scott R (1998) Efficient shoot regeneration and somatic embryogenesis from immature cotyledons of *Brassica napus* L. *Plant Breed* 117:503–503
17. Raemakers CJM, Jacobsen E, Visser RGF (1995) Secondary somatic embryogenesis and applications in plant breeding. *Euphytica* 81:93–107
18. Vasic D, Alibert G, Skoric D (2001) Protocol for efficient repetitive and secondary somatic embryogenesis in *Helianthus maximiliani* (Schrader). *Plant Cell Rep* 20:121–125
19. Martinelli L, Candioli E, Costa D et al (2001) Morphogenic competence of *Vitis rupestris* S. secondary somatic embryos with a long culture history. *Plant Cell Rep* 20:279–284
20. Ahloowalia BS (1991) Somatic embryos in monocots. Their genesis and genetic stability. *Rev Cytol Biol Veg Bot* 14:223–235
21. von Arnold S, Clapham D, Egertsdotter U et al (1996) Somatic embryogenesis in conifers—a case study of induction and development of somatic embryos in *Picea abies*. *Plant Growth Regul* 20:3–9
22. Henry RJ (1998) Molecular and biochemical characterization of somaclonal variation. In: Jain SM, Brar DS, Ahloowalia BS (eds) *Somaclonal variation and induced mutations in crop improvement*. Kluwer Academic Publishers, Dordrecht, Boston, London, pp 485–499
23. Germanà MA, Hafiz IA, Micheli M et al (2007) In vitro and ex vitro conversion of encapsulated somatic embryos of *Citrus reticulata* Blanco, cv. Mandarino Tardivo di Ciaculli. *Plant Cell Tiss Org Cult* 88:117–120
24. Germanà MA, Piccioni E, Standardi A (1999) Effects of encapsulation on *Citrus reticulata* Blanco somatic embryos conversion. *Plant Cell Tiss Org Cult* 55:235–237
25. Singh B, Sharma S, Rani G (2007) In vitro response of encapsulated and non-encapsulated somatic embryos of Kinnow mandarin (*Citrus nobilis* Lour × *C. deliciosa* Tenora). *Plant Biotechnol Rep* 1:101–107
26. Germanà MA, Lambardi M, Ozudogru EA (2011) Preliminary results on desiccation and PVS2-vitrification of carrizo citrange synthetic

- seeds as pre-treatments to cryopreservation. *Acta Hort* 892:311–318
27. Malik SK, Chaudhury R (2006) The cryopreservation of embryonic axes of two wild and endangered *Citrus* species. *Plant Genet Resour Charact Utilizat* 4:204–209
  28. Haccius B (1978) Question of unicellular origin of non-zygotic embryos in callus culture. *Phytomorphology* 28:74–81
  29. Toonen MAJ, Hendriks T, Schmidt EDL et al (1994) Description of somatic-embryo forming single cells in carrot suspension cultures employing video cell tracking. *Planta* 194:565–572
  30. De Vries SC, Booij H, Meyerink P et al (1988) Acquisition of embryogenic potential in carrot cell-suspension cultures. *Planta* 176:196–204
  31. Lee KS, Zapata-Arias FJ, Brunner H et al (1997) Histology of somatic embryo initiation and organogenesis from rhizome explants of *Musa* spp. *Plant Cell Tiss Org Cult* 51:1–8
  32. Chan JL, Saenz L, Talavera C et al (1998) Regeneration of coconut (*Cocos nucifera* L.) from plumule explants through somatic embryogenesis. *Plant Cell Rep* 17:515–521
  33. Rugkhla A, Jones MGK (1998) Somatic embryogenesis and plantlet formation in *Santalum album* and *S. spicatum*. *J Exp Bot* 49:563–571
  34. Nonohay JS, Mariath JEA, Winge H (1999) Histological analysis of somatic embryogenesis in Brazilian cultivars of barley, *Hordeum vulgare* L., Poaceae. *Plant Cell Rep* 18:929–934
  35. Canhoto JM, Cruz GS (1996) Histodifferentiation of somatic embryos in cotyledons of pineapple guava (*Feijoa sellowiana* Berg). *Protoplasma* 191:34–45
  36. Choi YE, Yang DC, Park JC et al (1998) Regenerative ability of somatic single and multiple embryos from cotyledons of Korean ginseng on hormonefree medium. *Plant Cell Rep* 17:544–551
  37. Williams EG, Maheswaran G (1986) Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. *Ann Bot* 57:443–462
  38. Vasil IK (1999) Molecular improvement of cereal crops, vol 5, *Advances in cellular and molecular biology of plants*. Kluwer, Dordrecht
  39. Reinert J (1958) Morphogenese und ihre Kontrolle an Gewebekulturen aus Carotten. *Naturwissenschaften* 45:344–345
  40. Steward FC, Mapes MO, Mears K (1958) Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cell. *Am J Bot* 45:705–708
  41. Muir WH, Hildebrandt AC, Riker AJ (1954) Plant tissue cultures produced from isolated single cells. *Science* 119:877–878
  42. Bergmann L (1959) A new technique for isolating and cloning cells of higher plants. *Nature* 184:648–649
  43. Jones LE, Hildebrandt AC, Riker AJ (1960) Growth of somatic tobacco cells in microculture. *Am J Bot* 47:468–475
  44. Vasil V, Hildebrandt AC (1965) Differentiation of tobacco plants from single, isolated cells in microcultures. *Science* 150:889–892
  45. Vasil V, Hildebrandt AC (1967) Further studies on the growth and differentiation of single, isolated cells of tobacco in vitro. *Planta* 75:139–151
  46. Villalobos VM, Engelmann F (1995) Ex situ conservation of plant germplasm using biotechnology. *World J Microbiol Biotechnol* 11:375–382
  47. Castillo P, Marquez J, Rubluo A et al (2000) Plant regeneration from callus and suspension cultures of *Valeriana edulis* ssp. *procera* via simultaneous organogenesis and somatic embryogenesis. *Plant Sci* 151:115–119
  48. Bakos A, Borcis T, Toldi O et al (2000) Evidence for somatic embryogenesis during plant regeneration from seedling-derived callus of dodder (*Cuscuta trifolii* Bab. et Gibs). *Plant Cell Rep* 19:525–528
  49. Ma G, Xu Q (2002) Induction of somatic embryogenesis and adventitious shoots from immature leaves of cassava. *Plant Cell Tiss Org Cult* 70:281–288
  50. Vikrant A, Rashid A (2003) Somatic embryogenesis or shoot formation following high 2,4-D pulse-treatment of mature embryos of *Paspalum scrobiculatum*. *Biol Plant* 46:297–300
  51. Redenbaugh K, Paasch BD, Nichol JW et al (1986) Somatic seeds: encapsulation of sexual plant embryos. *Biotechnology* 4:797–801
  52. Levi A, Sink KC (1990) Differential effects of sucrose, glucose and fructose during somatic embryogenesis in asparagus. *J Plant Physiol* 137:184–189
  53. Park SU, Facchini PJ (2000) High-efficiency somatic embryogenesis and plant regeneration in California poppy, *Eschscholzia californica* Cham. *Plant Cell Rep* 19:421–426

54. Vlasinova H, Havel L (1999) Continuous somatic embryogenesis in Japanese maple (*Acer palmatum* Thunb.). *J Plant Physiol* 154:212–218
55. Carman JG (1997) Asynchronous expression of duplicate genes in angiosperms may cause apomixis, bispory, tetraspory, and polyembryony. *Biol J Linn Soc* 61:51–94
56. Koltunow AM (1993) Apomixis: embryo sacs and embryos formed without meiosis or fertilization in ovules. *Plant Cell Environ* 5: 1425–1437
57. Ueno I, Iwamasa M, Nishiura M (1967) Embryo number of various varieties of citrus and its relatives. *Bull Hort Res Stn Jpn Ser B7*:11–22 (In Japanese with English summary)
58. McMeniman S, Lubulwa G (1997) Project development assessment: an economic evaluation of the potential benefits of integrating apomixis into hybrid rice. Economic Evaluation Unit Working Paper. Australian Centre for International Agricultural Research, Canberra
59. Dwivedi SL, Perotti E, Upadhyaya HD et al (2010) Sexual and apomictic plant reproduction in the genomics era: exploring the mechanisms potentially useful in crop plants. *Sex Plant Reprod* 23:265–279
60. Vielle Calsada JP, Crane CF, Stelly DM (1996) Apomixis: the asexual revolution. *Science* 274(5291):1322–1323
61. Soost RK, Roose ML (1996) Citrus. In: Janick J, Moore JN (eds) *Fruit breeding: tree and tropical fruits*, vol 1. John Wiley and Sons Inc., New York, pp 257–323
62. Rangan TS, Murashige T, Bitters WP (1969) In vitro studies of zygotic and nucellar embryogenesis in citrus. In: Chapman HD (ed) *Proceedings of the first international citrus symposium*. University of California, Riverside, pp 225–229
63. Gmitter FG Jr, Moore GA (1986) Plant regeneration from undeveloped ovules and embryogenic calli of citrus: embryo production, germination, and plant survival. *Plant Cell Tiss Org Cult* 6:139–147
64. Starrantino A, Russo F (1980) Seedlings from undeveloped ovules of ripe fruits of polyembryonic citrus cultivars. *HortScience* 15: 296–297
65. Button J (1978) The effect of some carbohydrates on the growth and organization of Citrus ovular callus. *Z Pflanzenphysiol* 88:61–68
66. Kayim M, Koc NK (2006) The effects of some carbohydrates on growth and somatic embryogenesis in citrus callus culture. *Sci Hortic* 109(1):29–34
67. Koc NK, Can C, Cinar A (1992) Effects of some culture media on somatic embryogenesis and rooting in ovular callus of “Shamouti” orange (*Citrus sinensis* Osb.). *Tree J Agric Forests* 16:140–147
68. Kochba J, Spiegel-Roy P (1973) Effects of culture media on embryoid formation from ovular callus of ‘Shamouti’ orange *C. sinensis*. *Z Pflanzenzuecht* 69:156–162
69. Tomaz MR, Mendes BMJ, Moura O, Filho FA et al (2001) Somatic embryogenesis in citrus spp.: carbohydrate stimulation and histodifferentiation. *In Vitro Cell Dev Biol Plant* 37:446–452
70. Gmitter FG Jr, Ling XB, Deng XX (1990) Induction of triploid citrus plants from endosperm calli in vitro. *Theor Appl Genet* 80(6): 785–790
71. Nito N, Iwamasa M (1990) In vitro plantlet formation from juice vesicle callus of Satsuma (*Citrus unshiu* Mare.). *Plant Cell Tiss Org Cult* 20(2):137–140
72. Chaturvedi HC, Sharma AK (1985) Androgenesis in *Citrus aurantifolia* (Christm.) Swingle. *Planta* 165:142–144
73. Chiancone B, Tassoni A, Bagni N et al (2006) Effect of polyamines on in vitro anther culture of *Citrus clementina* Hort. ex Tan. *Plant Cell Tiss Org Cult* 88:145–153
74. Calovic M, Vilorio Z, Nielsen B et al (2003) Somatic embryogenesis from lemon styles and analysis of genetic stability in regenerated plants using RAPD and flow cytometry. *Proc Int Soc Citricult*, 131–134
75. Carimi F, De Pasquale F, Crescimanno FG (1995) Somatic embryogenesis in citrus from styles culture. *Plant Sci* 105:81–86
76. Carimi F, Tortorici MC, De Pasquale F et al (1998) Somatic embryogenesis and plant regeneration from undeveloped ovules and stigma/style explants of sweet orange navel group [*Citrus sinensis* (L.) Osb.]. *Plant Cell Tiss Org Cult* 54:183–189
77. D’Onghia AM, Carimi F, De Pasquale F. et al (2003) Somatic embryogenesis from style culture: a new technique for the sanitization, conservation and safe exchange of citrus germplasm. *Proc Int Soc Citricult*, 147–149
78. Rangan TS, Murashige T, Bitters WP (1968) In vitro initiation of nucellar embryos in monoembryonic citrus. *HortScience* 3:226–227
79. Bitters WP, Murashige T, Rangan TS et al (1970) Investigations on established virus-free plants through tissue culture. *Calif Citrus Nursery Soc* 9:27–30
80. Button J, Bornman CH (1971) Development of nucellar plants from unpollinated and

- unfertilized ovules of the Washington navel orange in vitro. *J S Afr Bot* 37:127–134
81. Litz RE, Moore GA, Srinivasan C (1985) In vitro system for propagation and improvement of tropical fruits and palm. *Hortic Rev* 7:157–200
  82. Hidaka T, Yamada Y, Shichijo T (1981) Plantlet formation from anthers of *Citrus aurantium* L. *Proc Int Soc Citricult* 1:153–155
  83. De Pasquale F, Carimi F, Crescimanno FG (1994) Somatic embryogenesis from styles of different cultivars of *Citrus limon* (L.) Burm. *Aust J Bot* 42:587–594
  84. Gill MIS, Singh Z, Dhillon BS et al (1995) Somatic embryogenesis and plantlet regeneration in mandarin (*Citrus reticulata* Blanco). *Sci Hortic* 63:167–174
  85. Navarro L, Ortiz JM, Juarez J (1985) Aberrant citrus plants obtained by somatic embryogenesis of nucelli cultured in vitro. *HortScience* 20:214–215
  86. De Carlo A, Lambardi M, Ozudogru EA (2011) Cryogenic technologies for the long-term storage of Citrus germplasm. In: Yeung E, Thorpe TA (eds) *Plant embryo culture: methods and protocols*, vol 710, *Methods in molecular biology*. Springer, New York, pp 185–200
  87. Grosser JW, Deng XX, Goodrich RM (2007) Somaclonal variation in sweet orange: practical applications for variety improvement and possible causes. In: Kahn IA (ed) *Citrus genetics, breeding and biotechnology*. CAB International, Cambridge, MA, pp 219–234
  88. Grosser JW, Gmitter FG Jr, Tusa N et al (1990) Somatic hybrid plants from sexually incompatible woody species: *Citrus reticulata* and *Citropsis gilletiana*. *Plant Cell Rep* 8(11):656–659
  89. Vardi A, Galun E (1988) Recent advances in protoplast culture of horticultural crops: Citrus. *Sci Hortic* 37:217–230
  90. Germanà MA (1997) Haploidy in citrus. In: Jain SM, Sopory SK, Veilleux RE (eds) *In vitro haploid production in higher plants*, vol 5. Academic, Dordrecht, pp 195–217
  91. Germanà MA (2006) Doubled haploid production in fruit crops. *Plant Cell Tiss Org Cult* 86:131–146
  92. Germanà MA (2009) Haploid and doubled haploids in fruit trees. In: Touraev A, Forster B, Jain M (eds) *Advances in Haploid Production in Higher Plants*. Springer, Dordrecht, pp 241–263
  93. Germanà MA (2003) Somatic embryogenesis and plant regeneration from anther culture of *Citrus aurantium* and *Citrus reticulata*. *Biol Bratislava* 58(4):843–850
  94. Chaturvedi HC, Sharma AK (1985) Production of androgenic plants of *Citrus aurantifolia*. *J Plant Physiol* 119:473–477
  95. Ling J, Iwamasa M, Nito N (1988) Plantlet regeneration by anther culture of Calamondin (*C. madurensis* Lour.). In: *Proceedings of the Sixth International Society Citriculture*, vol 1, p 251–256
  96. Germanà MA, Wang YY, Barbagallo MG et al (1994) Recovery of haploid and diploid plantlets from anther culture of *Citrus clementina* Hort. ex Tan. and *Citrus reticulata* Blanco. *J Hort Sci* 69(3):473–480
  97. Deng XX, Deng ZA, Xiao SY et al (1992) Pollen derived plantlets from anther culture of *Ichang papeda* hybrids No. 14 and Trifoliate orange. *Proc Int Soc Citricult* 1:190–192
  98. Kobayashi S, Ikeda I, Nakatani M (1981) Role of the primordium cell in nucellar embryogenesis in citrus. *Proc Int Soc Citricult* 1:44–48
  99. Carimi F (2005) Somatic embryogenesis protocol: Citrus. In: Jain SM, Gupta PK (eds) *Protocol for somatic embryogenesis in woody plants*. Springer, Netherlands, pp 321–343
  100. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497
  101. Murashige T, Tucker DP (1969) Growth factor requirements of citrus tissue culture. In: *Proceedings of the first international citrus symposium*, p 1155–1161
  102. Moore GA (1986) In vitro propagation of Citrus rootstocks. *HortScience* 21:300–301
  103. Grosser JW, Calovic M, Louzada ES (2010) Protoplast fusion technology—somatic hybridization and cybridization. In: Davey MR, Anthony P (eds) *Plant cell culture*. John Wiley & Sons Ltd., New York
  104. Larkin PJ, Scowcroft WR (1981) Somaclonal variation—a novel source of variability from cell cultures for plant improvement. *Theor Appl Genet* 60:197–214
  105. Duncan RR (1997) Tissue culture induced variation in crop improvement. In: Sparks DL (ed) *Advances in agronomy*, vol 58. Academic, New York, pp 201–240
  106. Brar DS, Jain SM (1998) Somaclonal variation: mechanism and applications in crop improvement. In: Jain SM, Brar DS, Ahloowalia BS (eds) *Somaclonal variation and induced mutations in crop improvement*. Kluwer Academic Publishers, Dordrecht, Boston, London, pp 16–37

107. Cassells AC, Curry RF (2001) Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. *Plant Cell Tiss Org Cult* 64: 145–157
108. Kaeppler SM, Phillips RL, Olhoft P (1998) Molecular basis of heritable tissue culture-induced variation in plants. In: Jain SM, Brar DS, Ahloowalia BS (eds) *Somaclonal variation and induced mutations in crop improvement*. Kluwer Academic Publishers, Dordrecht, Boston, London, pp 465–484
109. Caplan A, Berger PH, Naderi M (1998) Phenotypic variation between transgenic plants: what is making gene expression unpredictable? In: Jain SM, Brar DS, Ahloowalia BS (eds) *Somaclonal variation and induced mutations in crop improvement*. Kluwer Academic Publishers, Dordrecht, Boston, London, pp 539–562
110. Jain SM (2001) Tissue culture-derived variation in crop improvement. *Euphytica* 118:153–166
111. Remotti PC (1998) Somaclonal variation and in-vitro selection for crop improvement. In: Jain SM, Brar DS, Ahloowalia BS (eds) *Somaclonal variation and induced mutations in crop improvement*. Kluwer Academic Publishers, Dordrecht, Boston, London, pp 169–201
112. Kochba J, Ben-Hayyim G, Spigel-Roy P et al (1982) Selection of stable salt-tolerant cell lines and embryos in *C. sinensis* and *C. aurantium*. *Z Pflanzenphysiol* 106:111–118
113. Spiegel-Roy P, Ben-Hayyim G (1985) Selection and breeding for salinity tolerance in vitro. *Plant Soil* 89:243–252
114. Ben-Hayyim G, Goffer Y (1989) Plantlet regeneration from a NaCl-selected salt tolerant callus culture of Shamouti orange (*Citrus sinensis* L. Osbeck). *Plant Cell Rep* 7:680–683
115. Nadel B, Spiegel-Roy P (1987) Selection of *Citrus limon* cell culture variants resistant to the Mal secco toxin. *Plant Sci* 53:177–182
116. Gentile A, Deng ZN, Tribulato E et al (2000) Evaluation of lemon somaclones for tolerance to mal secco disease by artificial inoculation. *Acta Hort* 535:259–263
117. Grosser JW, Chandler JL, Gmitter FG Jr (2003) Development of improved sweet oranges via somaclonal variation. In: *Proceedings of the international society citriculture*, p 42–45
118. Attree SM, Bekkaoui F, Dunstan DI et al (1987) Regeneration of somatic embryos from protoplasts isolated from an embryogenic suspension culture of white spruce (*Picea glauca*). *Plant Cell Rep* 6:480–483
119. Attree SM, Dunstan DI, Fowke LC (1989) Plantlet regeneration from embryogenic protoplast of white spruce (*Picea glauca*). *Biotechnology* 7:1060–1062
120. Gupta PK, Durzan DJ (1987) Somatic embryos from protoplast of loblolly pine pro-embryonal cells. *Biotechnology* 5:710–712
121. Grosser JW, Chandler JL (1987) Aseptic isolation of leaf protoplasts from Citrus, Poncirus, Citrus×Poncirus hybrids and Severinia for use in somatic hybridization experiments. *Sci Hort* 31:253–257
122. Kobayashi S, Uchimaya H, Ikeda I (1983) Plant regeneration from ‘Troviita’ orange protoplasts. *Jpn J Breed* 33:119–122
123. Vardi A, Spiegel-Roy P, Galun E (1982) Plant regeneration from *Citrus* protoplasts: variability in methodological requirements among cultivars and species. *Theor Appl Genet* 62: 171–176
124. Grosser JW, Ollitrault P, Olivares-Fuster O (2000) Somatic hybridization in citrus: an effective tool to facilitate variety improvement. *In Vitro Cell Dev Biol Plant* 36: 434–449
125. Grosser JW, Gmitter FG Jr, Tusa N et al (1996) Further evidence of a cybridization requirement for plant regeneration from citrus leaf protoplasts following somatic fusion. *Plant Cell Rep* 15(9):672–676
126. Guo WW, Cai XD, Grosser JW (2004) Somatic cell cybrids and hybrids in plant improvement. In: Daniell H, Chase CD (eds) *Molecular biology and biotechnology of plant organelles*. Kluwer Academic Publisher, Dordrecht, The Netherlands, pp 635–659
127. Davey MR, Cocking EC, Freeman J et al (1980) Transformation of petunia protoplasts by isolated Agrobacterium plasmids. *Plant Sci Lett* 18:307–313
128. Marton L, Wullems GJ, Molendijk L et al (1979) In vitro transformation of cultured cells from *Nicotiana tabacum* by *Agrobacterium tumefaciens*. *Nature* 277:129–131
129. Omar AA, Song W-Y, Grosser JW (2007) Introduction of the Xa21, *Xanthomonas* resistance gene from rice, into ‘Hamlin’ sweet orange [*Citrus Sinensis* (L.) Osbeck] using protoplast/GFP co-transformation and single plasmid transformation. *J Hortic Sci Biotechnol* 82(6):914–923

130. Paszkowski J, Shillito RD, Saul M et al (1984) Direct gene transfer to plants. *EMBO J* 3: 2717–2722
131. Vasil IK, Vasil V (1992) Advances in cereal protoplast research. *Physiol Plant* 85:279–283
132. Fleming GH, Olivares-Fuster O, Fatta Del-Bosco S et al (2000) An alternative method for the genetic transformation of sweet orange. *In Vitro Cell Dev Biol Plant* 36:450–455
133. Kao KN, Michayluk MR (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. *Planta* 126:105–110
134. Grosser JW, Collins GB (1984) Isolation and culture of *Trifolium rubens* protoplasts with whole plant regeneration. *Plant Sci Lett* 37:165–170
135. Myers JR, Grosser JW, Taylor NL et al (1989) Genotype dependent whole plant regeneration from protoplasts of red clover (*Trifolium pratense* L.). *Plant Cell Tiss Org Cult* 19: 113–127
136. Russell JA, McCown BH (1986) Culture and regeneration of *Populus* leaf protoplasts isolated from non-seedling tissue. *Plant Sci* 46: 133–142
137. Vardi A, Spiegel-Roy P, Galun E (1975) Citrus cell culture: isolation of protoplasts, plating densities, effect of mutagens and regeneration of embryos. *Plant Sci Lett* 4:231–236
138. Ohgawara T, Kobayashi S, Ohgawara E et al (1985) Somatic hybrid plants obtained by protoplast fusion between *Citrus sinensis* and *Poncirus trifoliata*. *Theor Appl Genet* 71:1–4
139. Sakai A, Kobayashi S, Oiyama I (1991) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb.) by a simple freezing method. *Plant Sci* 74:243–248
140. Navarro L, Olivares-Fuster O, Juarez J et al (2004) Applications of biotechnology to citrus improvement in Spain. *Acta Hort* 632:221–234
141. Grosser JW, Mourao-Fo FAA, Gmitter FG Jr et al (1996) Allotetraploid hybrids between citrus and seven related genera produced by somatic hybridization. *Theor Appl Genet* 92:577–582
142. Grosser JW, Jiang J, Mourao-Fo FAA et al (1998) Somatic hybridization, an integral component of citrus cultivar improvement: I. Scion improvement. *HortScience* 33: 1057–1059
143. Vilorio Z, Grosser JW (2005) Acid citrus fruit improvement via interploid hybridization using allotetraploid somatic hybrid and auto-tetraploid breeding parents. *J Am Soc Horti Sci* 130:392–402
144. Ollitrault P, Vanel F, Froelicher Y et al (2000) Creation of triploid Citrus hybrids by electro-fusion of haploid and diploid protoplasts. *Acta Hort* 535:191–198
145. Grosser JW, Omar AA, Gmitter JA et al (2012) Salinity tolerance of ‘Valencia’ orange trees on allotetraploid rootstocks. *Proc Fla State Hort Soc* 125:50–55
146. Guo WW, Prasad D, Cheng YJ et al (2004) Targeted cybridization in citrus: transfer of Satsuma cytoplasm to seedy cultivars for potential seedlessness. *Plant Cell Rep* 22: 752–758
147. Vardi A, Breiman A, Galun E (1987) Citrus cybrids: production by donor-recipient protoplast-fusion and verification by mitochondrial-DNA restriction profiles. *Theor Appl Genet* 75:51–58
148. Moreira CD, Chase CD, Gmitter FG Jr et al (2000) Transmission of organelle genomes in citrus somatic hybrids. *Plant Cell Tiss Org Cult* 61:165–168
149. Omar AA, Satpute A, Murata M et al (2014) Production of Three New Grapefruit Cybrids to Improve Citrus Canker Resistance. *Hort Sci* 49, S234 (Abstr.)
150. Kobayashi S, Uchimaya H (1989) Expression and integration of a foreign gene in orange (*Citrus sinensis* Osb.) protoplasts by direct DNA transfer. *Jpn J Genet* 64:91–97
151. Hidaka T, Omura M, Ugaki M et al (1990) Agrobacterium-mediated transformation and regeneration of Citrus spp. from suspension cells. *Jpn J Breed* 40:199–207
152. Vardi A, Bleichman S, Aviv D (1990) Genetic transformation of citrus protoplasts and regeneration of transgenic plants. *Plant Sci* 69:199–206
153. Davey MR, Rech EL, Mulligan BJ (1989) Direct DNA transfer to plant cells. *Plant Mol Biol* 13:273–285
154. Fromm M, Callis J, Taylor LP et al (1985) Electroporation of DNA and RNA into plant protoplasts. *Methods Enzymol* 153:351–366
155. Negrutiu I, Shillito R, Potrykus I et al (1987) Hybrid genes in the analysis of transformation conditions: I. Setting up a simple method for direct gene transfer in plant protoplasts. *Plant Mol Biol* 8:363–373
156. Potrykus I (1991) Gene transfer to plants: assessment of published approaches and results. *Annu Rev Plant Physiol Plant Mol Biol* 42:205–255

157. Guo W, Duan Y, Olivares-Fuster O et al (2005) Protoplast transformation and regeneration of transgenic Valencia sweet orange plants containing a juice quality-related pectin methylesterase gene. *Plant Cell Rep* 24(8): 482–486
158. Dutt M, Grosser JW (2010) An embryogenic suspension cell culture system for *Agrobacterium*-mediated transformation of citrus. *Plant Cell Rep* 29(11):1251–1260
159. Cervera M, Pina JA, Juarez J et al (1998) *Agrobacterium*-mediated transformation of citrange: Factors affecting transformation and regeneration. *Plant Cell Rep* 18(3–4): 271–278
160. Moore GA, Jacono CC, Neidigh JL et al (1992) *Agrobacterium*-mediated transformation of citrus stem segments and regeneration of transgenic plants. *Plant Cell Rep* 11(5–6): 238–242