Chapter 10
Applications of In Vitro Techniques in Plant Breeding

Zul Zulkarnain, Tanya Tapingkae, and Acram Taji

Abstract Although the Green Revolution of the 1960s tripled the world's food supply, food distribution remains uneven and the problem of chronic under-nutrition for millions of people in the world persists. In the face of predicted increases in the world population to around 10 billion by 2050 and the challenges faced by agriculture as a result of climate change, providing adequate food and fiber for humanity is a pressing issue requiring urgent attention. Since more and more of the arable land is being used to house the growing world population, provide feed for stock to supply animal protein and to grow crops for bio-energy, how could agriculture keep pace and remain productive without further degradation of the soil or damage to the environment? Could biotechnology be a key to solving world hunger given the challenges of climate change and immense population growth? This chapter examines recent advances in the application of a number of biotechnological techniques used in in vitro plant breeding including embryo rescue, somatic embryogenesis, in vitro pollination, flowering and fertilization as well as protoplast and somatic hybridization. A special focus has been given to exploitation of somaclonal variation in production of plants with better yield attributes as well as the ability to better cope with biotic and abiotic stresses. These techniques have the potential to increase food supply. The chapter overviews our collective experience working in this field over the past 30 years.

Keywords In vitro flowering • Protoplast • Somaclonal variation • Somatic embryogenesis • Somatic hybridization

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10.1 Introduction

Research in plant tissue culture over the past several decades has led to the development of techniques now used commercially across the globe to rapidly multiply a wide range of crops and improve their production systems. Plant tissue culture technology began with Gottlieb Haberlandt's theory of cell totipotency at the beginning of twentieth century (see Vasil 2008). Following on from this, the discovery of auxins by Frits Warmolt Went in 1926 (see Pennazio 2002), and cytokinins by Folke Skoog and colleagues in the 1950s (see Kieber 2002), led to the first success of in vitro techniques in plant tissues. Since then, the technology has developed considerably and now plays a key role in genetic engineering and crop improvement. Plant tissue culture offers an array of techniques that complement conventional plant propagation and plant breeding methods. The most common reasons for the use of in vitro techniques has been for plant propagation, but its most important application in recent years has been to crop improvement using gene technology (Khan 2009; Takeda and Matsuoka 2008; Thakur et al. 2012). Techniques such as in vitro fertilization and protoplast fusion enable the recombination of genotypes otherwise limited by incompatibility (Sri Rama Murthy et al. 2012; Tapingkae et al. 2012; Wang et al. 2006). Conventional breeding can be hastened by exploiting increased genetic diversity resulting from somatic variability (Bairu et al. 2011; Nwauzoma and Jaja 2013).

This chapter is aimed at introducing the reader to recent advances in the application of in vitro breeding techniques including embryo rescue, and utilizing somatic embryogenesis and somaclonal variation in plant breeding and mass propagation. In vitro flowering, pollination and fertilization as well as protoplast culture technologies and their relevance to plant breeding are also reviewed. These methods are now well-established technologies that have made significant contributions to plant improvement and mass propagation in horticulture, agriculture, and to some extent forestry.

10.2 Embryo Rescue

The term *embryo rescue* is used to describe the in vitro techniques aiming to encourage the development of immature embryos into complete plants. This technique has been widely used to avoid embryo abortion in regenerated plants from hybridization. The technique of embryo rescue has become an important tool in plant breeding, allowing the formation of many interspecific and intergeneric crop species.

Embryo rescue, also known as *embryo culture*, involves the excising of embryos and placing them onto sterile culture medium. The technique was first developed by Tukey in 1933 who successfully grew the embryo of cherry on an artificial medium. Since then, the procedure has been applied in embryo rescue of many other crops, such as *Lilium* (Chi 2002; Proseviçius and Strikulyte 2004), *Gossypium* (Mehetre

and Aher 2004), *Malus* (Dantas et al. 2006), *Prunus* (Kukharchyk and Kastrickaya 2006), *Elaeis* (Alves et al. 2011), various tree fruits (Fathi and Jahani 2012) and *Capsicum* (Debbarama et al. 2013).

Major application of embryo culture in plant breeding has been for interspecific or intergeneric hybridization, in which the endosperm develops poorly or does not develop at all due to hybridization barriers. Embryo culture can also be applied to shorten the breeding cycle by overcoming dormancy in seeds. Factors such as endogenous inhibitors, light, temperatures, humidity or embryo immaturity often cause seed dormancy to occur. By removing the embryos from the influences of these factors, they may germinate and grow quickly, and as a consequence the breeding cycle is shortened.

10.2.1 Interspecific and Intergeneric Hybridization

The endosperm of interspecific or intergeneric crosses, as well as crosses between diploids and tetraploids, often develop poorly or not at all, resulting in nonviable embryos. Such embryos, however, may have the potential for initiating growth and subsequent development. By aseptically isolating and culturing them in a nutrient medium, this hybridization barrier may be overcome. Stebbins (1950) proposed two types of hybridization barriers, pre-fertilization and post-fertilization barriers. The pre-fertilization barriers include those mechanisms which prevent fertilization to occur and can be the result of geographical distance, apomixes as well as pollen-pistil incompatibilities; whereas post-fertilization barriers can be caused by ploidy differences, chromosome elimination and seed dormancy.

The embryo rescue technique has been successfully applied to overcome the post-fertilization barrier which has been a great hindrance to hybridization in plant breeding. This technique has been applied in rescuing young embryos of successful interspecific crosses of *Lycopersicon esculentum* × *L. peruvianum* (Thomas and Pratt 1981) and *Medicago sativa* × *M. rupestris* (McCoy 1985). Fertile hybrid progenies were produced from the intergeneric cross between *Brassica napus* and *Sinapsis alba* by combination of ovary culture and embryo rescue techniques. The hybrids possess important traits such as resistance to major insect pests, tolerance to high temperatures and drought as well as resistance to shattering (Brown et al. 1997; Momotaz et al. 1998). Other recent examples of the application of embryo rescue technique in plant breeding are summarized in Table 10.1.

10.2.2 Overcoming Embryo Abortion

Embryo abortion is a common phenomenon and has been a major problem in conventional plant breeding. This is primarily due to the failure of the endosperm to properly develop as nutritive tissue surrounding the embryo (Bhojwani and

Table 10.1 Recent examples of the use of embryo rescue technology in plant breeding

Species	Use of embryo culture	References
Allium cepa × *A. roylei*	Introgression desirable traits of *Allium roylei* into the *A. cepa* genome	Chuda and Adamus (2012)
Arachis hypogaea × *A. paraguariensis, A. hypogaea* × *A. appressipila*	Regeneration of interspecific hybrids with resistance to early leaf spot	Rao et al. (2003)
Capsicum annuum, *C. chinense*, and *C. frutescens*	Interspecific hybridization for crop improvement	Debbarama et al. (2013)
Elaeis oleifera × *E. guineensis*	Optimizing media composition for immature embryo culture	Alves et al. (2011)
Gossypium spp.	Introgression of desirable characteristics into commercial cotton	Mehetre and Aher (2004)
Hylocereus polyrhizus × *H. undatus*	Developing efficient method to rescue embryos following interspecific crosses	Cisneros and Tel-Zur (2010)
Lilium spp.	Producing interspecific hybrids with useful traits for flower market	Prosevičius and Strikulyte (2004)
Lilium spp.	Optimizing efficiency of embryo rescue methods in interspecific hybridization	Chi (2002)
Malus pumila	Rescuing immature embryos for rootstocks	Dantas et al. (2006)
Phaseolus vulgaris × *P. coccineus*	Rescuing globular stage embryos following interspecific hybridization	Barikissou and Baudoin (2011)
Phoenix dactylifera × *P. pusilla*	Rescuing and multiplication of interspecific hybrid zygotic embryos	Sudhersan et al. (2009); Sudhersan and Al-Shayji (2011)
Prunus spp.	Rescuing genetically unbalanced hybrids	Kukharchyk and Kastrickaya (2006)
Salix viminalis × *Populus alba, S. viminalis* × *P. violascens, S. viminalis* × *P. tremula*	Overcoming post-zygotic barriers caused by the deficiency of endosperm as nutritive tissue	Bagniewska-Zadworna et al. (2010)
Solanum pinnatisectum × *S. tuberosum*	Introgression of resistance to late blight from *Solanum pinnatisectum* into *S. tuberosum* genome	Ramon and Hanneman Jr. (2002)
Triticale	Optimizing plant regeneration and the generation of genetic variability	Maddock (1985)
Vaccinium spp.	Overcoming post-zygotic barrier during the production of interspecific hybrids	Pathirana et al. (2013)

(continued)

Table 10.1 (continued)

Species	Use of embryo culture	References
Vitis vinifera	Application of CPPU and BA to enhance embryo recovery	Nookaraju et al. (2007)
V. vinifera × *V. amurensis*	Resistance to downy mildew and anthracnose	Tian and Wang (2008)

Razdan 1996; Hu and Wang 1986). For this reason, embryo rescue technology has been widely used to regenerate complete plants under in vitro conditions (Reed 2005).

This method can also be used to rescue young embryos from intraspecific hybrids that normally produce unviable seeds. For instance in seedless triploid embryos resulting from crosses between diploids and tetraploids of the same species. By in vitro culture on aseptic nutrient medium the embryos may develop and grow into complete plants, thus overcoming postzygotic barriers such as endosperm failure.

Recovery of triploid hybrids by embryo rescue has also been reported to be successful from intraspecific crosses between Fujiminori ($2n=4x=76$) × Jingxiu ($2n=2x=38$) grape varieties (Yang et al. 2007). Intraspecific crosses between diploid (2n = 22) and tetraploid (2n = 44) daylily (*Hemerocallis*) was also performed in vivo to produce triploid hybrids (3n = 33). Following from this, the embryo rescue procedure was applied to regenerate plants from immature triploid hybrid embryos, which then were propagated in vitro and successfully acclimatized to ex vitro conditions (Zhiwu et al. 2009). Further, Guo et al. (2011) reported successful embryo culture of triploid grapes obtained from crosses between diploid and tetraploid cultivars. More recent achievements in the use of embryo culture technology coupled with triploid plant regeneration have been reported by Aleza et al. (2010, 2012) in the breeding program of seedless mandarin oranges.

10.2.3 Shortening Breeding Cycle by Overcoming Seed Dormancy

Embryo rescue technique has also been used to shorten breeding cycle in a number of fruit crops by overcoming seed dormancy. In species which require enough time for embryo maturity, seedlings cannot be raised just after fruit ripening. In addition, some species need a longer period to break their seed dormancy. For example, seeds of Brussels sprouts, rose, apple, oil palm and *Iris* do not germinate after fruit ripening. Culturing immature embryos on proper growth medium will result in immediate germination, and therefore shorten the breeding cycle.

The occurrence of seed dormancy may be due to internal factors such as endogenous inhibitors and embryo immaturity, or external factors such as light, temperatures and humidity. Debbarama et al. (2013) claimed that seed dormancy may be localized

in the seed coat or in the endosperm, or both. Therefore, the embryos will germinate and grow successfully and more rapidly when removed from these factors.

Application of various embryo rescue technique to wide area of plant research have also been reviewed by Bridgen (1994), Sharma et al. (1996), Chuanen and Guangmin (2005), and more recently by Fathi and Jahani (2012).

10.3 Somatic Embryogenesis

The term *somatic embryogenesis* refers to the process of embryo development from cells other than gametes (somatic cells) without a normal fertilization process. Since the embryos developed by circumventing the normal fertilization process, they are genetically identical to their parent tissue, and as such they are clones.

The phenomenon of somatic embryogenesis was first reported by Steward et al. (1958) on suspension culture of *Daucus carota*, and by Reinert (1959) on callus culture of the same species. It is worth noting that Krikorian and Simola (1999), commented on the pioneer work of Harry Waris on somatic embryogenesis of *Oenanthe aquatica* (Umbelliferae). They emphasized that Waris was one of the first researchers to observe and recognize somatic embryo production in aseptic culture (see also Simola 2000).

Somatic embryogenesis can be used in a number of ways. For example, large scale-clonal propagation of elite cultivars (Ahmad et al. 2011), producing artificial seeds (synthetic seeds) (Pintos et al. 2008), gene transfer for genetic improvement (Li et al. 2002), in vitro selection approaches for various biotic and abiotic stresses (Ahmad et al. 2011), and providing potential models for studying molecular, regulatory and morphogenetic events during plant embryogenesis (Kamle et al. 2011; Ravi and Anand 2012).

Slater et al. (2003) claimed that somatic embryos may be produced indirectly by involving the dedifferentiation of organized tissue into the callus mass prior to embryo formation, or embryos may be produced directly from organized tissue without an intervening callus phase. The anatomical and physiological features of embryos derived from somatic tissues are highly comparable to zygotic embryos derived through normal fertilization (Dobrowolska et al. 2012; Mathew and Philip 2003; Palada-Nicolaua and Hausman 2001). They both proceed through a series of distinct stages which span a period of several days. There is no difference in embryogenesis of dicots or monocots up to the octant stage. Following from this, however, the embryogenesis takes different pathways (Raghavan 1986). In monocots Godbole et al. (2002) reported that beyond the octant, embryogenesis encompasses globular, elongated, scutellar and coleoptile stages; while in dicots Mandal and Gupta (2002) claimed that the stages are globular, heart, torpedo and cotyledon or plantlet stages.

10.3.1 Somatic Embryogenesis for Mass Propagation

Somatic embryogenesis is the most attractive and practical way for rapid mass multiplication of agricultural crops. Deo et al. (2010) suggested that this technology offers many advantages over conventional micropropagation such as the opportunity of producing somatic embryos; shoot and root of somatic embryos are formed simultaneously avoiding the requirement of a rooting phase as in conventional micropropagation. Embryo formation and germination can be synchronized to maximize plantlet regeneration, the dormancy of somatic embryos can be induced to make long-term storage become possible, and it is easy to scale-up somatic embryos with less labor inputs.

Research on somatic embryogenesis had been done on various plant species such as *Allium sativum* (Luciani et al. 2006), *Dioscorea alata* (Belarmino and Gonzales 2008), *Bactris gasipaes* (de-Alencar et al. 2010), *Agapanthus praecox* ssp. *Minimus* (Yaacob et al. 2012) and *Theobroma cacao* (Quainoo and Dwomo 2012). Somatic embryogenesis had also been successfully applied to *Alstroemeria* (Khaleghi et al. 2008), *Dianthus caryophyll*us (Ali et al. 2008; Karami et al. 2007) and *Bauhinia variegata* (Banerjee et al. 2012). Successful somatic embryogenesis has also been reported in *Coffea* (Neuenschwander and Baumann 1992; Priyono 1993; Sreenath et al. 1995), but the rate of success was relatively low and plantlets regeneration was found to be difficult. This technique has also been successfully applied in clonal propagation of various gymnosperms such as *Picea* (Ahmad et al. 2011), *Ephedra foliata* (Dhiman et al. 2010) and *Araucaria angustifolia* (Steiner et al. 2012).

In spite of its advantages, however, somatic embryogenesis also has limitations. There is the tendency of somatic embryo development to be nonsynchronous resulting in embryos of various developmental stages to be present in the culture system (Kong et al. 2012; Zegzouti et al. 2001). However, this limitation could be overcome by incorporating abscisic acid (ABA) and mannitol in culture medium (Torres et al. 2001). Another problem concerning somatic embryogenesis is the instability of cell lines in culture that may induce morphological abnormalities such as pluricotyledony, multiplex apex formation, fused cotyledons, fasciation (Singh and Chaturvedi 2013; Zegzouti et al. 2001), slender stems, stubby structures and non-functional leaves (Benelli et al. 2010). In gymnosperm, the regenerated somatic embryos lack a lipid- and protein-rich megagametophyte, from which amino acids and sugars are mobilized during germination (Bornman et al. 2001).

10.3.2 Somatic Embryogenesis in Plant Breeding

Somatic embryogenesis has become an indispensable modern plant breeding component since this system provides an alternative platform in the development of new crops with many valuable agronomic properties. One of the benefits is that the somaclonal variations (discussed later in this chapter) which arise from the process

of somatic embryogenesis could be utilized as a source of genetic variability for producing novel varieties. The use of somatic embryogenesis as a preferred method for genetic improvement of valuable germplasm of a number of important crops has been emphasized by many researchers (Ashakiran et al. 2011; Ji et al. 2011; Kamle et al. 2011).

Somatic cell hybridization through protoplast fusion (discussed later in this chapter) has proved useful in shortening the cycle of plant breeding. This technique was first developed by Melchers and Labib (1974) through their investigation on tobacco tissue culture. Since then, somatic cell hybridization has been applied to a wide range of crop species such as crosses between *Triticum aestivum* × *Haynaldia villosa* (Zhou et al. 1996, 2002), *Hordeum vulgare* × *Daucus carota* (Kisaka et al. 2001), *Triticum aestivum* × *Setaria italica* (Cheng et al. 2004) and *Carica papaya* L × *Vasconcellea cauliflora* (Dinesh et al. 2013). The rate of success, however, varied among crosses.

There are three types of hybrids formed through somatic cell hybridization: symmetric hybrids which contain somatic chromosomes of both parents, asymmetric hybrids which preserve the genetic material of one parent, and cybrids which consist of nucleus of one parent and cytoplasm from both parents. Among the three types, Zhou et al. (2001) claimed that asymmetric somatic hybridization is superior to symmetric because the resultants possess comparatively fewer chromosomes (genes) from the donor, avoiding too many wild traits being introduced into the acceptor, thus the traits of the hybrids were closer to the goal of breeding. Further, Miko (2008) suggested that a few characters are passed on from parent to offspring by genes which are not part of a nuclear chromosome, but located in cell organelles in the cytoplasm (cytoplasmic genes). This suggests that regeneration of cybrids with the mixture of cytoplasm from both parents but having only one nuclear genome will help in transfer of cytoplasmic genetic information from donor to offspring. Thus, somatic cybridization can be applicable in plant breeding program

10.3.3 Somatic Embryogenesis for Production of Synthetic Seeds

Ara et al. (2000) defined artificial seed as encapsulated plant tissue that can be used as a seed that will germinate and grow into a complete plant under favorable in vitro or ex vitro conditions, and this capability is preserved even after storage. Somatic embryos have the potential to be used in producing artificial seed as first demonstrated by Kitto and Janick (1982) in *Daucus carota*. In addition to somatic embryos, axillary shoots, apical shoot tips, buds or stem, root segments, embryogenic calli, as well as protocorms or protocorm-like bodies may be used to produce artificial seeds (Siong et al. 2012; Vdovitchenko and Kuzovkina 2011). However, of various micropropagules used, somatic embryos and axillary shoot buds have been mainly favored for synthetic seed production (Siong et al. 2012).

The development of synthetic seed production technology is considered an effective and efficient method of propagation in a number of important crops. Explants such as somatic embryos, shoot tips or axillary buds are encapsulated in coating material such as hydrogel, alginate gel, ethylene glycol, or dimethylsulfoxide (Asmah et al. 2011), which can be developed into plants. The coating protects the explants from mechanical damage during handling and allows germination to occur like true seeds and sprout into seedlings under suitable conditions. It is important to note that materials used to encapsulate embryos should also be nontoxic, biocompatible and water soluble (Ara et al. 2000). For this reason, Saiprasad (2011) suggested the use of alginate gel for encapsulating synthetic seed since it has moderate viscosity, low toxicity, rapid gelification, low cost and bio-compatibility properties. In addition, alginate may enhance capsule formation and its rigidity provides better protection for encased explants against mechanical injury.

The successful application of artificial seed technology can only be achieved when there is efficient upstream production of micropropagules (somatic embryos or axillary shoots) as well as downstream germination protocols for high percentage plant regeneration. In this regard, Ara et al. (2000) and Ravi and Anand (2012) proposed simplified schematic procedures for the production of artificial seeds, which is summarized in Fig. 10.1.

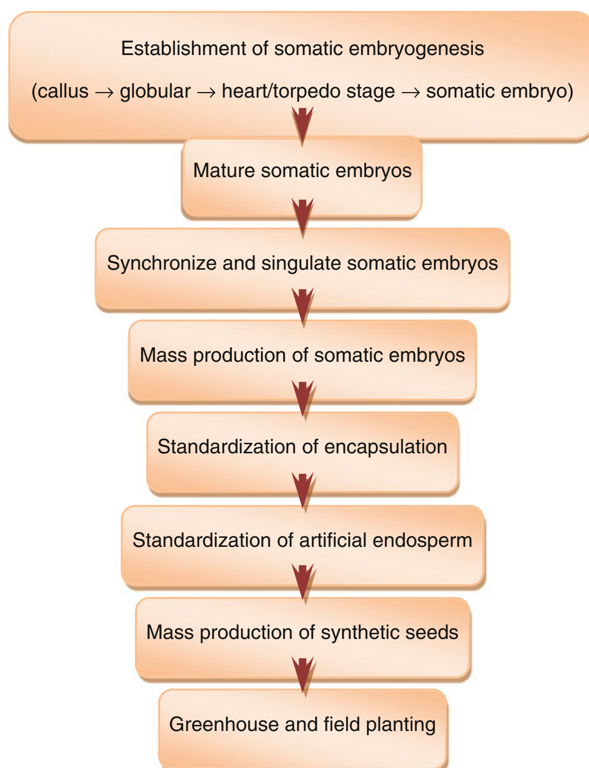

Fig. 10.1 Simplified procedure for the production of artificial seed

There are various advantages of artificial seeds such as: better clonal plants which could be propagated in large scale; preservation of elite and endangered or extinct or rare plant species; and consistent and synchronized harvesting of important agricultural crops (Khor and Loh 2005). In addition, ease of handling, potential long-term storage and low cost of production and subsequent propagation are other benefits (Bekheet 2006). Ravi and Anand (2012) suggested that artificial seeds can also be beneficial in understanding seed coat formation, fusion of endosperm in embryo development and seed germination, production of somatic hybrids in plants with unstable genotypes or those showing seed sterility; it can also be incorporated in embryo rescue technology.

Recent developments in tissue culture techniques have made synthetic seed technology considerably more diverse, and has been reported in a number of plant species, including *Asparagus officinalis* (Mamiya and Sakamoto 2001), *Geodorum densiflorum* (Datta et al. 2001), *Paulownia elongata* (Ipekci and Gozukirmizi 2003), *Dendrobium, Oncydium* and *Catlleya* orchids (Saiprasad and Polisetty 2003), *Gypsophila paniculata* (Rady and Hanafy 2004), *Cyclamen persicum* (Winkelmann et al. 2004), *Camellia sinensis* (Seran et al. 2005), *Allium sativum* (Bekheet 2006), *Pyrus communis* (Nower et al. 2007), *Oryza sativa* (Bidhan and Mandal 2008; Kumar et al. 2005) and *Brassica napus* (Zeynali et al. 2013). These results have shown that artificial seed production is potentially useful for the large scale propagation of superior hybrids of economically important species.

10.4 Somaclonal Variation

The existence of genetic variation is an important factor exploited in plant breeding. The desired variation, however, is often not available in the right combination or does not exist at all under in vitro conditions. Jain et al. (1998) suggested such variation can be induced in vitro from somatic cells or tissue resulting in somaclonal variation that could be used in combination with conventional breeding methods to create more genetic variability. Somaclonal variation has been a valuable tool in plant breeding; wherein variation in tissue culture regenerated plants from somatic cells can be used to develop crops with desirable traits. Characteristics for which somaclonal mutants can be improved during in vitro culture includes resistance to disease, herbicides and tolerance to environmental or chemical stress, as well as for increased production of secondary metabolites. Selection is done by employing a stress-causing agent in tissue culture containing dividing cells. An efficient method for obtaining plants with desired characteristics is to add selective agents that will alter other aspects of the phenotype is reviewed by Tapingkae et al. (2012).

Somaclonal variation has been associated with changes in chromosome numbers (polyploidy and aneuploidy), chromosome structure (translocations, deletions, insertions and duplications), point mutations, and DNA methylation (Nwauzoma and Jaja 2013; Rodriguez-Enriquez et al. 2011). The molecular basis of somaclonal variation is not precisely known; however, both genetic and epigenetic mechanisms

are suggested to play a role (Jiang et al. 2011). Changes in DNA methylation often give rise to epigenetic effects, which can affect expression of genes normally suppressed. Epigenetic variation is often unstable and can disappear either after plants are removed from culture or within a few clonal generations, whereas genetic variation is heritable (Biswas et al. 2009). Therefore, the success in applying somaclonal variation in plant breeding is dependent on the genetic stability of the selected somaclones.

10.4.1 Detection of Somaclonal Variation

The early detection of the presence of somaclonal variants could save valuable time and minimize the overall economic loss to the users of tissue-cultured planting materials. Various strategies were used to detect somaclonal variants, based on one or more determinants from among morphological traits, cytogenetic analysis (numerical and structural variation in the chromosomes) and molecular and biochemical genetic markers (Tan et al. 2013). Development and application of modern technologies based on molecular markers provide valuable tools for the detection of somaclonal variation. The use of molecular marker techniques to detect somaclonal variations has been applied successfully to several plant species, such as *Solanum tuberosum* (Ehsanpour et al. 2007), *Gossypium hirsutum* (Jin et al. 2008) and *Musa* spp. (Abdellatif et al. 2012). Research publications continue to increase rapidly and the field is gaining growing interest in a wide range of research areas. This has widely been covered in an extensive set of literature and the reader is referred to publications in broad reference works such as those of Davies (2010), Albrecht et al. (2012) and de Maagd and Hall (2013).

10.4.2 Genomics, Proteomics and Metabolomics

The technologies of genomics which assesses changes in the genome, proteomics which studies the total protein complement (the proteome) and the metabolomics which investigates the complement of small molecules (low molecular weight, <1500 Da) (Davies 2010), collectively are referred to as *omics*, have the potential to be utilized in the detection of somaclonal variation.

10.4.2.1 Genomics

Genetic fingerprinting is a powerful tool in the field of plant science, to be used, for example, for correct germplasm identification. When linked to metabolomics and proteomics (fingerprinting techniques on the plant's metabolites or protein composition) has the potential to elucidate data on phenotypic variation, caused by growth

conditions or environmental factors, and yield data on the genes involved in the pathways and enzymes involved in the synthesis of natural products (Terryn et al. 2006).

The development of high throughput genome technologies in the past decade has permitted a number of options to profile the epigenome of several organisms including plants such as Arabidopsis. These new methods are able to provide a detailed characterization of genomic DNA methylation and histone modifications at an unprecedented resolution, which can be integrated with transcriptomics data including the smRNA transcriptome (Wang et al. 2009).

Randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) markers have been employed to assess the genetic fidelity or genetic variability in regenerated plants. Among the markers, ISSR and RAPD have been mostly preferred and well established in many plant species, including *Vitis* (Nookaraju and Agrawal 2012), *Musa* sp. (Aremu et al. 2013), *Cynara scolymus* (Rey et al. 2013), *Olea europaea* (Leva et al. 2012), *Pongamia pinnata* (Kesari et al. 2012) and *Punica granatum* L. (da Silva et al. 2013).

ISSR markers involve PCR amplification of the region between two closely placed simple repeat sequences that are inversely oriented. They are identified using primers designed from within the repeated region (Zietkiewicz et al. 1994). This technique is based on PCR amplification of inter-microsatellite sequences remaining popular due to their relative simplicity, reliability, cost effectiveness and highly discriminative nature (Agarwal et al. 2008). Hence it has been widely used in numerous fields such as genetic diversity, phylogenetic studies, ecology and evolutionary biology (Aremu et al. 2013; Aruna et al. 2012). However, reports about somaclonal variation are still controversial. For example, RAPD and ISSR analyses of leaf genomic DNA were able to distinguish between date palm (*Phoenix dactylifera*) cultivars (Zehdi et al. 2002), but according to Fki et al. (2011), they failed to reveal polymorphisms in genomic DNA that could be associated with somaclonal variations in tissue culture-derived date palms.

10.4.2.2 Proteomics

Modern technologies of gene sequencing, microarray experiments, gene and protein expression within the cell of an organism and information on molecular markers have been very useful in identifying regions on chromosomes that bring about variation in a trait, thereby providing tools that can lead to far more accurate selection processes for genetic improvement (Nimisha et al. 2013). Using proteomic analyses it is possible to identify proteins, their functions and interactions as well as their subcellular localization in a tissue. This can be useful for the determination of protein alteration during plant development, including somaclonal variation. Accordingly, use of proteomics has become increasingly common in cellular, genetic and physiological research (Miransari and Smith 2013). For example, the proteomic analysis of Arabidopsis cells using Polyacrylamide Gel Electrophoresis

(2D-PAGE) and matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF) (MALDI-TOF) peptide mass fingerprinting indicated the presence of 663 different proteins originated from 2943 2D-PAGE spots (Giavalisco et al. 2005).

Currently, three main techniques are used for quantitation in proteomics: two-dimensional 2D-PAGE linked to mass spectrometry for protein identification and stable-isotope labelling-free shotgun proteomics. With 2D-PAGE, proteome differences can be identified by gel-gel comparisons but care needs to be taken to ensure reproducibility, to avoid variable protein staining efficiency of individual gels, and to take account of bias against some protein classes such as membrane proteins. One can expect ca. 1000–2000 proteins to be separated and detected in a single gel. Another technique (shotgun proteomics) involves tryptic digestion of a complex protein sample followed by peptide analysis using liquid chromatography-mass spectrometry (LC–MS) techniques. In this case, the increased complexity of the sample caused by the high number of peptides produced is the primary challenge, but the capacity for throughput is high compared with that of 2D-PAGE with less bias against specific protein classes, as in the case of 2D-PAGE. Protein identification is difficult where whole genome sequences are not available. However, standard protein profiling technologies have two major limitations. First, only the most abundant proteins can be detected in a complex protein mixture and second, the high throughput analysis is mostly restricted to proteins and peptides in protein databases (Davies 2010).

More recently a comprehensive proteomic assay was conducted trying to find embryogenic-specific proteins. Proteomic approaches have been applied to investigate the protein profiles and their changes during callus differentiation of *Vanilla planifolia* (Palama et al. 2010). Morel et al. (2012) found 100 significantly- and differentially-expressed proteins in somatic embryogenesis of *Pinus pinaster*. They were mainly involved in carbohydrate or lipid metabolism and genetic information processing. Many storage proteins were identified (vicillin-like, legumin-like, LEA proteins); some of them were observed from the beginning of maturation. In *Cyphomandra betacea*, Correia and Canhoto (2012) reported that a better ability of the embryogenic cells to regulate the effects of stress conditions relied on an increase in metabolism, protein synthesis and stress related proteins.

10.4.2.3 Metabolomics

Metabolomics is the systematic characterization of small molecules and their concentrations in cells, tissues, or organisms, in ever more detail. The basis of metabolomics is making unbiased observations with highly reproducible analytical tools, followed by a biostatistical analysis to find correlations between all the available data. This can be illustrated by the identification of the active compounds in medicinal plants. Metabolomics intends to give a wide view of all metabolites in a specific sample at a specific time. Applications of metabolomics in the plant sciences are already extensive and broad. Information on the biochemical composition of plants can help identify biochemical changes as a potential basis of causality for

phenomena of interest such as visible (phenotype) or invisible (chemical/physiological) differences (reviewed by de Maagd and Hall 2013).

Most metabolomics experiments involve a combination of separation and detection technologies that can be used in serial or in parallel combinations, often referred to as *hyphenated approaches*. Therefore these analyses never give a complete picture, and one needs to know beforehand what class of compounds is considered relevant in order to choose the proper methodology. High performance liquid chromatography (HPLC) with UV, liquid chromatography-Mass spectrometry (LC-MS) or Gas chromatography-mass spectrometry (GC-MS) are most commonly used to monitor the presence of secondary metabolites (Moco and Vervoort 2012). Whereas, GC-MS is particularly appropriate for the analysis of volatile organic compounds and derivatives of primary and secondary metabolites, LC-MS is highly applicable to the analysis of a wide range of semi-polar compounds including many secondary metabolites with nutriceutical proprieties (Rea et al. 2011). Detailed coverage of the various technologies used for metabolomics can be found in Schauer and Fernie (2006) and Bowne et al. (2011).

Terryn et al. (2006) developed a comprehensive profiling approach that is based on functional genomics. This approach integrates cDNA-AFLP-based transcript profiling and targeted metabolic profiling. As this method requires no prior genetic knowledge or sequence databanks, it is appropriate to any plant species to unravel the biosynthesis of any metabolite of interest. This knowledge will then allow for metabolic engineering, as well as pave the way for so-called *combinatorial biochemistry*, with which novel metabolites could be produced in plants. However, this technology is quite expensive and needs specialized researchers for correct application as well as for interpretation of the data (de Maagd and Hall 2013). All these limitations are expected to decrease in the near future as the technology continues to develop fast.

New tools of functional genomics combined with metabolomics and proteomics will revolutionize the knowledge of pathways and enzymes involved in the synthesis of natural products, and thus allow a more focused approach for their production. With the increasing need for novel drugs, selection of somaclones with desired traits, using omics technologies will play an increasingly important role in the large-scale production of pharmaceuticals in plants.

10.5 In Vitro Flowering

An in vitro plant production system is considered a convenient tool to study the flowering mechanism, as the conditions can be manipulated and so provide a controlled system. It is possible to use in vitro flowering in genetic studies, biochemistry and studies of environmental factors affecting flower formation. It can also be applied as a tool to shorten breeding programs or can be adjusted to commercial production of specific compounds (Lin et al. 2003). In vitro flowering has been reported from both monocotyledonous (Joshi and Nadgauda 1997; Lin et al. 2003,

2004; Nadgauda et al. 1990; Ramanayake 2006) and dicotyledonous (Al-Khayri et al. 1991, 1992; Kanchanapoom et al. 2009; Yadav and Singh 2011) families. Several factors, such as genotype, culture medium, physical and environmental growth conditions, and factors inherent to the explants, affect in vitro flowering. More general reviews of in vitro flowering include Lakshmanan and Taji (2004), Ziv and Naor (2006) and Sri Rama Murthy et al. (2012). Successful in vitro flowering in *Dendrobium* orchid and *Dendrocalamus hamiltonii* has been reviewed by Zulkarnain et al. (2012).

Novel approaches involving in vitro flowering and molecular techniques offer unique opportunities to investigate the flowering process from new perspectives. For example, flowering time in Rosaceae species, such as apricot, sour and sweet cherry, apple, peach, almond and rose, were identified in breeding for climatic adaptation (reviewed by Campoy et al. 2011). These data may facilitate the development of breeding strategies aimed at releasing early or late flowering cultivars and controlling the phenological adaptation of new cultivars to climate change.

The endogenous signaling pathways responding to gibberellic acid (GA_3) (autonomous and age) and environmental signals (photoperiod and temperature) converge on genes that then activate floral homeotic genes (reviewed by Pineiro and Jarillo 2013; Pose et al. 2012; Song et al. 2013; Taoka et al. 2013). In vitro flowering can reduce the influence of environmental factors and allow for precise control of environmental factors and the application of plant growth regulators. It may also offer a means for studying the mechanism of flowering. Recently Chavan et al. (2013) in *Ceropegia panchganiensis* observed varied flower induction to BAP and sucrose concentrations and combinations.

10.5.1 In Vitro Pollination and Fertilization

In angiosperms, pollination is defined as the transfer of pollen grains from an anther to the stigma of the same or different flowers on the same plant (self-pollination) or flowers of other plants (cross-pollination). When a pollen grain makes contact with the stigma, it adheres, hydrates, and germinates, resulting in the growth of a pollen tube which convey the sperm cells to the embryo sac and egg in the ovule (Lord and Russell 2002). Following from this, the plant undergoes fertilization through the fusion of sperm and egg cells.

Pollination and fertilization are crucial events contributing to genetic diversity and providing a basis for the improvement of important agricultural crops. However, the possibilities for cross combination is sometimes limited by sexual incompatibility and incongruity, restricting successful interspecific hybridization (Vervaeke et al. 2002; Zenkteler 1970). Incompatibility may occur in intraspecific crosses as a result of the activity of S-alleles, whereas incongruity operates in interspecific crosses due to a lack of genetic information to complete pre- and post-pollination processes (Hogenboom 1973).

Stebbins (1958) separated such sexual barriers preventing interspecific hybridization into pre-fertilization (factors hindering effective fertilization) and post-fertilization (barriers occurring during or after syngamy) barriers. The pre-fertilization barrier is mostly confined within the style (Vervaeke et al. 2002), for example the failure of pollen to germinate on stigma, or failure of pollen tube to reach the ovule due to very long style, or slow growth of pollen tube which fails to reach the ovule before the ovary abscises (Taji et al. 2002). To overcome this type of sexual barrier, a number of techniques such as bud pollination, stump pollination, use of mentor pollen and grafting of the style have been developed (Van Tuyl and De Jeu 1997).

On the other hand, the post-fertilization barrier is mainly caused by the failure of hybrid embryo to gain its maturity because of embryo-endosperm incompatibility or poor embryo development (Taji et al. 2002). To overcome the post-fertilization barriers, Van Tuyl and De Jeu (1997) proposed ovary and ovary-slice culture, ovule culture and embryo culture.

Ovary and ovary-slice culture are applied when abortion takes place at a very young stage of seed development. In this technique, embryos are dissected or in some crops, where the ovary is large, slicing the ovary in small parts is a better option for rescuing the young seedlings in vitro. Ovule culture is applied when the mismatch between embryo and endosperm development occurs very early and ovary culture and/or ovary slice culture fails. In this method, ovules are dissected out of the ovaries and are cultured in vitro under controlled environment. Meanwhile, embryo rescue method may be applied when young fruits remain for a long time on the mother plant, and it is necessary to excise the entire embryo to prevent abortion. However, it is technically difficult to isolate the intact embryos due to their very small size, therefore ovaries with young embryos or entire fertilized ovules are often cultured (Van Tuyl and De Jeu 1997).

The technique of in vitro fertilization, in which isolated sperm and egg cells are induced to fuse under controlled conditions, removes much of the interfering barrier. In vitro pollination and fertilization have been used to overcome pre- and post-fertilization barriers in a number of genera. The use of in vitro fertilization in higher plants, therefore, has become an important contemporary research area in plant developmental and reproductive biology with potentially significant scientific applications (Wang et al. 2006).

Since it was first introduced by Kanta et al. (1962) on *Papaver somniforum*, the technology of in vitro pollination and fertilization have been successfully applied in various important agricultural crops including *Lycopersicon esculentum* (Sheeja and Mandal 2003), *Helianthus annuus* (Popielarska 2005) and *Melandrium album* (Zenkteler et al. 2005).

In plant breeding the technique of in vitro pollination and fertilization has potential applications at least in the following areas: (a) production of interspecific and intergeneric hybrids, (b) production of haploid plants through parthenogenesis, (c) overcoming self-incompatibility, and (d) overcoming cross-incompatibility. In addition, Taji et al. (2002) proposed that in vitro pollination and fertilization is also important in the study of pollen physiology and fertilization.

10.5.2 Production of Interspecific and Intergeneric Hybrids

In vitro pollination and fertilization have been successfully used in interspecific and intergeneric hybridization to produce novel cultivars with blended traits of both parents and to introgress useful traits of one species to another. This is made possible because under in vitro condition environmental factors can be controlled and optimized, while in nature such hybrids would not be formed readily. In vitro interspecific hybridization progenies have been created within the genus *Helianthus* (Weber et al. 2000), *Lilium* (Lim et al. 2008; Wang et al. 2012) and *Cucumis* (Skálová et al. 2010). Meanwhile, successful intergeneric hybridization has been achieved in *Brassica napus* using *Brassica elongata*, *B. fructiculosa, B. souliei, Diplotaxis tenuifolia, Hirschfeldia incana, Coincya monensis* and *Sinapis arvensis* (Siemens 2002). Further, intergeneric hybrids have also been successfully obtained between *Sandersonia aurantiaca* × *Gloriosa rothschildiana* (Nakamura et al. 2005), between rare and endangered orchids, *Renanthera imschootiana* × *Vanda coerulea* (Kishor and Sharma 2009) and between *Cyamopsis tetragonoloba* × *Cyamopsis serrata* and C. *tetragonoloba* × *C. senegalensis* (Ahlawat et al. 2013).

10.5.3 Production of Haploid Plants

The potential of haploid technology for plant breeding arose in 1964 with the first report by Guha and Maheshwari (1964) on haploid embryo production from in vitro culture of *Datura* anthers. This was followed by the successful effort of Nitsch and Nitsch (1969) on the regeneration of haploid plants from microspore culture of tobacco. Since then, many attempts have been made on over 250 plant species belonging to almost all families of the plant kingdom (Maluszynski et al. 2003).

As with anther or microspore culture, in vitro pollination and fertilization may also be used to produce haploid plants through parthenogenesis. This has been achieved in *Mimulus luteus* when the ovule was pollinated with pollen from *Torenia fournieri* (both from family Scrophulariaceae) (Taji et al. 2002). More recent achievement on the production of double haploid plants through ovule cultures resulted from the hybridization reported in cultivated species of *Cucurbita pepo* with three other Cucurbita species i.e. *C. moschata*, *C. ficifolia* and *C. martinezii* (Rakha et al. 2012). Though ovules offer possible alternative source for haploid or doubled-haploid production, the exploration of this tissue in producing haploid or doubled-haploids in breeding programs is still very limited. For more in-depth discussion on haploid plant production the reader is referred to Chap. 5 in this volume. Further reading on haploid higher plant production via in vitro technique can be found in Jain et al. (1996a, b, c, d, e).

10.5.4 Overcoming Self- and Cross-Incompatibility

Self-incompatibility is a physiological barrier preventing fusion of male and female gametes from the same individual (self-fertilization), thus preventing inbreeding and encouraging outcrossing. On the other hand, cross-incompatibility occurs when male and female gametes from different individual fail to fuse, and as such, prevent outcrossing and encourage inbreeding to take place. In vitro pollination and fertilization can be used to overcome these physiological barriers.

In this method, the entire ovule mass from an ovary, intact on the placenta, is cultured in vitro just after pollination and fertilization is completed. For example, *Cucumis sativus* which is sexually incompatible with almost all *Cucumis* species due to different chromosome number, n = 7 in *C. sativus* versus n = 12 in *C. melo* and most wild *Cucumis* species. This obstacle can be overcome through the use of embryo rescue and/or ovule culture (Skálová et al. 2008).

10.5.5 Pollen Physiology and Fertilization

The technique of in vitro pollination and fertilization is an effective tool in studying pollen physiology and fertilization. This has been reported by Chapman and Goring (2010) who found that successful fertilization in the Brassicaceae was regulated by the cellular systems in the pistil that guide the post-pollination events, from pollen capture on the stigmatic papillae to pollen tube guidance to the ovule, with the final release of the sperm cells to effect fertilization. Study on in vitro pollination and fertilization would also reveal information about the requirement of different constituents on the germination of pollen grains as demonstrated in *Solanum macranthum* (Mondal and Ghanta 2012), *Withania somnifera* (Ghanta and Mondal 2013) and *Cajanus cinereus*, *Rhyncosia rothii* and *R. aureus* (Jayaprakash and Sabesan 2013). Specific temperature requirement for in vitro pollen germination and pollen tube growth of cotton has also been reported by Kakani et al. (2005).

10.6 Protoplast Culture Technology

Protoplasts are described as naked plant cells obtained through the removal of the cellulosic cell wall. Removing the cell walls could be performed through enzymatic procedures as pioneered by Cocking (1960) using root tips of tomato (*Lycopersicon esculentum* Mill. var. Sutton's Best of All) seedlings and a fungal cellulase obtained from *Myrothecium verrucaria*. Since then, the technology has been applied in breeding programs aiming at producing progenies with useful agronomical traits.

The potential use of protoplast technology for the genetic improvement of many agricultural crops is immense. This technology has allowed not only intraspecific

hybridization to take place, but also the creation of interspecific and intergeneric hybrids as well as cybridization. Various desirable traits from donor plants have been successfully transferred to hybrids and cybrids using this technology (see Tables 10.2 and 10.3).

Table 10.2 Recent examples of the transfer of useful traits by protoplast fusion

Species	Useful traits transferred	References
Aspergillus niger (+) *A. ficuum*	Enhanced phytase production	Gunashree and Venkateswaran (2010)
Brassica napus (+) *B. rapa*	Increased biomass and yield	Qian et al. (2003)
Brassica napus (+) *Crambe abyssinica*	Increased erucic acid content in seeds	Wang et al. (2003)
Brassica napus (+) *Sinapsis arvensis*	Enhanced resistance to blackleg (*Leptosphaeria maculans*)	Hu et al. (2002a)
Brassica napus (+) *Orychophragmus violaceus*	Improved fatty acid composition in seeds	Hu et al. (2002b)
Curvularia lunata (+) *Helminthosporium gramineum*	Improved biocontrol efficiency against rice weeds	Zhang et al. (2007)
Citrus sinensis (+) *Fortunella crassifolia*	Increased plant vigor	Cheng et al. (2003)
Citrus sinensis (+) *Clausena lansium*	Production of triploid plants	Fu et al. (2003)
Raphanus sativus (+) *Brassica campestris*	Resistance to atrazine	Pelletier et al. (1983)
Saccharomyces cerevisiae (+) *Kluyveromyces marxianus*	Enhancing production of temperature-tolerance ethanol	Krishnamoorthy et al. (2010)
Solanum melongena (+) *S. aethiopicum*	Resistance to bacterial wilt (*Ralstonia solanacearum*)	Collonnier et al. (2001)
Solanum melongena (+) *S. sisymbrifolium*	Resistance to bacterial and fungal wilts	Collonnier et al. (2003)
Solanum nigrum (+) *S.tuberosum*	Resistance to atrazine	Binding et al. (1982)
Solanum nigrum (+) *S.lycopersicum*	Resistance to atrazine	Jain et al. (1988)
Solanum tuberosum (+) *S. tuberosum*	Resistance to potato virus Y	Gavrilenko et al. (2003)
Solanum tuberosum (+) *S. nigrum*	Resistance to late blight (*Phytophthora infestens*)	Zimnoch-Guzowska et al. (2003)
Solanum tuberosum (+) *S. stenotomum*	Resistance to bacterial wilt (*Ralstonia solanacearum*)	Fock et al. (2001)
Solanum tuberosum (+) *S. chacoense, S. tuberosum* (+) *S. cardiophyllum, S. tuberosum* (+) *S. pinnatisectum*	Resistance to late blight and Colorado potato beetle	Chen et al. (2008)
Trichoderma reesei (+) *T. harzianum*	Enhancing enzyme production and bio-control activity against soil-borne pathogens	Srinivasan et al. (2009)

Table 10.3 Recent examples of the applications of plant protoplasts technology

Species	Application	References
Arabidopsis thaliana	Mechanisms of gene recognition in plant pathogenicity	Leister and Katagiri (2000)
A. thaliana, Zea mays	Elucidation of plant signal transduction mechanisms	Sheen (2001)
Brassica carinata, B. rapa	Cell wall regeneration, cell division and production of microcolonies	Beránek et al. (2007)
B. carinata, B. napus	Studies on regenerative capacity and reliable protoplast culture protocol	Klíma et al. (2009)
B. chinensis	Electrophysiological studies of outward K+ channels	Fan et al. (2003)
B. napus	Resistance to *Leptosphaeria maculans*	Hu et al. (2002a)
B. oleracea ssp. *capitata*	Cold-tolerant cytoplasmic male-sterile	Sigareva and Earle (1997)
B. oleracea ssp. *italica*	Herbicide (glufosinate) resistance	Waterer et al. (2000)
B. rapa	Resistance to bacterial soft rot	Ren et al. (2000)
Cucurbita pepo	Viral pathogenicity	Choi et al. (2003)
Centella asiatica	Basis for the development of protoclones with high asiaticoside productivity	Aziz et al. (2006)
Citrus spp.	Production of tetraploids and triploids for scion and rootstock	Grosser and Gmitter Jr. (2010)
Helianthus annuus	Synthetic peptide import through the plasma membrane	Cormeau et al. (2002)
Hibiscus cannabinus	Viral replication processes	Liang et al. (2002)
Muscari neglectum	Somatic embryo development and plant regeneration	Karamian and Ranjbar (2010)
Nicotiana benthamiana	Viral recombination and replication	Shapka and Nagy (2004)
N. plumbaginifolia	Genetic basis of developmental regulation and specificity	Chesnokov et al. (2002)
N. tabacum	Regulation of osmotic water transport across cell membranes	Ding et al. (2004)
Phaseolus vulgaris	Electrophysiological studies of inward-rectifying K+ channels	Etherton et al. (2004)
Raphanus sativus	Immunocytochemical evaluation of aquaporin accumulation	Suga et al. (2003)
Vicia faba	Fluorometric analysis of photosynthetic electron transport	Goh et al. (2002)
Vigna radiata	Intracellular responses to drought and salinity stress	Kim et al. (2004)
V. unguiculata	Studies on plasma membrane organization	Vermeer et al. (2004)
Zea mays	Studies on transient gene expression and proteomics	Cheng et al. (2001)

10.6.1 Protoplast Isolation

Before dealing with protoplast culture, it is important to understand the method of protoplast isolation. The material should be handled carefully and gently in order to obtain uninjured and viable protoplasts as they are prone to breakage or injury during isolation. Protoplast isolation is achieved via mechanical and enzymatic procedures.

Mechanical isolation of protoplast was first demonstrated by von Klercker (1892) who successfully isolated protoplast from plasmolyzed cells of *Stratiates aloides.* Following from this, the method was then used to isolate protoplasts from onion bulb tissues. Onion scales were immersed in 1.0 M sucrose solution to induce plasmolysis and therefore protoplasts shrunk away from their enclosing cell walls. The plasmolyzed tissue was carefully cut at such a thickness, so that only cell walls are cut without disturbing the protoplasts. The shrunken protoplast were released by osmotic swelling when the tissue is placed in sucrose solution at a low concentration.

The enzymatic isolation of protoplast involves the use of enzymes to dissolve the plant cell wall. The wall consists of primary components such as cellulose, hemicellulose and pectic substances. Cellulose is a simple, linear polymer of D-glucose with β-1,4 linkage, and having molecular weight of 50,000–2,500,000 Dalton (depending on species). Hemicelluloses are less well defined and appear to be mixed polymers of glucose, galactose, mannose, arabinose and xylose, and most of them are linked with both β-1,4 and β-1,3 linkages. Meanwhile, pectin substances mostly found in middle lamella, which holds the cells together. Pectin is a polymer of methyl-D-galacturonate with α-1,4 linkages and having molecular weights of 25,000–360,000 Da. Therefore, cell wall degrading enzymes such as cellulases, hemicellulases and pectinases are used to treat source tissues in enzymatic protoplast isolation method.

The enzymatic method could be used as a two-step procedure (sequential method) or single-step procedure (direct method). In the two-step procedure, single cells are isolated from source tissues using pectinase, then cellulase is added to this cell suspension to digest the cell wall and release the protoplasts. However, with further refinement, a single-step procedure was developed for cell separation and cell wall degradation. In this method protoplasts are isolated directly by using two enzymes, cellulase and pectinase, simultaneously. Protoplast yield through direct method is high and, now this is the most frequently used method (Pati et al. 2008).

10.6.2 Protoplast Purification

Following isolation, protoplasts need to be purified by removing undigested materials (debris), burst protoplast and enzymes. This step is required to purify the population to obtain intact and viable protoplasts and to ensure the successful culture.

Debris can be removed by filtering the suspension through a steel or nylon mesh of 100 μ pore size, whereas enzymes are removed by centrifuging the solution at 600 rpm for 5 min. The protoplasts settle to the bottom of centrifuge tube while supernatant is removed using a pipette. The protoplast are then resuspended in a washing medium containing osmoticum with or without nutrient medium. The suspension is recentrifuged to allow the protoplast to settle down and the washing medium is decanted. Removing traces of enzyme by washing the protoplasts is carried out two or three times. Intact protoplasts are separated from broken ones by suspending the preparation in 20–40 % sucrose solution and centrifugation at 350 rpm for 3 min. Intact protoplasts are carefully removed from the surface of the solution using a pipette (Tomar and Dantu 2010).

The viability of the protoplasts is examined using a fluorescent microscope. For this reason, fluorescein diacetate (FDA) solution in acetone (5 mg L^{-1}) is added to the suspension to give final concentration of 0.01 %. After 5 min at room temperature, the protoplasts are examined. Only viable protoplasts can fluoresce under the fluorescent microscope (Tomar and Dantu 2010).

10.6.3 Protoplast Fusion

Protoplast fusion is known as a physical event, during which two or more protoplasts come in contact and adhere to one another, either spontaneously or with the aid of electrofusion procedure (De Filippis et al. 2000; Rakosy-Tican et al. 2001) or in the presence of fusiogenic agents such as polyethylene glycol (Klíma et al. 2009; Zhou et al. 2001) or by using positively charged ions (such as Ca^{++}) in a high pH solution (Tomar and Dantu 2010).

Protoplast fusion technology offers the possibility to transfer useful traits from one species to another, such as disease resistance, nitrogen fixation, rapid growth rate, more productivity, better protein quality, frost hardiness, drought resistance, herbicide resistance and heat and cold resistance. Specific genera have been targeted in somatic hybridization through protoplast fusion to transfer useful agronomic traits, as indicated in Table 10.2. Thus, this technology has the potential to improve commercially-important plant species. However, Taji et al. (2002) suggested that protoplast fusion should not be intended to replace conventional plant breeding methods, but rather to complement them. The main obstacles are the difficulty in regenerating fusion products and low fertility of recovered plants.

The fusion of cytoplasms from two protoplasts may or may not be followed by the fusion of their nuclei. When the fusion of nuclei does not take place, the cells become binucleate known as heterokaryon. However, when the nuclei fuse together the cells are known as hybrid or synkaryon or synkaryocyte. On the other hand, when cytoplasm fuse but genetic information from one of the two nuclei is lost, the cell becomes cybrid (cytoplasmic hybrid). Figure 10.2 represents schematic diagram of protoplast fusion resulting in hybrid and cybrid cells.

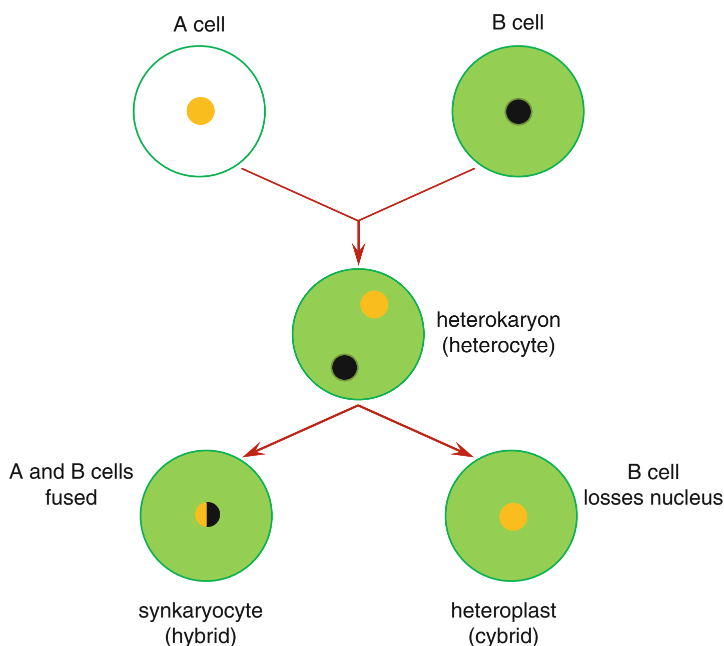

Fig. 10.2 Schematic diagram of hybrid and cybrid production through protoplast fusion

There are exceptions to the rule of chromosome theory of inheritance. One is that inherited characters may not be determined by genes located in chromosomes within the nucleus. A few characters are passed on from parents to offspring by genes which are not part of a nuclear chromosomes, but located in cell organelles in the cytoplasm (cytoplasmic genes) (Miko 2008). Tao et al. (2004) indicated that cytoplasmic and cytoplasmic-nuclear genomes interaction played important roles in yield, low temperature tolerance, and some important agronomic traits in *japonica* rice. Further Tao et al. (2011) detected that cytoplasmic genes had a significant effect on grain weight and filled-grain ratio on *indica* rice. In maize, Tang et al. (2013) reported that plant height and ear height are also controlled by cytoplasmic genes. These suggest that the selection of appropriate cytoplasmic germplasm is broadly important in plant breeding. Therefore, regeneration of cybrids with only one nuclear genome but having the mixture of cytoplasm from both parents will be very helpful in transfer of cytoplasmic genetic information from one plant to another. Thus, the production of cybrids can be applicable in plant breeding program.

Protoplast fusion may be used in somatic hybridization to create hybrid plants that may not be achieved through in vivo crossing techniques due to taxonomic or sexual barriers. However, it is important to note that even the creation of somatic hybrids from completely unrelated and incompatible species through protoplasts fusion is possible, the end products are often unbalanced (sterile, malformed and unstable), and therefore unviable (Chawla 2002).

10.6.4 Application of Protoplast Technology

Protoplasts provide a basic experimental system for ultrastructural, genetic and physiological studies. In addition, isolated protoplasts are also exploited in numerous studies involving synthesis of pharmaceutical products, and toxicological assessments. Several recent examples of the application of protoplasts technology are summarized in Table 10.3.

Cybrids produced as a result of protoplast fusion between *Nicotiana plumbaginifolia* TBR2 mutant and *N. tabacum* were resistant to high levels of herbicide atrazine (Menczel et al. 1986). They also found that these plants were male sterile due to the protruding stigma and shorter than normal filaments of the cybrid plants. Melchers et al. (1992) showed that when mitochondrial-inactivated tomato protoplasts were fused with nuclear-inactivated *Solanum* spp. protoplasts, cytoplasmic male sterility occurred in the resultant plants. Furthermore partial genome transfer can be obtained through asymmetric somatic hybridization when utilizing protoplast culture technology. Yamashita et al. (1989) produced asymmetric somatic hybrids of *Brassica* by fusing the inactivated *B. oleracea* protoplasts with X-irradiated *B. campestris* protoplasts.

10.7 Conclusion and Prospects

Advances in agricultural biotechnology are the cornerstone for feeding the growing world population, estimated to approach 10 billion by 2050. The impact of agriculture biotechnology has been immense since the first products of this technology were released, including crops that exhibited resistance to certain insects, tolerance to selected herbicides, and increased resistance to viral diseases, improving the yield of many crops dramatically and positively affecting the environment, the consumer and the farming companies. The next wave of agriculture biotechnology is further focused on producing crops that withstand biotic and abiotic stresses, as well as value adding to crops through increased levels of nutrients, vitamins and minerals. However, safety of products produced through genetic engineering has been a point of immense debate and hence such products, as yet, have not been widely embraced. The technologies described in this chapter have been utilized to complement classical plant breeding achieving the desired traits without the controversial method of genetic engineering. These technologies will further enhance crop productivity and produce novel plant-based biomaterials including potential energy resources in the face of limitations in natural resources and the inevitable impact of climate change on agriculture productivity.

In vitro techniques are now applied extensively in breeding programs because of their convenience and because of the potential of decreasing the breeding cycle. However, the lack of well-established in vitro techniques often prevents its practical use. A complete operation of in vitro screening and breeding involves variant or

mutant induction, selection, plant regeneration, acclimatization and assessment of in vivo plants. Extensive research into the establishment of each in vitro technique protocol for the success of plant screening and breeding programs is required. Recently, climate change has been affecting the growth and yield of plants all over the world. Growers may be forced to change to a new crop that is suitable for new environmental conditions such as extreme temperatures, drought, or chemical accumulated/contaminated soil. The endless possibilities for developing improvements in plants through technologies described in this chapter have the potential to help solve world hunger and problems faced in agriculture as a result of climate change.

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