Isolated Microspore Culture and Its Applications in Plant Breeding and Genetics

21

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Abstract

Isolated microspore culture (IMC) represents a unique system of single cell reprogramming in plants wherein a haploid male gametophyte, the microspore, switches its default gametophytic developmental pathway toward embryogenesis by specific stress treatment. The application of a stress treatment(s) is necessary for efficient embryogenesis induction. Depending on species, microspores are often induced by cold and heat shock, osmotic stress, starvation, anti-microtubular agents, stress hormones, antibiotics, or polyamines. This technique (IMC) is likely to remain as a well-known method in plant breeding since it allows for the rapid production of completely homozygous lines while, in the context of developmental biology, it allows for in vitro embryogenesis to be explored in greater detail. Isolated microspores also represent ideal recipients for several gene transfer techniques including electroporation, microprojectile bombardment, and *Agrobacterium*-mediated transformation. IMC is also extensively used for genetic studies, i.e., studying inheritance of quantitative traits, quantitative trait loci (QTL) mapping, and genomics and gene identification, for mutation and selection and also used for producing reversible male-sterile lines. Male sterility avoids the labor costs of manual emasculation and serves as a molecular strategy for transgene containment by preventing pollen release to the environment. Combination of this technique with doubled haploid (DH) production leads to an innovative environmentally friendly breeding technology. In addition, the usefulness of DHs for reverse breeding program, an applied plant breeding technique introduced to directly produce parental lines for any hybrid plant, is also generally discussed.

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M. Anis, N. Ahmad (eds.), *Plant Tissue Culture: Propagation, Conservation and Crop Improvement*, DOI 10.1007/978-981-10-1917-3_21

Keywords
Doubled haploid • Microspore embryogenesis • Stress • Breeding • Genetics

21.1 Introduction

Haploid is a general term describing sporophytes with single set of parental chromosomes. Haploid and DH plant production technique offers an excellent system to speed up plant breeding programs, genetic analysis, physiological studies, gene transformation, QTL mapping, and reverse breeding programs (Liu et al. 2005; Wijnker et al. 2012; Brew-Appiah et al. 2013; Ilyas et al. 2014).

Haploids occur rarely in nature and therefore of limited practical value. To be useful, they must be produced in large numbers. Haploids/DHs are mainly produced via: (1) parthenogenesis, (2) wide hybridization followed by chromosome elimination, and (3) androgenesis/gynogenesis (Asif 2013; Mishra and Goswami 2014). Parthenogenesis is a type of asexual reproduction wherein haploid plants develop from unfertilized egg cells (Bohanec 2009). Elimination of one parental genome after fertilizing the ovule by pollen of another species can occur in intraspecific, interspecific, intergeneric, or more distant hybrids leading to haploid plant formation of only one parent (Dunwell 2010). The DH production method used in barley (*Hordeum vulgare* L.) is a typical example of selective chromosome elimination in the cross between *H. vulgare* and diploid *H. bulbosum*, so that the chromosomes of *H. bulbosum* will be finally eliminated (Jauhar 2003). Gynogenesis, haploid regeneration from un-pollinated female gametophytes, is another method for haploid production that has been used in many agronomically important species such as sugar beet and onion (Portemer et al. 2015). Nowadays androgenesis, regeneration of haploid or DH plants through the anther or isolated microspore culture, has attracted the interests of many researchers and become the method of choice for haploid production in a wide variety of species.

Microspores, immature pollen grains, are produced within the anthers (microsporangia or pollen sacs) of the flower. Microsporogenesis, the process of formation of haploid unicellular microspores, commences with dividing microsporocytes (pollen mother cells or meiocytes) by meiosis into the four haploid microspores. Under normal conditions (in vivo), microspores are programmed to develop into the mature pollen grains in a process called "microgametogenesis" (Ma 2005). However, by manipulating the environment of the gametic cells under specific in vitro conditions, it is possible to divert the default developmental pathway toward embryogenesis giving rise to haploid plants rather than mature pollen grains (Shariatpanahi et al. 2006).

The first successful regeneration of haploid plants through the in vitro cultured anthers was reported by Guha and Maheshwari (1964) in *Datura innoxia*. Soon later, androgenic haploid plants were achieved by Bourgin and Nitsch (1967) from cultured anthers of *Nicotiana sylvestris* and *N. tabacum*. During the past four decades, many improved methods have been developed to increase the efficiency of

haploid production from cultured anthers and isolated microspores in various species (Ferrie and Caswell 2011).

DH production through the IMC technique drastically reduces the time period needed to develop new cultivars (Germana 2006). It generally takes 10–15 years to release a new cultivar through the conventional breeding programs, i.e., the pedigree or bulk methods in self-pollinating crops, which includes selfing and subsequent selection. On the other hand, regeneration of haploid plants followed by chromosome doubling and selection of superior lines reduces the time frame by 3–4 years. In cross-pollinating heterozygous crops, which often express a high degree of self-incompatibility and inbreeding depression, IMC is a rapid method to produce homozygous lines that can further be used in the development of synthetic varieties or hybrids (Immonen and Anttila 1996). Because microspore-derived DH plants are genetically fixed and stable (true breeding lines), they can be replicated in trials allowing better estimates of within and between line variation and also improve the selection efficiency (Collard et al. 2005). In addition, the phenotype of DH plants is not masked by dominance effects, and traits encoded by recessive genes can be easily identified (Szarejko and Forster 2007; Ferrie and Möllers 2010). IMC also provides tremendous potential for studying quantitative inheritance. By using F2-derived DH population, genetic parameters and number of segregating genes can be estimated. Also, a smaller population of DH plants is needed for screening desirable recombinants than would be the case for conventional diploid populations (Martinez et al. 2005; Hussain et al. 2012). DH populations are available for DNA extraction and mapping 1.5 years after the initial crosses, i.e., almost as quick as an F2 or BC1 population and definitely much faster than a pedigree inbred or single seed descent population. DHs can be re-grown and distributed in seed form so that it is comfortable to screen with many markers. Map construction from a DH population derived from the F1 of a cross is relatively simple because the expected segregation is that of a backcross (Forster and Thomas 2003). DH populations are of favor in the QTL identification as multisite replicated trials can be grown 3 years after the first cross (Forster and Thomas 2003; Collard et al. 2005).

In this chapter, common stresses used for microspore embryogenesis induction are generally discussed. Then, the latest progresses in the induction and regulation of microspore embryogenesis and also its applications in plant breeding programs and genetic studies have been argued in a greater detail.

21.2 Induction of Microspore Embryogenesis

Application of a stress(s) is necessary to induce microspore embryogenesis. The stresses required to switch the default developmental pathway of microspores toward embryogenesis vary with the species and even within a species and among its genotypes or varieties also. Nevertheless, several inducing factors have been identified that positively influence the efficiency of microspore embryogenesis in diverse species (reviewed by Shariatpanahi et al. 2006). Heat shock is usually conducted at 30–37 °C for a varying duration from few hours up to several days,

whereas cold treatment is carried out at 4–10 °C from few days up to several weeks. It has been indicated that heat treatment significantly affects the expression of Rho-GTPases (ROP) in isolated microspores of *B. napus* which is involved in the cytoskeletal rearrangement (Chan and Pauls 2007). Heat shock influences microtubule distribution, leading to more symmetrical division in microspores, and blocks further gametophytic development (Dubas et al. 2011). Heat shock not only induces microspore embryogenesis but also accelerates the process of embryo formation (Ahmadi et al. 2012b). Heat/cold treatment also affects auxin distribution and therefore the fate of embryogenesis either via or without suspensor formation (Prem et al. 2012a; Dubas et al. 2014). Auxin mainly localizes in a polar way already in the uninucleate microspores subjected to the mild (1 day at 32 °C) or without (18 °C) heat treatment, which is essential for initiation of suspensor-bearing MDEs. While microspores are treated to a prolonged heat (32 °C for 5 days), auxin polarization arranges at a suspensor-free few-celled embryos (Dubas et al. 2011, 2014). Culture of isolated microspores in the induction medium containing non-metabolizable carbon sources, e.g., in mannitol or polyethylene glycol, has also proved to be useful in many species (Muñoz-Amatriaín et al. 2006), hot pepper (Kim et al. 2008), eggplant (Bal et al. 2009), wheat (Ayed et al. 2010), and oil palm (Indrianto et al. 2014). Transcriptome analysis of barley microspores revealed that 4-day mannitol treatment substantially affected the expression of 2673 genes. Upregulation of transcription factors related to stress responses and changes in developmental pathway of treated microspores toward embryogenesis took place during the pretreatment (Muñoz-Amatriaín et al. 2006). In addition, colchicine, a microtubule-depolymerizing agent, has been used as a stress pretreatment for embryogenesis induction in various species (Soriano et al. 2007; Klima et al. 2008; Dubas et al. 2010; Islam 2010; Li et al. 2012). Application of anti-microtubular agents, e.g., colchicine, cytochalasin D, trifluralin, or oryzalin, leads to cytoskeletal rearrangement which plays a key role in determining the developmental fate, since the disruption of these networks triggers or is sufficient to induce gametic embryogenesis in the absence of a stress treatment (Soriano et al. 2013). Recently, several novel inducers have been reported to switch gametophytic developmental pathway of microspores toward embryogenesis which are discussed below.

21.2.1 Antibiotics

Microbial contamination is a serious and often unavoidable problem in the field of microspore culture. Contaminations introduced to in vitro cultures can be exogenous or endogenous originating from explants' surface, intracellular spaces within the plant tissues, or poor aseptic conditions during manipulation in laminar flow hoods. Endogenous bacterial contamination has long been considered as one of the major bottlenecks in androgenesis induction in vegetables such as *Capsicum annuum* L. which drastically losses the number of isolated cultures (Lantos et al. 2012; Asif et al. 2013b). Such contaminations may be substantially reduced or eliminated during the in vitro stages of plant tissue culture using antimicrobial treatments such

as antibiotics. In the *Agrobacterium*-mediated gene transformation, it is also essential to use antibiotic for eliminating the remained *A. tumefaciens* cells after cocultivation. Despite their common and successful application to minimize bacterial growth in the cell and tissue cultures, antibiotics are shown to retard/inhibit or stimulate explant growth and development. Their role(s) in affecting the developmental events is not well understood, but it has been assumed that the antibiotics mimic plant hormones since some of them possess an auxin-like activity (Qin et al. 2011). The number of MDE formation from wheat isolated microspores decreased with increasing level of Timentin in the induction medium (Brew-Appiah et al. 2013). 100–400 mg l^{-1} Timentin was recognized as the optimal concentration so that a reasonable number of MDEs were produced while their regeneration efficiency was retained. Optimal antibiotic agent, on the one hand, should provide reliable defense for bacterial infections and, on the other hand, keep microspores intact and viable. Reported by Asif et al. (2013b), 24 bacterial isolates were strongly inhibited using vancomycin and cefotaxime (both 100 mg l^{-1}) in microspore culture of triticale and wheat. In addition, microspore embryogenesis induction medium fortified with the same antibiotic treatments resulted in increased embryo-like structure (ELS) formation, green and albino plant production in triticale. In wheat, significant genotype-dependant effect of cefotaxime was noticed for ELS, green and albino plant regeneration. Vancomycin, on the other hand, was not advantageous to MDE formation so that it significantly reduced ELS number and green plant regeneration in all wheat genotypes tested (Asif et al. 2013b). Exogenously applied cefotaxime (50 mg l^{-1}) and vancomycin (100 mg l^{-1}) for 24 h and 48 h, respectively, profoundly enhanced microspore embryogenesis induction in *B. napus*. Higher levels and durations were detrimental so that normal plant regeneration substantially decreased and resulted in callusing (Ahmadi et al. 2014).

21.2.2 Antioxidants

Isolation and stress treatment of microspores cause oxidative damage to the cells in the form of lipid peroxidation, protein oxidation, nucleic acid damage, and appearance of apoptotic-like bodies, all eventually lead to cell death and thus decrease in viability of cultured microspores (Żur et al. 2009; Rodríguez-Serrano et al. 2011; Sharma et al. 2012). Despite having a quick harmful effect on microspore viability, the increase in reactive oxygen species (ROS) production has been reported to be favorable to microspore embryogenesis induction if firmly adjusted (Hoseini et al. 2014; Żur et al. 2014) so that treatment of barley (*H. vulgare* L.) microspores with ROS scavengers resulted in decreased cell death during early stages, but later reduced the total number of MDEs produced (Rodríguez-Serrano et al. 2011). In plant cells, the precise equilibrium between ROS generation and decomposition depends on the activity of enzymatic and nonenzymatic components of the antioxidative system. Major ROS-scavenging enzymes include superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT), whereas ascorbic acid, glutathione, proline, α-tocopherol, carotenoids, and flavonoids belong to the most important

nonenzymatic antioxidants (Gill and Tuteja 2010). Reported by Żur et al. (2009), induction medium, as the major inducer of microspore embryogenesis in triticale, had a temperature-dependant manner effect on the activity of antioxidative enzymes, i.e., SOD, POX, and CAT, so that the activity of all enzymes under low-temperature (5 °C) regime was similar as in the control subjected only with the stress connected with the establishment of in vitro culture. By contrast, the type of induction medium exhibited drastic effect on the activity of all abovementioned enzymes when cultures were incubated at higher-temperature (32 °C) regime (Żur et al. 2009). Low-temperature pretreatment (3 weeks at 4 °C) of freshly excised triticale tillers significantly increased the activity of enzymatic antioxidants in the anthers of responsive DH lines when compared with the recalcitrant ones (Żur et al. 2014). Principal component analysis (PCA) revealed that the activity of POX, CAT, and SOD was positively correlated with the efficiency of embryogenesis (Żur et al. 2014). Antioxidative enzymes play important roles in microspore embryogenesis induction which cannot be replaced even by highly active nonenzymatic antioxidants (Żur et al. 2014). The ability to sustain antioxidative enzyme activity under inductive stresses is also an important factor needed for highly effective microspore embryogenesis induction, allowing for the generation of signals initiating microspore reprogramming and simultaneously protecting the cells from further toxic effects of ROS accumulation (Żur et al. 2014). Plastids and mitochondria are the major target/production sites of ROS during abiotic stresses. Highly increased levels of ROS in plastids not only decrease the viability of microspores but also are correlated with albinism, as albino plants are deprived of chlorophyll (Asif et al. 2013a). Exogenously applied mitochondrial or plastid antioxidants, e.g., glutathione in the induction medium, significantly increased embryogenesis and green plant regeneration in contrast to the untreated cultures in wheat and triticale (Asif et al. 2013a). Glutathione and ascorbate are nonenzymatic antioxidants that detoxify and scavenge free radicals and regulate the redox cellular state. Synergistic effect of ascorbate and glutathione has been observed in microspore embryogenesis of *B. napus* in a dose-dependent manner. Microspore embryogenesis was enhanced using 10 mg l^{-1} glutathione in combination with 5 or 10 mg l^{-1} ascorbic acid. However, abnormal embryos were observed when glutathione level was increased by 100 mg l^{-1} (Hoseini et al. 2014). Ascorbate and glutathione are also involved in the plantlet regeneration from MDEs. Buthionine sulfoximine (BSO) treatment which affects glutathione and ascorbate metabolism produced lower ascorbate levels in the regenerated MDEs and activated expression of meristem-specific genes including *ZWILLE*, *SHOOTMERISTEMLESS*, and *ARGONAUTE 1* (Stasolla et al. 2008). Expression of these genes is required for shoot meristem formation and correct embryo patterning via activating phytohormones, e.g., cytokine biosynthesis (Scofield et al. 2013). The enhancing effect of α-tocopherol as intercellular and intracellular physiological antioxidant which maintains homeostasis of labile metabolites in the cells was also reported in isolated microspore culture of *B. napus* (Hoseini et al. 2014). However, the effect of α-tocopherol on microspore embryogenesis follows a temperature-dependant manner so that it was more effective on microspore embryogenesis at 30 °C in comparison with those cultures treated at 32.5 °C.

21.2.3 Phytohormones

Phytohormones also known as "plant regulators" including auxins, cytokinins, gibberellic acid, brassinosteroids, ethylene, abscisic acid, jasmonic acid, and salicylic acid are chemical messengers that coordinate cellular activities, i.e., cell division and differentiation. Last decade has witnessed astonishing progresses in the induction and regulation of MDE formation using these phytohormones. Current achievements in this area are discussed below.

21.2.3.1 Auxins

Auxins have cardinal roles in coordination of many growth and behavioral processes and are essential for plant body development. Auxins also have an outstanding role(s) during the somatic/gametic and zygotic embryogenesis. HPLC analysis of endogenous auxin level revealed that auxin content was increased about 100-fold in microspore-derived proembryos when compared with the auxin content detected at the single cell level (Dubas et al. 2014; Rodríguez-Sanz et al. 2014). Such high auxin levels could characterize dividing cells being stimulated to grow and divide. Differential distribution of the plant hormone auxin is also important and prerequisite for normal MDE formation. Using the reporter β-glucuronidase (*GUS*) and the green fluorescent protein (GFP) markers under control of synthetic auxin-responsive DR5 or DR5rev promoters, Dubas et al. (2014) observed polar auxin distribution after exine rupture, which lasted at the late globular stage under the prolonged heat stress condition. This polarity is not only the effect of heat stress but also reflects genome-regulated pattern of *B. napus* MDE development. The apical-basal embryogenic axe formation can be probably marked by such polar pattern of reporter gene activity (Dubas et al. 2014). Treatments with the inhibitor of the polar auxin transport 1-N-naphthylphthalamic acid (NPA) or high doses of antiauxin *p*-chlorophenoxyisobutyric acid (PCIB, chemical compound that competes with auxin for binding sites) affected embryogenesis initiation and further MDE development in contrast to untreated cultures. Neither new MDEs nor development of embryo-like structures were detected in the treated cultures, whereas in the embryogenic cultures without NPA and PCIB, embryogenesis was normally initiated and progressed (Ahmadi et al. 2012a; Rodríguez-Sanz et al. 2014).

MDEs are mainly formed via two major routes: a zygotic-like pathway characterized by initial suspensor formation followed by embryo proper formation and the second route characterized by initially unorganized embryos lacking a suspensor (Dubas et al. 2014; Soriano et al. 2013). The pattern of embryogenic development is highly based on the intensity and distribution of the plant hormone auxins during the embryogenesis process (Dubas et al. 2014). Polar auxin transport is needed for embryo proper specification from the suspensor in the zygotic-like pathway. Tracking this pathway revealed higher auxin concentration in one pole of initially inducted uninucleate microspores. After asymmetric transverse division, higher auxin activity was noted in the basal cell, recognized as the place of auxin biosynthesis from which the hormone is transported to the apical cell. After a series of basal cell transverse divisions and suspensor formation, directional auxin flow takes

place from the suspensor cells to apical cells. As the proembryo progresses, auxin mainly accumulates at the places where the cotyledon primordium and provascular strands began to form. On the contrary, in the suspensor cells except for the region of the hypophysis, auxin activity decreases. In the suspensor-lacking pathway, on the other hand, no auxin polarity was observed in uninucleate microspores and after symmetrical divisions. When a multicellular structure was released from the exine, auxin was observed at the only one pole. This polar auxin distribution lasted up to the late dermatogen stage. Such polar pattern of auxin distribution probably marks the apical-basal embryogenic axe formation (Dubas et al. 2014).

21.2.3.2 Stress Hormones

Microspores under certain stress conditions in vitro can switch their developmental programmed pathway toward embryogenesis. Stress hormones are produced by plants in response to a number of environmental stresses, including some that are used to induce androgenesis. Applying low-temperature pretreatment to excised inflorescences of triticale led to intensive ABA accumulation in comparison with untreated tillers (Żur et al. 2012).

However, there has been no correlation between ABA concentration and androgenesis induction efficiency, whereas negative correlation was seen between regeneration ability and concentration of ABA collected from cold-treated tillers. Monitoring changes in plasma membrane fluidity and ABA content associated with embryogenesis induction in *B. napus* microspores revealed that plasma membrane remodeling followed by inductive stress (heat shock at 32 °C for 24 h) is coincided with changes in ABA content in both microspores and induction medium (Dubas et al. 2013). Heat shock causes microspores' plasma membrane more rigid and increases ABA level in the induced microspores. However, heat treatment also increased ABA content in microspores of nonresponsive cultivars which had no clear-cut impact on androgenesis induction efficiency, suggesting a more complex mechanism of initiation process. On the contrary, promotive effects of stress hormones, i.e., ABA, salicylic acid, and jasmonic acid, were observed when exogenously applied to the induction medium (Ahmadi et al. 2014). ABA at 0.5 mg l^{-1} for 12 h enhanced microspore embryogenesis by about threefold compared with untreated cultures. High embryogenesis frequency was also observed when cultures were exposed to 1.0 mg l^{-1} jasmonic acid for 12 h. However, microspore embryogenesis and plantlet regeneration significantly decreased as jasmonic acid level was increased. SA treatment (0.2 and 0.5 mg l^{-1}) during the 6 h exposure also caused a profound increase in the number of embryos generated relative to untreated cultures, but treatment at longer durations decreased MDE formation, so that 2.0 and 5.0 mg l^{-1} SA for 12 and 24 h completely inhibited embryogenesis induction. Nevertheless, initial sporophytic divisions were observed in the treated microspore at those toxic levels, but all failed to proceed further into the fully developed MDEs (Ahmadi et al. 2014).

21.3 Applications of Microspore Embryogenesis

21.3.1 Gene Transformation

Microspores contain gametic number (n) of chromosomes. Both the haploid chromosome number and embryogenic capability exhibit microspores, one of the most interesting cells to be stably transformed. Based on the developmental stage of microspores, transforming procedures can be classified into two major categories, gametophytic and sporophytic (Resch and Touraev 2011). The gametophytic route includes (i) transforming mature pollen wherein a recombinant DNA is incorporated into the pollen genome before pollination or introduced to stigma before/after pollination and (ii) transformations based on microspore maturation where the foreign DNA is delivered into the microspores, cultured and matured under in vitro condition, and then used for pollination to achieve transformants (also known as male germ line transformation). The sporophytic route is based on transforming embryogenic microspores, wherein the default gametophytic fate of isolated microspores is shifted toward sporophytic pathway to produce MDEs instead of mature pollen grains (Brew-Appiah et al. 2013). Once transgenes are incorporated into the haploid genome of targeted cells prior to S phase (DNA synthesis) and genome doubling, the DHs may become completely homozygous for the transgenes. Thus, IMC not only provides an ideal system for gene transformation but also is amenable for transgene to be readily selected under in vitro conditions. Isolated microspores also provide an excellent system to avoid hemizygousity. On the contrary, identification of homozygous transgene(s) derived from somatic (diploid) targets requires the assessment of two additional segregating generations bred from hemizygous transformants (Hansel et al. 2012). Nowadays, many researchers have recognized the great benefit(s) that a functional microspore transformation system would offer and tried to transform microspores in diverse species using eletroporation, microprojectile bombardment, and *Agrobacterium tumefaciens*-mediated methods demonstrating stable integration of DNA.

Electroporation has been reported to be an efficient tool for foreign DNA delivery into the plant cells. The first report of electroporation-mediated delivery of DNA to the microspores arose from detection of *chloramphenicol acetyltransferase* expression in maize microspores by Fennell and Hauptmann (1992). Soon after, the delivery of *β-glucuronidase* (*GUS*) to the microspores of *B. napus* (Jardinaut et al. 1993) and maize (Jardinaut et al. 1995) was reported. Obert et al. (2004) conducted an experiment to optimize the electroporation medium, concentration of donor DNA, voltage, and pulse duration in order to gain a reproducible protocol for maize microspores. Taken together, they found that the highest amount of *GUS*-expressing microspores was achieved when a combination of 200 mg ml^{-1} DNA, three pulses with frequency of 0.1 Hz, and field 400 V/cm during 20 ms was applied (Obert et al. 2004).

Ingram et al. (1999) transformed MDEs obtained from in vitro cultured anthers of wheat using microprojectile bombardment. They found that paper supports during the bombardment significantly improve the efficiency of transformation so that

bombardment of MDEs on semiliquid regeneration medium without a filter paper on the surface of the medium led to a 45 % reduction in the mean number of GUS foci/MDE at 1-day post-bombardment. The presence of semiliquid medium over the MDEs could act as a protective film, with its surface tension reducing the impact of gold particles (Ingram et al. 1999). Type and concentration of carbohydrate in the culture medium also affect the expression of transgene. Significantly longer *GUS* expression in the transformed MDEs was noted in the presence of high amounts of maltose (0.4 M) in the culture medium. High osmoticum medium is thought to protect tissues during bombardment by reducing cell turgor, causing plasmolysis. This leads to reduced leakage of cell contents following bombardment. High osmoticum medium may also induce membrane changes, leading to increased cell tolerance to microprojectile impact (Clapham et al. 1995; Ingram et al. 1999). Transgenic plants could be produced by the direct delivery of plasmid DNA into the isolated microspores using high-velocity microprojectiles (Yao et al. 1997; Nehlin et al. 2000). Higher rate of transformation was observed when 48 h incubated microspores were used as the targets. Size of gold particles is another determining factor which severely affects the efficiency of transformation. Bombardment of incubated microspores with particle sizes of 1.5–3.0 μm resulted in a four- to sixfold increase in *GUS* expression compared with particles of 1.0 μm (Nehlin et al. 2000). Drastic decline in viability of microspores was observed within the first week of culture which could adversely affect the efficiency of process. Renovation of culture medium 24 h, 48 h, or 72 h after initial culture showed beneficial effects on microspore viability (Nehlin et al. 2000). Working on isolated microspores of wheat, Mentewab et al. (1999) and Folling and Olesen (2001) observed transient expression of marker genes, but transgenic wheat plants were not obtained, probably due to the inefficient microspore regeneration protocol in the experiments. Less than 100 MDEs were produced from isolated microspores of several spikes in the controls. The efficiency is likely to be further reduced with the introduction of genes by microprojectile bombardment since microspores are very sensitive to physical damage. The success in microspore transformation fairly relies on the effectiveness of microspore regeneration protocol. Thus, a highly efficient microspore culture system is required as a basis for transformation experiments.

Agrobacterium-mediated transformation is in general a much more efficient way of introducing foreign genes into some species, e.g., *Brassicas*, than microprojectile bombardment or other techniques (Takahata et al. 2005). A comparison between *Agrobacterium*- and biolistic-based transformation methods in *H. vulgare* highlighted some of the advantages of the *Agrobacterium* system such as higher efficiency of transformation, lower copy number of transgene(s), and more stable inheritance with fewer rates of the transgene(s) silencing (Travella et al. 2005). Being potentially useful recipient of foreign genes, Cegielska-Taras et al. (2008) transformed *B. napus* MDEs using *A. tumefaciens* strains EHA105 and LBA4404, both carrying the binary vector pKGIB containing the *uidA* gene and the *bar* gene for resistance to phosphinothricin as a selectable marker. Transformed MDEs expressed *GUS* and regenerated plants exhibited resistance to herbicide Basta. Since the physical barrier imposed by the thick microspore wall constitutes an obstacle for

efficient transforming of isolated microspores, Abdollahi et al. (2009) implemented additional methods to overcome this drawback. They obtained the best result through the co-transformation by microspore bombardment with DNA-coated microprojectile particles, followed by *A. tumefaciens* infection, a process called "agrolistic." The "agrolistic" method combines the benefits of the *Agrobacterium* transformation system with that of the biolistic DNA delivery method (Ziemienowicz et al. 2012). Although profound progresses in gene transformation methods based on IMC technique have been made in the last decades, there is still demand for further investment and improvement in both model and crop plant species.

Nevertheless microspore transformation is widely used to avoid hemizygousity; surprisingly this system has confronted with the regeneration of hemizygous transgenics in some cases (Shim et al. 2009; Brew-Appiah et al. 2013). This dilemma appears when microspores targeted to transformation have progressed into the G2 stage or going into mitosis becoming binucleate (Shim et al. 2009). The type of microspore pretreatment has a great influence of rate of hemizygous regeneration. Mannitol with cold treatment for 4 days and mannitol treatment for 7 days at 28 °C followed by biolistic bombardment led to a few hemizygous transgenic plants in barley. Conversely, a cold pretreatment for 28 days, which slows but does not arrest the cell cycle, led to a few homozygous transgenics using biolistic bombardment. Reported by Shim et al. (2009), microspores in cold plus mannitol pretreatment for 4 days were held in the G1 and S cell cycle stages during pretreatment, while in the cold pretreatment for 28 days, they progressed slowly into the G2 stage or some going into the mitosis. It has been hypothesized that inducing a transgene(s) at the G1 or early prior to S phase, following pretreatment, should produce homozygous transformants, whereas introduction at the G2 phase should lead to regeneration of hemizygous transgenic plants (Shim et al. 2009).

21.3.2 Induced Mutation and Selection

Plant breeding programs are based on creating genetic variation, selection, evaluation, and multiplication of desired genotypes. Microspore culture provides an ideal method for creating variation via induced mutation (Seyis et al. 2014). Any DNA alteration imposed to haploid cells would be homozygous in the DH plants facilitating the identification of recessive mutants. On the other hand, recessive mutations with deleterious effects cannot be recovered using this method (Ferrie and Möllers 2010). The new genetic variability from cultured haploid cells will be increased by the application of physical, chemical mutagens, T-DNA insertion mutagenesis (Jung and An 2013), AC/DS transposon mutagenesis (Wang et al. 2013), and site-directed mutation using transcription activator-like effector nucleases (TALENs, Gurushidze et al. 2014). Induced mutation and selection has been extensively used to improve yield, quality, disease, and pest resistance in crops such as *Brassicas*, wheat, rice, barley, cotton, peanuts, and beans, which are seed propagated (Barro et al. 2002; Seyis et al. 2014). Many selected mutants have been released as cultivars; several others have been used as parents in the basic breeding programs, i.e.,

pedigree of some of the leading cultivars. The release of high-yielding and short-height barley mutants as varieties "Diamant" and "Golden Promise" have had a major impact on the brewing industry in Europe. These mutants have been used as the parents of many leading barely cultivars released in Europe (reviewed by Ahloowalia and Maluszynski 2001).

Availability of refined protocols for MDE production and manipulation of haploid tissues in *Brassicas* has attracted the attention of breeders to induce mutation and in vitro selection in this responsive species. Thanks to rapid generation of large MDE haploid populations, IMC has become a useful tool for mutation and selection. Polsoni et al. (1987) accomplished a successful rapid isolation of oilseed rape (*B. napus* L.) microspores by mechanical homogenization (maceration) of whole fluorescence with floral buds no longer than 4.5 mm, also designated as large-scale microspore culture. With this method, millions of microspores can be isolated in each trial, resulting in a final yield of several hundred MDEs within 1 month, so they outlined the efficiency of this technique for induced mutation and in vitro screening (Polsoni et al. 1987). Soon later, Swanson et al. (1988) selected mutant DH lines which were tolerant to chlorsulfuron herbicide about 10–1000 times more than the corresponding plants. Imidazolinone-tolerant canola DH plants were also obtained with five to ten times improved tolerance to the field-recommended rates of Pursuit and Scepter (Swanson et al. 1989). Ultraviolet (UV) light has been widely used as a physical mutagenic agent. DH plants of *B. napus* regenerated from microspores exposed to UV irradiation exhibited changes in the level of resistance to herbicides, pathogens, abiotic stresses, and saturated fatty acid profile. Irradiation of *B. napus*-isolated microspores led to regeneration of DH plants exhibiting increased resistance to the chlorsulfuron and *Alternaria brassicicola*, suggesting the generation of novel heritable resistance to this herbicide and pathogen (Ahmad et al. 1991). Also, *B. campestris* DH plants resistant to soft rot disease were produced by UV irradiation (Feng-Ian and Takahata 1999). EMS, as a chemical mutagen, has also been utilized to construct mutant library. From a population of nearly 400 DH plants derived following EMS treatment, nine lines were identified that exhibited promising useful changes in erucic acid content in the seed oil (Barro et al. 2001). Isolated microspores of *B. napus* were exposed to EMS mutagen and then screened on the culture medium supplemented with oxalic acid as a selection agent of *Sclerotinia sclerotiorum* (Liu et al. 2005). Of the 54 DH lines produced, two DH lines of resistant mutants exhibited greater resistance when compared with donor lines and the resistant control. In addition, induced mutation followed by in vitro selection in the media containing trans-4-hydroxy-l-proline as a selecting agent resulted in high cold tolerance in the regenerated DH plants of *B. napus* (McClinchey and Kott 2008; Janska et al. 2010). Furthermore, EMS treatment efficiently affected the fatty acid profile of regenerated mutant DH plants (Ferrie et al. 2008). Fatty acid analysis of *B. napus* DH lines indicated a range of 5.0–7.7 % in saturated fatty acid proportions. In *B. juncea*, saturate proportions ranged from 5.4 to 9.5 %, and of the 7000 *B. rapa* lines that were analyzed, 197 lines exhibited raised oleic acid (>55 %), 69 lines showed reduced linolenic acid content (<8 %), and 157 lines had low saturated fatty acid proportions (<5 %), when compared with the parental lines (Ferrie et al.

2008). Male-sterile DH lines with cytoplasmic inheritance or dominant genetic male sterility were obtained using ^{60}Co γ-ray irradiation which provides valuable resources for developing male sterility in *Brassicas* (Huang et al. 2014). Instead of treating isolated microspores, Prem et al. (2012b) subjected germinating seeds to EMS and ENU in order to obtain mutant donor plants. Irrespective of genotype, isolated microspores cultured from ENU-treated donor plants produced no MDE. In addition, the response of mutant plants derived from EMS mutagen was about 100 times lower than nontreated donor plants. They concluded that instead of having detrimental effects on efficiency of MDE production, valuable mutants can be recovered from mutated donor plants (Prem et al. 2012b). All together, chemical mutagens have several advantages over physical ones. For instance, the rate of mutagenesis is much higher and the variety of point mutations produced is vast, but majority of mutants created by chemical mutagenesis have indicated to exhibit adverse agronomic traits (Han et al. 2007; Xu et al. 2010).

21.3.3 Quantitative Trait Loci (QTL) Mapping and Marker-Assisted Selection (MAS)

DH populations suggest tremendous material for constructing genetic linkage maps and QTL analyses. The major advantage of DHs over other commonly used populations such as F2 and backcross is that they produce completely homozygous lines which can be easily multiplied without any genetic alteration occurring and thus facilitates conducting replicated trials across different locations and years for various agronomic traits (Collard et al. 2005). In addition, DH populations allow for assignment of components of environmental variation, which can be valuable for analysis of physiological effects (Pink et al. 2008). To date, the QTLs responsible for many important agronomic traits including seed yield and oil content (Lionneton et al. 2002; Chen et al. 2010; Cloutier et al. 2011; Yang et al. 2012), glucosinolate accumulation (Lou et al. 2008), chlorophyll content (Ge et al. 2012), bolting time (Ajisaka et al. 2001; Nishioka et al. 2005; Yang et al. 2007; Chen et al. 2010), and resistance to clubroot (Suwabe et al. 2003), blackleg (Yu et al. 2005, Yu et al. 2008), *Verticillium longisporum* (Rygulla et al. 2008), downy mildew (Yu et al. 2009), *Sclerotinia* stem rot (Wu et al. 2013), and Turnip mosaic virus (Zhang et al. 2008; Jin et al. 2014) have been surveyed and analyzed with the molecular map constructed from DH populations resulting from IMC technique.

The construction of linkage maps and QTL analysis is often costly and time-consuming; therefore, any alternative system(s) that can save time and expenses would be highly appreciated, especially if resources are limited. One "short-cut" method used to identify markers that tag QTLs is bulked segregation analysis (BSA, Collard et al. 2005). In BSA, a population is screened for a trait, and individuals at the two extreme ends of the distribution formed into two contrasting bulks. The DNA samples of two pools or "bulks" are then combined. By making DNA bulks, all loci are randomized, except for the region containing the gene of interest. Polymorphic markers, after screening two bulks, may indicate marker(s) that are

linked to a gene or QTL of desired trait (Collard et al. 2005). This technique is dependent upon accurate phenotyping, and DH populations have a particular advantage here in that they can be repeatedly tested. Although DH populations proved to be valuable segregant population for QTL analyses, they may not be applicable to outbreeding crops where inbreeding depression can cause nonrandom changes in gene frequency and loss of vigor of the lines (Collard and Mackill 2008).

The markers that have preliminary identified genetic mapping studies are also quite suitable for MAS. Most characters of economic importance are quantitative traits which are affected by environment and numerous loci throughout the genome with individually small effects and thus exhibit low heritability. In such situations, selection is often postponed until the lines become more homozygous in later generations (F5 or F6). Visual assessment for agronomic traits is carried out for resistance to stresses, as well as laboratory tests for quality or other traits to select superiors. The homozygous breeding lines can be harvested in bulk and assessed in replicated trials. The entire process takes considerable amount of time (often 5–10 years) and cost (Collard and Mackill 2008). In order to improve efficiency of selection, integrating molecular genetics with artificial selection known as MAS has been arose (Pink et al. 2008). DNA markers used for MAS should be highly reliable (reproducible), codominant in inheritance, relatively simple and cheap to be used, and generally highly polymorphic. Once tightly linked markers that reliably predict a trait phenotype have been identified, they may be used for MAS. For many agronomic traits, homozygous and heterozygous plants cannot be distinguished by conventional phenotypic screening. This technique allows breeders to select individuals based on their genotypes and thus accelerate the breeding process (Collard et al. 2005; Collard and Mackill 2008).

21.3.4 Reversible Male Sterility

F1 hybrid cultivars often represent high heterosis and therefore of great commercial value and the protection of breeder's rights (Birchler et al. 2003; Ribarits et al. 2007). Commercial production of hybrids is only feasible if a reliable and cost-effective pollination control system is available. Various procedures have been reported to block self-pollination of the female flowers such as mechanical removal of anthers or male flowers, application of male-specific gametocides, or application of male sterility (Parez-Prat and van Lookeren Compagne 2002).

Male sterility, as an efficient system for hybrid production, avoids the manual emasculation labor and serves as a molecular strategy for transgene containment by preventing pollen release to the environment, but most crops lack a naturally occurring system (Parez-Prat and van Lookeren Compagne 2002; Ribarits et al. 2007; Toppino et al. 2011). In addition, the achievement of complete male sterility in the female parent and the restored fertility in F1 hybrids is the major bottleneck in the commercial hybrid seed production.

Thanks to recent progresses in the area of metabolic engineering in pathways of microgametogenesis, biotechnology has added new possibilities to obtain reversible

male-sterile plants (Ribarits et al. 2007; Toppino et al. 2011). It has been indicated that glutamine plays an essential role during pollen development, as isolated and in vitro cultured microspores are unable to develop into the functional pollen grains in a medium lacking glutamine (Ribarits et al. 2007). Transformation of *Nicotiana tabacum* L. with mutated tobacco *glutamine synthetase* genes, which synthesize glutamine from glutamate, fused to the tapetum- and microspore-specific promoters caused pollen abortion closed to the first pollen mitosis and thus resulted in male sterility. Completely homozygous male-sterile lines were rapidly produced by microspore embryogenesis technique. Furthermore, foliage sprays with glutamine and in vitro maturation restored the fertility of male-sterile lines (Ribarits et al. 2007). Using this technique, F1 hybrid seed production technology can be applied in many commercially important crops.

21.3.5 Reverse Breeding

Reverse breeding is a novel breeding technique which allows for production of new hybrid plant varieties in a much shorter time frame and ambient numbers compared to conventional plant breeding techniques (forward breeding). The term "reverse breeding" was originally introduced to describe a technique in plant cell cultures, where homozygous lines are produced from heterozygous parent lines (Dirks et al. 2009; Wijnker et al. 2012). Here, the term "reverse breeding" includes the earlier proposed usage but goes beyond the original definition by widening the methods used to produce homozygous lines (Palmgren et al. 2014).

Generally, reverse breeding comprises two essential steps: (i) the suppression of crossover based in a selected hybrid plant (chosen for its elite quality) and (ii) regeneration of DHs from microspores containing nonrecombinant chromosomes. The knockdown of gene expression involved in homologous recombination, essential for reverse breeding, can be achieved by targeting genes using RNA interference (RNAi) or siRNAs, resulting in predominantly posttranscriptional gene silencing. Alternatively, dominant-negative mutations of the target gene can be used (Dirks et al. 2009). Silencing *DISRUPTED MEIOTIC cDNA1* (*DMC1*) gene resulted in nonrecombined parental chromosome segregation during meiosis in *A. thaliana* gametes (Wijnker et al. 2012). The protein encoded by *DMC1* is essential for meiotic homologous recombination which plays a central role in generating diversity of genetic information and also is essential for the reductional segregation of chromosomes that must occur in order to produce gametes during the sexual reproduction (Cloud et al. 2012). The second step in reverse breeding is to convert microspores, carrying nonrecombined chromosomes, into the DH plants through the IMC technique, as an efficient method, or other techniques depending on the plant species (Forster et al. 2007). From the regenerated DHs, complementary parents can be screened for reconstituting the initial heterozygote (Dirks et al. 2009). It seems that many plant breeding enterprises would benefit from this novel technique to deal with market demands and develop new varieties that are more sustainable or productive in a wide range of crops, including fruits and vegetables in the near future.

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