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REVIEW

Gametic embryogenesis and haploid technology as valuable support to plant breeding

Maria Antonietta Germanà

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Abstract Plant breeding is focused on continuously increasing crop production to meet the needs of an ever-growing world population, improving food quality to ensure a long and healthy life and address the problems of global warming and environment pollution, together with the challenges of developing novel sources of biofuels. The breeders' search for novel genetic combinations, with which to select plants with improved traits to satisfy both farmers and consumers, is endless. About half of the dramatic increase in crop yield obtained in the second half of the last century has been achieved thanks to the results of genetic improvement, while the residual advance has been due to the enhanced management techniques (pest and disease control, fertilization, and irrigation). Biotechnologies provide powerful tools for plant breeding, and among these ones, tissue culture, particularly haploid and doubled haploid technology, can effectively help to select superior plants. In fact, haploids (Hs), which are plants with gametophytic chromosome number, and doubled haploids (DHs), which are haploids that have undergone chromosome duplication, represent a particularly attractive biotechnological method to accelerate plant breeding. Currently, haploid technology, making possible through gametic embryogenesis the single-step development of complete homozygous lines from heterozygous parents, has already had a huge impact on agricultural systems of many agronomically important crops, representing an integral part in their improvement programmes. The aim of this review was to provide some background, recent advances, and future prospective on the employment of haploid technology through gametic embryogenesis as a powerful tool to support plant breeding.

Keywords Anther culture · Doubled haploids · Gynogenesis · Homozygosity · Isolated microspore culture · Pollen embryogenesis

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M. A. Germanà (✉)
Dipartimento DEMETRA, Facoltà di Agraria, Università degli Studi di Palermo, Viale delle Scienze, 11, 90128 Palermo, Italy
e-mail: agermana@unipa.it

Introduction

The first ambitious objective, among the Millennium Development Goals, consists in the eradication of extreme poverty and food shortage by the 2015 target date. Nowadays, for fighting hunger and malnutrition using a sustainable and low-input farming system, plant breeding rather than agrochemistry and mechanization seems to be able to more efficiently increase food and feed production on less land and often in a more environment-friendly way.

Plant breeders use traditional and biotechnological techniques to create and use novel genetic variations, aimed at selecting new elite and suitable varieties, with improved traits to satisfy both farmers and consumers (in terms of productivity, agricultural and quality performance, adaptability to marginal lands and regional environments, biotic and abiotic stress tolerance, etc.). Particularly, recent advances in biotechnology represent a valuable and powerful tool to enhance the efficiency and shorten the time required to reach the fixed purposes in a breeding programme, as well as to address economic and ecological goals. Among the biotechnological methods, haploid (H) and doubled haploid (DH) technology through gametic

embryogenesis has long been recognized as a valuable tool to help plant improvement. Haploids (Hs) are sporophytic plants with the gametophytic chromosome number, while doubled haploids (DHs) are haploid plants that underwent spontaneous or induced chromosome duplication. Dihaploids are haploids ($1n = 2x$) obtained from an autotetraploid genotype ($2n = 4x$).

Already in 1974, at the time of the First International Symposium on Haploids in Higher Plants, held in Guelph, Canada, the 'Utilization of haploidy in plant breeding' was discussed (Kasha 1974), and the first report of a doubled haploid rapeseed cultivar (Maris Haplona) was already known (Thompson 1972). Even prior to 1974, it was generally accepted that the application of DH technology to crop breeding had several advantages compared to conventional methods of producing homozygous lines (Forster and Thomas 2005). After a slow introduction of DH technology in plant breeding, in the past few decades there has been a rapid expansion, mainly due to the advances in the tissue culture abilities, so that, gametic embryogenesis, other than being just a fascinating developmental phenomenon (Dunwell 2010), has been described as "an additional weapon in the armoury of the breeder" (Forster et al. 2007b). Therefore, both the Food and Agriculture Organization of the United Nations (FAO) and the International Atomic Energy Agency (IAEA) have supported and coordinated research focused on the development of more efficient DH production methods and their application in plant breeding in developing countries (Dunwell 2010). Furthermore, a European Union-funded research network, the COST 851 programme, entitled 'Gametic cells and molecular breeding for crop improvement', was carried out from 2001 to 2006. The first crop plant released by DH technology was the rapeseed (*Brassica napus*) Maris Haplona (Thompson 1972), followed by the cultivar Mingo in barley (*Hordeum vulgare*), obtained in Canada in the late 1970s (Ho and Jones 1980), even if Khush and Virmani (1996) reported improved rice and tobacco cultivars previously developed in China, through doubled haploid breeding. Worldwide, the use of DH technology has become routine by many breeding companies and laboratories involved in the improvement of a wide range of crops (Tuvesson et al. 2007), resulting in the development of almost 300 new varieties (these data are probably underestimated because of the scarce information from some parts of the world and from some private companies), mainly including wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), *Brassica* spp., eggplant (*Solanum melongena* L.), pepper (*Capsicum annuum* L.), asparagus (*Asparagus officinalis* L.), and tobacco (*Nicotiana tabacum* L.) (Thomas et al. 2003; Dunwell 2010). The largest number of the varieties derived from doubled haploids is in barley, followed by rapeseed and wheat (Thomas et al. 2003). Regeneration from gametes has been reported in numerous species, and over the past few years, practical applications of DHs have been extended to a wide range of varieties, mostly in the model species, such as barley (Jacquard et al. 2003), rapeseed (Custers 2003), and wheat (Tuvesson et al. 2003). In fact, in these species, this technology has become a standard research tool for breeding, and thousands of DHs are produced annually routinely using these methods. In other crops, such as rye (Immonen and Tenhola-Roininen 2003), oat (Kiviharju et al. 2005), triticale (Immonen and Robinson 2000), wild barley (Islam et al. 1992), potato (Rokka 2009), and cabbage (Hansen 2003), DH methods are somewhat less advanced, even if hundreds of DHs are obtained and DH technologies can be applied, not for the whole breeding programme, but only for selected parts, for example stabilizing semi-dwarfness in a rye breeding programme (Tenhola et al. 2001). Moreover, recently newcomers, previously considered rather recalcitrant, such as forage, vegetable crops, legumes, aromatic, medicinal, and woody plants, are now objects of successful studies on the induction of gametic embryogenesis aimed at their genetic improvement and genomic studies (Bajaj 1990; Dunwell 2010; Ferrie 2007, 2009; Germanà 2006, 2009; Raghavan 1990; Sangwan-Norrel et al. 1986; Wenzel et al. 1995). In fact, during the past few decades, remarkable progress has been made in this research area: the frequency of haploid production has been increased, reliable tissue culture protocols have been set up for a wider range of species and agricultural crops, thanks to recent technological innovations and to a greater understanding of underlying control mechanisms, and new cultivars, obtained through gametic embryogenesis, have been released. Nowadays, the realization of the potential in these areas, alongside real and tangible results, is causing a resurgence of interest in haploids of higher plants (Forster et al. 2007a).

The aim of this review was to provide some background, recent advances, and future prospective regarding the employment of haploid technology through gametic embryogenesis as powerful tool to support plant breeding.

Gametic embryogenesis

A sporophytic ($2n$) and a gametophytic (n) generation generally characterize the life of higher plants, where the gametes are products of meiosis and the sporophyte, mostly diploid, develops from the zygote produced by the fertilization. In comparison with the sporophytic generation, the gametophytic generation has been progressively reduced both in terms of size and lifespan throughout evolution, and therefore, its importance is usually underestimated.

Heslop-Harrison (1979) termed this step of angiosperm life as the "forgotten generation".

Recent basic and applied studies, which have increased the knowledge of gamete biology and biotechnology, make the manipulation of their development a reliable tool for crop improvement (Mulchay 1986). The most important application of gamete biotechnology in breeding and genetic studies is the ability to obtain haploids and doubled haploids. In fact, immature plant gametophytes can switch from their normal developmental gametophytic pathway towards a sporophytic one, in a process termed "gametic embryogenesis", one the different routes of embryogenesis present in the plant kingdom. Particularly, gametic embryogenesis, a fascinating example of cellular totipotency, is named "gynogenesis" when it initiates from a female gamete and "pollen embryogenesis" (sometimes referred to as androgenesis) when it starts from a male gamete (microspore or immature pollen grain). Unless spontaneous or induced chromosome duplication, plants obtained through gametic embryogenesis are haploid because they are regenerated from the products of meiotic segregation. The production of homozygosity in one generation, rather than several generations of selfing, accelerates the improvement of breeding. Moreover, it is possible to reach about 98% homozygosity after 6 years of selfing of an annual self-pollinating crop, while 100% can be achieved in one-step through haploidy techniques. Furthermore, gametic embryogenesis is an indispensable tool to obtain homozygosity in species suffering from inbreeding depression (implying therefore that they cannot easily produce fertile homozygous lines by self-pollination), such as rye (*Secale cereale*) (Immonen and Anttila 1996) and forage grasses *Festuca* and *Lolium* (Nitzche 1970), as well as in perennial woody species, generally characterized by a long juvenile periods, a high degree of heterozygosity, large size and, often, self-incompatibility (Germanà 2006, 2009) and generally in plants with barriers to repeated selfing (e.g. dioecy, self-incompatibility and long juvenile periods), where it is not possible to obtain homozygous breeding lines through conventional methods.

DH technology

A lot of research has been carried out since the 1970s to obtain haploids for breeding through gametic embryogenesis. H and DH production has been reviewed in several reviews and volumes (Andersen 2005; Dunwell 2010; Germanà 1997, 2006, 2007, 2009, 2011; Jain et al. 1996; Kasha 1974; Magoon and Khanna 1963; Maluszynski et al. 2003a, b; Palmer et al. 2005; Seguì-Simarro and Nuez 2008a; Seguí-Simarro 2010; Smykal 2000, Touraev et al. 2009; Zhang et al. 1990; Xu et al. 2007, etc.). There are many economically important crop species, including major cereals and cabbages, in which doubled haploid techniques have been well established (Wedzony et al. 2009). Nevertheless currently, there is a great interest to expand the application of DH technology to other high value crops, such as medicinal and aromatic plants (Ferrie 2009), legumes (Grewal et al. 2009; Skrzypek et al. 2008), and woody plants (Germanà 2006, 2009).

To produce Hs and DHs, various DH techniques, such as in vivo modified pollination methods (selection of twin seedlings, chromosome elimination subsequent to wide hybridization, "*bulbosum*" method by Kasha and Kao 1970, in situ pollination with irradiated pollen, pollination with pollen from a triploid plant, etc.) and in vitro culture of immature gametophytes (gynogenesis and pollen embryogenesis through in vitro anther or isolated microspore culture) can be employed (Fig. 1) (Forster and Thomas 2005). The most suitable method of DH production changes depending on the crop. For example, for barley, it is the wide crossing, for rapeseed it is the microspore culture, and for wheat, it is the anther culture (Thomas et al. 2003). However, there is a tendency towards isolated microspore culture application at the same time as new tissue culture protocols are developed. These methods will be briefly summarized here, but are more fully detailed in the exhaustive manual of Maluszynski et al. (2003a), which reports on 44 protocols of doubled haploid production, related to at least 23 plant species.

There is a strong commercial interest in methods for the production and exploitation of haploid plants, verified by the extensive application of intellectual property (IP) protection and patenting system to haploid plants both in the USA and elsewhere (Dunwell 2009, 2010).

Selection of seedlings

The production of spontaneous haploids in over 100 angiosperm species has been reported by Kasha (1974). Sources of spontaneously occurring haploids can be parthenogenesis (the production of an embryo from an egg cell, without the participation of the male gamete) or apogamy (the production of an embryo from a gametophytic cell other than the ovum).

Although DH lines derived from spontaneously occurring haploids have previously been successfully exploited for commercial production in maize (Chase 1951) and the first released DH cultivar, the tomato 'Marglobe', was obtained by Morrison in this way many years before (Morrison 1932), the frequency of Hs spontaneously produced is too low to allow that the selection of seedlings can be used routinely in crop breeding programmes.

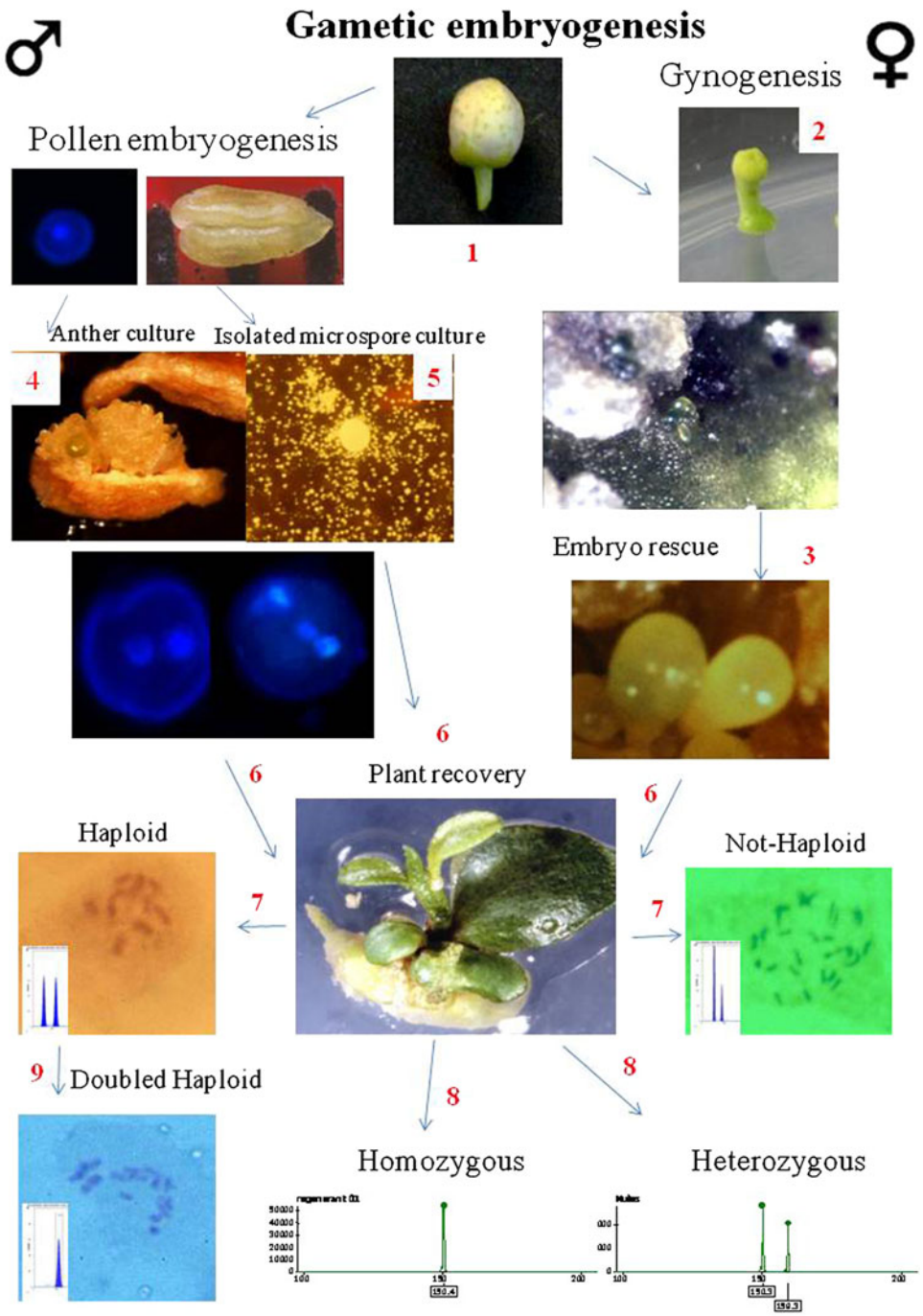

Fig. 1 Schematic representation of the gametic embryogenesis: *1* selection of floral buds, *2* gynogenesis, *3* embryo rescue, *4* anther culture, *5* isolated microspore culture, *6* plant recovery, *7* characterization of regenerants: ploidy analysis, *8* characterization of regenerants: detection of homozygosity (microsatellites), *9* induced chromosome doubling

Wide hybridization: interspecific hybridization (the *bulbosum* method)

Kao and Kasha (1969) first reported monoploid barley induction by crossing tetraploid *Hordeum vulgare* ($2x = 4x = 28$) as the female and teraploid *H. bulbosum* ($2x = 4x = 28$) as the male. Pollen grains of *H. bulbosum* fertilize *H. vulgare*, but afterwards, chromosomes of *H. bulbosum* are eliminated from cells of developing embryo. It was demonstrated as embryo rescue, and growth of donor plants in controlled artificial conditions allow high frequencies of monoploid production (Jensen 1986).

In situ gynogenesis

Maternal haploids can be induced through pollination when specific haploid inducer lines are available. For example in maize (*Zea mays* L.), where the utilization of maternal haploid plants has great potential for breeding and genetics, genotypes related to the line Stock 6 (Eder and Chalyk

2002) and the line RWS (Geiger and Gordillo 2009) are able to induce maternal haploid production on a large scale from different genotypes. Also in potato, that was one of the first crop plants in which haploid breeding techniques were used (Hougas and Peloquin 1958; Chase 1963), (di)haploid ($2n = 2x$) lines are produced by pollination of cultivated potato with specific haploid inducer clones of *Solanum phureja* as well as by in vitro anther culture (Rokka 2009).

In vivo induction of maternal haploids can also be obtained through pollination performed with irradiated pollen (usually by gamma rays from cobalt 60) or pollen from triploid plants. This kind of pollen can germinate on the stigma, grow within the style and reach the embryo sac, but they are not able to fertilize the egg cell and the polar nuclei stimulating therefore the development of haploid embryos from ovules (Musial and Przywara 1998). Parthenogenesis induced in vivo is usually followed by in vitro culture of embryos. In the case of irradiated pollen, the success of this technique is dependent on the choice of the radiation dose, the developmental stage of the embryos at the time of culture, the culture conditions, and the media composition.

In situ gynogenesis induced by irradiated pollen, first discovered by Raquin (1985) in *Petunia*, has also been successfully used for *Cucumis melo* (Sauton and Dumas de Vaulx 1987), *Daucus carota* (Rode and Dumas de Vaulx 1987), *Malus domestica* (Zhang and Lespinasse 1991; Hofer and Lespinasse 1996), *Pyrus communis* (Bouvier et al. 1993), *Actinidia deliciosa* (Pandey et al. 1990; Chalak and Legave 1997), *Citrullus lanatus* (Sari et al. 1994), *Citrus* hybrids, and *Citrus clementina* Hort. ex Tan. (Froelicher et al. 2007; Aleza et al. 2009). Gynogenic embryos have been also obtained in *Citrus clementina* Hort. ex Tan., through parthenogenesis induced by pollination with pollen from a triploid plant, both in vivo by Oiyama and Kobayashi (1993) and in vitro by Germanà and Chiancone (2001).

In vitro culture of immature gametophytes

Gametic embryogenesis, consisting in a change in the normal gametophytic development into a sporophytic one, can be induced through ovule and ovary in vitro culture and through anther or isolated microspore in vitro culture. Numerous endogenous and exogenous factors affect the embryogenic response of gametes in culture (Atanassov et al. 1995; Datta 2005; Smykal 2000; Wang et al. 2000). Particularly, genotype, physiological state, and conditions of growth of donor plants, stage of gamete development, pre-treatment of flower buds and in vitro culture media and conditions, together with their interactions, are all factors that greatly affect the gamete response to the in vitro culture. There is no single standard condition or protocol to induce gamete-derived plant formation, and it is possible that gametes, not only of different species, but also of different cultivars within a species, have very diverse requirements to undertake a sporophytic development. For example, screening through microspore culture response using the *Brassica napus* microspore culture protocol (Ferrie and Keller 1995) over 80 species from 24 families known to have beneficial properties, Ferrie et al. (2005) observed differences among the families in terms of response with the majority of the species giving no response, a few species showing induction (i.e. swelling and initial divisions), but no further development, and some members of the *Apiaceae* and *Caryophyllaceae* families in which embryos were produced. Despite the progress that has been achieved, many genotypes are still considered recalcitrant. In addition, some process difficulties, such as low frequencies of embryo induction, albinism, low plant regeneration, little plant survival, and the genotype- and season-dependent response can be encountered in tissue culture protocols aimed to regenerate from gametes of many genotypes. Many research studies have been carried out, focusing on increasing the frequency of embryogenesis with responsive species and on developing new protocols for recalcitrant ones.

Ovule and ovary culture

Gynogenic haploid regeneration or haploid parthenogenesis using the un-pollinated female gametophytics (ovules, ovaries or floral buds) is a method for haploid induction used in several agronomically important species, such as sugar beet and onion (Bohanec 2009). Ovules in an immature developmental stage are required, and for most of the species, the egg cells are usually the predominant source of haploid embryo. Currently, gynogenesis is the least favoured technique because of the low efficiency, but it is applied to species that do not respond to more efficient methods (Forster et al. 2007a).

Anther and isolated microspore culture

Extensive research has been carried out on in vitro regeneration from the male gamete, since the discovery made about 40 years after the first natural haploid finding, by Guha and Maheshwari (1964) in the solanaceous species *Datura innoxia* and consisting in the production of embryos and plants with a haploid chromosome number through in vitro culture of immature anthers. A decade later, Nitsch (1974) cultured *Nicotiana* microspores naturally released from anther tissue ('shed microspore' culture) and only in 1982, Lichter mechanically isolated microspores from *Brassica* buds prior to culturing them,

paving the way for further and numerous studies on microspore culture. While in anther culture, the whole immature anthers are in vitro cultured, in microspore culture, the immature male gametes are isolated prior to culture, removing somatic anther tissue by different methods. Although anther culture is often the method of choice for doubled haploid production in many crops, because of its higher efficiency and advantageous simplicity (Germanà 2011; Sopory and Munshi 1996), the technique of the isolated microspore culture provides a better way of investigating the cellular, physiological, biochemical, and molecular processes involved in pollen embryogenesis (Nitsch 1977; Reinert and Bajaj 1977) even if it requires better equipment and more skills than it does in anther culture. Moreover, isolated microspore culture avoids the regeneration of diploid embryos and plants from somatic anther tissue (Ferrie and Caswell 2010; Germanà 2005, 2011). In laboratories around the world, isolated microspore culture protocols are well established and routinely used for developing new varieties, as well as for basic research in areas such as genomics, gene expression, and genetic mapping (Ferrie and Caswell 2010). Specifically, very efficient and replicable protocols for the production of microspore embryos have been set up in rapeseed, tobacco, and barley (Jähne and Lörz 1995; Touraev et al. 1997), where up to 70% of the cultured microspores can be induced to divide initially, and embryos usually develop by direct embryogenesis, i.e. without a callus phase (Indrianto et al. 2001). Essentially, the steps involved in an isolated microspore culture protocol are: growing donor plants, harvesting floral organs, isolating microspores, culturing and inducing microspores, regenerating embryos, and doubling the chromosome number (Ferrie and Caswell 2010).

Characterization of regenerants: ploidy analysis

Conventional cytological techniques can be employed to count chromosome numbers from root tip cells of regenerated embryos and plantlets, even if, more recently, ploidy level can be more easily assessed by flow cytometry analysis (Bohanec 2003). Other indirect methods have been used to estimate ploidy level, such as counting the chloroplast number in stomatal guard cells and determining plastid dimensions (Lee and Hecht 1975; Qin and Rotino 1995; Yuan et al. 2009).

Not only haploids or doubled haploids have been obtained by gametic embryogenesis. Actually, non-haploid (diploid, triploid, tetraploid, pentaploid, and hexaploid) embryos and plantlets have been obtained via anther culture of different genotypes (D'Amato 1977; Dunwell 2010). The occurrence of polyploids seems to be related to microspore developmental stages, and according to Engvild et al. (1972), anthers with young uninucleate microspores give rise to haploid plants, anthers with older uninucleate microspores give rise to diploid plants, and anthers with bicellular microspores give rise to triploid plants. Specifically, triploids regenerated from anther culture have been reported in *Datura innoxia* (Sunderland 1974), in *Petunia hybrida* (Raquin and Pilet 1972), and in several fruit species (Germanà 2006, 2009). More particularly, regenerants with different ploidy levels obtained through anther culture have been reported in various plant species, e.g. 6% haploids, 70% diploids, and 24% triploids in *Datura metel* L. (Narayanaswamy and Chandy 1971); 34% haploids, 65% diploids, and 0.8% triploids in *Capsicum annum* L. (Dolcet-Sanjuan et al. 1997); 7% haploids, 43% diploids, 29% triploids, and 21% tetraploids in apple (De Witte et al. 1999); 14.3% diploid, 71.4% triploids, and 14.3% tetraploids in *Petunia axillaris* (Engvild 1973); 2.1% haploids, 9.6% diploids, 81.9% triploids, 3.2% tetraploids, and 3.2% hexaploids in *Citrus clementina* Hort. ex Tan. (Germanà et al. 2005), and 96.3% triploids and 3.7% tetraploids in *Carica papaya* L. (Rimberia et al. 2006). Non-haploids may arise from: (a) somatic tissues, (b) endoreduplication and fusion of nuclei, (c) endomitosis, and (d) irregular gametes formed by meiotic irregularities (D'Amato 1977; Sangwan-Norreell 1983; Sunderland and Dunwell 1977; Narayanaswamy and George 1982). In some cases, the origin of non-haploids seems to derive from the incomplete cell wall formation (Dunwell and Sunderland 1974a, b, 1975, 1976a, b, c). The dynamics and mechanisms of diploidization at early stages of microspore-derived embryogenesis have been studied in barley by Gonzalez-Melendi et al. (2005) and reviewed in Seguì-Simarro and Nuez (2008b). Anyway, the diploid or polyploid plants originated in this way are completely homozygous.

Characterization of regenerants: detection of homozygosity

Because of the spontaneous chromosome doubling occurring in the haploid calli and embryos, ploidy level analysis cannot always identify pollen-derived plants. In fact, when in vitro anther or ovary cultures are performed, diploid plants can be homozygous doubled haploids or heterozygous diploids produced by the somatic tissue. For example, anther culture can be employed to obtain somatic embryos and clonal plant propagation in many genotypes (Germanà 2003, 2005).

Isozyme analyses, RAPD markers, sequence characterized amplified region (SCAR), and simple sequence repeats (SSRs) have been utilized to assess homozygosis and to determine the gametic origin of calluses and regenerated plantlets, irrespective of their ploidy. Other systems have

been used for identifying haploids, such as the use of dominant anthocyanin marker genes, described and widely applied in maize by Nanda and Chase (1966) and Chase (1969), in which, the *R1-nj* gene is used to distinguish haploid and diploid plants. When this gene is expressed, it provides an anthocyan pigmentation of the embryo and the endosperm and kernels with a pigmented endosperm and a non-pigmented embryo were selected as haploids (Eder and Chalyk 2002). Moreover, in apple, Verdoodt et al. (1998) used the single multi-allelic self-incompatibility gene to discriminate homozygous from heterozygous individuals obtained by anther culture as well as by in situ parthenogenesis.

Spontaneous or induced chromosome doubling

To convert sterile haploids, in vitro regenerated from gametes, into fertile, homozygous doubled haploid plants, spontaneous or induced chromosome doubling process is required. To use haploid plants in breeding programmes, it is necessary to have high chromosome duplication rates of haploid plants. Other than the widely used colchicine, other doubling agents, such as oryzalin and trifluralin, can be employed. The method of application for effective duplication varies depending on the genotype.

Hs, DHs, and plant breeding

Speed and efficiency play a very important part in plant improvement due to the need for cost control. The value of haploids in accelerating plant breeding and genetic research was quickly recognized with the discovery of the first natural haploid in *Datura stramonium* and *Nicotiana* (Blakeslee et al. 1922; Blakeslee and Belling 1924; Kostoff 1929), but long before techniques for producing haploids by in vitro gametic embryogenesis became available. Since the first report in 1949, by Chase, regarding a spontaneous parthenogenesis system to produce the first maize doubled haploid inbreds, numerous have been the applications of haploidy to crop breeding, as short-cut method in establishing homozygous lines (Khush and Virmani 1996). The organization of the First International Symposium 'Haploids in Higher Plants', which took place at Guelph (Canada) in 1974 (Kasha 1974), verified the, already great, interest in haploids.

Selected DH lines were then used to produce commercial hybrids (Chase 1974), and DH cultivars are nowadays a feature in many crop species, consisting in more than 280 varieties produced by different DH methods, as reported on the COST Action 851 website (http://www.scri.sari.ac.uk/assoc/COST851/COSThome.htm). Actually, doubled haploid breeding has been successful in developing cultivars in less time than conventional methods. In fact, doubled haploid technology can accelerate the breeding of new varieties, and the time to develop new cultivars may be reduced by 50% in winter-grown crops and somewhat less in spring grown crops when DH technology is used for fixation of traits following F1 crosses compared to classical pedigree breeding (Forster and Thomas 2005). For example, 3–7 years can be saved in a winter-grown crop, such as a winter wheat (Tuvesson et al. 2007). The reduction of time is relative to the conventional breeding method used and the generation from which the line is derived. Doubled haploid lines are homozygous and homogeneous, and by coupling rapid line development with homogeneity, doubled haploid breeding is ideal for countries requiring a high level of cultivar purity (Baenziger 1996). Moreover, in herbs, spices, nutraceuticals, and medicinal plants, where much of the industry relies on ''wild'' plants, individual variation can cause problems for those who conduct clinical trials as well as for consumers, who are expecting a uniform product (Ferrie 2007). In such cases, uniform varieties would be beneficial for use in agronomic and clinical trials. Doubled haploidy is also ideal for a rapid response to changing conditions, such as when a new pest biotype occurs or it is introduced from abroad. The major limitation appears to be the cost of producing doubled haploid lines in sufficient quantities for a breeding programme.

The most important use of haploids is based on the fact that remarkable improvements in the economics of plant breeding can be achieved via DH production as selection and other procedural efficiencies can be markedly improved by using true-breeding (homozygous) progenies (Nei 1963; Snape 1989; Jauhar et al. 2009; Dunwell 2010). For crop improvement purposes, DH lines are developed mainly to achieve homozygosity in diploid or allopolyploid species, saving several generations in a breeding programme and producing new homozygous cultivars or parental lines for F1 hybrids (Veilleux 1994). The advantage of haploid breeding is displayed in crops with a variety of genome complements (Ortiz et al. 2009) and breeding systems (Kruse 1980). The distinction between self- or cross-pollinated species and whether the inheritance is disomic or polysomic has been made by Khasha and Sequin-Swartz (1983) and later by Khush and Virmani (1996). New superior cultivars produced via gametic embryogenesis (above all through anther culture) have been reported for rice, wheat, tobacco, maize, and pepper (Evans 1989), and doubled haploids are being routinely used in breeding programmes for new cultivar development in many crops like eggplant, pepper, barley, and rape (Veilleux 1994). For instance, the DH technique is routinely applied in winter rapeseed breeding programmes for the generation of completely homozygous lines (Mollers

and Iqbal 2009), and it is estimated that in Europe 50% of contemporary barley (*Hordeum vulgare*) cultivars are produced via a doubled haploid method (Dunwell 2010). Consistently, through DH technology, barley varieties with a better agricultural and quality performance and better suited to marginal zones, such as the Peruvian highlands, have been obtained (Gomez-Pando et al. 2009), and some DH wheat cultivars have become dominant in specific regions of the world (Jauhar et al. 2009). Once again with barley, doubled haploids developed by the *H. bulbosum* method have been shown to be similar to lines developed by pedigree and single seed descent methods (Park et al. 2009; Choo et al. 1982), and DHs have been used for recurrent selection in winter barley (Forughi-Wehr and Wenzel 1993), as well as in maize (Chalyk and Rotarenco 1999).

In terms of maize, Eder and Chalyk (2002) reported that the selection of haploid plants, obtained from two synthetic populations, produced an increase in the grain yield and influenced some other traits of diploid plants, hypothesizing that the selection of large ears in haploid plants possibly increased the frequency of genes providing a significant contribution to plant vigour and viability and that Hs with unfavourable or harmful genes either die or are weak and sterile and do not form seeds. As a result of the reduction of the frequency of harmful genes by means of natural selection, haploid plants can be considered as a natural filter that discards harmful genes from a population, or any other breeding material, from which haploids were obtained (Eder and Chalyk 2002).

Courtois (1993) reported a statistical analysis of the field performance of anther culture-derived doubled haploids in rice, comparing the mean, genetic variance skewness and kurtosis of three doubled haploid populations to their respective single seed descent populations and verifying that both methods were equally effective for developing new lines.

In many cases, through gametic embryogenesis, an enhanced gene expression was produced for complex traits, such as drought resistance or freezing-tolerance, in excess of that found in the parent genotypes, allowing for the exploiting of the genetic potential of a genotype that, otherwise in its unreduced disomic state, was underexpressed due to combinations of control by recessive alleles, or possible pleiotropic or epistatic interactions (reviewed by Humphreys et al. 2007). Likewise, Lotfi et al. (2003) reported the production of haploid and doubled haploid plants of melon (*Cucumis melo* L.) for use in breeding for multiple virus resistance.

Among the application of Hs and DHs to plant breeding, specific opportunity in fruit crop improvement can be found in the use of the reduced size of Hs compared with diploids and heterozygous in the field of the ornamental plants or of the dwarfing rootstocks or of the tree cultivation in greenhouse (Germanà 2006, 2009; Rimberia et al. 2006). Moreover, triploid regenerants often obtained via gametic embryogenesis may have great commercial potential in those crops, for which the fruit seedlessness is required (Germanà 2006, 2009). Furthermore, gametic embryogenesis, particularly obtained via anther culture, seems to contribute directly to the production of female pure lines in papaya (*Carica papaya* L.), where female cultivars usually have higher productivity compared to the hermaphrodite ones (Rimberia et al. 2006).

Heterosis and haploidy

Homozygous DH plants can possibly directly become new varieties or they can be valuable to produce F1 hybrid plants, performing crosses between selected homozygous males and females. The F1 plants often exhibit the so-called hybrid vigour or heterosis, a phenomenon for which hybrids between two inbred varieties or lines often exhibit greater biomass and other superior characteristics than those of the best parent (Shull 1908; Maluszynski et al. 2001; Hochholdinger and Hoecker 2007). For example, in vegetable crops, one of the main uses of DHs is as parents for F1 hybrid seed production. There are different explanations regarding the occurrence of heterosis: one, for example, is dominance, i.e. slightly deleterious alleles, which are homozygous in the respective parents, are complemented in the hybrids with superior alleles (Bruce 1910; Jones 1917). Another potential basis for heterosis is overdominance. In this case, unlike alleles result in a stimulating effect, i.e. that genetic heterozygosity per se results in greater genetic activity (East 1936; Hull 1945). In inbreeding depression, which is the reverse concept to heterosis, quantitative trait values decrease as homozygosity increases in successive generations, even though the allele frequency as a whole for the successive populations remains unchanged. Inbreeding depression is often attributed to the fixation of deleterious alleles. Recently, the elucidation of heritable epigenetic phenomena raises the possibility that epigenetic effects contribute to heterosis and/or inbreeding depression. Some transcription factor loci in maize show differential expression, depending on the fact that they are present as a homozygote or heterozygote (Maluszynski et al. 2001).

Doubled haploids provide a unique system to attempt the ‘fixing’ of hybrid performance in homozygous lines and to avoid the step of hybrid seed production (Maluszynski et al. 2001). Since the second half of the last century, the genetic phenomenon of heterosis has been the basis for a strategy of the hybrid corn companies. The assumption on the ‘fixability’ of hybrid yield in homozygous lines comes from reports that large additive genetic variance is

responsible for yield heterosis in wheat or barley, and it was proven also for mutant crosses (Maluszynski et al. 2001; Auger et al. 2004). Kasha et al. (1977) carried out the first attempt to use doubled haploid procedures for fixation of heterosis in barley, identifying at least one DH line per cross similar to or higher in yield than the heterotic F1. Ba Bong and Swaminathan (1995) produced DH lines by anther culture from the heterotic F1s of three commercial *indica* rice varieties. Similarly, DH lines significantly surpassing the best parent were obtained from F1 hybrids expressing high-yield heterosis in wheat (Maluszynski et al. 2001).

Mutation and haploidy

Induced mutations have been used mainly to improve particular characters in well-adapted local varieties or to generate variation, which is difficult to be found in germplasm collections. Desired mutant traits commonly exploited in crop plants are, for example: higher yield and better quality, earliness and lateness, semi-dwarfness, lodging resistance, herbicide resistance, and improved disease resistance (Maluszynski et al. 1995, 1996). About 70% of mutant cultivars were released as direct mutants, i.e. without any further breeding; the remaining 30% were developed through cross-breeding programmes where mutants or mutant varieties served as a source for desirable alleles (Maluszynski et al. 2000). Doubled haploidy can also be useful in isolating and purifying desired mutations from the other mutations and in reducing the mutational load, instead of the costly methods of repeated backcrossing to the parental lines (BC) and/or single seed descent (SSD). In fact, DH systems have many attractive benefits for inducing, selecting, and fixing mutations (Szarejko 2003; Szarejko and Forster 2006). Actually, haploid cells provide an ideal target for mutation induction and selection, for the possibility to screen for both recessive and dominant mutants in the first generation after mutagenic treatment, avoiding chimerism and shortening the breeding times, especially as induced mutations are predominantly recessive and are normally selected in the second or third (M2 and M3) generation after mutagenic treatment. In fact, mutation techniques applied to haploid cells enable the immediate expression of recessive mutations and the possibility to obtain, by doubling chromosomes, complete homozygous diploids (Howland and Hart 1977). Application of DH systems enhances the effectiveness of the selection of desired recombinants, especially when quantitative traits (e.g. yield and quality) are evaluated, shortening the time employed in the process of mutation detection and evaluation (Forster and Thomas 2005). Really, if the mutagenic treatment is carried out on quiescent seed as normal and in addition the M1 plants are used as gamete donors for DH production, many generations normally needed to produce pure breeding lines are saved.

Evidently, the availability of an efficient protocol for doubled haploid production is necessary to take advantages of combining mutation techniques with DH systems, and for this reason, it has been mostly applied to barley as it is a model species for both mutation studies and DH production. Mutant DH lines of barley were found at a frequency of 25% in a DH population derived from anther culture of M1 (Szarejko et al. 1995). However, this method has also been used successfully in rice and wheat, and several true-to-type mutant lines that exceeded the yield of the parental line were identified (Szarejko and Forster 2006). Moreover, since the recent progress in developing effective protocols for DH production through isolated microspore culture (Maluszynski et al. 2003a, b; Ferrie and Caswell 2010), the DH systems themselves provide an opportunity to target haploid cells for mutation treatment and capture the mutation in a homozygous, pure line, making possible the application of mass scale in vitro mutagenesis/selection methods in major cereals, in rapeseed and other Brassicas (Szarejko and Forster 2006). When the uninucleate microspores are used as a target for mutagenic treatment, it is important that the mutagens are applied shortly after microspore isolation or after pre-treatment before the first nuclear division to avoid chimeric and heterozygous plant formation. This is particularly crucial in genotypes, such as barley, where spontaneous doubling occurs shortly after the first divisions in culture (Chen et al. 1984; Kasha et al. 2001). Other than gametes (notably embryogenic microspores), haploid cells, tissues, organs, and explants have also been used as targets for mutagenic treatments, applying both chemical and physical mutagens. For instance, Castillo et al. (2001) produced through chemical mutagenesis of anther and microspores cultures, developmental mutants in *Hordeum vulgare*, while Lee and Lee (2002) in *Oryza sativa*.

In vitro selection during gametic embryogenesis

Plant breeding programmes are costly and time-consuming: large population sizes are needed to obtain the required combination of characters in the new genotypes. Angiosperm pollen has great potential by culturing the isolated microspores and exerting stress during culture. In this way, microspores, which result from recombination, are subjected to selection, and it is possible to recover, via gametic embryogenesis, mutants for physiological and biochemical traits (Evans et al. 1990). Male Gametophytic Selection (MGS), based on the overlap (about 70%) in genetic expression between the gametophytic and the sporophytic generation (Mascarenhas 1990) and on the similarity in

their response in the presence of several external factors (Hormaza and Herrero 1992, 1996), presents several advantages deriving from the characteristics of the gametic phase: haploidy and large population size. In fact, the advantage of performing selection during in vitro microspore embryogenesis among hundreds of thousands of synchronously developing embryos, instead of on several thousand lines, is cleary evident. For this aim, however, a well-defined procedure of regeneration through gametic embryogenesis is necessary. For example, in rapeseed, mutants resistant to herbicides have been developed by adding after mutation treatment, the selective agent to the culture medium, usually at a dose close to LD100 and testing in field conditions the next generation of plants obtained from survived embryos (Beversdorf and Kott 1987; Kott 1998; Palmer et al. 1996; Swanson et al. 1988; 1989). Likewise, similar in vitro mutagenesis and selection systems (adding to the media, as selective agent, a pathogen-derived toxin) were developed for obtaining DH lines with improved tolerance to biotic stresses, such as to *Sclerotinia sclerotiorum* in *Brassica napus* (Liu et al. 2005) and *Erwinia carotovora* in *B. campestris* (Zhang and Takahata 1999). With the aim of producing doubled-haploid maize plants tolerant to oxidative stress, in vitro selection was carried out in maize anther culture with reactive oxygen species (ROS) progenitors such as paraquat, menadione, tert-butylhydroperoxide (t-BHP), and methionine combined with riboflavin and several fertile doubled-haploid plants were produced (Ambrus et al. 2006; Darko et al. 2009). As well, mutants for seed quality traits have been also selected in rapeseed as these are also expressed in the embryo, e.g. fatty acid composition. Because the processes of lipid biosynthesis in microspore-derived and zygotic embryos are similar, one cotyledon collected from a gamete-derived embryos can be used to determine the fatty acid composition, providing an early selection of rapeseed mutants with improved oil quality, i.e. a higher concentration of oleic acids and decreased levels of saturated fatty acids (Huang 1992; Kott 1998; Turner and Facciotti 1990; Wong and Swanson 1991). Besides, among 834,000 mutagenized rapeseed embryos, Rahman et al. (1995) selected ten microspore-derived embryos that survived lethal doses of NaCl in medium and one DH line that expressed salt tolerance also at the plant level.

In the 1980s, haploid protoplast cultures obtained from haploid calli derived from mutagenically treated microspores of *Nicotiana* species were used for the selection of cell lines tolerant to salt or amino acid analogues (reviewed by Maluszynski et al. 1996). More recently, microspore-derived cell lines of rice have been developed, showing tolerance to the herbicide cyhalofop butyl (Bae et al. 2002) or tryptophan and lysine analogues (Kim et al. 2003).

Genetic transformation and haploidy

The haploid chromosome set, added to embryogenic competence, makes microspores one of the most attractive cellular targets for the transfer and stable integration of recombinant DNA into plant genomes in both dicotyledous as well as in monocotyledonous, via direct methods (microinjection, electroporation, and particle bombardment) or *Agrobacterium tumefaciens*-mediated gene transfer (Kumlehn 2009; Shim and Kasha 2003; Resch et al. 2009; Touraev et al. 2001). In fact, if transgenes can be incorporated into the haploid microspore genome before DNA synthesis and chromosome doubling, the DHs may also be homozygous for the transgenes (Shim and Kasha 2003).

Not only the unicellular microspores, but also haploid cells or embryos at all stages from gametic embryogenesis and regeneration have been used as recipients for gene delivery to directly obtain DH plants homozygous for the transgene. Plant genome doubling after transgene integration allows the production of homozygous, true-breeding for the transgene plants, with high transgene dosage (often resulting in a more conspicuous phenotype) and with the availability of genetically fixed plant materials for the proper evaluation. The direct DNA-transfer method has been applied to many crops, such as rapeseed (Neuhaus et al. 1987; Miki et al. 1990; Jones-Villeneuve et al. 1995), barley (Bolik and Koop 1991; Olsen 1991), maize (Gaillard et al. 1992), and tobacco (Resch et al. 2009). Instead, polyethylene glycol-mediated poration and electroporation has been used for genetic transformation of isolated barley (Kuhlmann et al. 1991; Vischi and Marchetti 1997), maize (Fennel and Hauptmann 1992), and rapeseed (Jardinaud et al. 1993) microspores.

Gametoclonal variation

"Gametoclonal variation", the variation often observed among plants regenerated from cultured gametic cells and consisting in genetic (chromosome number and structures), morphological, and biochemical differences (Evans et al. 1984; Morrison and Evans 1987), is another opportunity to use Hs in crop breeding. While "somaclonal variation" is due only to the in vitro plant regeneration procedure from cultured cells or tissue (Larkin and Scowcroft 1981), "gametoclonal variation" results from both meiotic and mitotic division, and it can be induced by the cell culture and chromosome doubling procedures by segregation and by independent assortment (Morrison and Evans 1987; Huang 1996). Moreover, due to their homozygosity, the gametoclones display the direct expression of both dominant and recessive characters. When the new variation is

induced at the diploid level, the result will be a heterozygous plant.

A comparison of doubled haploid plants coming from male or female gametes is interesting because the method of generating doubled haploids seems to produce differences in doubled haploid field performances (Sam Noeum and Ahmedi 1982; San and Demerly 1984). Usually, the maternally derived homozygous regenerants show less gametoclonal variation for agronomical performances when compared to doubled haploids obtained from male gametes, other than to be generally more vigourous (Wernsman et al. 1989; Snape et al. 1988).

Marker-assisted selection (MAS) and haploidy

Haploid technology is particularly effective in accelerating breeding when combined with other biotechnologies, such as MAS (Forster et al. 2007b; Tuvesson et al. 2007; Werner et al. 2007). SSR-, SCAR-, CAPS-, RAPD-, ISSR-, AFLP-, retrotransposon-based markers and SNP, and also isoenzymes and protein profiles, are among the most used markers by breeders (de Vienne 2003). Co-dominant markers are particularly useful as they allow us to discriminate homo- and heterozygous individuals. In fact, other than being a powerful tool for genetic analysis and protection of germplasm, characterizing through fingerprinting unknown germplasm and protecting the new varieties, molecular markers are useful for planning new crosses, predicting novel useful gene combinations (Tuvesson et al. 2007). It is important that markers used in breeding should be closely linked to the target gene or, better yet, if it is directly in the gene/allele (Tuvesson et al. 2007). Furthermore, the introgression of desired traits from new gene sources can be accelerated applying marker-assisted backcrossing (MAB).

Marker-assisted selection combined with doubled haploidy, provides a short-cut in backcross conversion, a plant breeding method, effective but time-consuming, to select an elite line defective in a particular trait (Toojinda et al. 1998; Tuvesson et al. 2007; Werner et al. 2007). The elite line is crossed with a donor line possessing the trait to be introgressed; the F1 hybrid is then backcrossed to the elite line, and subsequent backcrosses clear most of the donor genome, except the portion carrying the desired trait. Doubled haploidy can be performed at different generations, depending on the efficiency of DH production: the more efficient the DH system, the earlier it can be applied (Forster et al. 2007b). The method has been used successfully to introgress stripe rust resistance in barley (*H. vulgare*) (Toojinda et al. 1998).

Werner et al. (2005) demonstrated that, by combining molecular markers and DHs, it is possible to stack resistance genes on top of each other, the so called pyramiding. Usually, pyramiding genes is a difficult and time-consuming task, without the use of molecular markers, but it is becoming increasingly important as new genes and markers become available.

The DHs are also advantageous in the technique of the bulked segregant analysis (BSA), which compares individuals from two extremes of a population distribution for a given trait (for example, disease resistance and susceptibility) with the aim of establishing marker–trait associations. As with quantitative trait mapping, the precision of BSA is dependent on robust phenotyping and the possibility to repeatedly analyse and genotype selected DHs to allow for the collection of consistent data from trials, replicated in the time and in the space. BSA and DH analysis has been successful in establishing MAS for disease and pest resistance, as well as for quality traits (Michelmore et al. 1991).

With the aim of establishing guidelines for the breeder to optimally balance competing economic efficiency and biological constraints, Campbell et al. (2001) have tested in wheat a genetic model for predicting the minimum acceptable population sizes required for minimal loss of genetic variation through genetic drift and needed to maintain genetic variation among DH plants following marker selection.

Somatic hybridization and haploidy

Haploid technology combined with somatic hybridization can help the breeders in different ways, for instance, to select resistant genotypes. Actually, dihaploids obtained from anther culture of somatic hybrids may be employed in the backcrosses for introgression of different pathogen resistance. For example, this particular method has been employed by Rokka et al. (1995) to produce, through anther culture, haploids derived from somatic hybrids between *Solanum brevidens* and *Solanum tuberosum* and by Rizza et al. (2002) to obtain androgenic dihaploid plants from somatic hybrids between eggplant (*Solanum melongena*) and *Solanum aethiopicum* as a source of resistance. Moreover, ploidy manipulation through anther culture of interspecific hybrids between *Helianthus annuus* and two wild hexaploid species (*H. tuberosus* and *H. resinosus*) was performed to transfer resistance to *Alternaria helianthi* from the two hexaploids to the cultivated sunflower (Sujatha and Prabakaran 2006).

Alternatively, protoplast fusion techniques can be utilized to combine the complete genome (nuclear and cytoplasmic) including all the dominant characters of different haploid lines. There is an important application for use of haploids in interspecific hybridization to overcome incompatibility barriers caused by the differences in ploidy levels and endosperm balance numbers. For instance in

potato (*Solanum tuberosum* L. ssp. *tuberosum*), the gene pool can be broadened and certain valuable traits, such as disease resistance characters from the wild solanaceous species, can be more efficiently introgressed into cultivated potato (Rokka 2009). Recently, sources of durable resistance to late blight are derived from *Solanum bulbocastanum* (Helgeson et al. 1998) and *S. nigrum* (Zimnoch-Guzowska et al. 2003). Väänänen et al. (2005) reported that even completely novel compounds could be introduced by use of combined genomes of haploid clones.

Genetics, genomics, and haploidy

Doubled haploidy is important in basic and applied genetic studies (Forster and Thomas 2005). In out-pollinating species, genetic analysis can be simplified by using at least one DH parent in the initial cross to produce a segregating population, as has already been successful done in vegetables such as the *Brassica oleracea* complex, namely cabbage, cauliflower, broccoli and Brussels sprouts (Sebastian et al. 2002), and the fodder grass *Lolium perenne* (Bert 1999). DH lines are a key feature in genomics, particularly in integrating genetic and physical maps, thereby providing precision in targeting candidate genes (Kunzel et al. 2000; Wang et al. 2001), and they have widespread application in fundamental quantitative genetics (Pink et al. 2008) and SNP discovery (Trick et al. 2009). Actually, the use of DHs have been very advantageous in establishing chromosomes maps in many species, notably barley (*H. vulgare*), rice (*Oryza sativa*), rapeseed (*B. napus*), and wheat (*T. aestivum*) (Forster and Thomas 2005) and in providing the greater part (in some cases more than 90%) of mapped genetic markers. In responsive crop species, such as barley, the process of producing a segregating DH population from an F1 hybrid to find marker–trait associations has become routine (Tuvesson et al. 2007).

To identify genes controlling a trait, it is possible to trawl through expressed sequence tags (ESTs) and to map their chromosome position relative to the examined trait. Actually, DHs have also played a pivotal role in establishing chromosomes maps in a range of species, particularly barley, rice, rapeseed, and wheat (Forster and Thomas 2005). Hs and DHs provide excellent material to obtain reliable information on the location of major genes and QTLs for economically important traits (Khush and Virmani 1996). For example, as reviewed by Dunwell (2010) in cereals, DH lines have been used in QTL mapping to study laccase enzymes in maize (Andersen et al. 2009), *Fusarium* head blight resistance in barley (Marchand et al. 2008), arsenic accumulation (Zhang et al. 2008a), agronomic traits (Lapitan et al. 2009), stem height (Ma et al. 2009), and cooking quality (Govindaraj et al. 2009) in rice, and agronomic traits (Chu et al. 2008), plant height (Zhang et al. 2008b), heading date (Zhang et al. 2008b), flour colour (Zhang et al. 2008c), phosphorus use efficiency (Su et al. 2009), zinc deficiency (Genc et al. 2009), and *Septori tritici* blotch resistance (Raman et al. 2009) in wheat.

Moreover, several genome sequencing programmes are using a haploid genome because of its simplified assembly, such as those regarding many perennial plants, like peach, coffee, pear, apple, and citrus (Dunwell 2010). Further, Yu (2009) stated that the exploitation of the genomic revolution in plant breeding also requires efficient protocols of haploid induction.

The DHs have an important role in the development and exploitation of structured mutant populations for forward and reverse genetics (Szarejko and Forster 2006). The reverse genetics strategy called Targeting Induced Local Lesions In Genomes (TILLING, McCallum et al. 2000; Perry et al. 2003) provides another method of linking genes to phenotypes, inducing a high frequency of mutations by chemical mutagen application, combined with the high throughput screening method for single nucleotide polymorphisms (SNPs) in the targeted sequence. Actually, mutants provide an effective means of discovering and validating gene function and to prevent the detection of false positives owing to inherent variation in the starting material; it is much better if the mutant populations derive from a homozygous line (Forster et al. 2007b). The basic strategy is to apply a mutagen (chemical mutagens are preferred because they produce point mutations and thereby functional mutants) to a selected line, usually to seed samples (M0). Once the mutagenic treatment has been applied, the material becomes the M1 generation, from which structured M2 plant and M3 family populations are developed (e.g. barley, Caldwell et al. 2004). DNA is normally extracted from M2 plants and scanned for mutation events (reverse genetics), and M3 families are screened for phenotypic variation (forward genetics). The strategy is dependent upon the detection of rare mutations within large populations, often comprising several thousand plants/families. It is therefore desirable that the starting material is as genetically pure as possible to minimize the detection of false positives (genetic variation/contamination in the starting material), and for this reason, DHs are ideal. To be employ for forward and reverse genetics, a DH population has to exhibit some characteristics: a DH responsive genotype; careful selection of mutagenic dose (to provide mutation events that can be captured in a reasonably sized DH population) and, if physical mutagens are used, co-location of tissue culture and mutation facilities (Forster et al. 2007a). Reversible male sterility and doubled haploid production are two effective technologies required for F1 hybrid breeding (Ribarits et al. 2009). Specifically, DHs are valuable in the innovative technology of reverse breeding (Dirks et al.

2003), involving the production of DHs from microspores of plants with no or limited recombination (obtained suppressing or preventing recombination, for instance, by gene knockout of key meiotic genes). The resulting recombinant inbred populations can be screened via molecular markers to identify those with complementary combinations of chromosomes to allow an original heterozygous parent of the DH to be reconstructed by hybridizing the two individuals. Consequently, different parents with different chromosome constitutions can be identified to reconstruct existing F1 hybrids. An innovative F1 hybrid seed technology, combining reversible male sterility and DH production and based on metabolic engineering of glutamine in developing tobacco anthers and pollen, has been developed (Ribarits et al. 2009).

Differently from the conventional use, aimed to developing uniform and homogeneous genotypes (Nitsch 1974), haploid technology has been recently used to exploit the inherent genetic diversity and high chromosome recombination within gametes with the purpose of studying the genetic and physiology of complex traits. For example, gametic embryogenesis described by Humphreys et al. (2007), particularly pollen embryogenesis, has been employed in grass breeding programmes to dissect complex genetic and physiological controls and to select useful gene combinations for breeding freezing tolerant grasses. Specifically, the use of pollen embryogenesis allowed the exploitation of the inherent genetic diversity and high chromosome recombination within gametes of *Lolium multiflorum* × *Festuca* spp. hybrids to deliver plant populations with a vast range of genotypic and phenotypic variations, producing regenerants with gene combinations difficult to recover through conventional backcross breeding programmes (Humphreys et al. 1998, 2001).

Conclusions

In a world in which almost a billion people suffer from food shortage and in a market constantly requiring new and different varieties, speed and efficiency are becoming increasingly important features for plant improvement. Haploid technology is a rapid system for developing homozygous lines, which are important biotechnological tools in breeding programmes, as well as in genetic and developmental studies. Research on gametic embryogenesis and H and DH production in plants is several decades old, and in those genotypes where these technologies have been successfully applied, improvement programmes are receiving significant advantages from them, shortening the time required to achieve the fixed goals. The exploitation of haploid and doubled haploids as a powerful breeding tool requires the availability of reliable and cost-effective tissue culture experimental procedures, and although significant advances have also been achieved in vegetable, fruit, ornamental, legume, woody, and medicinal species, there is not yet a universal protocol suitable for all crops, and the development of new techniques for doubled haploid induction is still required for many still recalcitrant genotypes.

However, recent renewed interest in haploidy research, due also to innovative end-use applications for DH technology, leading to a deeper understanding of the fundamental mechanisms of embryogenic induction in in vitro cultured male and female gametes and of formation of haploid plants, will contribute to improve the efficiency of gametic embryogenic for DH production in species already considered responsive and to reduce the number of recalcitrant genotypes.

In conclusion, gametic embryogenesis and haploid technology hold great promise for making a significant, low cost, and sustainable contribution to plant breeding, aimed at increasing farm productivity and food quality, particularly in developing countries and in an environmentally friendly way, helping to reduce the proportion of people suffering from chronic hunger and from diseases due to malnutrition. Therefore, it is desirable that significant investment in agricultural research be focused to support studies allowing for the effective deployment of gametic embryogenesis and haploid technology in the improvement of all species.

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