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Use of 2n Gametes in Plant Breeding

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1. Introduction

Genome doubling (polyploidization) has played a major role in the evolution and diversification of the plant kingdom and is regarded as an important mechanism of speciation and adaptation in plants (Otto & Whitton, 2000). The term ploidy refers to the number of basic chromosome sets (represented by 'x') present in a somatic plant cell (2n) or gamete (n). Scaling whole sets of chromosomes up or down is a powerful and commonly applied strategy to produce altered genotypes for breeding purposes.

Polyploids differ from their diploid progenitors in morphological, ecological, physiological and cytological characteristics. Their wider geographical distribution reflects the variety of their ecological tolerances (Carputo et al., 2003; Chen, 2007; Knight et al., 2005; Otto & Whitton, 2000; Soltis & Soltis, 2000; Thompson et al., 2004). Polyploids have breeding value as they can have broader and thicker leaves, larger flowers, longer internodes, fewer stems per plant, fewer inflorescences, higher vegetative yields, more compact plants, greater tolerance to environmental stress, higher (additive) resistance to several diseases, increased seed weight but fewer seeds or higher chlorophyll production. Doubling the chromosome number can rescue and stabilise interspecific hybrids that would otherwise show a high degree of sterility due to failure in meiosis. Furthermore, polyploidy might result in the development of sterile cultivars (e.g. triploids), loss of self-incompatibility, flowering time variation, changes in growth vigour or shifts in reproductive system (e.g. to asexual reproduction). Apomixis, an extreme form of reproductive modification, is commonly associated with polyploidy. Negative side effects of polyploidization might be infertility (which can also be a desired trait), brittle wood, watery fruit, stunting and malformation (Andruezza & Siddiqi, 2008; Baert et al., 1992; Barringer, 2007; Bretagnolle & Thompson, 1995; Briggs & Walters, 1997; Chen, 2007; Chahal & Gosal, 2002; Comai, 2005; Contreras et al., 2007; De Nettancourt, 1997; Eeckhaut et al., 2004; Grant, 1981; Gu et al., 2005; Kermani et al., 2003; Lamote et al., 2002; Otto & Whitton, 2000; Stebbins, 1971; Thomas, 1993; Van Huylenbroeck et al., 2000; Van Laere et al., 2011).

Several phenotypic characteristics have been used as an indirect measure of ploidy level. Polyploids frequently have larger pollen sizes, stomata sizes, and seeds than diploids but have slower developmental rates. Further, the number of chloroplasts in guard cells, leaf

area index, stomatal density, or pollen fertility might be related to the ploidy level (Aryavand et al., 2003; Kessel & Rowe, 1975; Mishra, 1997; Otto & Whitton, 2000; Vandenhout et al., 1995; Zlesak, 2009).

In general, polyploidy is accompanied by genome-wide changes in gene expression and epigenetic modifications. The genotypic and phenotypic differences are caused mainly by the increased cell size, gene dosage effect, allelic diversity (level of heterozygosity), gene silencing and genetic or epigenetic interactions (Andruezza & Siddiqi, 2008; Jovtchev et al., 2006; Kondorosi et al., 2000; Leitch & Bennett, 1997; Levin, 1983; Lewis, 1980; Mittelsten Scheid et al., 1996; Osborn et al., 2003; Pikaard, 1999).

Polyploids can be induced by two mechanisms. First, mitosis spindle inhibitors such as colchicine, oryzalin and trifluralin can be applied to create artificial (mitotic) polyploids (for a review see Dhooghe et al., 2011). Second, gametes with somatic chromosome numbers, also referred to as $2n$ gametes or (numerically) unreduced gametes are considered to be the driving force behind the formation of polyploids in nature (Bretagnolle & Thompson, 1995; Otto & Whitton, 2000).

Harlan & De Wet (1975) showed that almost all plant species produce $2n$ gametes in some frequencies and argued the importance of $2n$ gametes in the origin of polyploids. Although efforts to synthesize polyploids through the use of $2n$ gametes were performed much earlier (e.g. Skiebe 1958), it was assumed that $2n$ gametes occur only rarely and with little contribution to the origin of polyploids (Ramanna & Jacobsen, 2003). The use of $2n$ gametes in plant breeding, resulting in the establishment of sexual or meiotic polyploids, has been very useful for crop improvement (Ramanna & Jacobsen, 2003). These gametes combine the genetic effects of raised ploidy level with meiotic recombination, which makes them an attractive alternative for mitotic chromosome doubling. $2N$ gametes are an effective and efficient way to transmit genetic diversity (allelic variation) to cultivated forms, including both valuable qualitative and quantitative traits (Peloquin et al., 1999). More recently plant breeders have become interested in the practical use of $2n$ gametes in breeding programmes due to the new tools available for $2n$ gamete manipulation and insights into the genetic background of their formation.

This chapter presents a review of the recent advances in the practical breeding applications of $2n$ gametes. It addresses tools for detection, induction, and enrichment of $2n$ pollen, as well as the potential to engineer $2n$ gamete production in agricultural crops.

2. $2n$ gametes: mechanisms and genetic background

$2n$ gametes originate from meiotic aberrations. In a normal meiosis, one mother cell ($2n$) divides in 4 (n) daughter cells (tetrad formation). Aberrations during chromosome pairing, spindle formation (parallel spindle, tripolar spindle, fused spindle, sequential spindle or lack of spindle) or cytokinesis might result in the formation of triads, dyads or monads (Bretagnolle & Thompson, 1995; Dewitte et al., 2010c; Taschetto & Pagliarini, 2003). This finally results in pollen grains with the same DNA content ($2n$ in the case of dyads or triads) or a doubled DNA content ($4n$ in the case of monads) compared to the somatic $2n$ plant cell. Premeiotic and postmeiotic chromosome doubling and cytomixis have also been proposed as possible mechanisms for the production of $2n$ gametes (Bastiaanssen et al., 1998; Falistocco et al., 1995; Ghaffari, 2006; Lelley et al., 1987; Singhal & Kumar, 2008).

Two main categories of 2n gamete formation have been described: first division restitution (FDR), and second division restitution (SDR) (Bretagnolle & Thompson, 1995; Ramanna & Jacobsen, 2003; Zhou et al., 2008). In FDR, the pairing and/or the separation of the homologous chromosomes at meiosis I does not occur (univalent formation) or occurs only at low frequencies, while the second division occurs normally with the two sister chromatids of each chromosome moving to opposite poles. With the exception of cross-over segments, the resulting FDR gametes retain all parental chromosomes. In SDR, the pairing and the separation of the homologous chromosomes during meiosis I occurs normally (bivalent formation). In meiosis II, the centromeres of the half-bivalents divide, but the chromatids do not migrate to the poles. Finally, SDR gametes contain only half of the parental chromosomes as in normal gametes (random combinations), but each of these chromosomes is present twice. Besides these two main categories, several other meiotic aberrations exist where the final chromosome constitution in the microspores is equivalent to the FDR or SDR pathways as described above (Bretagnolle & Thompson, 1995). Less frequently reported are indeterminate meiotic restitution (IMR) and post meiotic restitution (PMR). IMR has been detected in lily (Lim et al., 2001), and shows characteristics similar to both SDR and FDR. PMR, where chromosomes duplicate after meiosis, was observed by Bastiaanssen et al. (1998) in potato. Genomic *in situ* hybridisation (GISH) has made detection of the restitution mechanism possible by monitoring the meiosis of hybrids and identifying the chromosomes of individual genomes in the sexual polyploid progeny.

Examples in maize (*Zea mays*; Rhoades & Dempsey, 1966), potato (*Solanum tuberosum*; Mok & Peloquin, 1975b; Veilleux, 1985; Watanabe & Peloquin, 1989), red clover (*Trifolium pratense*; Parrot & Smith, 1986), rye (*Secale cereale*; Lelley et al., 1987), alfalfa (*Medicago sativa*; Barcaccia et al., 2000; Ortiz & Peloquin, 1991) and banana (*Musa*; Ortiz, 1997) have shown the complexity of the genetic base of 2n gamete formation. Often, one major locus is responsible for 2n gamete formation while several other genes controls its frequency. Some authors tried to map 2n gamete production or developed molecular markers associated with 2n gamete production (e.g. Barcaccia et al., 2000, 2003; Zhang et al., 2007). However, research on the model plant thale cress (*Arabidopsis thaliana*) has led to many recent advances in elucidating the molecular mechanisms as well as the first genes in which mutations result in the production of viable 2n gametes (for a review see Brownfield & Köhler, 2011). d'Erfurth et al. (2008) were the first to successfully isolate and characterize a gene involved in 2n gamete production. The *Arabidopsis thaliana* *Parallel Spindle1* (*AtPS1*) gene is involved in abnormal orientation of spindles at meiosis II, which controls diploid 2n gamete formation in *Arabidopsis thaliana*. Parallel, fused or tripolar spindles are different phenotypic expressions of this gene. A similar male-specific dyad-triad phenotype was observed and characterised in another mutant called *jason* (De Storme & Geelen, 2011; Erilova et al., 2009). Mutants of two other genes, OSD and TAM (CYCA1;2), were shown to omit the second meiotic division in both male and female sporogenesis at high frequency resulting in the formation of both 2n pollen and egg cells (d'Erfurth et al., 2009, 2010).

3. Sources of 2n gametes

Three important sources of 2n gametes are commonly reported (Bretagnolle & Thompson, 1995), beginning with interspecific hybrids. In many genera interspecific or intergeneric hybrids have produced 2n gametes e.g. lily (*Lilium*; Barba-Gonzalez et al., 2005a; Lim et al.,

2001; Lim et al., 2004), Peruvian lily (*Alstroemeria*; Ramanna et al., 2003), wheat (*Triticum*; Xu & Joppa, 1995; Xu & Joppa, 2000; Zhang et al., 2010), *Impatiens* (Stephens, 1998), *Citrus* (Chen et al., 2008), rose of sharon (*Hibiscus*; Van Laere et al., 2009), clover (*Trifolium*; Meredith et al., 1995), and the *Festuca/Lolium* complex (Gymer & Whittington, 1973; Morgan et al., 1995). Frequently, these interspecific hybrids show abnormal or absent chromosome pairing and the presence of univalents, lagging chromosomes and chromosome bridges (Islam & Shepherd, 1980; Del Bosco et al., 1999; Trojak-goluch & Berbeć, 2003). Interspecific hybrids usually share two important features. First, both 2n eggs and 2n pollen are produced simultaneously by the same hybrid. Second, neither the two parents of the F₁ hybrids nor their (F₂) sexual polyploid progenies can produce 2n gametes in any notable frequencies (Ramanna & Jacobsen, 2003).

Meiotic mutants are a second source of 2n gametes. A mutation in a gene active in meiosis might disturb during spindle formation or cytokinesis, resulting in 2n gametes. As different genes are active within the micro- and macrosporogenesis, 2n pollen can be formed independent from 2n egg cells and vice versa. Meiotic mutants have been described in potato (Jongedijk et al., 1991; Peloquin et al., 1999; Ramanna, 1983), red clover (Parrot & Smith, 1984), alfalfa (Barcaccia et al., 2003), wheat (Jauhar, 2003; Roberts et al., 1999) and *Arabidopsis* (d' Erfurth et al., 2008; Yang et al., 1999), among others.

A third source are odd polyploids. For instance, crosses with triploids revealed that euploid gametes of triploids can be 1x, 2x or 3x (Husband, 2004; Van Huylenbroeck et al., 2005). Although these 2x gametes are not exactly 2n gametes (they are 3x gametes), they result in higher ploidy levels of the progeny and mostly act as a bridge between diploids and tetraploids (Köhler et al., 2010).

4. Practical use of 2n gametes in plant breeding

4.1 Detection of 2n gametes

2n gametes must be correctly identified when used in ploidy breeding programmes. Most detection techniques focus on pollen, since it is more convenient to isolate than egg cells. 2N pollen can be detected in four ways (Bretagnolle & Thompson, 1995): pollen size measurements, flow cytometric detection of pollen DNA content, analysis of the microsporogenesis and ploidy analysis of the progeny. In Table 1, an updated overview is given of the different methods used in several crops since Bretagnolle & Thompson's 1995 review. Only the last two of the above-mentioned techniques (ploidy and macrosporogenesis analysis) can be used to detect 2n egg formation. The frequency of 2n egg formation has often been estimated after crosses between 2x x 4x plants. A cross between a diploid seed parent and tetraploid pollen parent will only result in good tetraploid seed when 2n egg cells are present (Conicella et al., 1991; De Haan et al., 1992; Erazzu & Camadro, 2007; Estrada-Luna et al., 2004; Jongedijk, 1987; Lamote et al., 2002; Ortiz & Peloquin, 1991; Veronesi et al., 1986; Van Laere et al., 2009; Werner & Peloquin, 1987). In *Triticum-Aegilops* hybrids, Zhang et al. (2007a, 2010) used the selfed seedset as a good indication for the formation of 2n gametes.

An easy and commonly used method to screen for 2n pollen is searching for large pollen within a population. Large pollen has frequently been attributed to 2n pollen in many genera. This association is caused by the positive correlation between DNA content and cell

Detection method				Crop	Reference
PS	FC	AP	MS		
x			x	<i>Achillea borealis</i>	Ramsey, 2007
		x	x	<i>Actidinia spp.</i>	Yan et al., 1997
x		x	x	<i>Alstroemeria spp.</i>	Ramanna et al., 2003
x				<i>Anthoxanthum alpinum</i>	Bretagnolle, 2001
x	x	x	x	<i>Begonia spp.</i>	Dewitte et al., 2009
			x	<i>Brachiaria spp.</i>	Gallo et al., 2007
	x			<i>Brassica napus</i>	Pan et al., 2004
x		x		Cacti hybrids	Tel-Zur et al., 2003
		x	x	<i>Capsicum annuum</i>	Yan et al., 2000
x	x			<i>Cupressus spp.</i>	Pichot & El Maataoui, 2000
x	x	x		<i>Diospyros kaki</i>	Sugiura et al., 2000
x			x	<i>Diospyros spp.</i>	Xu et al., 2008
	x	x	x	<i>Durum wheat</i>	Jauhar, 2003
x	x	x	x	<i>Hibiscus spp.</i>	Van Laere et al., 2009
x			x	<i>Ipomoea batatas</i>	Becerra Lopez-Lavalle & Orjeda, 2002
	x	x	x	<i>Lilium spp.</i>	Lim et al., 2004
x	x	x		<i>Lilium spp.</i>	Akutsu et al., 2007
x		x		<i>Lilium spp.</i>	Barba-Gonzalez et al., 2004
x		x	x	<i>Lotus tenuis</i>	Negri & Lemmi, 1998
x				<i>Musa spp.</i>	Ortiz, 1997; Ssebulita et al., 2008
			x	<i>Paspalum spp.</i>	Pagliarini et al., 1999
			x	<i>Pfaffia spp.</i>	Taschetto & Pagliarini, 2003
			x	<i>Populus tomentosa</i>	Zhang & Kang, 2010
x			x	<i>Populus hybrid</i>	Wang et al., 2010
x			x	<i>Rhododendron spp.</i>	Jones & Ranney, 2009
x			x	<i>Rosa hybrida</i>	Crespel et al., 2006
x		x	x	<i>Rosa hybrida</i>	El Mokadem et al., 2002a
	x			<i>Rosa rugosa</i>	Roberts, 2007
x				<i>Rosa spp.</i>	Zlesak, 2009
x		x		<i>Trifolium pratense</i>	Simioni et al., 2006
x	x	x		<i>Tulipa spp.</i>	Okazaki et al., 2005
x			x	<i>Ziziphus jujube</i>	Xue et al., 2011

Table 1. Overview of studies on the detection of 2n pollen since Bretagnolle & Thompson's 1995 review. FC = Flow cytometry on pollen; PS = Pollen size measurements; AP = Ploidy analysis of progeny; MS = Microsporogenesis analysis

volume which in turn influences pollen diameter. In crops as Japanese persimmons (*Diospyros kaki*), banana, rose (*Rosa*) and sweet potato (*Ipomoea batatas*), the diameter of the 2n pollen was approximately 30% larger than that of the n pollen (Becerra Lopez-Lavalle & Orjeda, 2002; Crespel et al., 2006; Ortiz, 1997; Sugiura et al., 2000). In Chinese jujube (*Ziziphus jujube*) 2n pollen was more than 1.5 times larger compared to n pollen (Xue et al., 2011). The presence of large 2n pollengrains results in a bimodal pollengrain size

distribution instead of a normal distribution. Although the size distribution of normal and giant pollen grains show some overlap, a threshold value of the pollen grain size is often used to select individuals that produce $2n$ gametes (e.g. Crespel et al., 2006; Ortiz, 1997; Sugiura et al., 2000). The presence of large pollen only indicates the presence of $2n$ pollen but does not present proof of doubled DNA content. Another disadvantage of this screening technique is the broad overlap in size distribution between small and large pollen in some genera such as grasses. In these cases, the frequency of $2n$ pollen based on pollen size only is difficult to determine (Jansen & Den Nijs, 1993). A bimodal size distribution, on the other hand, can also be related to a population of small unviable and normal pollengrains as was observed in *Hibiscus* (Van Laere et al., 2009). Other methods are necessary to confirm the association between large and $2n$ pollen, and supplementary evaluation of pollen viability is necessary for breeding purposes. Besides pollen size also pollen shape (spherical instead of ellipsoidal) has been associated with the ploidy level, which simplifies the determination of $2n$ pollen (Akutsu et al., 2007; Dewitte et al., 2009; Ramanna et al., 2003).

A more stringent method is the direct quantification of nuclear pollen DNA using flow cytometry. To determine the DNA content of pollen, nuclei have to be released from pollen and purified from the pollen wall. Several enzymatical, chemical, mechanical and osmotical methods for nuclear isolation of pollen have been used in different plants, e.g. maize, *Plumbago zeylanica*, lily hybrids, tobacco (*Nicotiana tabacum*) and rape (*Brassica napus*) (Dupuis et al., 1987; Pan et al., 2004; Russel, 1991; Van Tuyl et al., 1989; Xu et al., 2002; Zhang et al., 1992). The presence of a complex outer exine layer on the pollen surface is the main obstacle in releasing the nuclei from pollen. This outer layer is a biopolymer that is highly resistant to enzymatic breakdown and hydrolytic decomposition in strong acid or alkaline media (Bohne et al., 2003). The isolation of nuclei from pollen is often difficult and the efficiency of nuclear isolation techniques must be investigated each time when a new genus is explored.

Flow cytometric analysis compares the DNA content of pollen nuclei to the DNA content of somatic leaf tissue. Pollen nuclei are expected to have only half of the DNA content (1C) compared to nuclei from somatic cells (2C) of the same plant. Consequently, $2n$ pollen have a nuclear DNA content equal to somatic cells. This is true for species as *Brassica napus* or *Triticum aestivum* (Pan et al., 2004). However, several reports on crops from the genus *Begonia* (Dewitte et al., 2009), *Lilium* (Van Tuyl et al., 1989), *Cupressus* (Pichot & Maâtaoui, 2000), *Hibiscus* (Van Laere et al., 2009) and *Rosa* (Roberts et al., 2007) have shown that pollen nuclei can be at the G2 phase of cell division, and have a temporary doubled DNA content (2C) which is equal to the DNA content of somatic cells. In *Begonia* for instance, analysis of normal (binucleate) pollen results in 2 peaks in a flow cytometric histogram at the 1C (vegetative nuclei) and 2C level (generative nuclei). If $2n$ pollen are present, a peak at the 4C level of the $2n$ generative nuclei can be observed (Dewitte et al., 2009).

Although flow cytometric screening of $2n$ pollen can be used routinely in breeding programmes, sample preparation for flow cytometric screening generally requires more time than for microscopic evaluation. Therefore, the use of flow cytometry can be limited to genotypes which produce pollen grains with highly variable sizes to confirm the occurrence of $2n$ pollen grains. Some quick physical techniques to isolate pollen nuclei, such as bead beating (Roberts, 2007) or chopping of pollen grains (Pichot & Maâtaoui, 2000; Sugiura et al., 2000; Van Laere et al., 2009; Van Tuyl et al., 1989), may speed up flow cytometric screening of $2n$ pollen.

Moreover, Dewitte et al. (2006, 2009) developed a nuclear isolation protocol which only releases nuclei from germination tubes. As a result, flow cytometric analysis is based only on viable pollen grains and no supplementary evaluation of pollen viability is necessary.

The presence of 2n pollen is associated with the occurrence of monads, dyads or triads during microsporogenesis, except when 2n gamete formation is the result of pre- or postmeiotic restitution. Analysis of microsporogenesis may therefore provide an alternative method to confirm the presence of 2n pollen but this method does not provide any information about pollen viability. Moreover, what is observed in the meiocytes is not necessarily reflected in the mature pollen (Dewitte et al., 2010a), since the production of n and 2n pollen also depends on balanced chromosome segregation during meiosis and further maturation steps after meiosis. Scoring the meiotic products is thus not the best method to determine the frequency of the final 2n pollen. Although these observations give no information about the viability of 2n pollen, they provide insight in the mechanisms (FDR, SDR) behind 2n gamete formation. The identification of the mechanisms behind the formation of 2n gametes is however complex, because different cytological mechanisms may operate within one individual. The use of molecular cytological techniques (genomic in situ hybridization, GISH or fluorescent in situ hybridization, FISH) or marker analysis (such as amplified fragment length polymorphism, AFLP) on meiocytes or polyploid progeny may provide more accurate or additional information on the mechanisms behind 2n gamete formation (Barba-Gonzalez et al., 2005b; Crespel et al., 2002; Lim et al., 2001). Molecular cytological approaches have been successfully used in the case of allopolyploids, where the constituent genomes can be clearly discriminated. This includes the unequivocal identification of not only genomes and individual alien chromosomes but also recombinant segments in the sexual polyploid progenies. Through DNA in situ hybridisation, genomes of allopolyploids can be more critically assigned and intergenomic translocations and recombinations can be detected such as in *Gasteria-Aloe* hybrids (Takahashi et al., 1997), *Alstroemeria* species (Ramanna et al. 2003) and *Lilium* species (Barba-Gonzalez et al., 2005; Karlov et al., 1999; Lim et al., 2001). As such, GISH can also be used to discover the mechanism of 2n gamete formation (Karlov et al., 1999).

Finally, ploidy analysis of the progeny (usually using flow cytometry) can reveal the presence of 2n gametes in parent plants. Progeny analysis has the advantage of indicating the existence of viable 2n pollen in parent plants, but 2n pollen in parent plants may remain unnoticed because of preferential pairing between normal gametes. This technique has the disadvantage of being very time consuming with no guarantee of information about the production frequency in the parent plant (Bretagnolle & Thompson, 1995) due to possible differences in pollen viability, germination speed or pollen tube growth between n and 2n pollen.

Although different techniques can be used to detect 2n pollen, frequencies should be considered carefully because they depend on the method used (Dewitte et al., 2009). Moreover, frequencies may vary in different populations, within a certain genotype and during time (season) due to environmental fluctuations (Bani-Aameur et al., 1992; Crespel et al., 2006; McCoy, 1982; Ortiz et al., 1998; Ortiz & Vulsteke, 1995; Parrott & Smith, 1984; Pécrix et al., 2011; Veilleux et al., 1982). In Table 2, some recently published data on 2n gamete frequency in different (hybrid and non hybrid) populations are given. In general, the proportion between normal and 2n gametes over an at random sampled population (the mean population frequency, F_m) is low, close to 1%. However, the proportion of plants

producing $2n$ gametes in a population (population frequency, F_p) as well as $2n$ gametes formation within an individual genotype (individual frequency, F_i) can be very variable. In several reports, F_p is higher than 10% and in some genera as *Ipomoea*, poplar (*Populus*) or *Pfaffia*, F_p values are noticed higher than 80%. A similar conclusion can be drawn from F_i , where values are reported from less than 1% up to 100% in *Begonia* (Dewitte et al., 2009).

As a general rule, it appears that approximately 0.1 to 2.0% of the gametes in a nonhybrid population are expected to be unreduced (Ramsey, 2007). However, the mean frequency of $2n$ gametes found in studies of hybrids (27.52%) was nearly a 50-fold greater than in nonhybrids (0.56%) (Ramsey and Schemske, 1998). Hence, the chance to find individuals that produce $2n$ gametes increases when hybrids are investigated.

Crop	No of genotypes	F_p^1 (%)	F_i^2 (%)	F_m^1 (%)	Reference
<i>Achillea borealis</i>	50-75	34.7-50.0	15.8	0.03-0.54	Ramsey, 2007
<i>Anthoxanthum alpinum</i>	31-78	9-12.8	39.5	0.8-1.3	Bretagnolle, 2001
<i>Begonia spp.</i>	70	14.3	100	-	Dewitte et al., 2009
<i>Hibiscus spp.</i>	15	13.3	10	-	Van Laere et al., 2009
<i>Ipomoea batatas</i>	64	86	84.2	-	Becerra Lopez-Lavalle & Orjeda, 2002
<i>Lolium perenne</i>	154	9.7	-	-	Lamote et al., 2002
<i>Musa spp.</i>	156	14-56	-	-	Ortiz, 1997
<i>Paspalum spp.</i>	112	46.4	-	-	Pagliarini et al., 1999
<i>Pfaffia spp.</i>	14	100	18.9	-	Taschetto & Pagliarini, 2003
<i>Populus tomentosa</i>	224	97.3	21.9	1.8-7.5	Zhang et al., 2007b
<i>Rosa spp.</i>	53	26.4-50.9	9.6	1-2	Crespel et al., 2006
<i>Solanum okadai</i>	118	20.3	5	-	Camadro et al., 2008

Table 2. Examples of $2n$ gamete frequencies in different crops (since Bretagnolle & Thompson's 1995 review). F_p : population frequency; F_i : individual frequency; F_m : mean population frequency. ¹Two values represent a frequency interval in which the value is dependent on the population or season. ²The highest reported value is represented

4.2 Induction of $2n$ gametes

To date, the major drawback to use $2n$ gametes in plant breeding is that only a minority of genotypes regularly produce $2n$ gametes. Identifying such genotypes in the breeding stock

thus requires a great deal of screening. Additionally, not many superior genotypes produce these gametes. Recently different attempts, with variable success, were made to induce these gametes in any genotype of interest. An overview of the methods used to induce 2n gametes is given in Table 3.

Induction method	Crop	Reference
N ₂ O	<i>Begonia spp.</i>	Dewitte et al., 2010
	<i>Lilium spp.</i>	Akutsu et al. 2007; Barba-Gonzales et al. 2006; Kitamura et al., 2008; Sato et al., 2010
	<i>Tulipa spp.</i>	Okazaki et al. 2005
Trifluralin	<i>Begonia spp.</i>	Dewitte et al., 2010
	<i>Zea Mays</i>	Kato, 1999
Colchicine	Chinese cabbage	Zhong et al., 2010
	<i>Lilium spp.</i>	Wu et al., 2007
	<i>Populus hybrid</i>	Li et al., 2008
	<i>Strelitzia reginae</i>	Xiao et al., 2007
Temperature	<i>Lilium spp.</i>	Lokker et al., 2004
	<i>Rosa spp.</i>	Pécricx et al., 2011
EMS	<i>Arabidopsis thaliana</i>	De Storme et al., 2007; De Storme & Geelen, 2011
GA ₃	<i>Carthamus tinctorious</i>	Kumar & Srivastava, 2009

Table 3. Methods used to induce 2n gamete formation

N₂O treatments have been reported to be an effective way to induce 2n gametes (both 2n pollen and egg cells) in tulip (*Tulipa*), *Lilium* and *Begonia* (Akutsu et al., 2007; Barba-Gonzales et al., 2006; Dewitte et al., 2010b; Okazaki et al., 2005). N₂O is suitable for treating organs inside tissues as the gas simply permeates the tissue, thereby protecting the tissues from harmful after-effects as soon as the gas is released (Kato & Geiger, 2002; Östergren, 1954). N₂O treatments are performed in a pressure tolerant vessel at 6 bar for 24 or 48h on bulbs (when microsporogenesis occurs inside the bulb) or flower buds at the stage of meiosis. In the latter case, the stage of meiosis can be estimated by the size of the flower bud (Akutsu et al., 2007; Barba-Gonzalez et al., 2006; Dewitte et al., 2010b). Consequently, bud size has been used as a criterium to start N₂O treatments. Akutsu et al. (2007) showed that effects were optimal when treatments started during pollen mother cell progression to metaphase I. By applying this technique, male sterile hybrids may produce fertile 2n gametes after treatments, but the efficiency of the treatment seems to be genotype specific (Barba-Gonzalez et al., 2006; Dewitte et al., 2010b). In *Begonia* for example, viable 2n pollen could be induced in the male sterile hybrid *B. schmidtiana* x *B. cucullata* in 14 of the 49 treated flowers, while no 2n pollen were induced in *B. fischeri* with a similar number of flowers treated.

The small number of studies on N₂O induction of 2n gametes calls for additional testing in other genera. Detailed studies on the exact mechanism of N₂O mediated chromosome doubling during meiosis are also limited, but it has been suggested that N₂O disrupts the spindle mechanism in both mitosis (Dvorak et al., 1973; Kato & Birchler, 2006; Kihari & Tsunewaki, 1960; Östergren, 1954) and meiosis (Akutsu et al., 2007; Barba-Gonzalez et al., 2006; Okazaki et al., 2005). Consequently, aneuploidy is frequently reported after treatments. In *Lilium*, Barba-Gonzalez et al. (2006) showed that N₂O fumigation produced mainly first division restitution (FDR) gametes, indicating a disruption in meiosis I. Furthermore, Kitamura et al. (2009) showed in *Lilium* that microtubules were depolymerised during metaphase I, which prevented chromosomes from moving to the poles.

Dewitte et al. (2010b) tried to induce 2n pollen by submerging flower buds of *Begonia* in a trifluralin solution. Their treatments resulted in a disturbed meiosis and finally in 4n gametes but no progeny with raised ploidy level could be obtained after crosses with these gametes. Another way to increase the ploidy level in pollen was achieved in maize by spraying tassels with a trifluralin solution before flowering. As such, the generative nucleus was mitotically arrested and viable bicellular pollen was obtained (Kato, 1999). In some genera, flower buds were treated with colchicine (Table 2). These treatments resulted in the induction of both 2n pollen and 2n egg cells, and polyploid progeny was established after crosses with the treated plants.

Some authors tried to induce 2n gametes by manipulation of the temperature. Lokker et al. (2004) exposed four complete sterile lily genotypes to heat shock treatments and observed that three of the four genotypes became fertile by the production of viable 2n gametes. Also Pécrix et al. (2011) observed a production of 2n gametes up to 24.5% in *Rosa spp.* through an exposition of a high temperature gradient, but the range of efficient temperatures is narrow and should be applied during early meiosis. The disturbed meiosis resulted in the production of dyads and triads which mainly resulted from spindle misorientations in meiosis II. This supports the hypothesis that polyploidization events could have occurred in adverse conditions and may be of importance during species evolution.

Interspecific hybridisation is another way to induce 2n gametes. The occurrence of 2n gametes has been reported frequently in interspecific hybrids (see above). For example, in a breeding program between the tetraploids *Hibiscus syriacus* and *Hibiscus paramutabilis*, 2 of the 5 F₂ hybrids produced between 6 and 10% 2n pollen. These hexaploid F₂ hybrids were all raised in ploidy level compared to the tetraploid F₁ hybrids as a result of 2n egg cells in the F₁ population (Van Laere et al., 2009). However, even in interspecific hybrids, 2n gametes are rather exception than rule and many hybrids may be screened to detect 2n gamete producing genotypes. In *Lilium*, only 12 of the 708 (1.2%) OA F₁ hybrids produce 2n gametes on a regular basis, while the other seedlings were sterile (Barba-Gonzales et al., 2004).

Different attempts were made to increase the frequency of 2n pollen in genotypes which produce a low number of 2n pollen. Specific efforts have been performed using temperature variation, genetic selection, velocity sedimentation or pollen sieving (Table 4). For example, by using genetic selection, Negri & Lemmi (1998) were able to increase the frequency 2n pollen (Fi) in *Lotus tenuis* from 0-13% in the natural populations to 47.5-77.6% in the selected individuals. A further increase in frequency with a factor 1.5 to 3 could be obtained in a warm chamber.

Increase method	Crop	Reference
Temperature	<i>Begonia spp.</i>	Dewitte, 2010a
	Loquat tree	Wang et al., 2003
	<i>Lotus tenuis</i>	Negri & Lemmi, 1998
	<i>Prunus persica</i>	Ye et al., 2010
	<i>Prunus spp.</i>	Zhang & Liu, 1998
	<i>Rosa spp.</i>	Crespel et al., 2006
Velocity sedimentation	<i>Solanum phureja</i>	McHale, 1983; Veilleux & Lauer, 1981; Werner & Peloquin, 1987
	<i>Solanum spp.</i>	Simon & Sanford, 1990
Pollen sieving	<i>Cyclamen persicon</i>	Takamura & Miyajima, 2002
	<i>Diospyros kaki</i>	Sugiura et al., 2000
	<i>Lilium spp.</i>	Okazaki et al., 2005
	<i>Solanum spp.</i>	Eijlander, 1988
Genetic selection	<i>Lotus tenuis</i>	Negri & Lemmi, 1998
	<i>Medicago sativa</i>	Tavoletti et al., 1991b; Calderini & Mariani, 1997; Mariani et al., 2000
	<i>Solanum</i>	Ortiz & Peloquin, 1992
	<i>Trifolium pratense</i>	Parrot & Smith, 1986

Table 4. Overview of methods used to increase the frequency of 2n gametes

4.3 Use of molecular tools to engineer 2n gamete formation

The isolation of genes involved in 2n gamete production opens the way for new strategies in plant breeding programmes. More specifically, techniques that involve knockdown of RNA levels, such as RNA interference (RNAi), virus induced gene silencing (VIGS) or mutagenesis of the encoding gene using techniques such as site-directed mutagenesis could be used to knock down the level of specific proteins which play a role in the unreduced gamete formation (Brownfield & Köhler, 2010). The translation of this knowledge from the model plant *Arabidopsis* into plant breeders work still has to start. However, the vision of crop generation via designed gametes is becoming realistic.

Potential strategies to mutate genes active in meiosis and induce 2n gamete producing genotypes also include general mutagenesis strategies using chemicals such as ethyl methane sulphonate (EMS) (used in *Arabidopsis* to create 2n gametes; De Storme et al., 2007), random insertional mutagenesis or irradiation of seeds or buds (Shu-Ye. & Srinivasan, 2010).

5. Role of 2n gametes in plant breeding

2N gametes have already been used to create new cultivars at higher ploidy levels as well as creating a bridge to transfer desirable genes from wild diploid species into the cultivated polyploid gene pool (Carputo et al., 2000 Peloquin & Ortiz, 1992). Although 2n gametes have been documented in several genera, they have been extensively investigated in potato (Den Nijs & Peloquin, 1977; Mok & Peloquin, 1975a; Peloquin et al., 1999), rose (Crespel et

al., 2002; Crespel et al., 2006; El Mokadem et al., 2002a; El Mokadem et al., 2002b;), lily (Barba-Gonzalez et al., 2005a, 2005b; Lim et al., 2001a; Lim et al., 2004), and alfalfa (Tavoletti et al., 1991a; Tavoletti et al., 1991b; Barcaccia et al., 2003). The $2n$ gametes have in turn contributed to these crops' breeding programmes. For example, ploidy manipulations have been used in potato (*Solanum tuberosum*) breeding for many decades. Cultivated potatoes are tetraploid but most wild species are diploid. Haploidisation techniques can create dihaploids from cultivated potatoes. Via a series of hybridisations between selected dihaploids and $2x$ species, desirable agronomic traits from wild and closely related species can be captured. These dihaploids (producing $2n$ gametes) can then be introduced to tetraploids via interploidy crosses between $2x$ and $4x$ plants. The most successful breeding scheme for potatoes involves obtaining $4x$ progeny from $4x-2x$ crosses, where the $2x$ parent forms $2n$ pollen via the meiotic mutant *ps* (Peloquin et al., 1999). Several ploidy series have been developed in potato by using haploidisation and sexual polyploidization techniques, ranging from the monoploid to the hexaploid level (Carputo & Barone, 2005).

Other examples of the usefulness of $2n$ gametes for crop improvement in plant breeding have been demonstrated in *Alstroemeria*, carnation (*Dianthus*), primrose (*Primula*), *Triticum*, cassava (*Manihot*), blueberry (*Vaccinium*), cocksfoot grasses (*Dactylis*), *Lotus* and *Trifolium* (Carroll & Borrill, 1965; Hahn et al., 1990; Hayashi et al., 2009; Lyrene et al., 2003; Negri & Veronesi, 1989; Nimura et al., 2008; Parrot & Smith, 1984; Ramanna et al., 2003; Zhang et al., 2010), among others. $2N$ gametes also occur after haploidisation, which is useful for scaling the ploidy level upward again without artificial ploidy doubling (e.g. El Mokadem et al., 2002b; Nelson et al., 2009).

One advantage of $2n$ gametes is one-step triploid generation. Breeding for triploids may become an aim in itself. In *Citrus* for example, seedlessness is one of the most important characteristics in mandarin oranges. *Citrus* triploid plants can be recovered by sexual hybridisation of diploid plants as a consequence of the formation of $2n$ gametes at low frequency (Aleza et al., 2010). If breeding for triploids is not the aim of the breeding programme, further crosses are required to achieve the tetraploid or higher ploidy levels. For this purpose, the production of $2n$ gametes should be inherited from the diploid to the triploid plant. Several examples have been described where triploid plants produce $2n$ gametes, and often these are the only viable source of gametes. Crosses with these triploids resulted in tetraploid progeny, and even in this tetraploid progeny, $2n$ gamete production was observed (Brown, 1988; Dewitte et al., 2010d; Dweikat & Lyrene, 1988; Mok et al., 1975).

Plant breeding seeks to maximize the heterozygosity in the generations used for commercial production, and also polyploid induction will only contribute to plant improvement if substantial heterozygosity can be incorporated (Sanford, 1983). Higher heterozygosity can be achieved at the polyploid rather than at the diploid level. This is because polyploids have a greater probability of possessing three or more different alleles at the same locus whereas diploids only have two (Bingham, 1980; Lewis, 1980; Sanford, 1983). The heterozygosity present in the polyploid depends on the heterozygosity present in the parent plants and the mechanism behind $2n$ gamete formation. In general, FDR pollen are more important in producing heterozygous hybrids because of the highly heterozygous $2n$ gametes formed (Bretagnolle & Thompson, 1995). In FDR, each gamete is, except for recombinant fragments, identical to the somatic cell and thus contains a high level of heterozygosity. In SDR, chromosome assortment is random (as in normal gametes) which results in a very

heterogeneous population of gametes, but with a lower heterozygosity within one cell. For breeding purposes, 2n gametes of the FDR type are more advantageous than those obtained by SDR for transferring parental heterozygosity (Barcaccia et al., 2000; Barcaccia et al., 2003), although Hutten et al. (1994) could not confirm this FDR superiority for all agronomic characteristics investigated in potato. By using molecular techniques as AFLP, random amplification of polymorphic DNA (RAPD) or isozyme analysis, the heterozygosity transmitted through 2n gametes can be calculated. Several reports on *Solanum spp.* indicate that FDR gametes transmit roughly 70-80% of the parental heterozygosity, whereas this is only about 30-40% for SDR (Barone et al., 1995; Douches & Quiros, 1988; Werner & Peloquin, 1991a; Werner & Peloquin, 1991b). These values were also reported in other crops: roses, ryegrass, *Begonia* and *Vaccinium darrowi* (Chen et al., 1997; Crespel et al., 2002; Dewitte et al., 2010a; Vorsa and Rowland, 1997; Qu & Hancock, 1995). In general FDR is more than twice as effective in transmitting heterozygosity as SDR (Peloquin et al., 2008).

Ramanna & Jacobsen (2003) report that sexual polyploids have been much more useful for crop improvement than mitotic polyploids. Beuselinck et al. (2003) has shown that 2n gametes from *Lotus glaber* may aid intercrossing with *Lotus corniculatus* to produce progeny with a greater heterotic advantage than progeny obtained from the colchicine induced tetraploid (4x) *Lotus glaber*. In alfalfa, McCoy & Rowe (1986) showed better breeding value of 2n gametes from the diploids than n gametes from chromosome doubled tetraploids. Lim et al. (2001) has shown that recombinant chromosomes were present in 2n gametes in contrast to 2x gametes from mitotically doubled plants. Hence, 2n gametes have great potential to selectively introgress certain characteristics. The genetic consequences of 2n gametes indicate that sexual polyploidization results in greater variability, fitness and heterozygosity than does somatic doubling (Carputo et al., 2003).

One potential strategy to maximize heterozygosity in polyploids is analytic breeding: breeding for superior genotypes at the diploid level followed through sexual hybridisation using 2n gametes. The potential of analytical breeding to maximize heterozygosity and intergenomic recombination has been shown in *Lilium* using GISH. In traditional methods, mitotically doubled tetraploids are crossed with a diploid to produce triploids. However, in the tetraploid no recombination is expected to occur due to autosyndetic pairing. This results in a narrow selection of cultivars from mitotic polyploidization. In contrast, analytic breeding strategies allow intergenomic recombination to produce triploid *Lilium* varieties (Khan et al., 2009; Khan et al., 2010). Other examples of analytical breeding for crop improvement are vegetatively propagated species such as potato, sweet potato, cassava, among roots and tubers, and plantain/banana (Ortiz, 2002).

6. Conclusions

The exploitation of 2n gametes creates a plethora of opportunities for practical breeding. In general, several reports show that a) 2n gametes are mostly easy to detect, although this depends on the method used, b) 2n pollen may transmit a high level of heterozygosity (genetic variation) to the progeny, c) the ability to produce 2n gametes may be passed on to the progeny and d) 2n gamete production can be induced by artificial techniques. Besides, recent progress in identifying the genes and molecular mechanisms involved in 2n gamete production offers breeders new opportunities to design new tools and techniques to engineer 2n gamete production in specific crops and plants. The increasing reports and

knowledge about the practical use of 2n gametes in several crops and their genetic consequences show that 2n gametes are recognized as a very important tool in plant breeding. With ongoing research at the molecular level and research towards efficient methods to induce 2n gametes, the importance of 2n gametes for plant breeding is sure to increase.

7. References

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Modern plant breeding is considered a discipline originating from the science of genetics. It is a complex subject, involving the use of many interdisciplinary modern sciences and technologies that became art, science and business. Revolutionary developments in plant genetics and genomics and coupling plant "omics" achievements with advances on computer science and informatics, as well as laboratory robotics further resulted in unprecedented developments in modern plant breeding, enriching the traditional breeding practices with precise, fast, efficient and cost-effective breeding tools and approaches. The objective of this Plant Breeding book is to present some of the recent advances of 21st century plant breeding, exemplifying novel views, approaches, research efforts, achievements, challenges and perspectives in breeding of some crop species. The book chapters have presented the latest advances and comprehensive information on selected topics that will enhance the reader's knowledge of contemporary plant breeding.

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