Chapter 10
Chromosome Engineering for High Precision Crop Improvement

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Abstract Logarithmically increasing population and steadily changing climatic conditions have created a threatening situation of food insecurity worldwide and pose a challenge to breeders. In view of the narrow genetic background of the cultivated crop species, it has become imperative to broaden their genetic base by introgressing alien genes. However, monitoring the introgression(s) is indispensable for accelerated and high precision crop improvement. This chapter reveals the application of various innovative approaches like haploid inducer genes and chromosome elimination-mediated doubled haploidy breeding in barley, maize, wheat and potato required for the acceleration of breeding endeavours. It also covers the strategic chromosome engineering techniques needed for the alien chromatin introgression in wheat and further monitoring by use of novel molecular cytogenetic tools, including GISH and FISH for the targeted genetic upgradation with high precision.

Keywords Wheat · Barley · Wheat × maize · Wheat × *Imperata cylindrica* · Haploid inducer genes · GISH · FISH

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10.1 Introduction

The world population is estimated to reach 9.1 billion by the middle of this century, 34 % higher than today, which will mainly comprise of an urban population (nearly 70 %) compared to 49 % today. Thus, the consumers will be far more than the food growers. Use of modern agricultural practices and development of elite wheat and rice cultivars revolutionized food production in the 1960s, resulting in a hike in crop yields. This period was commonly known as the 'Green revolution', but since the past few decades a plateau has been observed. Limitations of land resources, steadily changing climatic conditions and continuously evolving pathogens have further complicated the situation. To feed the logarithmically growing richer population, the scientific community across the world will have to join hands to produce elite crop cultivars with better adaptability in diverse environmental conditions. The focus of breeders is the sustainable genetic enrichment of crops in an accelerated and précised manner to address the upcoming issues of endlessly growing demands for food, feed, fodder, fuel, fibre and pharmaceuticals.

Traditionally, improvement in crop yield was achieved through hybridization among the related species and selection thereafter, however, the introduction of novel biotechnological tools has overcome crossing barriers and has led to the stable introduction of newer genes into the crops. Manipulation at the DNA level, commonly called 'genetic engineering', leading to the creation of improved plants with wider adaptability in diverse circumstances has instigated a new era in crop improvement. Introduction of a whole new genome (synthetic polyploids), addition or replacement of a complete chromosome (addition or substitution lines), or a small segment of alien chromatin (introgression) to the chromatin of crop species is collectively known as 'chromosome engineering', which creates variation at the chromosome level. Segregation in further generations can lead to loss of the newly introduced variations, so this has to be fixed immediately. Doubled haploidy breeding not only results in the attainment of a homozygous population in just one step but the manipulation is also integrated stably in the genetic complement of the crop. DH breeding accompanied with marker assisted selection can result in upgradation of elite cultivars with high precision in a very short time span.

This chapter summarizes haploid induction technology and chromosome engineering in a comprehensive manner, which may be of prospective use for future breeders.

10.2 Development of Haploid Plants via Chromosome Engineering

The sporophytes that contain gametic chromosome numbers are generally referred to as haploids, while the doubled haploids are the genotypes produced as a result of doubling of chromosomes in a haploid organism. Haploids originate either

spontaneously or can be induced artificially. The former case is a rare occurrence and is of less practical utility. Since the discovery of spontaneous haploid plants in *Datura* by Belling and Blakeslee in 1922 and induction of haploid plantlets via anther culture by Guha and Maheshwari in 1964, a lot of efforts have been invested to understand the phenomenon deeply and establish protocols for haploid induction in different crop species. Development of haploids and doubled haploids has various applications in crop improvement, the most important being the recovery of complete homozygous lines in just 2 years, thereby saving time and energy. Although haploids were first reported in the 1920s, they were not put to use till the 1950s due to unavailability of the chromosome doubling mechanism. After the discovery of colchicine as chromosome doubling agent the practical utility of haploids in crop improvement increased manifold.

10.2.1 Haploid Inducer Genes

Apart from spontaneous development of haploids as a result of meiotic irregularity, there are certain genetic factors identified that lead to development of haploids of higher frequency than expected via natural induction. Such genetic factors are commonly known as 'haploid induction genes' and the special genetic stocks or plants are referred to as 'inducers'. The haploid induction genes are commonly found and used in barley and maize.

10.2.1.1 Haploid Initiator Gene (*hap*) in Barley

The haploid inducing phenomenon in barley was described by Hagberg and Hagberg in 1980 after isolating a haploid inducing mutant from a mutant stock developed by Gustafsson and his associates (1960). The original mutant was developed in 'Bonus' variety of barley by giving 0.3 % ethyl methane sulphonate (EMS) treatment. The resulting mutant was a *tigrina* type chlorophyll mutant. The chlorophyll mutation was controlled by *tigrina* locus (*tig*) which revealed a balanced inheritance of 1:1 upon selfing of green plants due to the presence of another genetic factor responsible for male sterility (*let*). The cytological analysis of *tigrina* and green plants revealed that some of the plants were haploids and led to the isolation of haploid mutants having only the haploid induction factor (*hap*) and not the chlorophyll deficiency (*tig*) or male sterility (*let*) controlling factors.

The *hap* initiator gene is a mutation that leads to egg parthenogenesis accompanied by normal endosperm development producing normal appearing seed. After pollination, the male sperm nuclei take about 1 h to reach the synergid cells; other nuclei reach polar nuclei and form triploid endosperm. In *hap/hap* and *hap/+* genotypes, the egg cell is not always reached by the other sperm nucleus. Thus, in a *hap/hap* plant, evidently about half of the eggs stay unfertilized, and some of these

develop into haploid embryos. The *hap* initiator gene controls survival or abortion of abnormal embryos and endosperms.

The haploid initiator gene is active in both heterozygous and homozygous conditions, though the frequency of haploid plant production can be four times greater in homozygous *hap/hap* plants. Hagberg and Hagberg (1980) also reported that the *hap* mutation was an incomplete dominant mutation since the recovery of haploids in heterozygous (*hap*/+) mutants is nearly four times less than those of homozygous (*hap*/*hap*) plants. The *hap* gene was reported to influence double fertilization in 10–14 % (haploid induction frequency) of the selfed seeds (Hagberg and Hagberg 1981). The frequency of haploid occurrence is highly influenced by the genotype and also by the environment. Using the *hap* system with marker genes, breeders need not use the embryo culture technique. However, they have to make a large number of crosses—a greater number than is needed using the *Hordeum bulbosum* technique (Hagberg and Hagberg 1987).

A unique advantage of the *hap* method is that the haploid embryos do not require in vitro culture as seed development is relatively normal. However, the disadvantages of the technique are that (a) spontaneously doubled haploids cannot be easily distinguished from hybrid embryos and (b) the technique is limited to genotypes having the *hap* gene.

The technique of doubling the barley haploids is well developed and it should be a fairly simple procedure to isolate series of haploid plants in the F_2 generation and double their chromosome number. The question is whether the frequency of haploids will be sufficiently high to yield enough homozygous breeding lines for the selection programme. However, a further advantage of the *hap/hap* method over the *H. bulbosum* technique lies in the segregation of *hap/hap* genotypes in the F_3 and subsequent generations, with further possibilities for crossing over to occur between linked loci that are still heterozygous. This will allow for new recombinations to occur in each generation instead of just the F_1 generation, as is the case for the *H. bulbosum* technique. This is also under test at present.

10.2.1.2 Maize Haploid *Inducer* Line

Haploid induction in maize, particularly using in vivo mode of haploid induction, became a widely used tool in maize research and breeding during the past decade. The technology is used to develop doubled haploid (DH) mapping populations, and analyse linkage disequilibrium and haplotype/trait associations. The first haploid maize plant was reported by Randolph as early as 1932; however, it was only more than a decade later that Chase (1947, 1951) reported the spontaneous haploid induction rate in maize of 0.1 % and suggested that haploids could be used for line development in hybrid breeding. During initial works on induction of haploids in maize, the low recovery of spontaneous haploid induction posed a major hurdle in utilization of the available technique in development of maize haploids and their utilization in maize improvement programmes. However, looking into the potential of haploid plants in genetic studies as well as maize improvement, a number of

researchers worked on identification of efficient haploid inducing stocks. With more divergent germplasm involved and more refinement in the haploid generation protocols, the haploid recovery has been gradually yet significantly enhanced to the tune of 10 % (Chalyk 1999; Sarkar et al. 1994; Shatskaya et al. 1994; Zhang et al. 2008).

The in vivo haploid induction in maize can be categorized into two classes, maternal and paternal haploids. The genomes of maternal haploids originate exclusively from the seed parent plant where the pollen parent is the haploid inducer (Coe 1959). In the other case where paternal haploids are developed, the pollinator serves as the genome donor and the female parent acts as the inducer (Kermicle 1969). In the former case, i.e. induction of maternal haploids a male inducer line derived from Stock 6 line of maize is used, whereas for the latter, i.e. paternal haploids, the inducer line having *ig1* mutant is used as the female inducer.

10.2.2 Haploid Induction Using Indeterminate Gametophyte Mutant

Utilization of *ig1* mutant gene gives rise to indeterminate gametophyte as well as increases the frequency of haploid progenies when the line having this gene is crossed with any other normal maize line. Homozygous *ig1* mutants show several embryological abnormalities including egg cells without a nucleus. Post double fertilization of such eggs with normal male gametes, the cells give rise to haploid embryos. As only the pollen parent contributes towards the genetic composition of the haploid plants, the haploids are termed as paternal haploids. This system also provides a reliable system for conversion of an inbred line to its cytoplasmic male sterile form using *ig1/ig1* genetic stock; however, the low frequency of haploid recovery, as well as changes in the constitution of cytoplasm from the donor genotype, renders this system not so attractive as in vivo haploid generation in maize (Schneerman et al. 2000).

10.2.2.1 Induction of Maternal Haploids

Maternal haploids carry both the cytoplasmic and nuclear genome of the maternal (donor) parent, whereas the nuclear genome of the inducer line is gradually eliminated. Over a period of time the haploid induction efficiency of maternal haploids has been enhanced by identifying more efficient inducers from Stock 6, MHI, WS14, HZI1, etc. (Coe 1959; Lashermes and Beckert 1988). For easy identification of maternal haploids from diploids, some colour marker genes have been incorporated into male inducer lines for purple leaf, sheath and plants or purple endosperm crown and purple plumule colour to facilitate identification of haploid seeds at the ear level.

10.3 Mechanism of In Vivo Haploid Induction

Although the mechanism of induction of maternal haploids is poorly understood, some hypotheses have been put forward. Wedzony et al. (2004) proposed that one of the two sperm cells provided by the inducer is defective but still fuses with the egg cell. During subsequent cell divisions, the inducer chromosomes get gradually isolated from the maternal chromosomes and get gradually degenerated from the primordial cells. The other well functional sperm cells fuse with the polar nuclei and give rise to regular endosperm. Another hypothesis supported by Chaylk et al. (2003) suggests that one of the two sperm cells is not able to fuse with the egg cell but instead triggers haploid embryogenesis while the secondary fertilization goes normal. If the functional sperm fuses with the egg cell and the defective sperm fuses with the secondary nuclei, this will result in kernel abortion.

10.4 Chromosome Elimination Approaches of Haploid Induction

Elimination of chromosomes is a common phenomenon in wide hybrids, ranging from loss of one or two alien chromosomes to elimination of whole chromosome complement from one parent. Such elimination leads to the development of haploids which are then given chemical treatment to double the chromosome number. The doubled haploidy (DH) breeding following chromosome elimination approach has been used in crops such as barley, wheat, oats, triticale, rye and potato, where the other techniques of haploid induction like anther, pollen/microspore culture and ovule culture were not so efficient. In order to apply the DH systems successfully to a breeding programme, a technique should fulfill the following three criteria: (1) DH line(s) should be produced efficiently from all the genotypes, (2) DH should represent a random sample of the parental gametes and (3) DH should be genetically normal and stable (Snape et al. 1986).

Several explanations have been proposed to account for uniparental chromosome elimination, viz. difference in timing of essential mitotic processes attributable to asynchronous cell cycling (Gupta 1969) and asynchrony in nucleoprotein synthesis leading to loss of the most retarded chromosomes (Bennett et al. 1976; Laurie and Bennett 1989). Other hypotheses put forward are the formation of multipolar spindles (Subrahmanyam and Kasha 1973), spatial separation of genomes during interphase (Finch 1983; Linde-Laursen and von Bothmer 1999) and genome elimination by nuclear extrusions (Gernand et al. 2005, 2006). In addition, degradation of alien chromosomes by host-specific nucleases (Davies 1974), uniparental nondisjunction of anaphase chromosomes (Ishii et al. 2010) and parent-specific inactivation of centromeres (Finch 1983; Jin et al. 2004; Mochida et al. 2004) have been suggested. The actual cellular mechanism involved in the process of uniparental chromosome elimination remains poorly understood.

Various chromosome elimination-mediated approaches of doubled haploid production in various crops are given below.

10.4.1 Haploid Induction in Barley (*Hordeum vulgare*)

The first method in cereals based on wide crossing following chromosome elimination was *H. vulgare* × *H. bulbosum*, commonly known as 'bulbosum method' (Stephan 1969; Kasha and Kao 1970; Lange 1971). During early embryogenesis, chromosomes of the wild relative are preferentially eliminated from the cells of developing embryos leading to the formation of haploid embryos. The endosperm is frequently formed, but its development is usually disturbed, hence at 12–14 days of pollination, the embryos are excised from developing caryopsis and are cultured in vitro. The bulbosum method was the first haploid induction method to produce a large number of haploids across most genotypes and this method quickly entered into breeding programmes. Kasha and Kao (1970) presented evidence to show that these haploids are not caused by parthenogenesis but by the elimination of *H. bulbosum* chromosomes. This elimination is under genetic control (Ho and Kasha 1975). Haploids of *H. vulgare* are also obtained when it is used as a male parent in the wide hybridization programme. This method represents a considerably advanced approach in the production of barley haploids and it has a number of advantages over the anther culture. In particular, haploids can be produced from any cultivar of barley, whereas in the anther culture, success is dependent on the genotype. The parent-specific inactivation of centromeres during the mitosis-dependent process of chromosome elimination in *H. vulgare* × *H. bulbosum* hybrids was confirmed by Sanie et al. (2011). They reported that the loss of centromeric histone H3 (CENH3) from centromeres precedes uniparental chromosome elimination in interspecific barley hybrids. Gernand et al. (2006) studied the mechanism underlying selective elimination of the paternal chromosomes during the development of *H. vulgare* × *H. bulbosum* hybrid embryos that is restricted to an early stage of development. In almost all embryos, most of the *H. bulbosum* chromatin undergoes a fast rate of elimination within 9 days after pollination.

10.4.2 Haploid Induction in Wheat (*Triticum aestivum*)

10.4.2.1 Wheat × *Hordeum bulbosum*

Haploid wheat plantlets were obtained when 'Chinese Spring' variety of *Triticum aestivum* ($2n = 6x = 42$) was crossed with *H. bulbosum* ($2n = 2x = 14$ or $4x = 28$). This happened as a result of elimination of *H. bulbosum* chromosomes from the interspecific hybrid during its early embryogenesis (Barclay 1975; Zenketler and Straub 1979). However, this method was not successful with other wheat varieties

as in the anther culture due to the effect of dominant crossability inhibitor alleles *Kr1, Kr2, Kr3* and *Kr4* located on 5B, 5A, 5D and 1A chromosome arms (Riley and Chapman 1958; Krolow 1970; Sitch et al. 1985; Zheng et al. 1992), which prevent the entry of *H. bulbosum* pollen tube into the ovary of wheat. The 'Chinese Spring' variety of bread wheat possesses recessive crossability alleles, that is, *Kr1* and *Kr2*. Jalani and Moss (1980) reported that crossability genes have little effect on pollen germination and on the time taken for the pollen tubes to reach the micropyle. The number of pollen tubes reaching the micropyle is, however, affected by the *Kr* genes, as high crossable genotypes have more pollen tubes than low crossable ones. Factors affecting crossability between 'Chinese Spring' wheat and *H. bulbosum* were also found on chromosomes 3A, 3B and 3D (Miller et al. 1983). This system was hence useful to a limited extent due to the sensitivity of the *H. bulbosum* pollen to the crossability inhibitor genes.

10.4.2.2 Wheat × Maize

Zenkteler and Nitzsche (1984) reported for the first time that embryos were frequently formed when hexaploid wheat was pollinated with maize. Later, their results were confirmed by Laurie and Bennett (1986). They cytologically demonstrated that the maize pollen normally germinated and grew into the wheat embryo sac where the wheat egg was fertilized by the maize pollen. A hybrid zygote with 21 wheat chromosomes and 10 maize chromosomes was produced (Laurie and Bennett 1988). The hybrid zygotes were karyotypically unstable and the maize chromosomes failed to move towards the spindle poles during cell division. Possibly, their centromeres failed to attach to the spindle microtubules due to progressive loss of centromere activity. Resultantly, maize chromosomes were eliminated after three to four mitotic cell divisions forming wheat haploid embryo with $n = 21$ chromosomes (Laurie and Bennett 1989). Some earlier studies show that the wheat × maize system is more efficient in embryo formation compared to other techniques. For haploid embryo production a system of wheat × maize crossing is widely used due to higher production of haploid embryos compared to other grass species pollination systems (Inagaki and Tahir 1991; Kisana et al. 1993; Inagaki and Mujeeb-Kazi 1995). This system is fast, economically viable, easy for application and more efficient than others due to its low level of genotype specificity (Cherkaoui et al. 2000). The maize chromosome elimination system in wheat is insensitive to crossability inhibitor genes (Laurie and Bennett 1989) and enables the production of a large number of haploids from any genotype including those recalcitrant to androgenesis (Inagaki et al. 1998; David et al. 1999; Cherkaoui et al. 2000; Chaudhary et al. 2002; Singh et al. 2004; Pratap et al. 2006). Several other investigations of haploid wheat production through wide crossing have since been reported (Laurie and Bennett 1989; Laurie and Reymondie 1991; Matzk and Mahn 1994; Suenaga 1994; Morshedi and Darvey 1995). It appears that a wide range of wheat and maize genotypes can be used to produce haploid wheats, although there is evidence to suggest that the efficiency of production is variable (Suenaga 1994).

Haploid production efficiency is affected by the proportion of pollinated florets that develop haploid embryos. Yields of haploid embryos have been reported to be as high as 53 % (Morshedi and Darvey 1995) and as low as 1 % (Suenaga and Nakajima 1989), depending on a wide range of variables. Factors that affect the yield of haploid embryos include genotypic differences between individual wheat and maize lines (lnagaki and Tahir 1990; Suenaga 1994; Chaudhary et al. 2002; Sharma et al. 2005; Pratap and Chaudhary 2007; Dhiman et al. 2012), the timing and use of exogenous growth substances to stimulate ovule development (Suenaga and Nakajima 1989) and environmental factors (especially temperature) during and after pollination. Laurie and Bennett (1989) reported that all maize chromosomes were lost during the first three cell division cycles in most embryos. All embryos with four or more cells had micronuclei, showing that embryo development was dependent on fertilization. The only primary endosperm metaphase obtained in the experiment had 42 wheat and 10 maize chromosomes, and the presence of micronuclei in most developing endosperms showed that at least 85 % were of hybrid origin.

10.4.2.3 Wheat × *Imperata cylindrica*

Considering the above chromosome elimination-mediated haploid induction systems, no alternative pollen source was reported to overcome the problems of wheat × maize system, viz. non-synchronization of flowering with wheat naturally and poor performance in producing haploids from triticale × wheat and wheat × rye derivatives. These constraints made it imperative to search for some other pollen source. Professor H.K. Chaudhary and his associates (2005) pioneered in inventing wheat × *Imperata cylindrica*, a highly efficient doubled haploidy breeding system in wheat of the existing systems.

Among all the Gramineae genera, viz. *Zea mays, Sorghum bicolor, Pennisetum americanum, Setaria italica, Festuca arundinacea, Imperata cylindrica, Cynodon dactylon, Lolium temulentum* and *Phalaris minor* tested for haploid plant production, and *I. cylindrica* produced more embryos and haploids over others (Chaudhary et al. 2005). Cogon grass (*I. cylindrica*) (Fig. 10.1) is a wild weedy perennial grass ($2n = 2x = 20$) that does not require repeated sowing and its flowering coincides well with that of wheat and triticale under natural conditions. Furthermore, it is available under natural conditions in almost all parts of the world wherever wheat is cultivated. The *I. cylindrica*-mediated chromosome elimination approach (Fig. 10.2) of doubled-haploidy breeding is a non-specific genotype for hybridization with any variety of wheat (Chaudhary et al. 2005), triticale or their derivatives (Pratap et al. 2005).

Imperata cylindrica has been reported to perform significantly better than maize for all the haploid induction parameters in wheat and triticale and their derivatives (Chaudhary 2008a, b, 2012, 2013; Jeberson et al. 2012; Kishore et al. 2011; Chaudhary et al. 2013a, b; Badiyal et al. 2014). Cytological investigation of the wheat × *I. cylindrica* chromosome elimination system has shown that there is no

Fig. 10.1 Spike of a wildly growing plant of *Imperata cylindrica*, an efficient pollen source for haploid induction in wheat

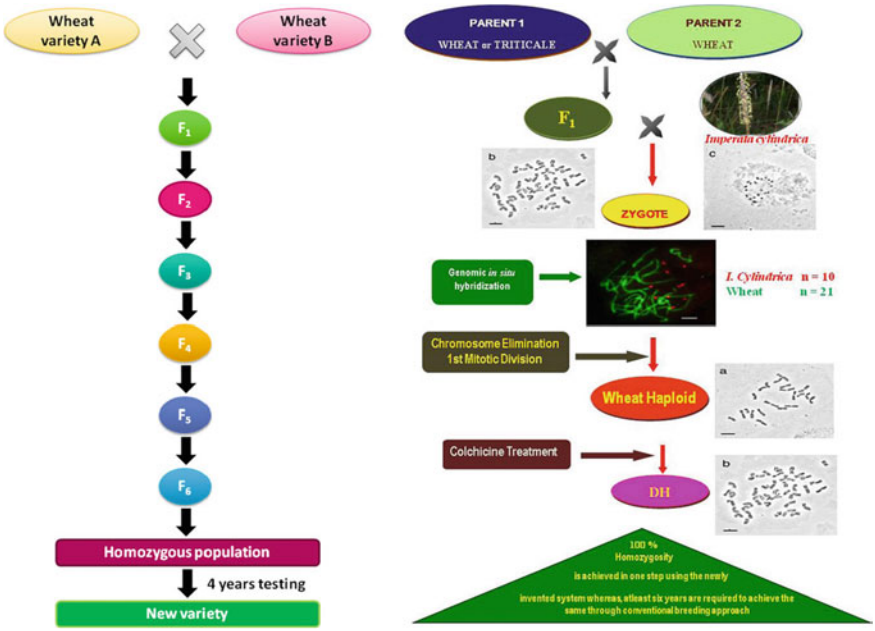

Fig. 10.2 Flowchart exhibiting comparison of the conventional (*left flank*) and DH breeding approaches (*right flank*) (Chaudhary et al. 2005; Komeda et al. 2007)

endosperm formation and the elimination of chromosomes of *I. cylindrica* takes place in the first zygotic division during the process of seed development, thus allowing the production of embryo-carrying pseudoseeds (Komeda et al. 2007;

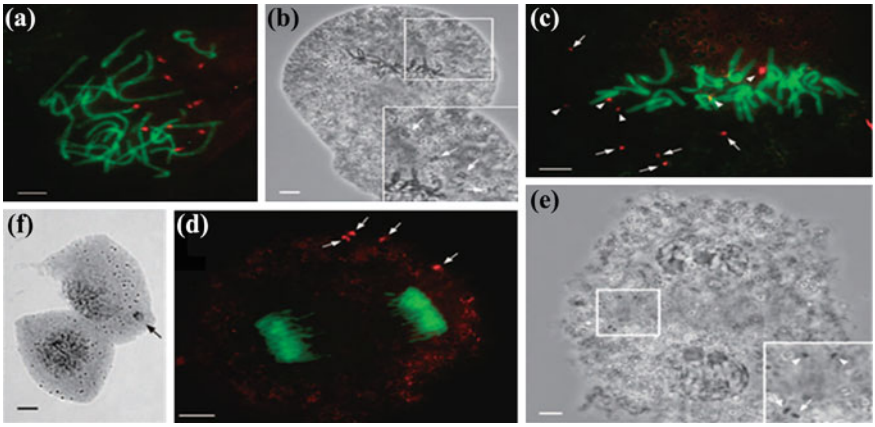

Fig. 10.3 Molecular cytological evidence of sequential elimination of alien chromosomes in wheat × *I. cylindrica* hybrids during first zygotic mitosis (*Green* Wheat chromosomes, *Red I. cylindrica* chromosomes). **a** Interspecific hybrid. **b**, **c** Aberrant movement of *I. cylindrica* chromosomes. **d** Anaphase cell showing elimination of *I. cylindrica* chromosomes. **e** Unsquashed zygote at telophase showing extruding *I. cylindrica* chromosomes. **f** Interphase daughter cells after 3–4 days of pollination showing extruded *I. cylindrica* micronucleus (Komeda et al. 2007)

Fig. 10.3). Recently, Tayeng et al. (2012) reported that in vivo application of colchicine (2,000 ppm) enhances the doubled-haploid production efficiency in wheat × *I. cylindrica*-mediated chromosome elimination approach of doubled haploidy breeding. The haploid chromosome set of wheat (n = 21) is obtained after wheat × *I. cylindrica* hybridization. Similar to wheat × maize system, the mean response of wheat and *I. cylindrica* to haploid induction varies from genotype to genotype (Rather et al. 2014). The morphological marker, that is, absence of endosperm in haploid embryo-carrying seeds developed from wheat × *I. cylindrica* hybridization, can be used efficiently to exploit the asynchronous behaviour of anthesis within wheat spikes (Chaudhary et al. 2013a, b) for undertaking this wide hybridization without emasculation. This endeavour has saved considerable time and energy required otherwise for emasculation in wheat × *I. cylindrica* hybridization.

10.4.3 Solanum tuberosum × S. phureja

Doubled haploids can be produced from tetraploid genotypes of *S. tuberosum* (cultivated potato) by pollination with the diploid potato species, *S. phureja* (Mendiburu et al. 1974; De Maine 2003). In about 0.5 % of pollinated ovules, both male sperm cells of *S. phureja* take part in the formation of functional endosperm. The best pollinator lines of *S. phureja* were bred for a dominant purple spot embryo marker; thus, seeds containing haploid embryos can be easily distinguished from hybrid *S. tuberosum* × *S. phureja* seeds. Methods of more effective chromosome

number duplication were developed more recently and production of potato can now be obtained by androgenetic methods with better efficiency (Jacobsen and Ramanna 1994; Rokka et al. 1996; Rokka 2003). Moreover, androgenesis is applicable to a wider range of *Solanum* species in comparison to crosses with *S. phureja* (Jacobsen and Ramanna 1994; Aziz et al. 1999; Rokka 2003). Montelongo-Escobedo and Rowe (1969) reported that the superior pollinator in potato haploidy breeding following chromosome elimination approach may be the one that produces a high frequency of restitution sperm nuclei. Dihaploid potatoes can be used for breeding purposes, including alien germplasm introgression or selection at the diploid level, but such plants are not homozygous. Haploids have a significant role in potato breeding programmes, as they enable interspecific hybridization which would not be otherwise possible due to differences in ploidy level and endosperm balance numbers. The gene pool of potato 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).

10.5 Centromere Sequences

Centromeres are the chromosomal loci that attach to spindle microtubules to mediate faithful inheritance of the genome during cell division. These are epigenetically specified by incorporation of CENH3, a histone H3 variant that replaces conventional H3 in centromeric nucleosomes16. Sanei et al. (2011) isolated CENH3-1, an embryo-lethal null mutant in *Arabidopsis thaliana* that allows us to completely replace native CENH3 with modified variants. CENH3-1 plants complemented by transgenic green fluorescent protein-tagged CENH3 (GFP–CENH3) have a wild-type phenotype. Komeda et al. (2007) reported that the loss of *I. cylindrica* chromosomes is due to a lack of kinetochore activity of the chromosomes as a result of which the chromosomes become laggards and get eliminated from the hybrid cell leading to formation of wheat haploids. In oat × maize hybrids, maize centromeres were reported to have lost the transcriptional activity in genetic background of oat. Synthesis of oat CENH3 proteins and their incorporation in one or a few maize kinetochores led to the normal segregation of maize chromosomes. Whereas the chromosomes without CEN proteins incorporated in their kinetochores fail to attach with the spindle fibres and hence became laggards and eventually got eliminated (Jin et al. 2004). Maruthachalam and Chan (2010) reported that CENH3 null mutants expressing altered CENH3 proteins can be successfully exploited for induction of haploids in *A. thaliana.* Similarly, the loss of centromeric histone H3 (CENH3) from centromeres of *H. bulbosum* was revealed in elimination of its chromosomes in *H. vulgare* × *H. bulbosum* hybrids. They revealed that centromere inactivity of *H. bulbosum* chromosomes was due to centromeric loss of CENH3 protein rather than uniparental silencing of CENH3 genes to trigger the mitosis-dependent process of uniparental chromosome elimination. They also revealed that the diploid barley

species encode two CENH3 variants, the proteins of which are intermingled within centromeres throughout mitosis and meiosis (Sanei et al. 2011).

10.6 Development of Recombinant, Addition and Substitution Lines

Wild relatives and related species are important resources for broadening the genetic variability of crop plants. The transfer of such desirable traits from wild species is possible at three levels:

(i) At the level of whole genome for the production of amphidiploids (*Raphanobrassica* and *Triticale*)
(ii) At the level of individual whole chromosome for the production of alien addition and substitution lines
(iii) At the level of chromosome segment as done in the production of intercalary and terminal translocations

As crop plants are often bred for specific quality attributes, such as high yield potential and plant type suited to specific agronomic practices, the amount of alien genetic material introduced into an elite cultivar has to be carefully controlled. The commercially viable and useful modes of enhancing genetic diversity using manipulations at whole chromosome and chromosome segment level are discussed under the following heads:

10.6.1 Alien addition and substitution lines
10.6.2 Translocation genetic stocks/lines.

10.6.1 Production of Alien Addition or Substitution Lines

The transfer of a single or pair of chromosome(s) from one species to another can be useful for the introduction of desirable traits such as resistance against biotic and abiotic stresses. For this, aneuploid plants are produced that contain an extra single chromosome or an extra chromosome pair from a donor plant, called monosomic or disomic addition lines, respectively. Such addition lines can be created by hybridization between donor and recipient plant lines followed by repeated back-crossing with the recipient plant line. Donor and recipient parents are usually from different species.

After achieving the objective of producing alien addition lines in different crops, their evaluation suggested that they were invariably unstable and at meiosis they exhibited a higher frequency of univalents than normal. In view of this problem with alien addition lines and due to their undesirable effects on the phenotype, the addition lines could not be considered suitable for commercial use. It was argued

that if chromosome number is maintained at normal euploid level by substituting a pair of alien chromosomes for a pair of normal recipient crop chromosomes, the product may be more desirable and, therefore, acceptable for cultivation.

One of the major achievements of alien addition and substitution lines has been the wheat–rye introgression in which individual whole chromosome alien addition lines were introduced into wheat, utilizing *Secale cereale* as donor species. An amphidiploid ($2n = 56$) between wheat and rye is first produced following the normal method of crossing the two species followed by doubling the chromosome number in the F_1 hybrid. The amphidiploid is backcrossed to wheat giving a heptaploid with $21^{II} + 7^{I}$, where the bivalents belong to wheat and univalents belong to rye. On selfing of these heptaploids ($2n = 7x$), monosomic ($21^{II} + 1^{I}$ rye) and disomic ($21^{II} + 1^{II}$ rye) are obtained. Whenever monosomic additions are available, these may be selfed to get the disomic addition lines.

The wheat–rye substitution lines are developed by crossing the alien addition lines as male parent with the monosomic lines of wheat. F_1 plants with $20^{II} + 2^{I}$ were selected, which either on selfing or backcrossing to the same addition line gave rise to monosomic or disomic substitution lines.

Other alien addition lines utilizing whole chromosomes from *Aegilops* or barley to wheat were developed by Islam et al. 1981. The success in wheat–barley cross was achieved for the first time by Kruse (1973) followed by Islam et al. (1975). Viable hybrid plants could be easily produced when barley was used as a female parent and when the developing embryos were treated with gibberallic acid and transferred to an artificial culture medium. The success was 5.8 % over all the crosses made by Islam et al. (1975), although 15.9 % success could be achieved when Chinese Spring wheat was crossed with Betzes barley. Substitution of one chromosome or a complete pair can also be useful, e.g. the introduction of disease resistance by the exchange of chromosome 1B of wheat for 1R of rye (Khush 1973). As discussed earlier, addition lines often show reproductive instability and are mostly not of direct use in breeding. However, they can be used for the localization of genes for particular traits to specific chromosomes. For this purpose, other variants can also be used, namely monosomic or trisomic lines within crop species. Such lines can be made by applying the mitotic inhibitor colchicine, radiation or selection in the progeny of triploid plants (Khush 1973). Sets of such lines have been compiled that together represent the complete haploid chromosome complement of a crop species. Such sets are for instance known for oat, barley, wheat, rice, sorghum, cotton, asparagus, pepper, tomato and tobacco.

Resistance to common root rot and black point, caused by *Cochliobolus sativus*, was evaluated in alien chromosome substitution and addition lines of the cultivars ‘Cadet’ and ‘Rescue’ (Conner et al. 1993). Substitution of chromosome 5B in ‘Rescue’ with 5Ag from *Agropyron elongatum* decreased root rot susceptibility to a level intermediate between that in the susceptible ‘Rescue’ and the resistant ‘Cadet’. The substitution of ‘Rescue’ chromosome 5A or 5D with 5Ag, or the addition of 5Ag to ‘Rescue’ complement had no consistent effect on root rot susceptibility. The root rot resistance of ‘Cadet’ was unaffected by substitution of chromosomes 5A, 5B, or 5D with 5Ag, or the addition of 5Ag.

Solanum brevidens is a wild diploid potato species possessing high level of resistance to several major potato diseases. Dong et al. (2005) developed fertile somatic hybrids between *S. brevidens* and the cultivated potato (*S. tuberosum*) in order to introgress disease resistance from this wild species into potato. A series of backcross progenies were developed from a hexaploid somatic hybrid A 206. Using a combination of *S. brevidens*-specific randomly amplified polymorphic DNA (RAPD) markers and a sequential genomic in situ hybridization (GISH) and fluorescence in situ hybridization (FISH) technique, they identified all 12 *S. brevidens* chromosomes in the backcross progenies. Seven potato *S. brevidens* monosomic chromosome addition lines (chromosomes 1, 3, 4, 5, 8, 9 and 10) and one monosomic substitution line (chromosome 6) were identified, and the remaining four *S. brevidens* chromosomes (2, 7, 11, and 12) were included in two other lines. These chromosomal addition/substitution stocks provide valuable tools for potato cytogenetic research, and can be used to introgress disease resistance from *S. brevidens* into potato.

The identification of addition or substitution lines for different alien chromosomes is achieved by any one of the following techniques:

(i) Morphology: Addition of different chromosomes sometimes leads to modification of morphology in different directions so that the different addition lines can be distinguished from each other. These addition lines also provide an opportunity to study the effect of individual alien chromosomes in the uniform wheat background.
(ii) Karyotype: The alien addition lines can also be identified on the basis of the chromosome size and morphology.
(iii) Intercrossing: Intercrossing between alien addition lines may help to find out if two plants have addition for the same or different chromosomes through the study of meiosis in F_1 hybrid, which should exhibit $21^{II} + 2^{I}$ if they differ.

10.7 Translocation Breeding

Reduced chromosome pairing and lack of recombination is an important problem in the production of interspecific hybrids. Pairing of chromosomes that normally show a low extent of pairing, that is, between the homoeologous chromosomes derived from the different parental species, can be induced by specific mutations. Recombination frequencies can also be increased artificially by use of various chemical agents, physical stress such as a temperature shock or by UV irradiation. In breeding with complex allopolyploids, there is also the possibility of suppression of genes favouring homologous pairing in order to induce exchange of segments

between homoeologous chromosomes. While irradiation produces random interchanges even between non-homoeologous chromosomes, recombination produces interchanges between homeologous chromosome segments. On the other hand, formation of multivalents during meiosis, particularly in newly created autopolyploids, is preferably avoided in normal propagation because of the consequent reproductive instabilities. This may be achieved by selection for improved fertility of progeny and/or induction of chromosomal changes leading to a 'diploid' (or 'allopolyploid') behaviour of the polyploid (diploidization), so that, in practice, mainly bivalents are formed during meiosis. Sometimes, supernumerary chromosomes (so-called B chromosomes, versus the normal A chromosomes complement) can also have an effect of favouring recombination between homologous chromosomes over that between closely related homeologous ones.

10.7.1 Translocations Using Irradiation

The first useful transfer by irradiation involved a segment from *Aegilops umbellulata* chromosome 6U carrying resistance to wheat leaf rust (*Lr 9*) to chromosome arm 6BL of wheat (Sears 1956). To achieve this goal, as cross of hexaploid wheat and *Ae. umbellulata* was not successful, an amphidiploid (*T. Dicoccoides* × *Ae. umbellulata*) with $2n = 21^{II}$ was used as a bridge. The amphidiploid was crossed with 'Chinese Spring' variety of wheat followed by two backcrosses with Chinese Spring and selection for leaf rust. This gave a plant with $2n = 43$, in which extra chromosome was from *Ae. umbellulata* and carried the gene for rust resistance associated with some undesirable characters. This plant was exposed to a heavy dose of X-rays before meiosis. Pollen from this irradiated plant was used for pollinating Chinese Spring wheat. Only balanced pollen with $n = 21$ would function and may or may not carry an intercalary translocation. Among 6091 offsprings, 132 were resistant, of which 40 had translocations and only one of them was intercalary. Plants for this intercalary translocation resembled Chinese Spring, except for rust resistance and slightly late maturity. The intercalary translocation was later found to be on a chromosome that was homeologous to the alien chromosome involved.

Following irradiation of seeds and plants of an alien substitution line carrying *Agropyron elongatum* chromosome, a segment carrying leaf rust resistance was transferred to the bread wheat variety 'Thatcher'. The stock was called *Translocation-4* (Sharma and Knott 1966).

In general, translocations can also be induced by irradiation of a trisomic addition line (with one extra homoeologous chromosome from another species) and by subsequent recovery of plants with chromosome segments of the added chromosome incorporated into their genome.

10.7.2 Translocations Through Recombination

10.7.2.1 Recombination via Manipulation of Diploidizing System

As mentioned above, translocations can also be obtained through recombination among the homeologous chromosomes which is facilitated by the suppression of genes that otherwise facilitate bivalent formation instead of multivalent formation. This type of recombination can be best explained by illustrating wheat genome. In wheat, there are various approaches as given below for manipulating the regulatory mechanism of homeologous recombination:

- Use of high pairing *Ph1* deficient stocks (*ph1b* in bread wheat and *ph1c* in durum wheat);
- Use of 5B-deficient stocks such as 5D(5B) substitution lines;
- Hybridization with alien species, which epistatically control the activity of *Ph* locus.

10.7.2.2 Use of High Pairing *Ph1* Deficient Stocks

Sears (1982) developed first mutant *Ph1b* at *Ph1* locus and later it was found to be deficient for dominant *Ph1* locus at intercalary position. Similarly, a mutant *Ph2b* was developed which was known to have *Ph1b*-like effect in wheat-alien bridge species (Wall et al. 1971; Sears 1977). The mutation in the *Ph1* locus strongly promotes homeologous chromosome pairing within wheat and between wheat and its relatives (Yu et al. 1995). Genetic transfers of alien genes from *Agropyron* and *Aegilops* spp. into wheat were achieved through use of *ph1b* stocks (Yu et al. 1995). Dundas et al. (2007) produced bread wheat introgression lines containing *SrR* (*Secale cereale*), *Sr26* (*Thinopyrum ponticum*), *Sr32* and *Sr39* (*Ae. speltoides*) and *Sr40* (*Triticum timopheevii*) using *ph1b* mutants. Similarly, *ph1c* mutant stocks in durum were used to introgress genes from *Aegilops* species such as *Sr39* from *Ae. speltoides* (Mago et al. 2009) as well as distant relatives such as *Secale cereale* (Giorgi and Barbera 1981) and *Th. bessarabicum* (King and Laurie 1993). Several other works also show the effectiveness of the recessive gene *ph1b* in inducing homeologous pairing of wheat with *Secale*, *Agropyron* and *Agrotricum* hybrids (Dhaliwal et al. 1977; Naranjo et al. 1988; Wu et al. 1989; Ahmad and Comeau 1991).

Mutants at *Ph* locus on 5BL chromosome arm of wheat were also isolated and used for facilitating homeologous recombination. To achieve this, special stocks (triple monosomics) with single doses each of 5B, the alien chromosome and a wheat homoeologue were prepared. This can be done by crossing monosomic 5B with an alien substitution line ($20^{II} + 1^{II}$ alien). This method was successfully utilized for homeologous recombination among wheat—*Agropyron* and wheat—rye chromosomes (Islam and Shepherd 1991).

10.7.2.3 Use of 5B-Deficient Stocks

As the dominant *Ph1* locus is situated at the long arm of 5B chromosome of wheat, 5B deficient stocks such as Langdon 5D (5B) disomic substitution lines can be utilized to promote intergenomic chromosome pairing leading to effective interspecific and intergeneric gene transfers (Sears 1981; Jauhar et al. 2009). Using this method, the genes for scab resistance were transferred successfully from diploid wheat grass *Lophopyrum elongatum* to durum wheat (Jauhar et al. 2009). The same strategy was employed for transferring genes from *Th. bessarabicum* and *Th. curvifolium* into durum.

Nullisomy for 5B was first used by Riley (1966) for transfer of segment from *Ae. bicornis,* but a more efficient method was suggested by Sears (1972), where a nulli-5B-tetra-5D line was crossed with an alien substitution line to produce plants which were nulli-5B-tri-5D and double monosomic for alien and wheat homeologues ($18I^{I} + 1^{III} + 2^{I}$). The absence of 5B allowed the alien chromosome to pair with its wheat homeologue and the progeny obtained by pollinating these plants by euploid wheat was tested for rust resistance. Using this method, Sears (1972, 1973) was successful in transferring segments from 3Ag (carrying *Lr 24* gene) and 7Ag (carrying *Lr 19* gene) to 3D and 7D, respectively.

10.7.2.4 Suppression of *Ph1* Activity Through Epistasis

Homoeologous pairing can also be enhanced among wheat and *Ae. speltoides* by suppressing the action of the *Ph1* gene due to the presence of the *Ph1* suppressor genes *Su1-Ph1* and *Su2-Ph1* (Dvorak et al. 2006). Leaf rust and stripe rust resistance genes from *Ae. umbellulata* (Chhuneja et al. 2008) and from *Ae. triuncialis* and *Ae. geniculata* (Aghaee-Sarbarzeh et al. 2002) were successfully transferred to wheat following this approach. A number of genes for disease resistance were transferred from wild relatives such as *Ae. ovata*, *Dasypyrum villosum* (Blanco et al. 1988), and *Thinopyrum curvifolium* (Jauhar and Almouslem 1998) to durum wheat utilizing this strategy. However, the existence of such genes is limited to some genotypes and the efficiency in inducing pairing between homeologous does not appear to be comparative to 5B deficient or *ph1b/ph1c* mutants (Jauhar and Chhibbar 1999). Thus this approach is the least exploited for alien gene transfers into wheat.

For a stable introgression, mere identification of the transferred gene/genes is not enough. The transferred genetic content should be inherited stably in the progeny to be manifested as the desired trait. Hence, the progeny is critically screened for the trait governed by the candidate gene. To identify the extent of homeologous recombination leading to successful alien introgressions in wheat, several techniques have been utilized. Earlier, chiasmata measurement and C-banding techniques were used by (Sears 1952, 1973; Gill and Chen 1987) to measure the homeologous recombination. But nowadays, the use of modern cytological procedures such as GISH and FISH (Schwarzacher et al. 1989; Yamamoto and Mukai 1989; Jenczewski et al. 2003; Ji and Chetelat 2003; Khrustaleva et al. 2005;

Schwarzacher et al. 2011) and novel biotechnological tools such as molecular markers have revolutionized the assessment of introgression breeding (Rogowsky et al. 1993; Lukaszewski et al. 2004). Works have identified wheat–rye translocations such as 1BL.1RS (Fig. 10.4) after successful wide hybridization among triticale and wheat genotypes (Chaudhary et al. 2004; Jeberson 2010). Simultaneously, GISH can also be used to identify the chromosomes of different species in an intergeneric cross such as wheat × *Imperata cylindrica* the wheat genetic background (Chaudhary et al. 2013a, b). The crop genome-specific DNA markers available today can easily detect the transferred genes into the crop background and, thus, they offer highly amenable and cost-effective tools for such assessments (Peng and Lapitan 2005).

Homoeologous recombination for transfer of alien segments can also be achieved by using certain strains of *Ae. speltoides* and *Ae. mutica* to suppress the effect of *Ph* in the appropriate hybrids. If the genes are to be transferred from the same species as that used for suppression, the transfer can be achieved by making and backcrossing the hybrids with wheat. In other cases, crosses are first made with the alien species and suppression of 5B system is achieved later by incorporating suitable genotype of *Ae. speltoides* or *Ae. mutica.* Riley et al. (1968) used this method for transfer of stripe rust resistance from *Ae. comosa* to wheat.

10.7.2.5 Recombination Without Manipulation of Diploidizing System

In some crops such as groundnut, a diploidizing genetic system is either absent or weak. Thus, in hybrids with related diploid species, occasional allo-syndetic pairing occurs and recombination is achieved through production of F_1 hybrids followed by backcrossing accompanied with selection for desirable traits. Alternatively, F_1 hybrids may be selfed to allow further recombination and stable tetraploids may be reconstituted through backcrossing. Using this approach, alien gene introgressions for different traits have been carried out in different crops such as groundnut (Singh et al. 1991), sugar beet (Nakamura and Tsuchiya 1982), etc.

10.8 Homologous Recombination for Chromosome Engineering

The recombinant lines developed after the successful introgression of alien chromatin into the wheat background, through homoeologous recombinations, are mainly agronomically inferior to elite cultivars. Hence, there is a need for transferring the desired genes (without linkage drag) into the elite background through homologous recombination using recurrent backcrossing (Valkoun 2001). Homology among recombinants can also be utilized by intercrossing them to generate secondary and tertiary recombinants, which are further screened for the introgressed trait. This

approach was followed by Lukaszewski and Xu (1995) to engineer 1BL.1RS translocation. Genes for leaf and stripe rust resistance were transferred from *Ae. umbellulata* accn. 3237 to durum wheat through the production of amphiploids (AABBUU) between *Ae. umbellulata* and T. *durum* cv. WH890 and were further crossed to CS *PhI* to promote homeologous pairing. The F_1s (AABBDU) thus generated were backcrossed to a susceptible hexaploid wheat cultivar WL711 and introgression lines carrying resistance to leaf and stripe rust were screened in the backcross progenies (Chhuneja et al. 2007). Genes conferring resistance against powdery mildew (*Pm13)* from the diploid grass *Aegilops longissima*, for leaf rust and yellow pigmentation (*Lr19 + Yp)* from the decaploid *Thinopyrum ponticum*, and the genes controlling gluten and gliadin (*Glu-D1* or *Gli-D1/Glu-D3)* from *T. aestivum* were separately introduced into durum wheat. Different strategies were employed for such transfers. Transfer of the *Pm13* gene was first obtained at the 6x level (Ceoloni et al. 1992) by use of the *ph1b* Chinese Spring mutant (Sears 1977) and then moved into a 4x background by homologous recombination (Ceoloni et al. 1996). For other transfers, wheat-alien chromosome recombination was induced using *ph1c* durum wheat mutants (Ceoloni et al. 2005). Several genes for disease resistance and quality attributes were transferred and stably inherited as reduced segments in the elite wheat background in backcrossed progenies. Such introgressions could be detected using GISH/FISH and molecular markers. The pattern of inheritance of the traits can be studied using F_2 population as the mapping population.

10.9 Reverse Breeding

In plant breeding, the importance of heterosis is well known where the hybrid (F_1) progeny is typically superior in comparison to its homozygous parents. The unpredictable nature of this phenomenon poses many problems for breeders (Fernandez-Silva et al. 2009). The performance of crop varieties cannot be optimized with the unknown constituents. Hence breeders evaluate heterosis by controlled crosses of inbred lines (i.e. by a prior selection and combination of unknown alleles). This hit-or-miss nature approach makes it difficult to optimize the effects of heterosis. Another barrier in plant breeding programmes is the reproduction of desirable heterozygotes by seeds. Favourable allele combinations of the elite heterozygote are lost in the next generation due to the segregation of traits.

Reverse breeding can be utilized as an alternative strategy to meet the challenge of fixing complex heterozygous genomes by constructing complementing homozygous lines (Dirks et al. 2009). In this method the order of events leading to the production of a hybrid plant variety is reversed (Fig. 10.4). It facilitates the production of homozygous parental lines that, once hybridized, reconstitute the genetic composition of an elite heterozygous plant, without the need for backcrossing and selection.

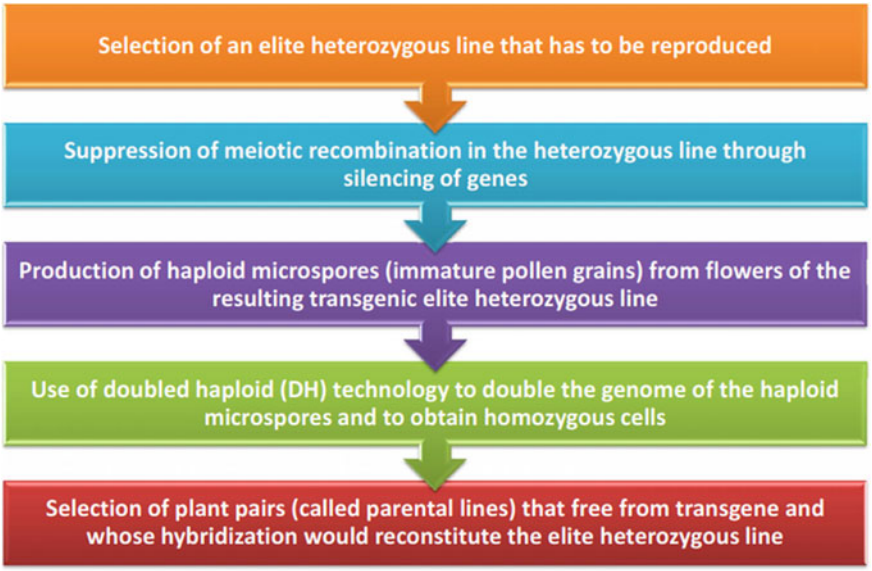

Fig. 10.4 Steps involved in reverse breeding

10.10 Crossover Suppression

The first step in reverse breeding is to produce gametes from the desired heterozygote without crossover recombination. To achieve this aim, genes controlling recombination are to be suppressed to ensure that gametes are formed without crossing over among the homologous chromosomes. There are a number of homologous chemicals available to suppress such genes, i.e. *MutL* homolog1 (suppresses *MutL*, which encodes a mismatch repair protein), *MutS* homolog2 suppresses *MutS* that encode mismatch repair proteins and promote homeologous recombination, RAD51 homolog (competes with RAD51, a gene involved in DNA repair) and homologs for proteins controlling crossing over such as SPORULATION-DEFICIENT11 (SPO11) a key protein for crossover initiation, univalent-producing mutants (desynaptic1 [*dsy1*], and meiotic prophase amonipeptidase1 [*mpa1*]) required for meiotic recombination. One of the successful examples of genome silencing is in the model plant *Arabidopsis* by Wijnker et al. (2012), who used RNA interference (RNAi) to knockdown the function of the *RecA* homolog DMC1, a meiosis-specific recombinase essential for the formation of crossovers. As RNAi is genetically dominant, it is easy to obtain progeny devoid of the RNAi cassette that would otherwise cause sterility in phenotypes among reverse-breeding offspring (Wijnker et al. 2012). RNAi silencing is easy to implement in many crops, and a single cassette targeting a well-conserved meiotic gene can be used across multiple crop species.

The other techniques for silencing of the gene include virus-induced gene silencing (VIGS) for induction of post-transcriptional gene silencing where a plant is infected with a virus that was modified to include a target gene RNA sequence. The plant breaks down the viral RNA using siRNA (small interference RNA) targeting simultaneously the plants' endogenous mRNA (Ruiz et al. 1998; Baulcombe 2004). The other technique used in reverse breeding is dominant negative mutations, where mutations in one allele may lead to a structural change in the protein that interferes with the function of the wild-type protein encoded by the other allele. These mutations are characterized by a dominant or semi-dominant phenotype (Rine and Herskowitz 1987).

The crossover can be suppressed by the silencing target genes using graft transmission method. Shoots of the plant in which genes are to be silenced would be grafted on transgenic rootstocks. The advantage of this method is that only a few transgenic rootstocks would be required to routinely apply reverse breeding in many crops (Shaharuddin et al. 2006).

The other methods include complete knockout of a gene by a recessive mutation, but this is not suitable for reverse breeding as it would reintroduce the same mutation into the offspring. There is a recent approach based on use of chemicals, i.e. 'mirin', an inhibitor for the Mre11-Rad50-Nbs1 (MRN) protein complex which plays a significant role in the processing of double strand breaks (Dupre et al. 2008). Other chemicals may be used, i.e. DIDS (Ishida et al. 2009), PSF (Morozumi et al. 2009), Halenaquinone (Takaku et al. 2011), Ri-1 (Budke et al. 2012) to inhibit RAD51. Exogenous application of compounds that cause inhibition or omission of recombination during meiosis would speed-up the application of reverse breeding enormously. A major advantage of using chemicals in reverse breeding is that their effect is not heritable hence the resultant reverse breeding products (DHs) are free of transgenes. This is important because the reverse breeding products are destined to be used in further breeding schemes, and should not have an achiasmatic phenotype. Perhaps contrary to intuition, DHs produced by transgene-mediated methods can be transgene-free. If a dominant knockdown construct is present in the hemizygous state, half of the gametes that are formed will not carry the transgene and, hence, are non-transgenic. Multiple transgenic lines with knockdown constructs on different chromosomes can be used to generate a full array of complementary DHs that do not carry transgenes (Wijnker and de Jong 2008). After the transformation non-recombinant the plant is subjected to the next step for production of doubled haploid.

10.11 Doubled Haploid Production

The second step in reverse breeding is to convert haploid gametes, carrying non-recombined chromosomes, into homozygous diploid adults. Efficient production of haploid plants that inherit a balanced number of chromosomes from gametes

can greatly accelerate reverse breeding. The probability of desired haploids in which at least one copy of all chromosomes present is quite low because achiasmatic chromosomes have high chances of making unbalanced univalents. However, these unbalanced chromosomes containing haploids cannot pass through all the developmental stages, from cell division and embryogenesis to plant regeneration. Haploids generated from non-recombinant individuals are converted to diploid instant homozygous lines, bypassing the generations of inbreeding. This can be achieved through different methods, depending on the plant species, as described earlier in the section on haploid induction. As reverse breeding is still in its infancy, most of the research is restricted to the model plant *A. thaliana.* The haploid plants were produced by microspore culture (Wijnker et al. 2012, 2014). Another approach to haploid production is through centromere-mediated genome elimination in *A. thaliana*. In this approach, manipulation of a single centromere protein, the centromere-specific histone CENH3, has been used. The mutant was crossed to wild type and the chromosomes from mutant were eliminated and haploids were generated. The success of reverse breeding is limited to those crops where DH technology is the common practice. For the great majority of crop species this technology is well established and professional breeding companies routinely use such techniques in their breeding programmes (Forster et al. 2007).

10.12 Applications

This technique can be used for preserving elite genotypes. Through reverse breeding homozygous parental lines can be produced from a heterozygous plant, which shows the potential of an elite variety. These parental lines can then be crossed to achieve hybrids which reconstruct the heterozygous genotype of the elite plant. With conventional methods it would not be possible to produce a variety that maintains the genotype of such an elite plant.

10.12.1 Production for Substitution Lines for Chromosomal Studies

Reverse breeding provides plant breeders with new possibilities for further applications in breeding. The chromosome substitution lines can be obtained when the F_1 hybrid is subjected to reverse breeding. As a resultant homozygous chromosome, substitution lines for single and multiple chromosomes are generated. Additionally, for generating heterozygous chromosome substitution lines, homozygous chromosome substitution lines are further backcrossed with one of the original parents. In *Arabidopsis*, a traditionally complete set of chromosome substitution lines have been generated using more generations of crossing and extensive genotyping.

Whereas with reverse breeding Wijnker et al. (2012) obtained a complete set of Landsberg erecta (*Ler*) chromosome substitutions in the Columbia (Col-0) background, as well as two substitutions of a Col-0 chromosome into a *Ler* background from the population of 36 *Arabidopsis* doubled-haploids just in two generation.

These chromosome substitutions have various potential applications, as in the generation of near-isogenic lines by recurrent backcrosses. Such lines are extremely valuable for mapping quantitative trait loci (QTL) and for advanced forms of marker-assisted breeding. It can be also applied to improve parental lines or for genetic studies (Driks et al. 2009). The quantitative traits are always a matter of interest in crops, as most agronomically important traits are polygenic in nature and are located on different chromosomes. The study of epistatic interactions between the background and genes contributed by the substitution chromosome are facilitated by reverse breeding. Offspring of plants in which just one chromosome is heterozygous will segregate for traits present on that chromosome only. Selfing plants that carry a substituted chromosome (or using recurrent backcrosses) allow breeders to fine-tune interesting characteristics on a single chromosome scale. This could bring forth improved breeding lines carrying introgressed traits. Reverse breeding provides the opportunity to have control over homozygosity or heterozygosity at the single chromosome level.

10.12.2 Production of Genetic Resources

The reverse breeding process provides a set of germplasm along with complementary parents to the hybrid. That germplasm may contain euploids, and can serve as potential germplasm for various agronomically important traits and their genetic interactions. Less number of doubled haploid plants is required, which is necessary to reconstruct the starting plant at different levels of probability. In maize ($x = 10$) just 98 DHs are expected to contain a set of two reciprocal genotypes ($P = 99$ %); the rest of doubled haploids can be used in crop improvement programmes.

10.12.3 Creation of Additional Hybrid Parental Lines

In vegetables crops, i.e. cauliflower, seed production problem hinders the commercialization of hybrid varieties. While applying reverse breeding to these heterozygous hybrids it is possible to produce the same variety with two other parental lines, with potentially better reproducibility. The additional parental line can provide better scope for adaptability of hybrids (Lussern et al. 2011).

10.12.4 Marker-Assisted Reverse Breeding (MARB)

Reverse breeding in combination with molecular markers can act as a more effective tool for plant breeding. Use of high throughput markers, i.e. SSR (simple sequence repeats) and SNPs (single nucleotide polymorphism) can speed-up the process of identification of complementary parents in populations of DHs in the early stages. Marker-assisted reverse breeding has far more importance in the study of gene interactions of the heterozygous inbred families (HIFs) produced from reverse breeding. It can be used in the screening of population segregate for the quantitative traits. Reverse breeding can be used to provide highly valuable insights into the nature of heterotic effects using HIFs for the generation of chromosome-specific linkage maps and the fine mapping of genes and alleles. In the case of *Arabidopsis*, Wijnker et al. (2012) use evenly spaced SNPs markers at approximately 4-Mb intervals to identify the reverse breeding F_1s from wild-type F_1s haploid lines.

10.13 Limitations

The major limitation of this method is that it cannot be applied to crops with large genome size because most of the resulting spores are unbalanced, containing either none, one or two copies of a given chromosome. However, balanced spores, containing one copy of each chromosome, will be formed at a probability of $(1/2)\ x$, where x equals the basic chromosome number. Consequently, the chance of obtaining balanced spores decreases exponentially with the chromosome number and seems feasible for species in which the chromosome number equals 12 or less. Unintended effects include the silencing of other homologous sequences in the genome as a result of the presence of the RNAi construct. This would not induce genomic changes, but could affect expression levels. Another unintended effect of the technique could be an incomplete suppression of meiosis. This would lead to some degree of meiosis and recombination, which are natural processes in plants.

10.14 Conclusion

In a world with a population of almost one billion endlessly struggling to fight hunger, the need for enhanced crop yields is a must within a short span of time. The integrated approach using haploid technology with the conventional breeding methods and molecular techniques has revolutionized crop improvement programmes across the world, though the technology is not available for all crops. During introgression breeding, the first and foremost requirement in the development of widely adaptable cultivars is to confirm the alien chromatin. In recent years,

it has been established that genetic and physical maps are not directly comparable to the chiasmata owing to their unequal distribution over the chromosomes. Molecular cytogenetic tools like genomic in situ hybridization (GISH) and fluorescence in situ hybridization (FISH) have been greatly used for the proper identification and localization of alien chromosomes or chromosome segments in the crop background. These techniques have undoubtedly revolutionized introgression breeding, but to get most of these tools, proper understanding of the gene interaction among themselves and with the environment is required.

In conclusion, haploid technology holds great promise for cost-effective highly efficient crop improvement, in a sustainable way. Use of this dynamic technology in alliance with other novel tools in chromosome engineering can boost the targeted farm productivity and food quality in a sustainable and eco-friendly way. Thus, greater emphasis and significant investment must be focused on the use of such innovative techniques for accelerated and high precision crop improvement.

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