Chapter 20
Haploid Technology

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20.1 Introduction

Haploid technology has proven its worth as a rapid means of effecting crop improvement. Since each doubled haploid plant originates from a different gametophytic cell, a population of doubled haploid plants represents a collection of meiotically recombined but genetically fixed individuals. Doubled haploid populations provide both an ideal source of genetic variation from which to select superior genotypes and a convenient resource for genetic mapping. The rapid attainment of homozygosity is also useful in the context of fixing the outcomes of interspecific recombination, induced mutagenesis and transgenesis. Barley (*Hordeum vulgare*) is amenable to at least two routes of haploid plant production, i.e. either via in vitro culture of immature pollen or via uniparental genome elimination following interspecific hybridisation. The use of haploid technology in the cereals was pioneered in barley and has enjoyed widespread use in both applied and basic barley research.

20.2 The Generation of Plants from Haploid Cells

The capacity of a haploid founder cell to successfully differentiate into a haploid plant forms the basis of haploid technology. Doubled haploids are not only homozygous at all loci, but in addition, since the founder cells are the product of meiosis, the doubled haploid derivatives will differ from one another genotypically (Fig. 20.1). In the flowering plants, female gametophytes (i.e. embryo sacs) develop

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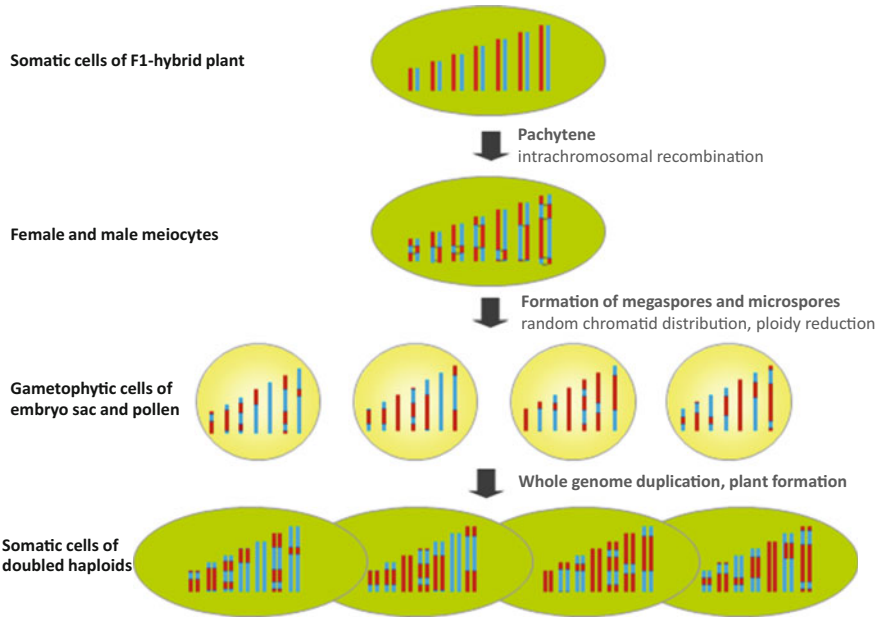

Fig. 20.1 Genetic recombination and fixation during doubled haploid production. The genetic constitution of the diploid donor plant (the chromosomes derived from its parents are indicated by different colours) is rearranged first by intrachromosomal recombination during the (meiotic) pachytene and then subsequently by random chromatid reassortment which is associated with the formation of the haploid megaspores and microspores, the immediate products of female and male meiosis, respectively. These spores are the founder cells of the female (embryo sac) and male (pollen) gametophytes, which themselves give rise to the egg and sperm cells. Whereas the pollen embryogenesis pathway of haploid plant formation involves the induction of cell proliferation and embryogenic development from the microspore or the vegetative cell of a young bicellular pollen, the parthenogenetic route originates either from the egg cell or possibly from another haploid cell type contained in the embryo sac. Haploids produced via uniparental genome elimination genetically derive from an egg or sperm cell, but embryogenic development is triggered by fertilisation, so that this pathway is associated with the transient presence of another haploid set of chromosomes (of *H. bulbosum* in the case of barley) that is lost again during the mitotic divisions taking place in the developing embryo (not shown for simplicity). Once haploid progeny have been successfully regenerated, either spontaneous or artificially induced whole genome duplication results in the restoration of diploidy and ensures that homozygosity prevails at all loci and that the plants are fertile

within the ovule, which forms part of the pistil, while the male gametophytes (pollen) develop within the anther. The result of a normal fertilisation is the fusion of the two gametes to form a diploid zygote, marking the beginning of the sporophytic phase. However, in some wide crosses, one of the two combined parental genomes is eliminated during the first few post-fertilisation mitotic cell divisions, resulting in a haploid embryo; the same process is also documented in intraspecific hybrids when one of the parents is a so-called inducer line (Ravi and Chan 2010). Whereas a zygote is programmed to undergo embryogenesis even

when one of its parental genomes has been lost, gametophytic cells have to be artificially triggered to proliferate, since the normal developmental programme of a gametophyte is strictly determined. The earliest identification of haploid barley plants was made by Johansen (1934), who noted that about 10 % of the seedlings developed from an unnamed cultivar had a distinctive morphology; their cytological behaviour later established that they were haploids. How such individuals arose spontaneously remains unknown.

20.2.1 Uniparental Genome Elimination Following Wide Hybridisation

The reproducible generation of haploid barley plants was first achieved by pollinating barley with *H. bulbosum*. Initial successes were achieved using autotetraploid accessions of both barley and *H. bulbosum* giving rise to dihaploid (diploid) plants (Davies 1958; Kao and Kasha 1969). It was later recognised that even diploid x diploid crosses were effective and that, following in vitro embryo rescue, the regenerated plantlets, which more resembled barley rather than *H. bulbosum* ones, carried just seven chromosomes, the haploid number of barley (Kasha and Kao 1970). Since the cells of developing hybrid embryos harboured up to 11 chromosomes, it was concluded that the formation of a haploid sporophyte relied on the gradual elimination of chromosomes rather than on a parthenogenetic process, as hypothesised by Davies (1958). Kasha and Kao (1970) understood the potential of barley haploids for varietal improvement. Pollination of diploid barley with a tetraploid accession (Lange 1971) or with more distantly related *Hordeum* species was later also shown to induce the formation of haploid embryos; these species include cereal rye (Fedak 1977; Forster and Dale 1983) and maize (Chen et al. 1991), albeit at a significantly lower level of efficiency than with diploid *H. bulbosum*. Surprisingly, perhaps, pollinating either *H. bulbosum* (Lange 1971) or *H. marinum* (Finch 1983; Jorgensen and von Bothmer 1988) with barley generated haploids carrying exclusively the barley nuclear genome, in this case within a non-barley cytoplasm. Ho and Kasha (1975) investigated the genetic control of chromosome elimination by pollinating each of the seven barley trisomic lines with tetraploid *H. bulbosum*, which showed genes on chromosomes 2H and 3H were involved in the elimination. Follow-on experiments based on monotelotrisomic lines indicated that the key genes mapped to both arms of 2H and to the short arm of 3H.

Cytological analyses have established that the full elimination of the *H. bulbosum* chromosomes is completed within nine days (Subrahmanyam and Kasha 1973; Bennett et al. 1976). Sanei et al. (2011) revealed that those *H. bulbosum* chromosomes which do not interact with the mitotic spindle and are therefore not transmitted to the daughter cells appear less condensed and lack a well-defined primary (centromeric) constriction during early mitotic anaphase.

The suggestion was therefore that asynchrony with respect to chromosome condensation must be a major contributor to the loss of chromosomes during cell division; the inability to form a functional kinetochore in a timely manner prevents the normal interaction with the mitotic spindle. Some corroboration has been provided by the observation that the centromeric histone H3 (CENH3) protein, an essential component of the kinetochore and a mediator between the centromere and the spindle microtubules, is present at levels below the detection limit in the centromeres of the lagging chromosomes, even though both *CENH3* mRNA is present and barley CENH3 can be taken up by *H. bulbosum* chromosomes (Sanei et al. 2011). The eventual elimination of the *H. bulbosum* complement is manifested by the formation of micronuclei, which are targeted for degradation. These micronuclei capture either entire or fragmented *H. bulbosum* chromosomes which lag during the separation of the two mitotic daughter cells, while highly condensed chromatin was also observed to be extruded from interphase nuclei (Finch 1983; Gernand et al. 2006).

The elimination process is efficient but not fully effective, as examples of incomplete genome elimination have been observed. Linde-Laursen and von Bothmer (1988) were able to show differences between the seven *H. bulbosum* chromosomes with respect to their propensity to be eliminated. Chromosome elimination is impaired at lower temperatures (Humphreys 1978), with a threshold of 18 °C during the early stages of embryo growth identified by Pickering and Morgan (1985). Whereas the incomplete elimination of the *H. bulbosum* genome can be regarded as a disadvantage in the context of doubled haploid production, the possibility of introgressing genes from *H. bulbosum*, the major representative of the secondary gene pool of cultivated barley, represents a valuable opportunity in barley improvement (Szigat and Pohler 1982; Xu and Kasha 1992). Some *H. vulgare* × *H. bulbosum* crosses have been performed with a view to producing introgression materials (Johnston et al. 2009, see also Chap. 17). Meanwhile, in breeding programmes employing doubled haploid technology, occasional hybrids can usually be recognised and discarded during embryo dissection on the basis of their distinctive shape, and any escapes from this selection step can be picked up at the seedling stage, since the leaves of hybrid plants are covered with soft hairs, a feature which is absent in cultivated barley.

Kasha and Kao (1970) assumed that the propensity to eliminate the *H. bulbosum* genome was genotype independent, but it is now known that some genetically determined variability does exist with respect to both fertilisation success and embryo development. This finding has driven the search for stocks which maximise the yield of haploid embryos (e.g. Simpson et al. 1980; Bjørnstad 1986; Devaux and Pickering 2005). The widespread deployment of the 'bulbosum' method during the last two decades of the twentieth century was promoted by the elaboration of robust protocols able to generate a regenerable haploid embryo in up to 30 % of florets pollinated (Kasha and Reinbergs 1976; Jensen 1976; Devaux and Pickering 2005). In a variation of the technique developed by Chen and Hayes (1989), a comparable level of efficiency was achieved by the in vitro culture of newly pollinated florets. Since the 2000s, however, the principle of pollen embryogenesis has largely

replaced uniparental genome elimination as a means of generating barley doubled haploids. The remaining interest in the latter route primarily rests on the finding that segregation bias is more intense in pollen-derived materials than in 'bulbosum'-derived ones (Devaux et al. 1995; Sayed et al. 2002; Cistué et al. 2011); this is of particular importance in the development of mapping populations (Johnston et al. 2009).

20.2.2 Parthenogenesis

Haploid plant formation is promoted in the *hap* mutant, which was induced by ethyl methane sulphonate treatment (Hagberg and Hagberg 1980). Up to 40 % of the embryos produced by self-pollinating a plant homozygous for the mutant allele are haploid progeny, while this frequency drops to 1–7 % in *hap* heterozygotes (Hagberg et al. 1985). The selfed progeny of a wild type x *hap* mutant hybrid include a significant proportion of haploid individuals, which implies that the genes underlying the trait are nuclear rather than cytoplasmic. A transmission electron microscopy-based investigation of the process of haploid embryo formation in the *hap* mutant has shown that the key event is the failure of fusion of the egg and sperm cell, even though the endosperm arises and develops normally (Mogensen 1982). What remains obscure is how the unfertilised *hap* egg cell is triggered to undergo embryogenesis. A scheme for exploiting the mutant for producing doubled haploid barley was elaborated by Hagberg et al. (1985). Its basis rested on the ability of *hap* heterozygotes to produce haploid progeny which harboured the wild type rather than the *hap* allele and further relied on the idea to develop a facile marker for these progeny (Hagberg and Hagberg 1987). The system has not so far been incorporated into a practical breeding approach, likely because of the low rate of haploid formation from *hap* heterozygotes and the risk of linkage drag associated with the mutant allele.

The successful regeneration of putative haploid barley plants from an unpollinated pistil explant cultivated in vitro has been reported on several occasions (San Noeum 1976; Wang and Kuang 1981; Huang et al. 1982; Castillo and Cistué 1993). A disadvantage of this approach is that it is difficult to exclude the products of accidental self-pollination, and furthermore none of these studies provided compelling evidence that the regenerants originate from the egg cell or another gametophytic cell type. Although an efficiency of up to ten plants per hundred cultured pistils has been reported, there are surprisingly no published examples of the use of this approach in either biotechnology or plant breeding practice.

20.2.3 Pollen Embryogenesis

The currently most widely employed method for producing barley haploids is based on the capacity to induce immature pollen to undergo cell proliferation and become embryogenic, a developmental route which has never been observed to occur naturally. The reprogramming of pollen development requires the imposition of a stress episode to abolish the cell's normal identity, followed by a period of culture in a medium which promotes cell proliferation and subsequent embryogenesis. Various stress conditions are effective, amongst which the commonest employed are low or high temperature and nutritional deficiency. The stress can be imposed either on the intact spike (Mordhorst and Lörz 1993), on dissected anthers (Roberts-Oehlschlager and Dunwell 1990; Hoekstra et al. 1992) or on isolated pollen (Kumlehn and Lörz 1999). Combinations of stress treatments have been reported to overcome the refractoriness of some cultivars (Coronado et al. 2005). Some anther culture protocols include no specific stress treatment but are nevertheless likely to expose the pollen to starvation given the temporary interruption in the supply of nutrients after anther dissection resulting from the time needed for components of the culture medium to diffuse through the anther wall. The developmental stage during which barley pollen is most readily triggered into embryogenic growth is around pollen mitosis I, between the time when the microspores are fully vacuolated (premitotic) and bicellular pollen grains were just formed. The physiological switch to amyloplast formation and starch accumulation which takes place in bicellular pollen grains is thought to be closely associated with the loss of the pollen's capacity of diverting its development away from its normal course towards cell proliferation and embryogenic growth (Daghma et al. 2014).

The formation of multicellular pollen structures can be achieved either by culturing an intact anther or by first releasing the immature pollen into the culture medium—both approaches are employed. Clapham (1973) was the first to describe the production of haploid barley plants following the pollen embryogenesis pathway, but at that time efficiency levels were low and a significant proportion of the regenerants lacked chlorophyll. Substantial progress has been achieved since this time by the use of mannitol solutions to expose spikes or anthers to carbohydrate starvation under appropriate osmotic conditions (Roberts-Oehlschlager and Dunwell 1990; Kasha et al. 2001), by optimising the severity of inductive stress (e.g. Coronado et al. 2005) and by developing protocols involving embryogenic pollen released at an early timepoint from anthers cultivated in liquid medium or by the isolation of immature pollen prior to cultivation (Ziauddin et al. 1990; Hoekstra et al. 1992). Major improvements of nutrient media for anthers or isolated pollen were associated with the use of maltose as a source of slowly accessible carbohydrate during embryogenic growth (Scott et al. 1995; Hunter 1989) and the use of significantly less ammonium as compared to the standard MS or B5 media (Mordhorst and Lörz 1993; Murashige and Skoog 1962; Gamborg et al. 1968). The capacity to regenerate plants from pollen-derived tissue has also been improved, e.g. by bringing forward the transfer of multicellular pollen structures

onto a solid medium and by increasing the concentration of copper sulphate in the medium (Kumlehn et al. 2006).

Further improvements in the efficiency of pollen embryogenesis have been hampered by a poor understanding of the underlying biological processes. A descriptive histological investigation based on fixed pollen structures was first conducted by Sunderland et al. (1979) and later extended by Ramírez et al. (2001). Meanwhile, Kumlehn and Lörz (1999) and Maraschin et al. (2005) tracked the development of living individual pollen grains by first immobilising them. The level of detail was limited by the length of the time interval between consecutive observations. Better definition has been achieved more recently by monitoring at 3 min intervals the development of vacuolated premitotic microspores up to the formation of multicellular, actively growing pollen structures (Daghma et al. 2012). Nine embryogenic and non-embryogenic types of pollen response to culture conditions were recognised. In the major embryogenic pathway, cell proliferation started from a symmetric mitosis (>50 % of pollen structures), whereas an asymmetric mitosis was associated with embryogenic development in less than 5 % of the test pollen. In the latter case, proliferation generally originated from a vegetative-like cell, while the generative-like one did not contribute to embryogenic development. Although generative-like cells occasionally divided, no further cell proliferation ensued (Daghma et al. 2014). In the same study, there was also evidence that the nuclear fusion of mitosis-derived pairs of daughter nuclei is the essential event for spontaneous whole genome doubling during barley pollen embryogenesis. Nuclear fusion events were observed throughout the process of pollen embryogenesis, explaining the known chimeric ploidy of microcalli and regenerants. The observations are consistent with those previously obtained using electron microscopy which have suggested that when nuclei coexist within a single cytoplasm following incomplete cytokinesis, their envelopes may fuse (González-Melendi et al. 2005).

The advent of 'omics' technologies has clarified some of the cellular processes associated with the initiation of pollen embryogenesis (Hosp et al. 2007). In barley, both *ECLTP* and *ECA1* are upregulated in early embryogenic pollen cultures (Vrinten et al. 1999); the former one shares sequence homology with a gene associated with embryogenic clusters in carrot cell cultures (Sterk et al. 1991), while an orthologue of the latter is highly activated in wheat egg cells (Sprunck et al. 2005). Nevertheless the product of neither of the two genes is likely to act as a sufficient trigger of the developmental switch from gametophytic to embryogenic pollen development. Other transcriptomic data sets have revealed at best genes playing a role in the cellular response to stress or genes which are in some way associated with embryogenesis rather than being essential factors for its initiation (Maraschin et al. 2006; Muñoz-Amatriaín et al. 2009). The major challenge which remains is that most of the cells present within an embryogenic pollen culture do

not differentiate into regenerable structures; rather they undergo programmed cell death. Consequently, there is a major risk of incorrect identification of genes, proteins and metabolites specifically associated with pollen embryogenesis.

20.2.4 Whole Genome Duplication

The frequency of spontaneous whole genome duplication in the product of an interspecific cross in which one parental genome has been eliminated is typically low, so progeny are mostly haploid. When both parental plants are auto polyploid, the resulting plants are typically, as expected, polyhaploid, yet those individuals are unlikely to be homozygous and thus will not be particularly useful for breeding purposes. In contrast, the majority of barley plants regenerated from embryogenic pollen cultures do experience whole genome duplication, as evidenced by the fact that at least some of their florets are self-fertile. Nonetheless, an artificial triggering of whole genome duplication can be desirable, e.g. when only few plants can be produced for some reason (Kumlehn et al. 2006). Colchicine-based whole genome duplication protocols, the use of which results in 50–80 % of doubled haploid regenerants, have been established for many years (e.g. Thiebaut and Kasha 1978), and no significant improvements in efficiency have been reported in the intervening years.

20.2.5 Evidence of the Cellular Origin and for the Homozygosity of Regenerants

Plants regenerated from cultured gametophytic explant material have not necessarily developed from a haploid cell, since the gametophyte can harbour some diploid cells following meiotic restitution events (Ramanna 1979; Bretagnolle and Thompson 1995) and also adjacent somatic (maternal) tissues may give rise to regenerants (Munyon et al. 1989; Arzate-Fernández et al. 1997; Bal et al. 2012). A thorough validation of a doubled haploid protocol is therefore needed before it can be extended to large-scale production. In some species, the homozygosity of doubled haploids has been confirmed using a variety of DNA-based markers (Meyer et al. 1993; Chani et al. 2000; Murovec et al. 2007; Nelson et al. 2009). More recently, Hofinger et al. (2013) have exploited to good effect an enzymatic mismatch cleavage assay to screen parental, F_1 hybrid and putative doubled haploids in barley.

20.3 Applications

20.3.1 Research and Pre-breeding

Populations of doubled haploids have been widely used to analyse the inheritance of quantitative traits, to produce genetic maps and to derive marker-trait associations (see also Chaps. 1, 15, 16, 18 and 19). Regenerable haploid cells are a particularly attractive target for recovering induced mutants, since it has the effect of fixing the mutated allele via whole genome duplication. The induction of mutants by irradiating barley spikes with γ-rays prior to anther dissection and culture has been reported by Szarejko et al. (1995). Castillo et al. (2001) mutagenised immature anthers and isolated microspores with sodium azide. Amongst the regenerants following pollen embryogenesis, some 15 % were reported to be morphologically altered in heritable fashion. Likewise, embryogenic pollen cultures have been successfully genetically transformed using both an *Agrobacterium*-mediated and a biolistics-based approach (Kumlehn et al. 2006; Shim et al. 2009). Kapusi et al. (2013) took advantage of the simplified segregation behaviour of doubled haploids (compared to that shown by sexually generated progeny) to produce selectable marker-free transgenic individuals. More recently, Gurushidze et al. (2014) have described a means to efficiently generate site-directed, true-breeding gene knockouts in barley using a customised transcription activator-like endonuclease. For more details on the synergy between haploid technology and transgenesis, see Chap. 21.

20.3.2 Practical Breeding

Haploid technology can enhance the efficiency of selection with regard to quantitative traits because it avoids the problem of genetic heterogeneity which is a feature of conventionally produced early generation material. It allows the bringing forward to a very early stage in the breeding programme of selection for traits which require replicated trialling—these traits are typically not testable before the F_5 generation in a pedigree-based conventional programme. The resulting savings in time and field resources can be substantial. The downside of the doubled haploid approach is the effort required to produce large numbers of progeny, which is a facile matter in conventional breeding programmes. Most current barley breeding programmes, at least in Europe, employ doubled haploid technology (see also Chap. 1). Six-rowed winter barley F_1 hybrid cultivars, which provide higher levels of yield and yield stability than conventional inbred cultivars (Mühleisen et al. 2014), are enjoying a steady increase in market share in Europe. An essential component of F_1 hybrid breeding is the maintenance of true-breeding parental lines, and doubled haploid technology provides a robust means to produce such materials.

Conclusion and Perspective

The key molecular mechanisms which trigger pollen embryogenesis and uniparental genome elimination—the two major props of haploid technology—are still obscure. Barley is a particularly useful experimental model for elucidating these mechanisms, since it enjoys a wealth of genetic and genomic resources, and is at the forefront of transgenic and mutation research. With respect to pollen embryogenesis, the stress treatments required to derail the normal developmental path in order to promote cell proliferation imply that the necessary cellular state lies on a knife edge between survival and death, and this perhaps reflects the continuing low frequency of embryo production achieved even from amenable genotypes. It is conceivable that the highest level of nonfatal stress which can be imposed may still be insufficient to induce embryogenic development in the most refractory cultivars. Since fertilisation success rates in interspecific crosses tend to be lower than in intraspecific ones, it would be advantageous to develop haploid inducer barley lines. The recognition of the CENH3 histone as a key determinant of the interaction between chromosomes and the mitotic spindle may pave the way towards this goal (Chan 2011). The greater our understanding of the cellular and molecular basis of haploid formation, the more feasible it will become to induce haploids under less stressful conditions than are utilised in current protocols, which should improve the efficiency of haploid plant production and avoid the problem of genotype dependency. Haploid technology already makes a major contribution to modern barley breeding but in addition has utility in the areas of marker discovery, genetic mapping, introgression breeding, mutagenesis and transgenesis. As such, its continuing use into the future seems to be assured.

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