II.3 Haploids in the Improvement of Poaceae

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1 Introduction

In 2001, cereals covered 45 % of the world's cultivated land, representing more than 0.67 billion ha producing 2.1 billion tonnes (from FAOSTAT, ,http://apps.fao.org/,). Although yield differed between developed and developing countries, 3.7 and 2.8 tonnes ha^{-1}, respectively, huge variations exist among countries, ranging from 0.12 to 7.6 tonnes ha^{-1}, which partly reflects the different environmental conditions over the globe. Despite the huge range, both yield and quality have greatly increased through the release of well-adapted new cultivars over the last few decades. In most of the cultivated Poaceae, the development of superior cultivars has been a continuing breeding process for many different characters. These include yield, quality of the harvested products, factors related to consistent yield and quality such as resistance to pests and diseases and tolerance to abiotic stress, which all contribute to more sustainable agriculture.

Among the Poaceae, there are both self- and cross-pollinated species, and landraces, natural populations and modern cultivars have been cultivated as synthetic, F_1 hybrids or homozygous selections, which require different breeding schemes depending on the species. Homozygosity is often a requirement for registering new self-pollinated cultivars and superior lines prior to crossing but is also useful as a tool to decrease the frequency of undesirable alleles in populations of cross-pollinated species by phenotypic selection. Homozygosity can be achieved by means of several inbreeding systems including selfing, full- and half-sib mating and backcrossing, all of which differ in the time needed to attain homozygosity and opportunities for selection (Hallauer and Miranda 1981). However, the fastest route to homozygosity is doubled haploidy and, therefore, doubled-haploid plants (DHs) have been very attractive to many scientists and breeders. DHs can be produced by several methods, which vary in their efficiency and are species-dependent. In some species, such as barley for which thousands of DHs can be produced

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routinely at moderate cost, doubled haploidy has been used widely in breeding programs and has contributed to the release of many cultivars. In other species, for example maize, low efficiency has limited its use. Homozygous DH populations have also enabled the mapping of many molecular markers as well as qualitative and quantitative trait loci (QTL) for subsequent marker-assisted selection (MAS). These have contributed to gene isolation. To a lesser extent haploidy has been used in mutation breeding and in genetic transformation.

Since we published a review on barley DHs 10 years ago (Pickering and Devaux 1992), several excellent books related to doubled haploidy have been published (Jain et al. 1996/1997; Chupeau et al. 1998). In this chapter, we focus on the latest developments in doubled haploidy of the Poaceae for optimum production and usage. In this respect, most of the cited references are more recent than 1992 since many important ones have already been noted in our 1992 review.

2 Doubled Haploid Production

2.1 Anther Culture

2.1.1 Donor Plant Growth Conditions

It has been widely recognized that the conditions under which the donor plants have been raised are critical to the success of tissue culture and more specifically of anther and microspore culture. To ensure better reproducibility or to eliminate some of the environmental parameters that adversely influence the technique, controlled environment growth chambers have been preferred (Afele and Kannenberg 1990; Hoekstra et al. 1992; Barceló et al. 1994; Orshinsky and Sadasivaiah 1997), although conventional greenhouses have been the most commonly used environment (Devaux et al. 1993b; Alemanno and Guiderdoni 1994; Saisingtong et al. 1996; González et al. 1997; Puolimatka and Pauk 2000). However, a few authors have collected their material from field-grown plants (Karsai et al. 1994; Lentini et al. 1995; Machii et al. 1998; Tuvesson et al. 2000) and perhaps, therefore, their investigation has been limited by availability of growth rooms and the success rates subject to more variation. Three optimal temperatures in growth rooms and greenhouses have been used depending upon the species. The lowest temperature range is for barley, usually between 12 and 15 °C (Ziauddin et al. 1992; Hou et al. 1994) with a possible reduction during the night (Kintzios and Fischbeck 1994). A higher range of temperatures, 15–20 °C, is more suitable for hexaploid wheat, triticale, rye and perennial ryegrass (Flehinghaus-Roux et al. 1995; Madsen et al. 1995; Redha et al. 1998; Immonen and Robinson 2000), although Orshinsky and Sadasivaiah (1997) reported more embryos

and green shoots when wheat donor plants were grown at high day/night temperature (25/18 °C) or when transferred from low (15/12 °C) to high temperature. The highest temperatures are most appropriate for maize (25–28/19–22 °C) (Afele and Kannenberg 1990; Saisingtong et al. 1996) and rice (28–31/18–20 °C) (Lentini et al. 1995). A 16-h day length regime is considered optimum for donor plant growth, with an irradiance ranging from 200 $\mu E\, m^{-2}\, s^{-1}$ for wheat (Henry et al. 1993) up to 600 $\mu E\, m^{-2}\, s^{-1}$ for maize (Martin and Widholm 1996).

2.1.2 Spike Sampling and Pretreatment

In most species, spikes were sampled just before the first microspore mitosis, i.e., from mid- to late-uninucleate stage, while maize tassels were collected when microspores were more advanced, from late uninucleate to early binucleate (Martin and Widholm 1996). The stage of microspores can easily be checked by squashing an anther taken from the middle of the spike in acetocarmine for microscopic examination. In the early uninucleate stage, the nucleus is near the pore and by late uninucleate it is opposite the pore (Kasha et al. 2001a). Pretreatment of whole spikes or isolated anthers is often required to switch the gametophytic pathway into a sporophytic development of the microspores (for review, see Reynolds 1997). The pretreatment induces stress to the microspore at a critical stage and can be applied as low or high temperature, osmotic shock, or chemicals to induce starvation and microtubule disruption chemicals. In cereals, a cold pretreatment has often been favored (Jähne and Lörz 1995) as it provides more flexibility and is less labor-intensive for routine production than some of the other pretreatments. Temperatures of about 4 °C have been used for barley, wheat, triticale and rye, while higher temperatures ranging from 7 to 10 °C are more desirable for maize and rice. However, for a hybrid between *Lolium multiflorum* × *Festuca arundinacea*, there is no beneficial effect of cold pretreatment on androgenesis response (Zare et al. 2002). The length of pretreatment also depends upon the species, with the longest time for barley, usually 28 days, intermediate for maize, triticale and wheat, and the lowest, for example 8 days and less, for rice and rye. Starvation on a 0.3- to 0.37-M mannitol solution was first reported for barley (Roberts-Oehlschlager and Dunwell 1990) and was progressively adopted by several laboratories (Ziauddin et al. 1992; Hoekstra et al. 1997; Caredda et al. 1999). Improvements were then achieved by incorporating culture media macronutrients in the mannitol solution (Hoekstra et al. 1997; van Bergen et al. 1999), by increasing the concentration of mannitol up to 0.7 M for low-responsive cultivars (Cistué et al. 1994, 1999; Castillo et al. 2001a) or by combining cold and starvation (Wojnarowiez et al. 2002). The effect of the hyperosmotic stress on the programmed cell death or apoptosis in the anther tissue of barley has been investigated by Wang et al. (1999) who found a more pronounced intranucleosomal cleavage of DNA in the pretre-

ated anthers than in the control. Using the TUNEL reaction, electron microscopy and RNA quantification, they described how the loculus wall and loculus tapetum cells were mainly affected by the treatment. The authors suggested that the stress conditions stimulated abscisic acid (ABA) production, which might inhibit apoptosis in ABA-susceptible microspores. Van Bergen et al. (1999) confirmed that high ABA content during anther pretreatment was correlated with subsequent high regeneration efficiency. Other chemicals such as colchicine added during the first days of anther culture have also improved microspore-derived embryo frequency in wheat (Szakács and Barnabás 1995) and maize (Saisingtong et al. 1996).

2.1.3 Induction Media and Culture Conditions

In barley, the most popular medium has been FHG (Hunter 1987), which differs from the original Linsmaier and Skoog (LS) medium (1965) by a tenfold reduced concentration of the ammonium nitrate, by omitting cobalt chloride and by adding 750 mg l^{-1} L-glutamine. The basic Potato-2 medium (Chuang et al. 1978) is popular for wheat (Henry and de Buyser 1990), triticale (Wang and Hu 1984), and ryegrass (Olesen et al. 1988) anther culture. To ensure more consistent results some researchers prefer synthetic media such as N6 (Orshinsky and Sadasivaiah 1994; González et al. 1997), FHG (Lashermes 1992), 190–2 (Brisibe et al. 1997), W14 (Immonen and Robinson 2000; Puolimatka and Pauk 2000; Zamani et al. 2000) and C17 (Machii et al. 1998; Arzani and Darvey 2001). However, the modified Potato-2 medium outperformed all synthetic media for wheat anther culture (Henry and de Buyser 1990). In maize, the basal YP medium of Ku et al. (1978), modified by Genovesi and Collins (1982), has been used by many researchers (Büter 1997), while in rice the N6 medium of Chu (1978) remains popular for japonica and the SK3 medium more suitable for indica/japonica hybrids (He et al. 1998). Several carbohydrates have been used depending upon the species. In barley, the most significant development for improving success rates has been the use of maltose (Hunter 1987), which has sometimes replaced sucrose in tetraploid and hexaploid wheat (Navarro-Alvarez et al. 1994; Otani and Shimada 1994, 1995; Stober and Hess 1997), triticale (Immonen and Robinson 2000), rice (Lentini et al. 1995), rye (Flehinghaus-Roux et al. 1995), and ryegrass (Opsahl-Ferstad et al. 1994). However, in maize, no other carbohydrate has been superior to sucrose for embryo and plant production (Büter 1997). Disaccharide concentration in induction media varies from 6% for barley and rice to 9–12% for wheat, triticale and maize (Barnabás et al. 1999). The effects of plant growth regulators in anther culture have been controversial, but there are some trends. Four main auxins have been specified, IAA, NAA, PAA and 2,4-D, at concentrations ranging from 0.5–2 mg l^{-1}; cytokinins are also added such as kinetin or 6-benzylaminopurine (BAP) at 0.1–1 mg l^{-1} (Cai et al. 1992). In general, weaker auxins at low concentration have been used for

barley more than in any other species. In some cases, the auxin has been omitted, leaving BAP as the sole growth substance (Kihara et al. 1994). Indeed, the anti-auxin 2,3,5-triiodobenzoic acid (TIBA) at low concentration (0.1 mg l^{-1}) may be suitable for maize (Dieu and Beckert 1986; Barnabás et al. 1999). Several other components have occasionally been added to the induction media. These include the ethylene antagonists silver thiosulfate or silver nitrate (Lashermes 1992; Evans and Batty 1994; Lentini et al. 1995), activated charcoal (Saisingtong et al. 1996) and L-proline to increase embryogenesis (Redha et al. 1998). Liquid, semi-liquid, and solid media have all been employed, and although Ficoll added to liquid medium enhanced embryo and plant production (Devaux 1992; Cistué et al. 1999; Immonen and Robinson 2000), solid media are easier to handle and cheaper and, therefore, they are preferred. Several gelling agents are available ranging from basic agar (0.7–0.8 %) to high-grade agarose (0.6 %), but gelrite (0.1–0.3 %) now usually replaces agar in the induction media as it yields better results. Membrane rafts have been proposed in conjunction with liquid media, but adverse effects have been obtained (Luckett and Smithard 1995). Anther cultures have been usually maintained at a range of temperatures between 21 and 29 °C often in darkness or with a 16-h photoperiod under dim light as embryogenic induction of microspore is inhibited by high-intensity white light (Reynolds and Crawford 1997). An increased (32 °C) (Brisibe et al. 1997) or reduced 14 °C temperature (Saisingtong et al. 1996; Redha et al. 1998) for the first 3–7 days may improve anther culture response.

2.1.4 Regeneration

Plantlet regeneration has been achieved by transferring the embryo-like-structures (ELS) and calli >1 mm in size to a fresh medium. The basal medium for regeneration may resemble or differ from the induction medium. A variety of similar synthetic media to those used for induction have been adopted for regeneration and solidified by agar or, more usually, gelrite. The most common carbohydrate used for regeneration is sucrose at 2–3 %, but some laboratories prefer maltose (Castillo et al. 2001a). Growth substances may be omitted, but an auxin (IAA, NAA or 2,4-D), a cytokinin (kinetin, BAP) or a mixture of the two has been used for regeneration at concentrations ranging from 0.05–1 mg l^{-1}. After transfer to a regeneration medium, the cultures are kept in growth rooms at temperatures of about 25–26 °C either in the dark or under a 16-h dim to mid-light regime. In most cases, the regenerated green plantlets are then transferred to the same solid medium but without any growth substances or with 1 mg l^{-1} IAA and 2 % sucrose before transplanting to potting compost.

2.1.5 Albinism

Almost every species in the Poaceae has been affected by albinism. In barley and rice, which have been more severely affected by albinism, green plant:albino plantlet ratio (G:A) is usually <1 (Caredda and Clément 1999) but fluctuates according to genotype, pretreatment and culture conditions. In wheat, the growth conditions of the donor plants are an important factor, too (Orshinski and Sadasivaiah 1997; Dogramaci-Altuntepe et al. 2001). Some genotypes regenerate few green plants (Tuvesson et al. 1989; Jähne et al. 1991; Lentini et al. 1995; Moieni and Sarrafi 1995; Stober and Hess 1997), which precludes the use of androgenesis when these genotypes are used in breeding programs. Plastid differentiation during anther culture was investigated in two barley cultivars, 'Cork' and 'Igri', which produce high and low proportions of albino plants, respectively (Caredda et al. 2000). Differences in proplastid differentiation, thylakoid development, and the ability of cells to divide were observed and the fate of microspore plastids could be predetermined as early as the microspore stage (Caredda et al. 2004). In contrast to Cork, plastids in Igri contained DNA at the time of culture, indicating that perhaps DNA degradation occurred earlier in Cork than in Igri. Deletions of genes related to chlorophyll and photosynthesis in the nuclear and plastid genomes occur in albino plantlets of Igri (Dunford and Walden 1991) and rice (Harada et al. 1991; Yamagishi 2002) and probably arise during regeneration (Mouritzen and Holm 1994). Pretreatment and culture conditions are also critical factors influencing green plant regeneration. In barley, Caredda et al. (1999) observed that organelle structure and G:A ratios were both improved by mannitol pretreatment rather than a period of cold. The ratio was further increased on an induction medium with mannitol as an osmotic pressure regulator (Wojnarowiez et al. 2004). However, in rye and in triticale, cold pretreatment seems optimal for green plant production (Immonen 1999; Immonen and Robinson 2000). G:A ratios for wheat were significantly improved when colchicine was added to the medium at the start of microspore induction (Zamani et al. 2000) and by replacing sucrose with maltose in the induction media for barley (Finnie et al. 1989), wheat (Navarro-Alvarez et al. 1994) and rice (Lentini et al. 1995).

2.1.6 Ploidy Level of Regenerants and Chromosome Doubling

The distribution of regenerated plantlets from anther culture according to their ploidy level differs among species. The rate of spontaneous chromosome doubling is on average 60 % in barley, 27 % in wheat, 17 % in triticale (Devaux 1992), 10 % in maize (Büter 1997), 50 % in rice (Guiderdoni et al. 1991) and 70 % in rye (Immonen 1999). Moreover, a relatively high proportion of polyploid, mainly tetraploid plants (8 %) are recovered from barley anther culture, the remaining plants being mainly haploid (Devaux 1992).

Fig. 1. Ploidy level determination of anther culture-derived plantlets by flow cytometry. Thirty plants can be checked in 1 h to identify spontaneous doubled haploid plantlets and to ensure an efficient chromosome doubling of haploid plants at an early stage

There are reports within a species of variations in spontaneous doubling according to the genotype (Alemanno and Guiderdoni 1994; Stober and Hess 1997), but ploidy level can be easily determined by flow cytometry (Coba de la Peña and Brown 2001) at a speed of 30 plants h^{-1} (Fig. 1). As an alternative, an indirect ploidy determination method, such as stomatal guard cell length (Borrino and Powell 1988) or stomatal chloroplast number (Ho et al. 1990), could be used. Following the analyses, polyploid plants can be discarded, diploid ones directly planted in the greenhouse and haploid plants treated with colchicine for chromosome doubling. Several factors influence the rate of spontaneous doubling in anther culture. These include the pretreatment stringency (Immonen and Robinson 2000), the carbohydrate source in the culture media (Navarro-Alvarez et al. 1994) and supplementing the medium with colchicine or antimicrotubule agents (herbicides) either during pretreatment (Antoine-Michard and Beckert 1997) or during incubation (Wan et al. 1991; Saisingtong et al. 1996; Redha et al. 1998; Barnabás et al. 1999).

2.2 Isolated Microspore Culture

Isolated microspore culture (IMC) is a development from anther culture in which the microspores are removed mechanically from the anther prior to in vitro culture. As haploid single cells, microspores are ideal for selection,

mutation, transformation and biochemical analysis. Furthermore, recent improvements in IMC efficiency enable the technique to be considered for routine production of DH, especially barley (Kasha et al. 2001a). IMC differs technically from anther culture in the following respects. Donor plants are raised either in controlled environments such as greenhouses or in growth rooms, under similar conditions to those for anther culture, although higher temperatures can be used especially for spring types (Kasha et al. 2001a; Ritala et al. 2001). In barley and wheat, spikes may be collected when microspores have reached the late-uninucleate to early-binucleate stage (Gustafson et al. 1995; Ritala et al. 2001). Excised anthers are usually pretreated in 0.3–0.7 M mannitol solution for 3–7 days in the dark either at 25–33 °C (Mouritzen and Holm 1994; Touraev et al. 1996; Castillo et al. 2000) or at cooler temperatures (Guo and Pulli 2000), while entire spikes are cold-pretreated (Mordhorst and Lörz 1993; Puolimatka and Pauk 1999) with starvation (Kasha et al. 2001a). A beneficial effect of macronutrients in the mannitol solution has also been reported (Hu et al. 1995; Li and Devaux 2001). In wheat, pretreatment of spikes with an inducing agent consisting of 0.1 $g\,l^{-1}$ of 2-hydroxynicotinic acid (2-HNA), 10^{-6} $mol\,l^{-1}$ of 2,4-D and 10^{-6} $mol\,l^{-1}$ of BAP converted up to 50 % of the microspores to the sporophytic pathway and resulted in high green plant production (Liu et al. 2002). Alternatively, a 2-HNA treatment can be applied to freshly isolated wheat microspores prior to culture (Zheng et al. 2001). Following pretreatment, microspores are released from anthers by blending (Mouritzen and Holm 1994; Gustafson et al. 1995), vortexing (Hu et al. 1995), stirring (Touraev et al. 1996), pestle, glass or Teflon rod maceration (Hoekstra et al. 1993; Cistué et al. 1995; Ritala et al. 2001) either in 0.3–0.4 M mannitol solution, washing solution or culture medium (Mejza et al. 1993; Salmenkallio-Marttila et al. 1995). Washing solutions differ from the induction medium by a reduced concentration of nutrients and by replacing maltose by sucrose (Kunz et al. 2000) or by adding 10 mM calcium chloride to the mannitol solution (Li and Devaux 2001). To save time, microspores can be isolated by blending wheat spikelets (Mejza et al. 1993) or barley spike segments (Mordhorst and Lörz 1993; Scott and Lyne 1994). The resulting microspore-containing slurry is filtered through a nylon mesh and subjected to several cycles of washing and centrifugation. To obtain consistent and viable cultures, dead cells and small debris can be removed by a density gradient of 18–21 % maltose (Ritala et al. 2001) or by a discontinuous Percoll gradient (Gaillard et al. 1991; Touraev et al. 1996) followed by centrifugation. In liquid media, microspores are cultured at densities ranging from 7×10^3 to 2×10^5 microspores ml^{-1} (Gustafson et al. 1995; Zheng et al. 2002), while between 3×10^3 and 6×10^5 microspores are deposited on filter papers (Hoekstra et al. 1996; Kasha et al. 2001a). Microspore culture media are basically the same as those used for anther culture except that some are conditioned with ovary pre- or co-culture, resulting in higher efficiencies and less genotypic influence (Hu and Kasha 1997; Zheng et al. 2002). Arabinogalactan protein (AGP) which has been identified in conditioned media with barley

IMC (Paire et al. 2003) or related hydroxyproline glycoproteins can be added to the induction medium to improve microspore response (Kasha and Simion 2001). The use of the auxin PAA in the induction medium has improved plant regeneration from barley microspore culture (Ziauddin et al. 1992). When liquid media are employed, 0.5–2 ml of fresh medium can be added 1–2 weeks after the beginning of the culture. Cultures are kept stationary and put onto a rotary shaker at ca. 70 rpm after 1–2 weeks (Salmenkallio-Marttila et al. 1995; Li and Devaux 2001) or continuously shaken (Scott and Lyne 1994). In a comparative study, Davies and Morton (1998) showed that IMC was much more efficient than anther culture with the barley cultivar Igri and to a lesser extent with an Australian spring F_1 hybrid. Although three of the 17 F_1 hybrids investigated by Castillo et al. (2000) responded better with IMC than anther culture, the average yield of anther culture was twice that of IMC. From the results of many studies, high yields of plant production, e.g., 50 green plants per anther, can be achieved by IMC with model genotypes, e.g., Igri (Hoekstra et al. 1996), but if the critical parts of the protocol have been followed similar yields can be expected from any other genotype (Kasha et al. 2001a). The rate of spontaneous chromosome doubling from IMC ranged from 5–83 % (Pauk et al. 2000; Kasha et al. 2001b). Hu and Kasha (1999) and Kasha et al. (2001b) observed that following the first mitotic division during pretreatment, the two daughter nuclei fused to form a diploid nucleus, which then undergoes rapid divisions. Increases in spontaneous chromosome doubling have been achieved by combining cold pretreatment of spikes with mannitol treatment of microspores (Li and Devaux 2003) or by adding colchicine or antimicrotubule agents to the culture medium (Hansen and Andersen 1998a,b).

2.3 Interspecific and Intergeneric Hybridizations

2.3.1 Barley × H. bulbosum

This cross was the preferred method for producing barley DHs before great improvements in androgenesis caused interspecific hybridizations to be superseded by anther and microspore culture. However, the interspecific cross is still used as an alternative method to obtain barley DHs from hybrids that are recalcitrant to androgenesis and also where an unbiased random sample of gametes is required for a mapping population. In general, 10–15 DHs per 100 pollinated florets can be achieved. The interspecific method was first described by Kasha and Kao (1970) who elucidated the mechanism by which barley haploid embryo formation occurred. Following fertilization of the *H. vulgare* egg by the *H. bulbosum* male gamete, the complete genome of the wild species is rapidly eliminated in the first few days after fertilization. The resultant haploid embryos must be rescued aseptically to a defined medium (for example, Gamborg's B5) prior to endosperm degeneration, usu-

ally about 12–14 days after pollination (d.a.p.). Haploid plantlets that develop can be treated with an aqueous colchicine solution (0.05%) + 2% dimethyl sulfoxide to restore the fertility by doubling the chromosome number. Chromosome elimination may not always take place and depends on parental genotype (Simpson et al. 1980; Pickering 1983; Pickering and Rennie 1990) and temperature during the first few days after pollination (Pickering 1984). To prolong seed development to the time of embryo culture, gibberellic acid (GA_3) at 75 mg l^{-1} with a wetting agent (Tween 20) is applied to florets 1 or 2 d.a.p. Occasionally, some cultivars do not respond well, for example, 'Magnum', and a combination of GA_3 and 2,4-D was developed as a postpollination spray for these cultivars (Pickering and Wallace 1994). Since then, GA_3 +

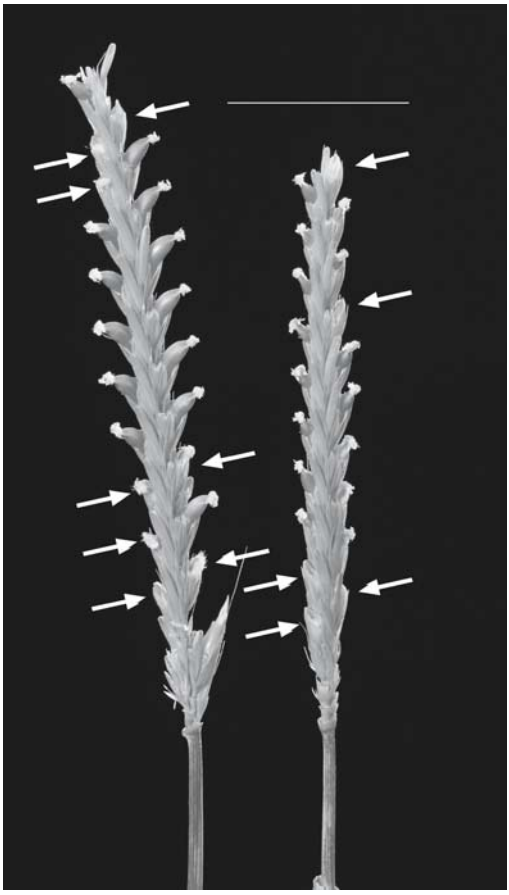

Fig. 2. Emasculated barley spikes 13 days after pollinating florets with *Hordeum bulbosum* L. *Left* Postpollination spray treatment 1 day after pollination with 75 mg l^{-1} GA_3 + 2 mg l^{-1} 2,4-D + 1 mg l^{-1} dicamba; *right* postpollination spray treatment 1 day after pollination with 75 mg l^{-1} GA_3. Note differences in seed size and unfertilized florets (*arrowed*) and the selfed seed (*lowermost floret on the left-hand spike*). *Bar* 20 mm

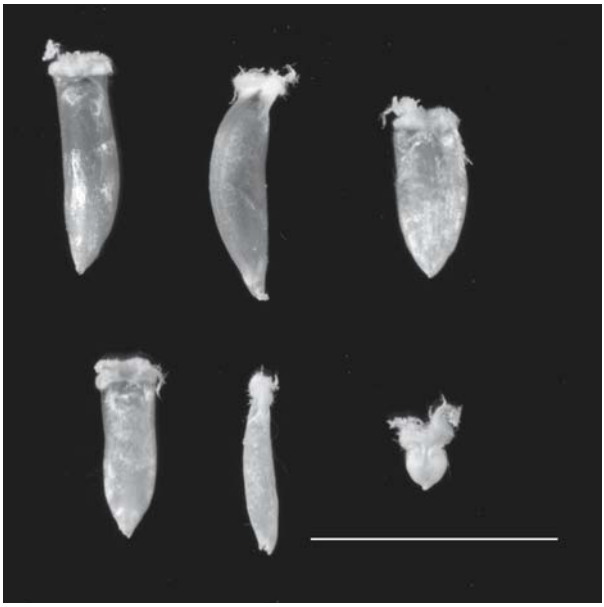

Fig. 3. Seeds and unfertilized florets removed from spikes illustrated in Fig. 1. *Top row*, *left* and *center* Seed development after postpollination spray treatment 1 day after pollination with 75 mg l^{-1} GA_3 + 2 mg l^{-1} 2,4-D + 1 mg l^{-1} dicamba; *right* unfertilized floret with the same postpollination treatment. *Bottom row* as for top row but spray treatment 75 mg l^{-1} GA_3 only. Note differences in seed development promoted by the addition of 2,4-D and dicamba. *Bar* 10 mm

2,4-D + dicamba at 75, 2 and 1 mg l^{-1}, respectively, has been used routinely 1 d.a.p. to reduce seed shrivelling (Figs. 2 and 3) without causing adverse effects on plant regeneration (Pickering, unpubl.). Apart from these minor refinements to the protocol since our previous review (Pickering and Devaux 1992), there have been no major reported improvements to the technique, so we refer the reader to this publication and to Devaux (2003) for more complete details.

2.3.2 Wheat × Maize; Triticale × Maize

To obtain DHs from tetraploid and hexaploid wheat via androgenesis has not been as successful as with barley. Initially, it was hoped that wide hybridizations between wheat and *H. bulbosum* might have been successful. However, unfortunately, because of a series of incompatibility (*Kr*) loci in most wheat cultivars, crossability has usually been very low (Laurie and Bennett 1987). Nevertheless, following the pioneering research of Laurie and associates (Laurie and Bennett 1988), wheat crossed with maize has been the method of choice for producing DHs and details of the many factors involved in DH production have been presented by Knox et al. (2000). In brief, a similar protocol

is adopted as for barley × *H. bulbosum* although a higher concentration of postpollination plant growth regulators (PGRs) is usually applied either as a spray or injected into the last internode; GA_3 can be omitted as it appears to be ineffective on wheat (Knox et al. 2000). Some of the other factors that are important in achieving successful production are growth conditions for donor plants, including light (Campbell et al. 2001), emasculation method and PGR applications (Knox et al. 2000). Genotypic influences have been reported, but these are generally not as important a factor as for androgenesis. Success rates with hexaploid wheat are lower than those obtained from barley × *H. bulbosum* (around 6 DHs per 100 pollinated florets; Lefebvre and Devaux 1996) and tetraploid wheat DH success rates are very much lower (<1 DH per 100 pollinated florets; Knox et al. 2000; P. Devaux, unpubl.). There have been no major improvements to the success rates for tetraploid wheats, although some minor modifications to the protocol, such as optimum PGR applications especially dicamba (Knox et al. 2000), have raised DH yields. Regarding DH production from hexaploid triticale using the wide hybridization technique, very little research has been undertaken to improve success rates, which are generally similar to those obtained with durum wheat (around 1–2 DHs per 100 pollinated florets; P. Devaux, unpubl.) and can be attributed to the absence of the D genome in durum wheat and hexaploid triticale (Inagaki and Hash 1998). Increases in DH efficiency were gained by modifying the PGR composition, especially dicamba, and concentrations of the components (Wedzony et al. 1998). For the future, therefore, optimizing PGR composition and concentrations should result in further improvements for both durum and triticale DH production.

2.3.3 Oat × Maize

There was some optimism that oat × maize hybridizations might have resulted in haploid oat embryo formation, but although very small numbers of oat haploids were obtained (Rines and Dahleen 1990) it later became clear that there were some anomalous plants formed after embryo rescue that contained additional chromosomes (Riera-Lizarazu et al. 1996). These were identified as maize chromosomes and since then the complete series of oat–maize chromosome addition lines have been obtained (Kynast et al. 2001). They are proving invaluable for research into species relationships and mapping experiments. The problem still remains of consistently obtaining oat DHs, and screening maize parental genotypes would be appropriate. However, since the oat crop is relatively minor compared with wheat and barley, the amount of research undertaken is consequently much less and funding harder to procure.

3 Use of Doubled Haploids

3.1 Breeding

DHs have long been used for breeding since they can easily be produced from a wide range of crosses. The advantages and limitations of doubled haploidy in breeding have been discussed many times (see, for example, Kasha and Reinbergs 1982; Devaux 1995; Steffenson et al. 1995). Gallais (1990) proposed the use of DHs for recurrent selection and line development as being one of the most efficient methods for low heritable traits. Thomas et al. (2003) pointed out that many factors influence the deployment of the DH method in breeding. Although DHs can be used at different stages of a breeding scheme and for several purposes, an appropriate way to assess the value of doubled haploidy in practical breeding is the number of cultivars released as DHs. In barley, wheat, rice and triticale nearly 150 cultivars were produced by doubled haploidy (www.scri.sari.ac.uk/assoc/COST851/) and the number is continuously increasing. With the development of linkage maps based on DHs, segregation distortions at marker loci were often detected (Devaux et al. 1995; Yamagishi et al. 1996; Dufour et al. 2001). Although skewed segregations were also reported in F_2 (Yamagishi et al. 1996) and single-seed descent populations (Bjørnstad et al. 1993), the complete occurrence of one parental allele has never been reported in any DH progeny derived from polymorphic parents. For example, the strongest distortion reported by Devaux et al. (1995) was 21 DHs with the Steptoe allele and 80 DHs with the Morex allele. Therefore if the DH production technique is efficient enough many DHs with the less frequent alleles can be produced and developed for breeding. When reliable molecular markers for the desired traits have been identified, selection pressure can be performed at an early stage, for example in vitro regeneration to eliminate undesirable recombinants among progenies of a breeder's crosses. Time and space can, therefore, be saved, enabling the identification of superior agronomic types very quickly. Furthermore, the molecular characterization of individual alleles at markers is simple and accurate with haploid and doubled-haploid tissues and will be discussed in more detail in the following section. By scoring the presence vs. absence of particular fragments, haploid and doubled-haploid tissues can be genotyped using PCR-dominant markers (Devaux 1995) such as sequence tagged sites (STSs) and amplified fragment length polymorphisms (AFLPs). Another application of molecular markers with regard to DHs as well as traditional lines is fingerprinting. Commercially promising DHs or lines can be genotyped with a set of molecular markers, most commonly simple sequence repeats (SSR), dispersed on each chromosome along with their parents and the most commonly grown cultivars. The genotyping data along with phenotype records help breeders in the choice of potential crosses by strengthening the characterization of new lines as well as determining their relative genetic distance from the current cultivars.

3.2 Molecular Genetics and Genomics

3.2.1 QTLs Influencing in Vitro Response

In the early 1970s when anther culture was in its infancy, genotypic differences in anther culture response were reported and well-designed experiments confirmed the occurrence of genes controlling this trait (see, for example, Charmet and Bernard 1984; Lazar et al. 1984; Powell 1988; Afele and Kannenberg 1990; Quimio and Zapata 1990). Consequently, those genes became targets for mapping. In maize, Cowen et al. (1992) detected two major and two minor QTLs accounting for 57% of the genetic variability for embryo-like structure (ELS) production. While investigating three crosses, Murigneux et al. (1994) found three to four QTLs related to percentage of responding anthers or embryo production specific to each cross, explaining 30–40% of the phenotypic variation. Using different material Beaumont et al. (1995) confirmed the perfect match of at least three QTLs identified by Murigneux et al. (1994) on chromosomes 5, 7 and 8. From a linkage map based on an anther culture-derived DH population of barley (Zivy et al. 1992), Devaux and Zivy (1994) hypothesized that their protein markers, which deviated from an expected 1:1 segregation ratio, could be linked to genes involved in anther culture response. They established that two QTLs were linked to genes involved in both ELS production and green plant regeneration, whereas the other two QTLs were linked to genes involved only in green plant regeneration. In a similar test, Manninen (2000) described 10 QTLs associated with percentage of responsive anthers, plants per responsive anther and spontaneous diploidization located on chromosomes 2H, 3H and 4H. Furthermore, not all regions associated with anther culture response matched regions where distorted segregation occurred. Using a DH population derived from an indica/japonica rice hybrid, He et al. (1998) identified five QTLs for callus induction frequency on chromosomes 6, 7, 8, 10 and 12, two QTLs for green plantlet differentiation frequency on chromosomes 1 and 9 and a major QTL for albino plantlet differentiation frequency on chromosome 9. Also in rice, a QTL associated with green plant regeneration on chromosome 10 was detected over three culture methods (Kwon et al. 2002). Hence, one marker identified genotypes with good and poor regenerability across many cultivars. Four QTLs explaining 80% of the genotypic variation for green plant regeneration were detected in wheat anther culture (Torp et al. 2001).

3.2.2 Mapping with Molecular Markers

Using genetic markers to manipulate loci controlling traits of interest and to understand gene organization in complex genomes represents a major breakthrough for plant geneticists and breeders. Advances in methods for assaying DNA polymorphisms have produced hundreds of segregating genetic mark-

ers in many species. In traditional linkage analysis the genetic markers have been arranged into dense genetic linkage maps (http://wheat.pw.usda.gov/ggpages/maps.shtml), enabling them to be used as selection criteria when they have been mapped near trait loci. Different types of segregating populations have been used in genetic mapping including F_2, F_3, backcross, recombinant inbred lines (RILs) and DHs. RILs and DHs become immortal populations once each individual has been selfed and the seeds stored properly. These populations remain stable for different purposes such as phenotypic evaluation under different selection pressures and for continuously mapping new markers and traits (Guyomarc'h et al. 2002; Thiel et al. 2003). Therefore, RILs and DHs represent an invaluable tool to identify QTLs and to assess their variability across environments and years. For example, the barley DH population derived from the F_1(Dicktoo × Morex) has been repeatedly phenotyped and genotyped over several years (Hayes et al. 1996). In some species, recombinant DH populations are easy to produce and have the important advantage over RILs that they are faster to generate. Consequently, in the four species, barley, wheat, rice and maize, at least 43 DH populations have been used for map construction and QTL mapping (Forster and Thomas 2003). From simulation studies, Martinez et al. (2002) showed that DH design is a very useful tool for QTL mapping, particularly when the effect of the QTL is low and the residual genetic variation from other chromosomes can be controlled by using multiple markers. Wu (1999) reported a new method for mapping segregating QTLs in an open-pollinated progeny population using dominant markers derived from haploid tissues. Using DH populations, many morphological, qualitative single locus and QTLs including genes conferring resistance to pests and diseases (Chen et al. 1994; Kicherer et al. 2000; Toojinda et al. 2000; Scheurer et al. 2001) and tolerance to abiotic stresses (Pan et al. 1994; Ellis et al. 2002; This et al. 2003), associated with heading time, photoperiod response (Sourdille et al. 2000), quality (Marquez-Cedillo et al. 2000; Perretant et al. 2000) and yield (Backes et al. 1995; Marquez-Cedillo et al. 2001) have been mapped. When no genetic linkage map is available or when a gene or genes controlling a trait have not yet been mapped, bulk segregant analysis (BSA) can be applied to identify markers for the genes (Michelmore et al. 1991). Precise phenotype characterization of each individual within the bulks has to be performed while markers are much easier to analyze when DH vs. F_2 lines are used in the bulks. BSA and DH quickly identified SSR markers linked with a new gene conferring resistance to barley mild mosaic virus (BaMMV) from the Japanese cv. Chikurin Ibaraki 1 and the locus was then mapped on the chromosome 6H (Le Gouis et al. 2004). Borovkova et al. (1995), using similar methods, identified RAPD and RFLP markers linked to the stem rust resistance gene *rpg4* in barley.

3.2.3 DHs and Expressed Sequence Tags (ESTs)

The development of expressed sequence tag (EST) sequencing projects has generated a large amount of sequence information. In wheat, the recent deposit of 200,000 ESTs into GenBank by DuPont has led to the availability of more than 500,000 genome data in the species (http://www.ncbi.nlm.nih.gov/dbEST/). From these sequences, molecular markers, such as SSR (Holton et al. 2002; Kantety et al. 2002) and single nucleotide polymorphisms (SNP), can be defined. SNPs are very powerful and abundant and have contributed greatly to allele diversity through evolution. They are usually discovered by sequencing PCR products generated from different individuals (Dietrich et al. 1999). Using this approach, Kota et al. (2001) identified SNPs between two barley accessions and subsequently mapped them using a DH population derived from the cross between the two accessions and denaturing high-performance liquid chromatography (DHPLC). The homozygous state of the DHs allowed simplified profile analyses; ESTs that were monomorphic at the RFLP level were then mapped.

3.2.4 Gene Cloning

Isolating important genes is a goal of many genomic projects, but progress has been slower in species with large genomes such as barley and wheat compared with rice, which is the model genome for grasses (Devos and Gale 2000). As a demonstration of this goal, Horvath et al. (2003) genetically engineered barley with the cloned *Rpg1* gene. DNA sequence comparisons in grass genomes have shown that coding regions are usually well conserved, but the distances between the genes seem to be correlated with genome size (Bennetzen 2000), hindering isolation of specific genes in large grass genomes. While it is possible to isolate a single gene using an F_2 population followed by inbreeding to obtain the homozygous recombinants, a large DH population is preferred when multiple gene isolations have to be carried out (A. Kleinhofs, pers. comm., 2003). Progress could be made in map-based cloning by further increasing the number of recombinant DHs in a population for fine mapping (Kilian et al. 1997). At this level, the complete homozygosity and immortality of the DH population are tremendous advantages for facilitating the strategy. Few genes related to microspore embryogenesis have so far been isolated. Early studies in functional proteomics had shown the involvement of extracellular proteins in the initiation of somatic embryogenesis in carrot (van Engelen and de Vries 1992). In maize, Vergne et al. (1993) found that a 32-kDa protein named MAR32 was induced and accumulated in the anthers during cold pretreatment of the tassels and that the amount of MAR32 was positively correlated with the proportion of responding anthers and the production of ELS. In androgenetic embryos of barley, the expression of two embryo-specific genes was detected more intensively at the globular

stage of the proembryos (Stirn et al. 1995). Reynolds and Crawford (1996) identified an ABA-responsive metallothionein (EcMt) gene expressed strongly in early stages of wheat anther culture, but the gene transcript was not detected in mature zygotic embryos, vegetative tissues or developing pollen. Five of the QTLs associated with anther culture in maize mapped near viviparous mutant loci, which are related to ABA production and regulation (Beaumont et al. 1995), confirming the importance of ABA for androgenesis. By differential screening, Vrinten et al. (1999) isolated three cDNAs in barley microspore culture that represented genes not previously identified in barley. Two of them showed homology with glutathione-S-transferases and lipid transfer protein genes, while the third had no homology to any isolated gene.

3.3 Mutation and Genetic Transformation

Increasing the genetic variability of crop species has been a goal of scientists and breeders in order to generate new and superior recombinants. Genetic modification of single haploid cells followed by regeneration enables the direct observation of recessive genes on the phenotype of a non-chimeric plant and can be induced by in vitro culture, mutagenic agents and by genetic transformation. In vitro culture has long been known to induce genetic changes (for review, see Karp 1991). Phenotypic as well as molecular changes have been occasionally reported in plants regenerated from haploid tissues (Snape et al. 1988; Devaux et al. 1993a; Wan and Widholm 1993). Unfortunately, most of these so-called gametoclonal variations negatively affect the agronomic performance of DHs (Powell et al. 1984; Snape et al. 1988). Although selection agents such as Na_2SO_3 or Al can be added to anther or microspore culture media to enhance the recovery of plants with high tolerance to the agent, there are indications that these plants can result from either recombination of genes (Ye et al. 1987) or from mutation (Barnabás et al. 2000). To further increase the rate of mutations, mutagenic agents can be applied to anther or microspore cultures (Castillo et al. 2001b) or to seeds from which plants are used for anther culture (Szarejko et al. 1995). In barley and wheat, both microspore-derived callus and isolated microspores have been used as explants for genetic transformation by particle bombardment (Jähne et al. 1994; Yao et al. 1997; Folling and Olesen 2001), yielding both homozygous (Jähne et al. 1994) and heterozygous transgenic plants (Yao et al. 1996) with improved phenotype (Leckband and Lörz 1998).

3.4 Other Research on Wide Hybrids in the Poaceae

Chromosome-engineered plants and introgressions obtained from wide hybrids via androgenesis is an important adjunct to DH production in the Poaceae, and although it is beyond the scope of this chapter to devote much

space to these topics, we will describe some recent developments. Anther culture has been successfully employed in the Poaceae to obtain novel genetic combinations derived from the parental genomes of interspecific and intergeneric hybrids. The aim was to regenerate plants with novel chromosomal and genetic constitutions that could not be obtained from conventional crossing procedures. Regenerants from cultured anthers of barley × *H. bulbosum* comprised a small number of chromosomally engineered derivatives (multiple chromosome substitution lines), which have proved useful in mapping introgressions from *H. bulbosum* into barley (Pickering and Fautrier 1993). A subsequent larger-scale experiment was carried out (Gilpin et al. 1997) and similar results obtained, but the number of novel plants obtained from androgenesis was far fewer than could be produced by conventional crosses between the two species (Pickering 1992; Zhang et al. 2001). Anther culture has also been used effectively in hybrids involving hexaploid and octoploid triticale × wheat (Wang and Hu 1985; Wang et al. 1996) and *Triticum–Agropyron* × wheat (Miao et al. 1988). Chromosome addition lines as well as translocations were obtained from these hybrids and this is an efficient way of obtaining novel chromosomally engineered plants. Similar results have been obtained with intergeneric hybrids involving the forage grass species *Lolium* and *Festuca*, and the results from these crosses have been recently extensively reviewed (Humphreys et al. 2000).

4 Conclusion

To summarize, in a relatively short time DH production in the Poaceae has reached the point where it is a routine procedure for several of the cereals. Improvements are still awaited in some of the less widely grown cereals, such as oats, and it is speculative whether such improvements will be forthcoming given the minor importance and lower research funding for such crops compared with the major cereals. The uses of DHs have lately expanded from being merely a breeding tool to achieve homozygosity from early generation hybrid material to playing a crucial role in marker-assisted selection, molecular mapping, and gene cloning. Haploids can also be used as a source of explants for mutation breeding and are eminently suitable in this role since there are no masking effects in haploid tissue that would confound and slow the pace of screening for recessive mutations. The future is exciting for breeders and geneticists alike, since further technical refinements can be expected and DH production will become even more of a routine procedure than it currently is. There will, of course, be expansion into the area of genomics and the possible use of haploid tissue in conjunction, for example, with microarrays, which will increase our knowledge of gene expression in plant ontogeny.

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