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Anther and microspore cultures of barley and wheat

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Abstract. This paper briefly cites the various procedures for the production of doubled haploids in barley and wheat. Various associated terms are defined and the factor involved in haploid production are outlined. Isolated microspore cultures offer some advantages over anther culture. Our current procedures for isolated microspore culture of barley and wheat are presented and compared.

Key words: barley, doubled haploids, isolated microspore, transformation, wheat.

Introduction

The production of haploid plants (having the gametic chromosome number) is of value to barley and wheat researchers and breeders. By doubling the haploid chromosome number to produce doubled haploids (DH), instant completely homozygous lines can be produced. This doubling can occur spontaneously during anther and microspore culture with high frequencies.

The use of doubled haploids in plant breeding has been summarized by NITZSCHE and WENZEL (1977), CHOO et al. (1985), MORRISON and EVANS (1988), PICKERING and DEVAUX (1992). The conclusions reached were that the major advantage was the time saved to produce pure lines at any stage in the breeding program. Other advantages were: the avoidance of environmental selection during inbreeding; the easier and simplified selection of traits using pure lines; and the simplification of the study of quantitative traits.

A recent important area of application of haploids is in genetic studies where segregation ratios are simpler and genetically homozygous lines are

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valuable. For example, in the development and utilization of molecular marker (RFLP) linkage maps, the production of pure lines from F_1 plants has been a major advantage in barley (HEUN et al. 1991, HAYES et al. 1993, KLEINHOFS et al. 1993). Not only are the gametic ratios simpler but the population of pure lines scored can be maintained and distributed to other researchers in such self-pollinated crops. SZAREJKO et al. (1991) have reviewed the applications of doubled haploids in mutation breeding.

However, haploid cells, either as microspores or as cell or protoplast cultures, provide ideal targets for mutation, selection and transformation (KASHA et al. 1992). This advantage only exists if plants can be regenerated from single cells and regeneration has been particularly difficult in cereal crops. This final report will mainly summarize the research on the regeneration of plants from haploid microspore cultures in barley and wheat and not serve to review our previous workshop reports (KASHA 1989, KASHA et al. 1991a,b, SZAREJKO, KASHA 1991).

Methods of producing haploids

There are a number of procedures by which haploid plants can be produced in cereals. These procedures may be grouped under five areas as: anther or microspore culture; ovule culture (SAN-NOEUM, AHMADI 1982); chromosome elimination following wide hybridization (KASHA, KAO 1970, LAURIE, BENNETT 1988); induction associated with alien cytoplasm (KOBAYASHI, TSUNEWAKI 1980); or haploid inducing genes (HAGBERG, HAGBERG 1987). The two procedures that have been extensively utilized are anther culture and chromosome elimination where new cultivars of barley and wheat have been produced (KASHA et al. 1990, PICKERING, DEVAUX 1992).

Since attention must be focused on the numbers of doubled haploids (DH's), the potential of anther/microspore culture is outstanding compared to the other procedures. In the former, the DH's come from the abundant male gametes per floret as compared to other procedures, which utilize the single ovule per floret.

The process of the development of a haploid plant from a male gamete is known as androgenesis. While such development is likely a continuous process, two phases appear to be critical for success, namely the initial induction phase and the subsequent embryo formation and regeneration. For cereals, the additional problem of albinism following androgenesis needs to be considered.

There are a number of factors influencing the success of androgenesis and these may be grouped as genotypic and environmental/physiological

(KASHA et al. 1991b). The latter includes donor plant growth conditions, preculture treatments, culture procedures, media components for both induction and regeneration, and culture conditions. Genotypic differences in response have been striking since the first attempts at cereal androgenesis (CLAPHAM 1971) and we have recently provided examples (KASHA et al. 1991b). Examples of environmental/physiological factors will be provided later in this text.

Microspore culture

The term microspore culture has been used in both a broad and narrow sense. In the broad sense it refers to the culture of any microspores including those in anthers. In the narrow sense, it refers to microspores isolated from the anther. While closely related, anther culture vs. isolated microspore culture can be quite different processes due to the major influence of the anther. Even in isolated microspore culture the anther may be influential during preculture treatments or when used to condition media. During the remainder of this paper, the term microspore culture in the narrow sense will be utilized and studied.

In efforts to understand the androgenetic process and for some uses, isolated microspore cultures have advantages over anther culture. These include: 1) easier manipulation of single cells for mutation, transformation and selection; 2) direct observation of microspore response and development; 3) more precise control in determining media requirements; 4) avoidance of confusion with potential regenerants from somatic anther tissue.

Barley microspore culture

Isolated microspore culture in barley has been investigated for a number of years and, as in anther culture, is highly variable with genotypes. A relatively simple shed microspore culture system has been described (ZIAUDDIN et al. 1990) to provide the rapid production of green plants. However, for mechanization purposes, large scale mechanical isolation would be preferred.

Microspore culture procedures contain a number of steps. First, the stage of microspore development is critical and must be examined carefully. The mid- to late-uninucleate stage of the microspores is usually examined under a microspore in a drop of acetocarmine stain. While induction response can be obtained with microspores from freshly collected spikes, pretreatments appear to be beneficial. This may be achieved by preconditioning culture media with anthers or ovules or pretreatment of anthers or spikes by cold or in mannitol solution. We find that pretreatment of anthers in 0.3 M mannitol for

3-5days is faster and provides more consistent results in barley microspore culture. After 3 days, microspores start to be shed into the mannitol solution. The anthers in mannitol are then placed in a glass homogenizer and macerated with a glass rod to release the microspores (ZIAUDDIN et al. 1992). The suspension is centrifuged at 50 g for 5 min. and the supernatant with anther debris is discarded. The microspore pellet is washed three times with 0.3 M mannitol by centrifugation at 50 g for 4 min. each time.

The microspore pellet is re-suspended in 1 mL of FHG induction media, separated into 0.3 mL aliquots to provide 3 replications, and placed on agarose solidified FHG induction media in 5.5 cm Petri dishes. The cultures are incubated in the dark for 28 days at 25°C. Embryos 1 mm are removed to regeneration media and the cultures continued (replenished with induction media if too dry).

The induction media is HUNTER's (1988) FHG liquid media with 6.2% maltose, 750 mg L^{-1} glutamine, 165 mg L^{-1} NH_4NO_3, 1 mg L^{-1} BAP and either no auxin or 10 mg L^{-1} PAA (phenyl acetic acid). The optimum auxin concentration may differ for various genotypes.

PAA is a naturally occurring auxin in plant tissues and may be one of the anther factors (XU, SUNDERLAND 1981, KÖHLER, WENZEL 1985) that is useful in preconditioned media. ZIAUDDIN et al. (1992) have examined the influence of PAA on barley microspore cultures and have shown beneficial effects. The main effect is the smaller embryo-like structures that develop and lead to much higher regeneration frequencies. For example, in cv. Igri we do not see more structures induced but regeneration is increased 3-fold. In cv. Bruce, a higher concentration of PAA (100 mg L^{-1}) is required to provide plant regeneration from microspore cultures. In contrast, induction in cvs. Igri and Sabarlis is completely inhibited at 100 mg L^{-1} PAA. With cv. Bruce, a difference between 10 vs. 100 mg L^{-1} PAA can be seen in the structure formed, with those at 100 mg L^{-1} having a smoother surface (KASHA et al. 1992).

We have worked with five spring growth habit barley genotypes in addition to the winter habit cv. Igri and we are able to get good induction and regeneration of all genotypes using the procedures that we have described. The main advantage of Igri is the very high frequency of green plants. On the other hand, with Igri, it is more difficult to obtain good anther donor plants due to its vernalization and subsequent low growth temperatures requirements. For Igri it is possible to get a high response and the best frequency represents 1 green plant per 100 microspores in an anther, which is comparable to the best results from dicot species.

We find that the frequency of completely fertile doubled haploids arising from barley microspore culture ranges from 60 to 80% of the plants. Since most of these plants are completely fertile, the chromosome doubling may have occurred at the initial nuclear division in the induced microspore. This supports our earlier hypothesis (CHEN et al. 1984) based upon cytological observation that embryo induction of microspores may involve disruption of nuclear and cell division.

Wheat microspore culture

There have been very few previous reports of microspore culture success in wheat (WEI 1982, DATTA, WENZEL 1987, TUVESSON, ÖHLUND 1993, MAJZA et al. 1993) and these have had limited success. Thus our goal has been to derive a more widely useful procedure for wheat, modelled along the lines of our barley microspore culture system.

The factors influencing isolated microspore culture of wheat appear to be the same as for barley microspore culture. These include genotype, donor plant growth conditions, preculture treatments, isolation of microspores, culture media and culture conditions. We have only worked with limited numbers of genotypes in spring habit hexaploid bread wheats and our data are very preliminary. However, we expect that these same factors would need to be examined for other wheat types, or species.

The temperatures at which the donor plants are grown have proven to be an important factor in our procedures. With the cv. Chris, we examined the results of two donor plant growth temperatures, namely 15°C day/12°C night and 18°C day/15°C night. We found that the frequency of viable microspores was higher and, more importantly, the percentage dividing microspore were about 5 times higher, when plants were grown at the higher temperatures. In contrast, most barley genotypes respond better at the lower plant growth temperature. The growth of donor plants at higher temperatures still needs to be examined in more detail with other wheat genotypes.

The procedures used for the isolation of microspores in wheat are more critical than in barley. We have examined shed microspores vs. those mechanically isolated and found the former to be beneficial for cv. Chris. The procedures for microspore isolation need to be gentler and we have found that placing the anthers in preculture solution on a stirrer plate with magnetic bar improves the release of microspores from the anthers. The anthers are then filtered off and the microspores washed after low speed centrifugation at 50 g for 5 min.

For the preculture of anthers in mannitol, we have found the addition of the macronutrients (salts) from MS media to be essential and the length of time in mannitol + salts is about 5-7days.

For wheat microspore culture, we are using liquid MS medium with 9% maltose, 1.0 mg L^{-1} IAA, 0.5 mg L^{-1} kinetin. The microspores are placed on 5 mL medium in a 5.5 cm Petri dish and incubated in the dark at 28°C on a shaker (75 rpm). The development of microspores and their frequency of multicellular structures is similar to barley but regeneration has not been as good.

For regeneration, we have found two steps are necessary compared to one in barley. The embryo structures 1 mm are transferred to solid MS induction media with maltose and hormones. Once shoots are formed, they are then transferred to MS regeneration media containing 3% sucrose and no hormones.

We have not examined the role of PAA relative to regeneration from wheat microspores. However Marsolais (ZIAUDDIN et al. 1992) found PAA to have beneficial effects on regeneration from wheat anther culture.

Regeneration from cv. Chris microspore cultures is in the range of 70 green plants from 100 anthers. Nearly all plants are green and from this brief experiment about 80% are fertile doubled haploids. MAJZA et al. (1993) observed an average of 51% fertile regenerated plants. We have found this procedure with cv. Chris to be repeatable and it appears promising for other spring wheat genotypes.

Transformation

One of our goals in developing a repeatable microspore culture system in cereals was to investigate their potential as targets for transformation. We are currently bombarding barley microspores with the Biolistic Particle gun PDS 1000^{TM} from DuPont. We have used plasmids supplied by Monsanto that contain the BAR gene for selection as well as the marker genes GUS and a corn anthocyanin pigment. We have observed transient expression of all three markers and have selected a number of seedlings on BASTA. However, we have not confirmed that they are transformed and BASTA does not appear to be a good selection agent in barley microspore transformation.

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