J Plant Growth Regul (1999) 18:135–143

Journal of
Plant
Growth
Regulation

The Role of Abscisic Acid in Induction of Androgenesis: A Comparative Study Between *Hordeum vulgare* L. Cvs. Igri and Digger

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Received May 21, 1999; accepted October 5, 1999

Abstract. Under the same mannitol pretreatment and culture conditions, regeneration efficiency in the barley cultivar (cv.) Igri was about 10 times higher than in the cv. Digger, a difference only partially reflected by a difference in viable microspores after anther pretreatment. Therefore, a comparative study between cvs. Igri and Digger was carried out under various pretreatment conditions. For both cultivars, under water, CPW buffer and mannitol pretreatment conditions, there was a positive correlation between microspore viability and regeneration efficiency in that mannitol > CPW buffer >> water. Mannitol pretreatment of cv. Igri produced a much higher endogenous abscisic acid (ABA) level than as to Digger. Addition of ABA stimulated both percentages of viability and regeneration efficiency except in the case of mannitol pretreatment. Under CPW buffer pretreatment conditions, addition of ABA significantly stimulated regeneration efficiency and was ABA concentration dependent. However, cv. Digger was less responsive to ABA than cv. Igri. In both cultivars, under less optimal pretreatment conditions (e.g., water and CPW buffer), the effect of ABA was to stimulate increased percentages of viability and/or to reduce the number of binucleate microspores. Moreover, in cv. Igri, direct culture of anthers for 4 days without pretreatment caused an increased number of binucleate microspores compared with microspores with pretreatment for 4 days. These binucleate microspores showed DNA degradation in the nuclei. However, with mannitol pretreatment binucleate microspores and DNA fragmentation in the nuclei of microspores was rarely observed. On the basis of our observations, we suggest that the difference in regeneration efficiency in cv. Igri and cv. Digger is related to the differences in endogenous ABA production levels under mannitol pretreatment and responsiveness to ABA. One of the effects of ABA is likely due to an inhibition of cell death.

Key Words. ABA—Androgenesis—Microspore culture—*Hordeum vulgare*

The application of microspore-derived plants in some species breeding programs is an established technique today. Advantages include accelerated and simplified breeding procedures (Morrison and Evans 1988). Microspores can be used as target cells for stable transformation, for example, by co-cultivation with *Agrobacterium* or particle bombardment (Creissen et al. 1990, Pechan 1989, Stöger et al. 1995, Twell et al. 1989). Successful use of microspores for such applications requires optimized culture procedures to achieve high regeneration frequencies. In addition, microspore regeneration systems can be used as model systems for studying cell differentiation and embryogenesis.

There are three prerequisites that determine optimal development of microspores to plants in barley. The stage of microspores should be at the verge of mitosis (Hoekstra et al 1992). Pretreatment of anthers of microspores and the use of the right hormone during culture of the pretreated microspores directly affect the regeneration efficiency (Hoekstra et al. 1992, 1993, 1996, Olsen 1987). The right pretreatment conditions are needed to switch the gametophytic pathway into sporophytic development of the microspores. Different pretreatments for different crops have been widely reported. For example, in *Brassica napus* it is known that shocking fresh microspores with heat is a very successful pretreatment (Custers et al. 1994). In tobacco, a pollen preculture in mannitol dissolved in so-called premedium is used as pretreatment (Touraev et al. 1996). The pretreatment of

Abbreviations: ABA, abscisic acid; ELS, embryo-like structures; FDA, fluorescein de-acetate.

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barley can be a cold treatment of spikes (Huang and Sunderland, 1982) or anthers can be pretreated on a mannitol solution (Roberts-Oehlschlager and Dunwell, 1990). This latter combination of salt stress and osmotic stress of anthers is the most successful way to achieve high frequencies of regeneration in barley.

Little is known about the processes that are effectuated by the pretreatment. Heberle-Bors (1989) suggests that during pretreatment starvation caused by absence of useful saccharides is the basis of certain degradation processes in pollen grains. This should result in the induction of pollen embryogenesis. Davies and Jones (1991) suggested that, in response to certain stress treatments like osmotic pressure and cold, abscisic acid (ABA) is produced. It has been reported that pretreatment of tobacco anthers by mannitol caused an increase in endogenous ABA levels (Imamura and Harada 1980). In addition, relatively high endogenous ABA levels are correlated with high embryogenic potential of *Pennisetum* and carrot cells (Kiyosue et al. 1992, Rajasekaran et al. 1987a,b). For barley, it is known that addition of the ABA synthesis inhibitor fluridone, during pretreatment, negatively affects regeneration efficiency (Hoekstra et al. 1997).

Apparently ABA may play an important role during anther pretreatment and subsequently affect the regeneration efficiency. To further verify this hypothesis, we studied two different barley cultivars (cv. Igri and cv. Digger) that showed very different regeneration efficiencies. In this study, ABA content in anthers of both cultivars was measured during pretreatment. The effect of external application of ABA on microspore viability and regeneration efficiency under various pretreatment conditions was also tested. On the basis of our experimental data, we conclude that a high ABA content during pretreatment is correlated with high regeneration efficiency. One of the effects of ABA is due to an inhibition of binucleate microspore formation.

Materials and Methods

Materials

Donor plants of *Hordeum vulgare* L. cvs. Igri and Digger were grown in a phytotron under conditions described previously (Hoekstra et al. 1992). Monoclonal antibody to free (+) ABA was purchased from Idetek, Inc. (San Bruno, CA). Rabbit anti-mouse alkaline-phosphatase conjugate, (+) ABA, and bovine calf serum (grade suitable for enzyme-linked immunosorbent assay; ELISA) and fluorescein di-acetate (FDA) were obtained from Sigma Chemical Company (St. Louis, MO).

Induction of Androgenesis

Selection of material (anthers containing late uninucleate microspores); mannitol pretreatment of anthers (in pretreatment solution called CPW buffer, containing 0.37 M mannitol, 10^{-2} M $CaCl_2$, 10^{-3} M $MgSO_4.7H_2O$, 10^{-3} M KNO_3, 2×10^{-4} M KH_2PO_4, 10^{-6} M KI, and 10^{-7} M $CuSO_4.5H_2O$, 440 mOsm $\cdot$ kg^{-1}); and culture conditions were as described previously (Hoekstra et al. 1993) with the following modifications. Anthers (without Ficoll) or microspores were cultured in medium I at 350 mOsm $\cdot$ kg^{-1}.

For microspore culture, anthers were pretreated on mannitol solution for 4 days and microspores in these anthers were isolated as described by Hoekstra et al. (1993). After isolation, 1×10^4 embryogenic microspores were plated in 1 mL medium I (Hoekstra et al. 1993) in a 3.5-cm Petri dish. After 14 days, the number of embryo-like structures (ELS) was counted. For anther culture, 30 anthers (equivalent to ½ spike) of several spikes were randomly used per parameter to be tested. After 4 days, the anthers were transferred to medium I without Ficoll at 350 mOsm $\cdot$ kg^{-1}. After 14 days of culture, the number of ELS was counted. For the effect of ABA during pretreatment, anthers were incubated in different pretreatment solutions in the presence of ABA (Sigma, St. Louis, MO). After a period of 4 days, the anthers were transferred to medium I (Hoekstra et al. 1993). For anther culture, after 4 days of pretreatment in mannitol, the anthers were transferred to medium I without Ficoll at 350 mOsm $\cdot$ kg^{-1}. The first 100 developing ELS were carefully transferred with forceps to medium III and the rest were plated directly, at the moment at which most ELS were at least 1 mm in diameter. After 3 weeks, plant production was assessed per group of 30 anthers. Medium I and III were reported in our previous study (Hoekstra et al. 1992).

Cytologic Staining

To detect DNA degradation in the nuclei of microspores, a TUNEL assay was used (Gavrieli et al. 1992). Barley anthers were fixed in 2% glutaraldehyde in PBS (pH 7.3) at room temperature and prepared for TUNEL staining as described before (Gavrieli et al. 1992, Wang et al. 1996, 1999). The samples were examined with a fluorescence microscope (Leitz Diaplan). Micrographs were made using a Kodak Gold film (400 ASA).

The number of viable microspores during anther pretreatment was determined by squashing the anther tissue in 1 mL pretreatment solution. The squashed material was stained with about 20 μL (5 mg/mL in acetone) FDA. In each experiment, at least 300 microspores were counted.

ABA Extraction and ELISA Assay

Anthers were put into Eppendorf vials and frozen with liquid N_2. Pestle maceration of the frozen anthers was not necessary for ABA extraction (unpublished results, M. Wang et al.). The contents of the Eppendorf vials were freeze dried. The samples were extracted on a rotation shaker (IKA) 150 rpm, 4°C with cold methanol (containing 100 mg/L butylated hydroxytoluene and 0.5 g/L citric acid monhydrate) twice each time in 0.5 mL for 24 h. The vials were centrifuged at 8,000 *g* for 15 min at 4°C, and the supernatant of the samples was pooled. The samples were concentrated in a Speed-vac system (Savant) and the volume adjusted to 25 μL with methanol. The samples were stored at −80°C until assay.

ABA levels in anthers and in pretreatment solutions were determined by ELISA as described by Wang et al. (1995).

Experimental Data

Mean values ± S.D. are presented unless stated otherwise. Significance of difference in mean values was tested with Student's *t* test.

Results

Regeneration Efficiency in Different Barley Cultivars

In the microspore culture system of barley, anthers containing uni-nucleate microspores were used for pretreatment and subsequently microspores were isolated from pretreated anthers for further culture. After 4 days of anther pretreatment on mannitol, both cv. Igri and cv. Digger microspores isolated from these anthers showed a nice round shape, with a red interference of the exine wall. After 3 days in culture, first divisions of microspores were visible. Between day 7 and 11 in culture, depending on the cultivar, the microspores broke out of their exine, and small compact structures were formed, the so called ELS. At day 14 most of the dividing microspores were out of their exine. On day 21 the first germinating embryos were visible. Furthermore, our regeneration experiments demonstrated that regeneration efficiency at days 1 and 2 of the 4-day mannitol pretreatment was less than at day 3 of the 4-day mannitol pretreatment (data not shown). There was almost no difference between the regeneration efficiency at day 3 and day 4 of the 4-day mannitol pretreatment (data not shown).

Difference between cultivars in various types of tissue culture systems are well known. We tested the cultivars Igri (winter type), Gimpel (spring type), and Digger (spring type) for their regeneration efficiency by counting the number of ELS on day 14 in cultures. Anthers from these cultivars were pretreated with mannitol for 4 days and microspores were isolated for further culture. The highest number of ELS (about 1,700 ELS/10^4 microspores) was produced by cv. Igri. Efficiency for cv. Gimpel was much lower (about 800 ELS/10^4 microspores) and cv. Digger produced only about 180 ELS/10^4 microspores. Regeneration efficiency of cv. Igri was nearly 10 times greater than for cv. Digger. The cultivars Igri and Digger, therefore, were used for further comparative study. After 3 weeks further culture, the production of plants in cv. Digger was only 15% per 30 anthers when we used cv. Igri as the standard for 100% production of green plants per 30 anthers. In addition, cv. Igri rarely produced albino plants but there were at least 20% to 40% of albino plants present in cv. Digger.

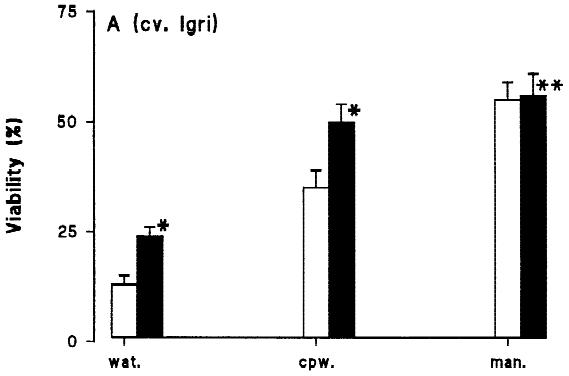
Microspore Viability and Formation of ELS

The viability of microspores in anthers before the start of pretreatment determines the quality of starting materials. For cv. Igri, the viability of microspores from freshly collected anthers was about 55% and after 4 days pretreatment with mannitol the viability remained at about the same level. For cv. Digger, the viability of microspores from freshly collected anthers was about 30% and after 4 days pretreatment with mannitol the viability also remained at about the same level. Thus, there was a clear difference in viability of microspores in freshly isolated anthers between cv. Igri and cv. Digger. Without pretreatment, the viabilities of microspores from both cultivars decreased significantly (van Bergen et al. 1999, Wang et al. 1999).

The regeneration efficiency of microspores from cv. Digger was about 10 times lower than that from cv. Igri and cannot be explained solely by microspore viability differences in freshly isolated anthers. We were interested in other differences between these cultivars after 4 days of pretreatment with mannitol, and thus continued the study. It has been demonstrated in cv. Igri that when anthers were treated with water or buffer (CPW) without addition of mannitol a much lower number of ELS was formed (Hoekstra et al. 1997). Therefore, we also questioned whether differences in viability of microspores after various pretreatments are responsible for differences in regeneration efficiency. Microspore viability was measured by staining the microspores with FDA. For cv. Igri, anthers were incubated in water, CPW buffer, or 0.37 M mannitol in CPW buffer for 4 days. Microspores from these anthers were isolated for determination of their viability. Mannitol pretreatment produced the highest viability (55%). CPW buffer resulted in about 10% lower microspore viability compared with mannitol treatment. In water, microspore viability was only about 13% (see Fig. 1*A*). When we order the effects of different pretreatments on microspore viability, we can conclude that mannitol (55%) > CPW buffer (41%) >> water (13%). This viability order agrees with the ELS formation order under similar conditions: mannitol (1900 ELS/10^4 microspores) > CPW buffer (900 ELS/10^4 microspores) >> water (190 ELS/10^4 microspores) (see Fig. 2*A*). However, CPW pretreatment only decreased microspore viability from 55% to 41% compared with mannitol pretreatment, whereas the number of ELS formed was about two times lower than mannitol pretreatment (Figs. 1*A* and 2*A*). For cv. Digger, microspore viabilities were about the same when anthers were incubated in CPW buffer (29%) or in mannitol (31%). However, when anthers were incubated in water, viability decreased to about 20% (Fig. 1*B*). The formation of ELS in cv. Digger followed the same order in conditions: mannitol (300 ELS/10^4 microspores) > CPW buffer (200 ELS/10^4 microspores) > water (100 ELS/10^4 microspores) (see also Fig. 2*B*). Our results demonstrate that viability of microspores after various pretreatment is not the only factor that determines regeneration efficiency.

ABA Levels During Pretreatment

It has been shown that mannitol pretreatment affects the viability of microspores and subsequently the number of ELS formed for both cv. Igri and cv. Digger (Figs. 1 and 2). Because it has been reported that pretreatment of tobacco anthers with mannitol caused an increase in en-



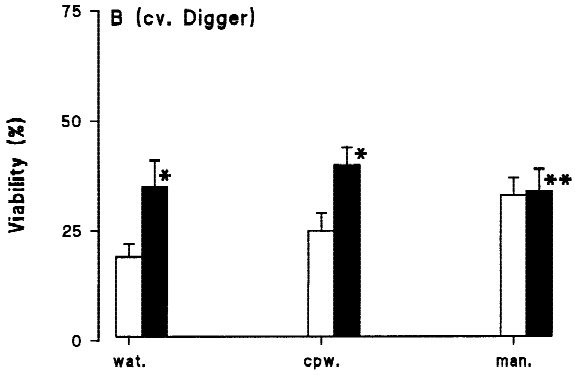
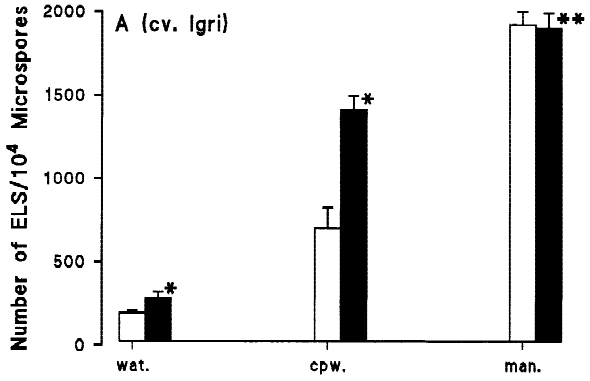


Fig. 1. Effects of various conditions on microspore viability in barley cultivars Igri and Digger. Barley anthers from the cultivars Igri *(A)* and Digger *(B)* were incubated under various conditions such as water, CPW buffer, and mannitol pretreatment (CPW buffer containing 0.37 M mannitol) in the presence *(closed bars)* and absence *(open bars)* of ABA (10^{-5} M) for 4 days. Subsequently, microspores from these anthers were isolated for determination of their viability by FDA. Means of four independent experiments are presented. *Mean values (in the presence of ABA) significantly different ($p < 0.05$) from value obtained without addition of ABA. **Mean values (in the presence of ABA) not significantly different ($p > 0.05$) from value obtained without addition of ABA.



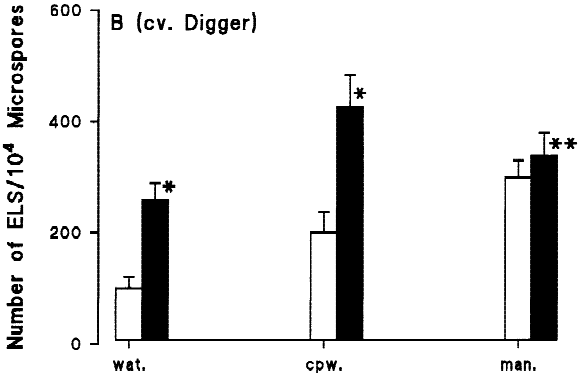


Fig. 2. Effects of various pretreatment conditions on regeneration efficiency in barley cultivars Igri and Digger. Barley anthers from the cultivars Igri *(A)* and Digger *(B)* were incubated under various conditions such as water, CPW buffer, and mannitol pretreatment (CPW buffer containing 0.37 M mannitol) in the presence and absence of ABA (10^{-5} M) for 4 days. Subsequently, microspores from these anthers were isolated for further culture for the formation of ELS. After 14 days of incubation, the number of ELS was counted. Means of four independent experiments are presented. *Mean values (in the presence of ABA) significantly different ($p < 0.05$) from value obtained without addition of ABA. **Mean values (in the presence of ABA) not significantly different ($p > 0.05$) from value obtained without addition of ABA.

dogenous ABA levels (Imamura and Harada 1980), the levels of ABA in both anthers and incubation media during pretreatment were investigated for both cultivars Igri and Digger. There was already a difference in ABA content in freshly isolated anthers between cv. Igri (510 ± 80 pg/30 anthers) and cv. Digger (350 ± 70 pg/30 anthers). For cv. Igri, after 1 day of pretreatment ABA content in anthers peaked (2,900 pg/30 anthers) and then gradually decreased to about 1,500 pg/30 anthers (Fig. 3*A*). Meanwhile, ABA levels in the incubation medium started to increase and reached about 2,500 pg/mL after 4 days of pretreatment (Fig. 3*A*). For cv. Digger, anther ABA content peaked at 1,000 pg/30 anthers after 1 day of pretreatment (Fig. 3*B*) and subsequently decreased sharply to about 100 pg/30 anthers after 4 days of pretreatment (Fig. 3*B*). At the same time ABA levels in the incubation medium increased (Fig. 3*B*). Thus, there are significant differences in the total amount of endogenous ABA production as well as the kinetics of either ABA degradation or secretion between cv. Igri and cv. Digger during pretreatment.

The Effect of ABA During Pretreatment

During pretreatment with mannitol, ABA levels in anthers and medium increased (Fig. 3). However, when anthers were incubated in CPW buffer without mannitol

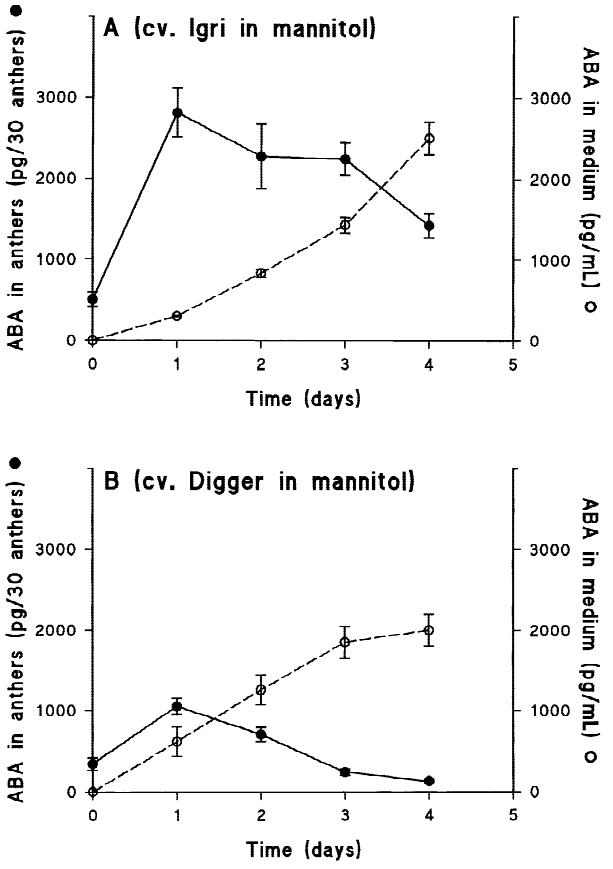


Fig. 3. Effects of pretreatment with mannitol on ABA levels in cultivars Igri and Digger.Barley anthers from the cultivars Igri *(A)* and Digger *(B)* were incubated under pretreatment conditions (CPW buffer containing 0.37 M mannitol) for 0, 1, 2, 3, and 4 days. At each time point, ABA level was determined in both anthers and incubation medium by ABA ELISA (see Materials and Methods). The means of three independent experiments are presented.

ABA levels were much lower and correlated with reduced microspore viability and low ELS production (van Bergen et al. 1999, Figs. 1 and 2). We tried to determine whether addition of ABA could overcome low ELS production under a CPW buffer pretreatment condition. Therefore, anthers from both cvs. Igri and Digger were incubated in CPW buffer containing various concentrations of ABA for 4 days. Then the microspores from these anthers were isolated and cultured further for 14 days. The ELS numbers were counted. For cv. Igri, stimulation of ELS formation started at 10^{-7} M ABA and reached a maximum at about 10^{-6} M ABA (Fig. 4). For cv. Digger, the stimulative effect on ELS formation was only observed when ABA was at least 10^{-6} M and even at 10^{-5} M ABA maximum induction of ELS was not reached (Fig. 4).

Because there was a positive correlation between microspore viability and the number of ELS formed, we tested the effect of ABA (10^{-5} M) in the incubation media on microspore viability after anthers were incubated for 4 days under various conditions (Fig. 1). For cv. Igri, the stimulative effects of ABA addition on microspore viability were observed only with water or CPW buffer–treated microspores but not with mannitol in CPW (pretreatment condition) (Fig. 1*A*), which was also true for cv. Digger (Fig. 1*B*). The effects of addition of ABA (10^{-5} M) on ELS numbers of Igri and Digger anthers after pretreatment with water or mannitol in CPW buffer were also tested (Fig. 2). For cv. Igri, the stimulative effects of ABA addition on ELS formation were observed only with water or CPW buffer but not with mannitol in CPW (pretreatment condition) (Fig. 2*A*). Similar effects of ABA addition were observed for cv. Digger (Fig. 2*B*).

Effect of ABA on the Number of Binucleate Microspores during Pretreatment

An interesting observation was made about the appearance of binucleate microspores after pretreatment. During pollen maturation, a uninucleate microspore will enter mitosis to form a binucleate microspore and further develop into a mature pollen. One of the functions of pretreatment of microspores that are just at the verge of mitosis is to switch the gametophytic pathway into a sporophytic development of the microspores. For cv. Igri, we seldom observed the appearance of binucleate microspores under mannitol pretreatment conditions (Table 1). Under water, CPW buffer pretreatment, or without pretreatment an increased number of binucleate microspores were observed (Table 1). These numbers could be sharply reduced by addition of ABA (Table 1). However, the appearance of binucleate microspores in cv. Digger was significantly higher than in cv. Igri (Table 1), and these binucleate microspores were positively stained with FDA, thus viable. For cv. Digger, because of the presence of binucleate microspores, the effective number of viable microspores that contribute to induction of androgenesis, as observed in Fig. 3, should be lower. Under pretreatment conditions of CPW buffer and water, addition of ABA was able to reduce the number of binucleate microspores significantly (Table 1). Thus the positive effect of ABA in these two pretreatment conditions is to increase the percentage of viable microspores and/or to reduce the number of binucleate microspores (Fig. 1 and Table 1). With mannitol pretreatment, addition of ABA had no effect on either aspect (Fig. 1 and Table 1).

Binucleate Microspores Show a Characteristic of DNA Degradation in Their Nuclei

For cv. Igri, it has been demonstrated that when anthers are directly cultured for 4 days without any pretreatment, both ABA levels in anthers and viabilities of microspores

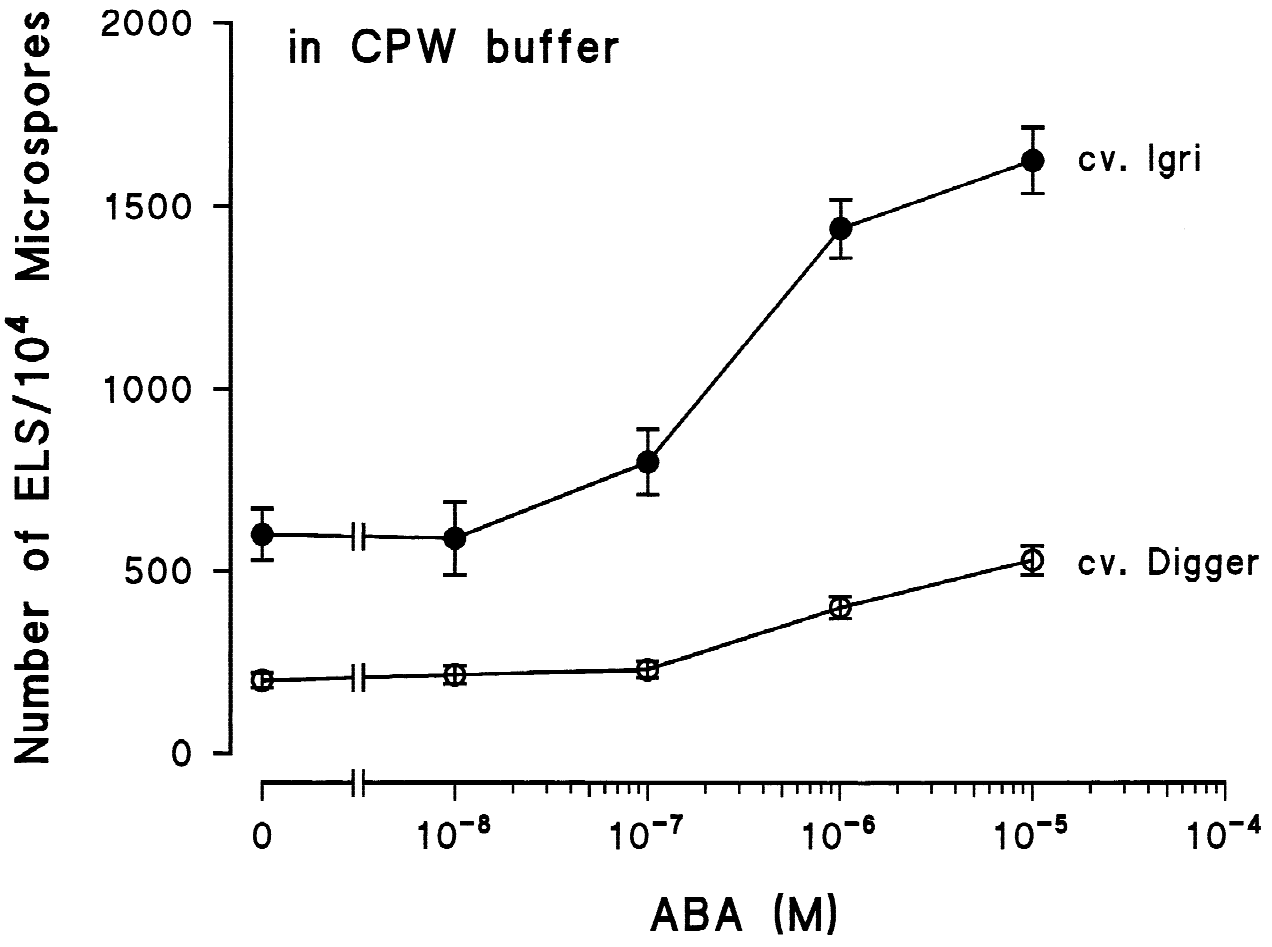


Fig. 4. Effects of various concentrations of ABA on regeneration efficiency in barley cultivars Igri *(A)* and Digger *(B)* were incubated in CPW buffer containing various concentrations of ABA (range from 0, 10^{-8} mM to 10^{-5} M) for 4 days. Subsequently, microspores from these anthers were isolated for further culture for the formation of ELS. After 14 days of incubation, the number of ELS was counted. Means of three independent experiments are presented.

from these anthers are at least two times reduced, which corresponds with a very low regeneration efficiency (Wang et al. 1999). In this study, we also observed that there were increased numbers of binucleate microspores in these anthers that were directly cultured without any pretreatment (Table 1). Microspore viability from these anthers decreased from 56% (t = 0) to 15.5% (t = 4 days). Moreover, when we applied a technique that specifically labels the broken ends of DNA and thus detects degradation of DNA within the nuclei of microspores (Gavrieli et al. 1992, Wang et al. 1999), we observed that almost all binucleate microspores were positive for DNA degradation (Fig. 5A). The consequence of DNA degradation is cell death (Wang et al. 1999). Although these binucleate microspores still appeared viable according to FDA staining, they were in the process of dying. However, on the other hand, when anthers received mannitol pretreatment for 4 days, there was no decrease in microspore viability (56% viable microspores on day 4), which corresponds with almost no binucleate microspores present (Table 1). Little or no DNA degradation in the nuclei of these microspores was observed (Fig. 5B).

Discussion

A stressful pretreatment is one of the requirements for doubled haploid production in barley. The difference in regeneration efficiency between cultivars Igri and Digger was correlated with both ABA content in anthers during pretreatment and microspore viability after pretreatment (Figs. 1 and 3). These results suggest that ABA production caused by stressful treatment may be essential for the viability of microspores and subsequently for androgenesis. Moreover, lower ABA production in cv. Digger during pretreatment compared with cv. Igri was also associated with production of albino plants. This observa-

Table 1. Effects of ABA on the percentages of binucleate microspores in cvs. Igri and Digger under various conditions.

Sample treatment	Binucleate microspores cv. Igri (%)	Binucleate microspores cv. Digger (%)
Mannitol	0.0 ± 0.0	13.5 ± 0.7
Mannitol + 10^{-5} M ABA	0.0 ± 0.0[b]	13.2 ± 0.8[b]
CPW buffer	9.3 ± 0.5	22.0 ± 1.4
CPW buffer + 10^{-5} M ABA	0.04 ± 0.01[a]	4.0 ± 5.0[a]
Water	14.2 ± 4.5	15.9 ± 10.0
Water + 10^{-5} M ABA	0.04 ± 0.01[a]	7.0 ± 0.8[a]
In medium (no pretreatment)	19.5 ± 2.0	ND
In medium + 10^{-5} M ABA	9.8 ± 1.5[a]	ND

Barley anthers from cultivars Igri and Digger were incubated under various conditions such as water, CPW buffer, and mannitol pretreatment (CPW buffer containing 0.37 M mannitol) in the presence and absence of ABA (10^{-5} M) for 4 days. For cv. Igri, anthers were also directly incubated in medium for 4 days without pretreatment. Subsequently, microspores from these anthers were isolated for determination of percentages of binucleate microspores. In each experiment, at least 500 microspores were counted. Means of four independent experiments are presented.

[a] Mean values (in the presence of ABA) significantly different ($p < 0.05$) from value obtained without addition of ABA.

[b] Mean values (in the presence of ABA) not significantly different ($p > 0.05$) from value obtained without addition of ABA. ND, Not determined.

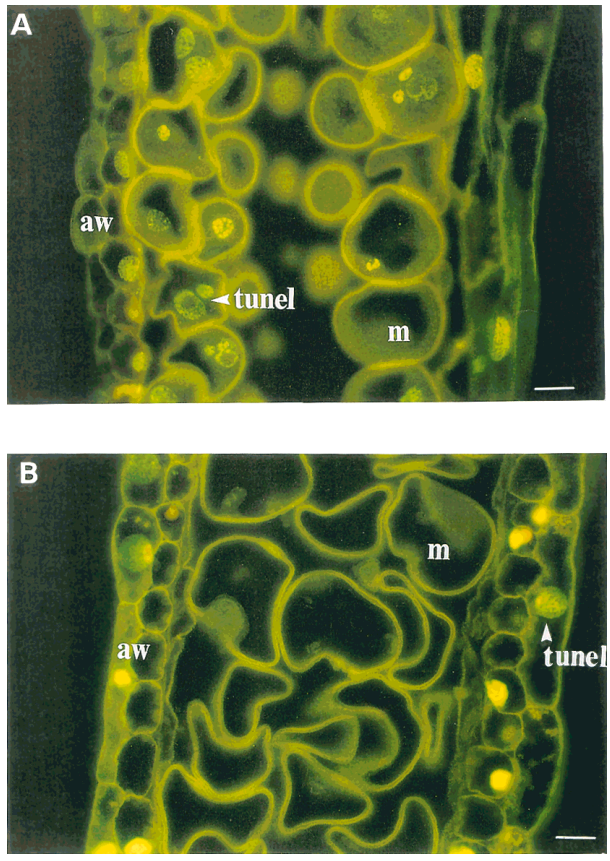


Fig. 5. TUNEL detection of DNA fragmentation in anthers with or without pretreatment for 4 days in cv. Igri. TUNEL staining of anthers with mannitol pretreatment *(B)* and anthers incubated in medium for 4 days without pretreatment *(A)*. For each sample, 5 to 10 anthers were analyzed, and representative examples are presented. *aw*, anther wall cell; *m*, microspore; *tunel*, TUNEL-stained nucleus/nuclei of microspore. Bars in *A* and *B* are 150 μm.

tion agrees with our previous observation that addition of the ABA biosynthesis inhibitor, fluridone, during pretreatment of cv. Igri causes production of albino plants as well (Hoekstra et al. 1997). Under suboptimal pretreatment conditions (CPW buffer without addition of mannitol), addition of ABA significantly stimulated regeneration efficiency (the formation of ELS) (Figs. 2 and 4). However, addition of ABA to an optimal pretreatment condition (mannitol pretreatment) on Igri or Digger anthers showed no stimulative effect on regeneration efficiency (Fig. 2). Earlier, we also demonstrated that the presence of fluridone during the first 24 h of pretreatment resulted in a large decrease in the number of plants obtained with mannitol pretreatment, suggesting that normally there is a need for *de novo* ABA synthesis during mannitol pretreatment (Hoekstra et al. 1997). So, an increase in endogenous ABA levels is likely required for plant production.

For both cultivars Igri and Digger, mannitol pretreatment resulted in increased production of endogenous ABA in contrast to without pretreatment. Subsequently, correlation with increased viability and regeneration efficiency was found (van Bergen et al. 1999). It is likely that under our experimental conditions (mannitol pretreatment), a stress-induced endogenous ABA level is sufficiently high to carry out its job; therefore, addition of ABA provided no further stimulative effects on either viability or regeneration efficiency (Fig. 1). On the other hand, for cv. Igri addition of ABA to both water and CPW buffer pretreatments could not restore regeneration efficiency to the level reached with mannitol pretreatment (Fig. 2), and ABA was not able to raise the regeneration efficiency of cv. Digger to the level of cv. Igri. So apparently, other factors besides ABA levels, such as ABA sensitivity or others, may also play an essential role in induction of androgenesis.

Correlation between androgenic capacity and ABA levels appears to be rather controversial from the literature (Johansson et al. 1992). Hu et al. (1995) report that appropriate androgenesis-inducing pretreatment of *Anemona canadensis* reduced endogenous ABA concentrations at the end of pretreatment by nearly four times, whereas Imamura and Harada (1980) demonstrate a peak in endogenous ABA levels after 24 h of mannitol pretreatment in tobacco anthers. Our results, demonstrating that for both cultivars there was a peak of ABA in anthers after the first 24 h of pretreatment with mannitol (Fig. 3), supports the idea that the *de novo* synthesis of ABA in

the first 24 h is important. Moreover, addition of ABA to premedium for the first 24 h resulted in the highest stimulatory effect on ELS formation (van Bergen et al. 1999). Our data agree with the observations of Imamura and Harada (1980). Of course, we should take the turnover of ABA during the different periods of incubation into consideration. Imamura and Harada (1980) proposed that a specific level of ABA was required for induction of androgenesis. These authors showed in a time range experiment using 10^{-5} M ABA, that a 1- to 3-day period of pretreatment stimulates plant production in tobacco androgenesis. For wheat androgenesis, Hu et al. (1995) observed stimulation by application of ABA (about 10^{-5} M) throughout the pretreatment. Kyo and Harada (1986) showed that application of 5×10^{-6} M ABA in the second half of the pollen pretreatment stimulated embryo production in tobacco.

Reynolds and Crawford (1996) demonstrated that there was a direct and positive correlation between increased ABA levels and expression of an early cysteine-labeled metallothionein gene in developing pollen embryoids. The importance of endogenous ABA levels has been determined for morphogenic competence in *Pennisetum* sp. by Rajasekaran et al. (1987b). Furthermore, these authors showed that application of ABA enhances somatic embryogenesis and that inhibition of plant production is observed by addition of fluridone in the induction medium. The use of fluridone on donor plants even causes the loss of embryogenesis capacity (Rajasekaran et al. 1987a)

In this study, a strong correlation between stress-induced ABA production and microspore viability and subsequently microspore regeneration efficiency was observed. It is likely that the high ABA levels induced by stress protect microspores from death (Fig. 1) and thereby increase the number of candidates for further cell division. In cv. Digger, under less optimal pretreatment conditions (e.g., water and CPW buffer), lower endogenous ABA levels were measured compared with mannitol pretreatment (van Bergen et al. 1999). Under such less optimal pretreatment conditions, the effect of ABA was not only to stimulate increased viability percentages but also to reduce the number of binucleate microspores (Fig. 1 and Table 1). In cv. Igri, we could find no binucleate microspores under mannitol pretreatment conditions, which might be explained by a relatively higher endogenous ABA level in cv. Igri than that in cv. Digger (see. Fig. 3). Also, ABA addition resulted in a much sharper reduction in the number of binucleate microspores in cv. Igri than in cv. Digger, which could be explained by different ABA responsiveness for the two cultivars (see Table 1 and Fig. 4). Because binucleate microspores showed DNA degradation (Fig. 5), even though still showing positive FDA staining (viable), they were in the processes of dying. Therefore, it is likely that the effects of ABA consist of at least two different aspects: (1) preventing microspore death and thus increasing the number of viable microspores during pretreatment and (2) repressing microspores in the further development into mature pollen. Moreover, under mannitol pretreatment conditions, the difference in regeneration efficiency between cv. Igri and cv. Digger might also reflect differences in ABA accumulation kinetics in anthers. For cv. Igri, a high peak level of ABA was reached at 1 day of mannitol pretreatment and subsequently decreased gradually in anthers (Fig. 3*A*). Although in cv. Digger a peak ABA level was also measured after 1 day of mannitol pretreatment (Fig. 3*B*), this peak value was much lower compared with cv. Igri (Fig. 3) and subsequently decreased sharply in anthers (Fig. 3*B*). Our near future studies will concentrate on the more detailed role of ABA in cell death and binucleate microspores.

Acknowledgements. We thank B. Van Duijn for critically reading the manuscript and suggestions. We express our gratitude to Gerda E. M. Lamers and K. Sierat for technical assistance and D. de Witt for growing the plants.

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