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Strong extracellular nuclease activity displayed by barley (*Hordeum vulgare* L.) uninucleate microspores

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Abstract Electroporation is becoming an increasingly important technique for plant transformation. Nevertheless, no positive results were achieved in barley when uninucleate microspores were used as target cells. Since it was previously demonstrated that electric shocks create pores in the microspore cell wall, experiments were designed to verify the presence of nucleases in the electroporation mix. Aliquots of all the solutions used for microspore extraction, purification and transformation were collected and analysed using supercoiled pBI 221 as a substrate; a nuclease activity was detected in all samples. Though microspore rinsing removed most nucleolytic activity in the supernatants, DNA preservation in the electroporation buffer was difficult to achieve, because microspores appeared capable of synthesising and releasing endonucleases at any time. Microspore chilling at 0°C was fairly effective in reducing nuclease secretion in the mix, whereas 1% PEG or 10 mM EDTA maintained most of the DNA in a supercoiled or circular relaxed form. EDTA effects were counterbalanced by Mg^{2+}, but not Ca^{2+} or Zn^{2+}, and enhanced by Mn^{2+}. Barley microspore nucleases actively degraded different DNAs as well as TMV RNA, and apparently had a molecular weight above 30 kDa. Nuclease inactivation with EDTA did not alter microspore viability and allowed a transient expression of the *uid*A gene in electroporated barley microspores.

Key words Barley · Microspores · Electroporation · Nucleases

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Introduction

Several techniques are now available to introduce foreign genes into barley: direct gene transfer to protoplasts via either PEG or electroporation (Junker et al. 1987; Lee et al. 1991; Teeri et al. 1989; Lazzeri et al. 1991), particle bombardment of suspension cells (Mendel et al. 1989), immature embryos (Kartha et al. 1990; Wan and Lemaux 1994), callus tissue (Kartha et al. 1990), endosperm (Knudsen and Muller 1991; Lee et al. 1991), microspores (Jähne et al. 1994) and microspore-derived embryos (Wan and Lemaux 1994).

Barley microspores are a very attractive target for genetic transformation for at least two reasons: firstly, the culture of isolated barley microspores is a consolidated method for the regeneration of large numbers of green plants (Olsen 1991; Hoekstra et al. 1993); secondly, microspore culture is the only system which consistently allows the regeneration of fertile plants from single cells in barley (Mordhorst and Lörz 1993). Furthermore, after chromosome doubling, transgenic homozygous plants can be readily obtained (Jähne et al. 1994).

An alternative method to particle bombardment is electroporation. This technique has been successfully applied over a range of bacterial species (Alexander et al. 1990; Kurusu et al. 1990; Elvin et al. 1991) and yeast strains (Rohrer and Picataggio 1992); in more recent times, transgenic rice, maize, rapeseed and soybean have been obtained (Dhir et al. 1992; Bergman and Glimelius 1993; Biswas et al. 1994; Laursen et al. 1994; Irie et al. 1996). Electroporation of barley microspores has been carried out in different laboratories but in no instance was a stable or transient expression of the transgene reported. On the other hand, it has been demonstrated that electric shocks create pores in the barley microspore cell wall (Joersbo et al. 1990) and that electroporation of *Brassica* microspores

causes a transient expression of the *uid*A reporter gene (Jardinaud et al. 1993).

It was hypothesised that one of the major problems in electro-mediated transformation of barley microspores could be represented by the presence of nucleases in the electroporation mix. The rationale for this is provided by the detection of pollen-associated nucleases in some dicot and monocot species (Matousek and Tupy 1985; Negrutiu et al. 1986; Roeckel et al. 1988; Van Wert and Saunders 1992).

Materials and methods

Plant material and microspore isolation

The experiments were carried out on the barley cultivar Igri. Seed germination and plant growth were performed according to Olsen (1987). The protocol for microspore isolation and purification was based on Mordhorst and Lörz (1993). Microspore concentration was determined with a haemocytometer, whereas viability was assessed with the fluorescein diacetate (FDA) staining method (Widholm 1972).

Nuclease activity tests

Nuclease activity was determined on microspores at the mid-to-late uninucleate stage. In all assays, microspores were re-suspended in electroporation buffer (10% glucose, 4 mM $CaCl_2$, 10 mM HEPES = 4-/2-hydroxyethyl/-1 piperazane-ethanesulfonic acid, 0.2 mM spermidine, pH 7.2) at a concentration of 3×10^5/ml; the target DNA was plasmid pBI 221 (Clontech), added to a final concentration of 20 μg/ml. Samples were incubated at 25°C for 15 min. After incubation, microspores were pelleted (80 g, 5 min) and supernatants were submitted to gel electrophoresis on a 0.8% agarose gel containing 0.5 μg/ml of ethidium bromide. Nuclease activity was recorded visually by observing the degradation of the plasmid band under UV light.

In order to test the nuclease activity in the protein fractions, 3–10 kDa, 10–30 kDa and > 30 kDa supernatants were ultrafiltrated employing Microcon™ devices (Amicon Inc) with suitable membrane cut-offs, incubated for 20 min at 37°C with 1 μg of SPP1 DNA cleaved with *Eco*RI (Marker VII, Boehringer Mannheim), and electrophoresed as above.

The RNase activity of the three fractions was assayed on purified TMV RNA (Boehringer Mannheim); RNA samples were incubated at 37°C for 10 min and eventually heated at 70°C for 5 min prior to electrophoresis. To avoid RNase contamination from other sources, all the equipment was washed with 0.1 M NaOH, 10% SDS for 30 min, rinsed with DEPC-treated water and dried with absolute ethanol.

Nuclease activity prevention

In order to determine the amount of reduction in the nucleolytic activity achievable with microspore rinsing, aliquots of all the solutions employed during microspore extraction and purification were collected and analysed. The substrate type and concentration, as well as the incubation time, were the same as those reported for the nucleolytic activity tests on electroporation buffers. In a separate experiment, incubation of plasmid DNA was carried out at 0°C, instead of 25°C, or in the presence of 0.1, 1 or 10% PEG. The effect of 0.1, 1, 10 mM EDTA [ethylene-diamine-tetra-acetic acid) or EGTA (ethylene glycol-bis (-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid] was also investigated; after treatment with metal-chelating agents, microspores were pelleted and scored for viability with FDA. To determine the type of divalent cation needed by the nucleolytic enzyme(s), the effects of a final 50 mM concentration of $CaCl_2$, $MgSO_4$, $MnSO_4$ and $ZnSO_4$ was separately tested on an electroporation buffer amended with 10 mM EDTA. Since target DNA was poorly visible in the gel when $ZnSO_4$ was used, a final 25 mM and 12.5 mM concentration of this salt was also tested. Furthermore, an attempt was made to degrade microspore-associated nucleases with papain, a typical plant proteinase. Papain (Sigma) was dissolved in 50 mM of potassium phosphate buffer, pH 6.8, and activated with 5 mM of cysteine for 15 min at 37°C; the activated enzyme was added to the electroporation buffer 30 min before the substrate DNA, at a final concentration of 200 μg/ml; in the control, papain was replaced by an equal volume of phosphate buffer plus cysteine.

Other experiments were carried out with *Sss*I methylase which transfers methyl groups to the cytosine residues (C^5) within the dinucleotide recognition sequence 5′ … CG … 3′, and with *Dam* methylase which modifies the adenine residues (N^6) in the sequence 5′ … GATC … 3′. DNA methylation was performed according to the manufacturer's instructions (New England Biolabs Inc). The extent of DNA protection was assayed on λ *Hin*dIII fragments (molecular weight marker II, Boehringer Mannheim). *Sss*I- or *Dam*-methylated λ DNAs were added to the electroporation mix to a final concentration of 20 μg/ml. Following a 2-h incubation at 37°C, supernatant fractions were extracted and electrophoresed on 2% agarose gel; methylated and unmethylated λ DNAs treated with the restriction enzyme *Cla*I (10 units endonuclease/μg DNA) were used as controls.

Electroporation of barley microspores

Barley microspores were re-suspended in the electroporation buffer with or without 10 mM EDTA, at a concentration of 3×10^5/ml; 300-μl aliquots of the microspore suspension containing 20 μg/ml of plasmid pBI 221 were pipetted in 4-well dishes (Nunc Denmark) and electroporated with Progenetor II (Hoefer Scientific Instruments) using a PG 220P-5 electrode. Different combinations of field strength, pulse duration and number were compared. After a post-pulsation period of 20 min at 22°C, the rate of microspore survival was determined. Microspores were then rinsed twice with 0.3 M mannitol, re-suspended in ACH medium (Shannon et al. 1985) and plated (3×10^5/ml) again in 300-μl aliquots. After 24 h of culture, a X-Gluc assay (Jefferson et al. 1987) was carried out; microspores expressing GUS activity were detected under a light microscope (400 × magnification, three replicates of 15 000 microspores each).

Results

After employment, all the solutions needed for microspore extraction, purification and transformation were found to contain nucleases. Nuclease activity was particularly high in the 0.3 M mannitol solution used for extraction and in the 19% maltose solution used for the isolation of viable microspores (Fig. 1, lane 2 and 3, respectively). Since a circular DNA was used as substrate, it can be assumed that cleavage was caused by one or more endonucleases. As shown in Fig. 1, DNA degradation in the mannitol solution, where viable microspores were eventually re-suspended, was much lower than that recorded in the electroporation buffer.

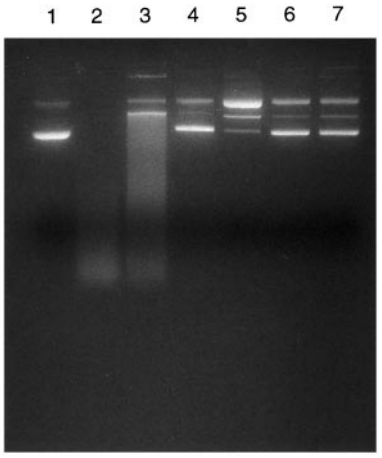
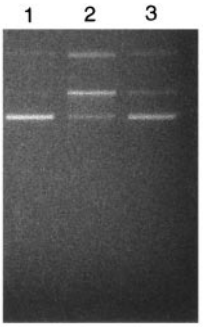


Fig. 1 Nucleolytic activity in the solutions used for microspore extraction, purification and transformation; 200 ng DNA in 10 µl of: *lane 1*: double-distilled water, *lane 2*: 0.3 M mannitol solution used for microspore extraction, *lane 3*: 19% maltose solution used for isolation of viable microspores, *lane 4*: 0.3 M mannitol solution used for microspore re-suspension, *lane 5*: electroporation buffer, *lanes 6 and 7*: the same as *lane 5* except one (*lane 6*) or two (*lane 7*) additional rinses of microspores with fresh electroporation buffer

Fig. 2 *Lane 1*: nucleolytic activity immediately after microspore re-suspension in the electroporation buffer, *lane 2*: nucleolytic activity after 30 min at 25°C, *lane 3*: nucleolytic activity after 30 min at 0°C



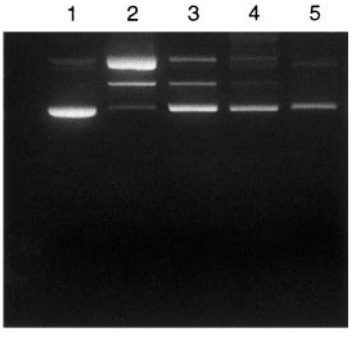


Fig. 3 Effect of PEG concentration on nucleolytic activity; 200 ng DNA in a 16-µl reaction mix (final volumes adjusted with water). *lane 1*: double-distilled water, *lane 2*: 10 µl electroporation buffer, *lane 3*: 10 µl electroporation buffer + 0.1% PEG, *lane 4*: 10 µl electroporation buffer + 1% PEG, *lane 5*: 10 µl electroporation buffer + 10% PEG

This fact can be easily explained in terms of lower microspore density and shorter incubation time.

Repeated washing of the microspores with fresh electroporation buffer prior to incubation with supercoiled pBI 221 allowed the removal of some nucleolytic activity in the supernatants but did not solve the problem of DNA preservation, mainly because the microspores appeared capable of synthesising and releasing endonucleases into the suspension buffer at any time (Fig. 1). The continuous secretion of nucleases into the surrounding medium was further demonstrated by scoring the level of DNA cleavage in supernatants taken from microspore suspensions kept at 25°C or 0°C (Fig. 2).

The addition of 0.1, 1 or 10% PEG to the electroporation buffer supernatants had positive effects on DNA integrity (Fig. 3); however, with PEG concentrations higher than 1%, little DNA was recovered Lowering the temperature of the supernatants to 0°C also proved effective; therefore, cool temperatures appeared to favour DNA preservation by limiting both the synthesis and activity of the enzyme(s). A limited DNA degradation was also noted when metal-chelating agents such as EDTA and EGTA were used. In particular, EDTA concentrations between 1 mM and 10 mM were able to maintain most pBI 221 in a supercoiled or relaxed circular form; however, it should be noted that, even at these EDTA levels, DNA degradation took place as demonstrated by the occurrence of the linear form. A nicking activity was also displayed at all EGTA concentrations, but less pronounced differences were noted by rising EGTA doses in the buffer. On the basis of our observations, it can be concluded that EGTA is less effective than EDTA in protecting substrate DNA from cleavage. It is worth noting that microspore viability was unaffected by EDTA or EGTA treatment; the percent viability for the untreated control, 10 mM EDTA, and 10 mM EGTA was 69.6, 66.0, and 69.2, respectively.

It should also be pointed out that these results were achieved with incubation times as short as 15 min and after having thoroughly rinsed the microspores during the process of isolation and purification. In the absence of any microspore wash, PEG, cool temperatures, and chelating agents had practically no effect on DNA preservation.

The separate addition of metal ions to an EDTA-amended electroporation buffer had distinct effects: Mg^{2+} greatly increased the nucleolytic activity whereas Mn^{2+} at the same molar concentration showed a strong inhibitory action (Fig. 4); Ca^{2+} or Zn^{2+} appeared to play no substantial role since their presence did not change nuclease activity in any direction (furthermore, at 50 mM or 25 mM Zn^{2+} concentrations, DNA recovery was poor).

To verify whether DNA methylation could represent an alternative to metal-chelating agents, we used two methylases which, due to their short-length recognition sites (CG for *Sss*I and GATC for *Dam*), are effective

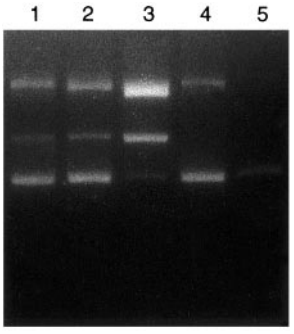


Fig. 4 Effect of different divalent cations on the nucleolytic activity displayed within: *lane 1*: electroporation buffer amended with 10 mM EDTA, *lane 2*: the same as *lane 1* plus 50 mM $CaCl_2$, *lane 3*: the same as *lane 1* plus 50 mM $MgSO_4$, *lane 4*: the same as *lane 1* plus 50 mM $MnSO_4$, *lane 5*: the same as *lane 1* plus 50 mM $ZnSO_4$

Table 1 Microspore viability as affected by electric field strength, pulse duration and number

Treatment	Electric field strength (V/cm)	Pulse duration (ms)	Pulse number	Viability (%)
Control	–	–	–	91.6
1	250	10.00	1	87.5
2	380	5.88	1	85.4
3	510	2.64	1	81.2
4	640	2.64	1	78.3
5	250	10.00	3	53.9
6	750	10.00	3	45.4

against a wide range of restriction enzymes. This study was carried out on the model system *Cla*I/λ DNA. As expected, *Cla*I cleavage was completely prevented when methylation was carried out with *Sss*I methylase (the *Cla*I recognition site, ATCGAT, contains the methylated sequence CG) and reduced to a few sites with *Dam* methylase (protection occurs only when the methylated GATC overlaps the *Cla*I recognition sequence). On the other hand, nucleases released by barley uninucleate microspores were absolutely unaffected by methylation. It should also be noted that, in a situation of partial DNA digestion, no additional bands were observed with respect to un-degraded DNA. Methylation experiments, together with the pattern of partial DNA digestion, suggest a rather unspecific nucleolytic activity for the enzymes considered.

Working with TMV RNA, it was possible to ascertain that the endonucleases released by microspores are also characterized by a strong ribonuclease activity. As for DNA cleavage, the enzymes appeared not to require specific sequences for starting degradation.

To prevent DNA degradation, we attempted nuclease digestion with activated papain; however, even if this proteolytic enzyme was present at a high concentration and the incubation time lasted 30 min, no positive results were achieved; on the other hand, biochemical analysis clearly indicated that the composition of the electroporation buffer was compatible with high levels of papain activity.

With regard to the significance of endonucleases for microspore electro-mediated transformation, we carried out some experiments with plasmid pBI 221 (containing the *uid*A reporter gene under the control of the CaMV 35s promoter). The size of this plasmid is 5.7 kb and the *uid*A cassette accounts for 53% of the total DNA; if nicking occurs at random, the sole linearization of pBI 221 would break the cassette in about half of the cases. In a preliminary test, the lack of any intrinsic GUS-like activity in barley microspores was verified; we eventually determined the type of relationship between microspore viability and electrical field strength (Table 1). Since 10 mM of EDTA had previously been shown to partially inactivate the endonucleases released by the microspores, the electroporation buffer was modified accordingly. GUS-expressing microspores were detected in the samples electroporated in the absence of DNA or in the absence of EDTA. By contrast, the histochemical assays gave some positive results when pBI 221 was protected by the metal-chelating agent (Table 1).

Table 2 Mean frequency of GUS-expressing microspores with 10 mM of EDTA in the electroporation buffer

Treatment	Electric field strength (V/cm)	Pulse duration (ms)	Pulse number	Frequency ($\times 10^{-4}$)
Control	–	–	–	0
1	250	10	1	0
2	250	100	1	0
3	250	10	3	2.0
4	250	100	3	0.7
5	750	0.5	1	1.8
6	750	50	1	2.0
7	750	0.5	3	2.2
8	750	50	3	0

Discussion

With regard to the importance of plant nucleases in the transformation process, it has been demonstrated that the exogenously applied DNA can be degraded not only in the apoplast but also within the cytoplasm or the nucleus (Ashraf et al. 1993); to avoid DNA cleavage, the use of liposomes (Gad et al. 1990), yeast spheroplasts (Hatsuyama et al. 1994) or inhibitory compounds has been suggested. Since pollen is an attractive target for transformation, but releases different nucleases into the surrounding medium, studies were carried out to find the best conditions for nuclease inactivation and the maintenance of good cell viability (Martin et al. 1992; Van Wert and Saunders 1992; Broglia and Corona 1995). As microspores are thought to be deprived of DNases, no particular care is currently taken during particle delivery or electroporation. However, we have clearly shown that one or more nucleases are associated with barley uninucleate microspores; in

contrast to other work, we have also demonstrated that their presence is not the effect of a biochemical contamination from adjacent tissues as found, or hypothesised, in other plant species (Booy et al. 1989; Van Wert and Saunders 1992; Jardinaud et al., 1993), but rather the result of a *de novo* synthesis carried out by the microspores themselves.

Barley microspore nucleases (BMNs) are extremely powerful enzymes, and an almost complete degradation of huge amounts of plasmid DNA can normally be achieved after a few minutes exposure. BMNs can also cleave barley DNA (data not shown) and, consequently, there must be some mechanism protecting the producing cells from suicide; in *Aspergillus nidulans* (Lamy and Davies 1991) a leader sequence was found to provide such a function. Based on the apparent molecular weight (>30 kDa), sugar unspecificity, insensitivity to DNA methylation, as well as a slower activity at cool temperatures or in the presence of PEG, BMNs should belong to the nuclease-I family, like pollen nucleases. However, it should be pointed out that, in contrast to observations with other species such as maize, wheat, alfalfa and tobacco (Roeckel et al. 1988; Martin et al. 1992; Van Wert and Saunders 1992; Matousek and Tupy 1985), BMNs seems to specifically require Mg^{2+} and not Zn^{2+} or Ca^{2+}; they are also resistant to enzymatic digestion. This evidence, together with the high activity shown by BMNs on dsDNA, would suggest some differences in the nucleolytic action. The purification of BMNs through affinity chromatography, coupled with the use of synthetic polynucleotides, will enable an elucidation of these aspects.

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