

A novel *in vitro* system for gamete fusion in maize

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ABSTRACT

Various systems by using electric pulse, calcium, or polyethylene glycol have been developed in the past decade for the *in vitro* fusion of plant gametes. These *in vitro* systems provide a new way to study the fertilization mechanisms of plants. In this study, we developed a bovine serum albumin (BSA)-mediated fusion system for the *in vitro* fusion of maize gametes. The *in vitro* fusion of the isolated single egg cell and sperm cell of maize was observed microscopically in the BSA solution and the fertilized egg cell showed normal cell wall regeneration and nuclear division. The effects of the BSA concentration, pH value and calcium level on the efficiency of the maize gamete fusion were also assessed. BSA concentration and pH value did significantly affect the efficiency of the gamete fusion. Calcium was not necessary for the gamete fusion when BSA was present. The optimal solution for the gamete fusion contained 0.1% BSA, pH 6.0. The fusion frequency was as high as 96.7% in that optimal solution. This new *in vitro* fertilization system offers an alternative tool for the *in vitro* study of fertilization mechanisms with much simpler manipulating procedure than PEG system, and it will be especially useful for the *in vitro* study of the calcium dynamics during plant fertilization.

Keywords: *in vitro* fertilization, bovine serum albumin, calcium, maize.

INTRODUCTION

Fertilization in higher plants occurs deeply in the maternal tissue and is therefore inaccessible for experimental manipulation [1]. The establishment of *in vitro* fertilization (IVF) techniques would greatly facilitate the investigation of the molecular and cellular events of fertilization. In the past decade, various *in vitro* fertilization techniques have been developed for higher plants. The fusion of isolated gametes was induced by using electric pulse [2], polyethylene glycol [3-5], or calcium [6-8]. With the help of the established IVF systems great progress has been made for some key events concerning fertilization mechanisms such as polygamy, gamete recognition and nuclear behavior in fused gametes [4, 9-11], which were actually unable to be investigated by traditional methods. However, IVF is still a challenge work and skill-dependent technique to researchers. Therefore it is still needed to further improve the technique of IVF, especially the technique of

gamete protection and gamete fusion, for further simplifying the procedure and making it easier to manipulators.

In this study, we investigated the possibility of the *in vitro* plant gamete fusion using BSA medium. BSA was previously used in medium as a protectant for the storage of isolated sperm cells in our experiments, and sperm cells was much more protected and was viable longer in BSA medium than in other medium [12]. We supposed that the gamete fusion in BSA medium may be possible if the concentration of BSA is adjusted to an appropriate level. The objective of this study was to develop a simple and efficient BSA-induced fusion system for the *in vitro* fertilization of maize gametes.

MATERIALS AND METHODS

Plant material

Maize (*Zea mays* L.) plants of inbred lines A 188 were grown in a greenhouse under 16 h photoperiod at 25°C. The ears of the plants were bagged before the silk emergence to prevent pollination. Ears were collected at a receptive stage when emerged silks reached 12-13 cm in length [13].

Isolation of egg cells and sperm cells

Egg cells were isolated from ovules dissected from mature ears of maize using a method modified from Kranz *et al* [2]. Half-ovules were

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Abbreviations: BSA (bovine serum albumin); IVF (*in vitro* fertilization); PEG (polyethylene glycol).

incubated for 5 min at room temperature in an enzymatic solution containing 0.5% Macerozyme Onozuka R10 (Yakult Honsha, Tokyo, Japan), 0.5% Cellulase Onozuka R10 (Yakult Honsha, Tokyo, Japan), adjusted to 725 mosmol/kg H₂O with mannitol, pH 5.7. Ovule pieces were then washed with 12% mannitol and egg cells were gently picked out from the embryo sac by manual microdissection under an inverted microscope (Olympus CK-30).

Sperm cells were released from freshly collected pollen grains after an osmotic shock in 12% mannitol, and isolated followed the method described by Kranz *et al* [2].

Gamete Fusion

The fusion of one egg cell and one sperm cell was performed in a 3.5 cm diameter plastic Petridishe that was previously filled with 2 ml BSA (Roche) fusion medium. Different BSA concentrations (0-0.6%), pH values (5-8) and calcium levels (0-2 mM or adding 2 mM EGTA) were assessed for their effects on the adhesion and fusion of the gametes. Based on the results of these tests, the standard optimal fusion medium used in this study contained 3 mM 2[N-morpholino] ethanesulfonic acid (MES), 0.1% (W/V) BSA, and adjusted to 750 mosmol/kg H₂O with mannitol, pH 6.0. As a control, the fusion of male and female gametes was also performed in PEG medium containing 5 mM calcium, 3 mM MES, 20% (W/V) PEG and 5% (W/V) mannitol, pH 6.0.

The fusion process was observed under an inverted microscope (Olympus CK-30). The individual pairs of male and female gametes were put into contact by a microcapillary. At least 20 pairs of cells were used for the fusion in each experiment.

Culture of the fusion products

Fertilized egg cells were cultured in droplets of the modified basic MS medium [14]. The medium were adjusted to 780 mOsmol/kg H₂O with 4% glucose and 11% mannitol, pH 5.7. Each of the droplets contained 2-4 μ l of medium and covered by mineral oil in a 3.5 cm diameter glass Petri dish. The culture dishes were kept in a culture chamber in dark at 25 °C to encourage the further development of fusion products.

Cell wall staining

For staining cell wall, zygotes were incubated in 0.001% Fluorescent Brightener 28 (Sigma) solution for 10 min. The Fluorescent Brightener 28 was dissolved in 12% mannitol. Then, the zygotes were washed three times in 12% mannitol before observation.

Microscopy and image processing

For visualizing the process of the gamete fusion, cells were examined under an inverted microscope (Olympus IMT-2), equipped with a cooled charge-coupled device (CCD) camera (CoolSNAP-HQ, Roper Scientific Inc.). Digital images from CCD were captured on a computer equipped with the software of Image-Pro Express (Media Cybernetics Inc.). Using the sequential image grabbing function, an image was acquired at a maximum rate of 0.4 s every frame.

Both Cultured cells and the cells after staining with Fluorescent Brightener 28 were observed under an epifluorescence inverted microscope (Leica DM IRE2), equipped with a cooled CCD (RTE/CCD-1300-Y/HS, Roper Scientific Inc.).

Digital images from CCD were captured on a computer equipped with the software of MetaMorph (Universal Imaging Corporation Inc.). Images were processed using Photoshop 6.0 software (Adobe

Systems, Mountain View, CA).

Online supplementary material

Video 1 (<http://www.cell-research.com/20059/video/video1.mpg>) shows the fusion process of the isolated sperm cell and egg cell of maize in the BSA fusion medium.

RESULTS

The development of BSA-mediated fusion system

No adhesion and fusion was observed when gametes were contacted in the fusion medium without BSA. When the concentration of BSA was 0.01%, the adhesion of gametes was accomplished in 10-30 min and in this case the fusion frequency was 15.0%. When the concentration of BSA was either 0.1% or 0.6%, the gametes adhere to each other in 10 s, and their fusion frequency was increased significantly up to 96.7% or 95.0% respectively (Fig. 1).

The role of BSA in gamete fusion was further investigated by altering the pH value of fusion medium. When the pH value was 8.0, no gamete adhesion was observed and consequently no fusion occurred. When the pH value was 7.0, the fusion frequency was 24.0%. When the pH value decreased further to 6.0, the fusion frequency was increased sharply to 96.7%. After 2 min in 0.1% BSA of pH 5.0 or lower, sperm cells and egg cells broke and lost their integrity. Sperm cells could be maintained the ability to fusion with egg cells more than 60 min in 0.1% BSA at pH 6.0 to 8.0 (Fig. 2).

Without adding calcium in the medium, 96.7% gametes could easily fuse in 0.1% BSA of pH 6.0. Adding 2 mM EGTA in the medium resulted in 60.0% gametes fusion. In contrast, adding 2 mM calcium made gamete adhesion more difficult and no fusion occurred. Adding 0.5 mM calcium resulted in the fusion of just 30.0% cell pairs. Whereas,

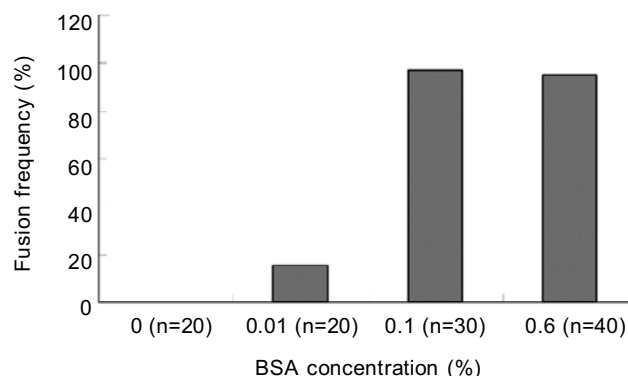


Fig. 1 Effects of different concentrations of bovine serum albumin (BSA) on the fusion frequency of the isolated sperm cell and egg cell of maize. The fusions were carried out in fusion medium of pH 6.0 without external calcium. The values of n represent the total number for fusion in each independent experiment.

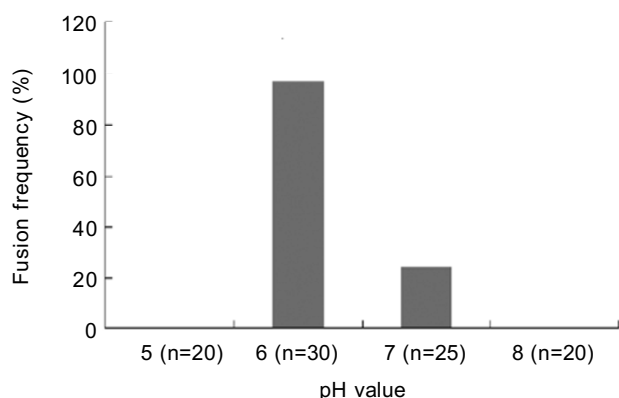


Fig. 2 Effects of different pH value on the fusion frequency of isolated sperm cell and egg cell of maize. The fusions were carried out in 0.1% BSA without external calcium. The values of n represent the total number for fusion in each independent experiment.

adding 0.1 mM calcium the fusion frequency could be up to 95% (Fig. 3).

Cytological characteristics of the *in vitro* fusion

Under the optimal BSA medium, the gamete fusions were usually occurred 7-10 min after adhesion. Two fusion processes were observed: the quick process and the slow process. During the quick fusion process, the sperm cell suddenly entered into the egg and the whole process completed within 1-2 s. In this process, the digital images of the process could not easily be captured. During the slow process, the sperm cell quickly pressed to the egg cell in the first 2 s (Fig. 4B-D). The sperm cell was tightly attached with the egg cell in the following 6 s (Fig. 4E and 4F) and seemed there was an interaction between the membranes of two gametes in this period. Then the sperm moved again to press the egg cell in successive 10 s (Fig. 4G and 4H) and the whole sperm cell finally entered the egg cell (Fig. 4I). The whole fusion process was finished

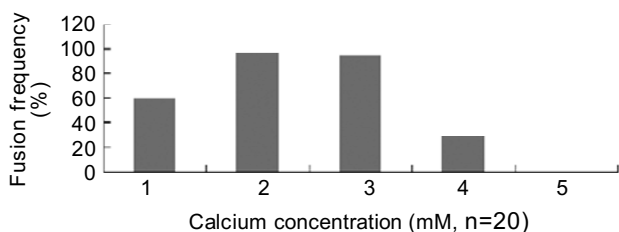


Fig. 3 Effects of calcium concentrations on the fusion frequency of isolated sperm cells and egg cells of maize. The fusions were carried out respectively. 1) 0.1% BSA with 2 mM EGTA; 2) 0.1% BSA; 3) 0.1% BSA with 0.1 mM calcium; 4) 0.1% BSA with 0.5 mM calcium; 5) 0.1% BSA with 2 mM calcium. The values of n represent the total number for fusion in each independent experiment.

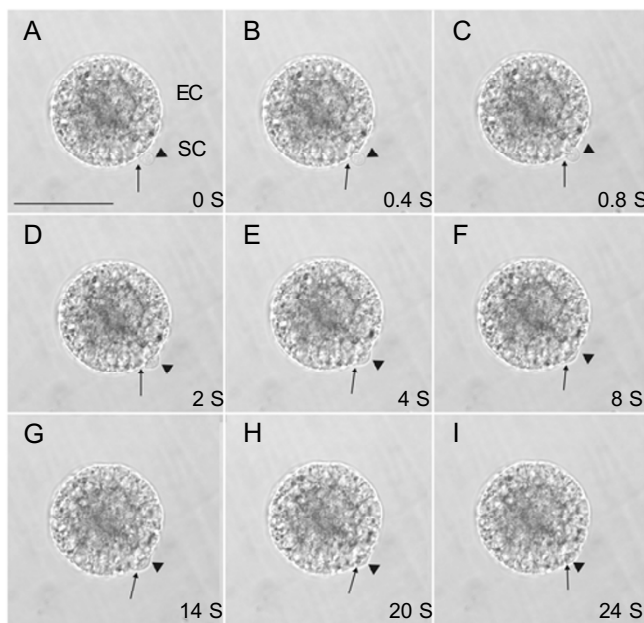


Fig. 4 Timelapse images of *in vitro* adhesion (A) and fusion (B-I) of the maize sperm cell (SC) and egg cell (EC) induced by 0.1% BSA medium (pH 6.0). Images were taken at varying time intervals over a period of 24 s, as indicated in right-bottom corners of each image. The arrows indicate fusion site and arrowheads indicate sperm cell. Bar= 50 μm.

in 24 s and was visualized (Fig. 4 and video 1).

Before fertilization the isolated egg cell was round-shaped (Fig. 5A) and its nucleus contained one nucleolus (Fig. 5B). Within several minutes after the fusion, the egg cell underwent a transient and reversible contraction (data not shown) as described by previous studies [15]. After incubation for 20 h in MS medium, some zygotes showed a little shrinkage and the cell wall could be clearly observed (Fig. 5C and 5D). After 24 h incubation in MS medium, fusion products showed two nucleoli in their nucleus (Fig. 5E), which is a marker showing nuclear fusion already completed. After 40 h incubation in MS medium, the nuclear division of zygotes was observed (Fig. 5F). These results indicated that the artificial zygotes produced by our IVF system were well developed.

In PEG control medium, 100% fusion frequency was observed among 20 pairs of gametes but the gamete fusion behavior was different from that in BSA fusion medium. Once the pair of gametes adhered, the sperm cell pressed to egg cell and whole sperm cell entrapped into egg cell in 10 s (Fig. 6) and it took another 3-10 min for the sperm cell to enter into the egg cell.

DISCUSSION

In this investigation, a novel *in vitro* system for maize

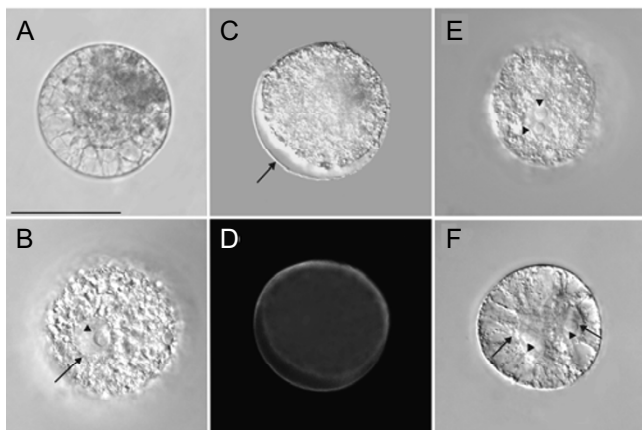


Fig. 5 The development of *in vitro* fertilized egg cell of maize. **(A)** A round-shaped egg cell. **(B)** The egg showing nucleus (arrow) and nucleolus (arrow head). **(C)** A shrank zygote 18 h after culture. The arrow indicates well regenerated cell wall of the zygote. **(D)** Fluorescent image showing cell wall of the same zygote as in C. The cell wall was labeled by Fluorescent Brightener 28. **(E)** A zygote 20 h after fusion, showing two nucleoli (arrow heads) already appeared in the nucleus. Note the diameters of the two nucleoli are different. **(F)** A zygote 40 h after fusion, showing nuclear division. The arrow heads indicate two nucleoli and arrows indicate two nuclei respectively. Bar=50 μ m.

gametes fusion was developed. The fusion frequency was as high as 96.7% in the solution containing 0.1% BSA at pH 6.0. The whole fusion process was observed and the zygotes were well developed.

BSA has been widely used in the media for the capacitation of spermatozoa and the acrosome reaction of mammalian sperm [16]. It is well known that mammalian sperm undergoes biochemical and physiological changes in the female reproductive tract, termed capacitation, which enables sperm fertilization [17]. However, it was not clear whether angiosperm sperm cells also undergo capacitation for fertilization. In this investigation, we showed, to our knowledge, in the first time that BSA did have the capacity to induce gametes adhesion and fusion *in vitro*. Previous study indicated that BSA conformation could change in different pH value [18]. Our result showed that the frequency of gamete adhesion and fusion decreased with the decrease of pH value (Fig. 5). The effect of pH value on the fusion frequency may result from its effects on BSA conformation and therefore influence its function in mediating cell fusion.

Generally, BSA induced gamete fusion shows several advantages over PEG induced fusion. In BSA medium both isolated gametes and fusion products showed vigorous viability demonstrated by FDA test and their fusion behavior. In fact, BSA was previously used as a protectant and could obviously prolong the storage of generative cells

and sperm cells of higher plants [12]. The isolated gametes could be kept in BSA for hours before and after fusion. However in PEG medium the gametes should be fused as soon as possible to avoid harmful effect of PEG on the membrane of the fusion products. BSA fusion system is also simpler than PEG system. It takes 3-10 min to bring the gametes into contact in PEG medium for its viscosity. In contrast, the cells can settle down quickly at the bottom of Petri dish in BSA medium and it is easy to move them close to each other afterwards. It takes less than 10 s to bring the male and female gametes into contact for the fusion.

As described in our previous work [4], adding low concentration of calcium in PEG can obviously enhance gamete fusion, however, it also makes cells easy to stick on the bottom of Petri dish and thus they are no longer to be moved for fusion. In this case, it needs to disturb the cells all the time to keep them being suspended in the medium. In BSA medium, however, the cells can be moved freely in the medium and once the pairs of gametes are adhered in BSA medium, they can be left there more than 10 min for observation of their fusing process without worrying their sticking on glass bottom. This is especially useful for less experienced researchers to manipulate.

It has been shown that calcium plays a pivotal role in

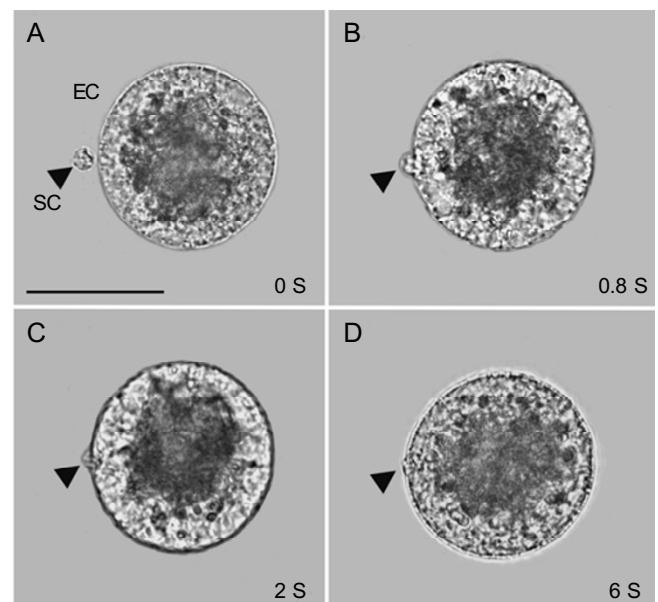


Fig. 6 Timelapse images of *in vitro* adhesion process **(A-D)** of maize sperm cell (SC) and egg cell (EC) induced by PEG medium containing 5 mM calcium, 3 mM MES, 20% (W/V) PEG and 5% (W/V) mannitol, pH 6.0. Images were taken at varying time intervals over a period of 6 s, as indicated in right-bottom corners of each image. The arrowheads indicate sperm cell. Bar= 50 μ m.

membrane fusion in many plant cell systems [19]. For IVF, calcium had been used at high concentrations (50 mM) to induce gamete fusion of maize [7]. Faure *et al* [6] showed that the fusion efficiency of IVF of maize in low calcium (1mM) was much lower than that in high calcium (5 mM). Although fusion could happen at even lower calcium concentrations (around 100 μ M), the fusion frequency was much lower [20]. It seemed that calcium at millimolar levels would benefit IVF. In deed, our study also showed that the PEG medium containing calcium was much better than that of without calcium to induce fusion of maize gametes. Adding calcium in fusion medium obviously shortened the duration of gamete adhesion and fusion (data not shown). However, in our present experiments, 97.6% gametes could easily fuse without adding calcium and 60% gametes could easily fuse with the 2 mM EGTA in the medium. The results indicated that adding calcium was not necessary for gamete fusion with the presence of BSA in the medium, although the low concentration of calcium at micromolar level might benefit gametes for their adhesion and fusion.

It was reported that maize gamete fusion could trigger a calcium influx, which was then followed by an increase of cytoplasmic calcium [21]. In this process, the extracellular calcium that penetrated the egg cells may partly contribute to the cytosolic calcium increase. In BSA induced gamete fusion, as demonstrated in present work, gamete fusion could also occur in presence of 2 mM EGTA. This new fusion system therefore offers an alternative way and useful control for investigating the link of the calcium influx to the cytosolic calcium increase during IVF.

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