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Full Length Research Paper

Using orthogonal design to determine optimal conditions for intergeneric protoplasts fusion between *Mingxian169* and *Y2155a*

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This study is important for the optimization of protoplast fusogen and washing solution system suitable for protoplast fusion between the *Triticum aestivum* and *Aegilops*. By enzymolysis, the result shows that more than 90% viable protoplasts of *Mingxian169* (common wheat) and *Y2155a* (*Aegilops*) were efficiently obtained and fused. The greatest of protoplast fusogen and washing solution condition was developed using an orthogonal experimental design, L25 (5^5), where L=orthogonal table; 5=factors; 5=five levels of each; and 25=experimental number. It was shown that the greatest protoplast fusogen was found at PEG6000 content (w/v) 25%, 0.50 M sucrose, pH 6.0 and washing solution was 5.0 mM $CaCl_2$. Over 10% viable heterokaryons was produced using different fusion condition. Because of the narrow genetic diversity of common wheat and elite agronomic traits of many wild relatives, it is very important and helpful for the improvement of common wheat through somatic hybridization between wheat and wheat wild relatives,

Key words: Protoplast, fusion, orthogonal design method, *Mingxian 169*, *Y2155a.*

INTRODUCTION

Wheat (*Triticum aestivum*) is one of the world's most important food crops. New varieties cultivating is the most effective, economical, and environment-protected method for coping with abiotic and biotic stresses. One way of increasing the genetic diversity of wheat is by somatic hybridization, which can help to introduce new characters from other species or genera (Dudits et al., 1987; Cox et al., 1990; Song et al., 1999). *Aegilops*, wild relatives of wheat, shows several elite agronomic traits for disease resistance. Protoplast fusion and somatic hybridization is a way to obtain such intergeneric hybrids (Negrutiu et al., 1989). It has been completed successfully for the work of protoplast isolation from wheat tissues (Evans et al., 1972; Maddock, 1987; He et al., 1992). Many scholars established the system of callus regeneration, some obtained shoots (Mikós and Erhard, 1995; Li et al., 2004), while whole plants regeneration was rarely reported (Xia et al., 2003).

Techniques for protoplast isolation and fusion are extensively studied on wheat (Cheng et al., 2004; Xiang and Xia GM FN, 2003), but it has not been reported for the protoplast fusion between *T. aestivum* and *Aegilops*. For the establishment of optimization system, an orthogonal experimental design was conducted in order to find out suitable system for protoplast fusogen and washing solution between *T. aestivum* and *Aegilops*. It was very important and helpful for the improvement of common wheat through somatic hybridization between wheat and wheat wild relatives, because of the narrow genetic diversity of common wheat and elite agronomic traits of many wild relatives. The protocol described by Xiang FN and Xia GM (2003) for intergeneric fusion of *T.*

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Abbreviations: MES, 2-N-Morpholinoethanesulphonic acid; **FDA**, fluorescein diacetate; **UV**, ultraviolet; **PEG6000**, polyethylene glycol 6000.

Table 1. Factors and levels of fusogen and washing solution.

Factor	PEG6000 content (w/v)	Osmoticum	Osmoticum content(M)	pH	Washing solution $CaCl_2$ (mM)
Level1	25	Mannitol	0.40	5.4	4.0
Level2	30	Sucrose	0.45	5.6	4.5
Level3	35	Glucose	0.50	5.8	5.0
Level4	40	Proline	0.55	6.0	5.5
Level5	45	KCl	0.60	6.2	6.0

aestivum L. and *Avena sativa* L. protoplasts was adopted.

MATERIALS AND METHODS

Plant material

Seeds of *Y2155a* were planted in vermiculite in large (11 cm diameter) plastic pots under 24/22°C, with a 16/8 h(light/dark). Well expanded second leaflets were collected from plants treated under short photoperiods and surface-sterilized with 0.53% sodium hypochlorite and 70% ethanol.

Young (3-5 mm) shoots from embryo axes of *Mingxian169* were used as the source of material for protoplast isolation. Dry seeds were surface-sterilized and treated with water for imbibition overnight. Embryo axes were then excised and prepared for the experiment.

Standard protoplasts isolation

Second leaflets of *Aegilops* and embryo axes of *Mingxian169* were finely chopped and pretreatment plasmolysed for 1 h in 5 mL CPW medium with 13% mannitol for pretreatment. Then tissues were digested 5.5 h in the constant temperature foster box(25°C) in an enzyme solution based on CPW 13M and 10 mM MES containing 1% Cellulase R-10(Japan), 0.1% Pectinase (Japan). Protoplasts were absorbed by pipette and precipitated. Each pellet was resuspended in 1 mL of the base solution and then repeat the last step two times. The protoplast density was detected and the viability evaluated with fluorescein diacetate (FDA) under UV light (BX51TF) as described earlier (Widholn, 1972).

Isolation of protoplasts for fusion

The method of plasmolysis, digestion and precipitation were identical to standard protocol, but, before the fusion, pellets were stained with FDA (*Y2155a*) and Rhodamine B (*Mingxian169*). Two staining solutions were prepared by adding 20 µL from a stock (of 5 mg FDA per mL of acetone and 5 mg Rhodamine B per mL of de-ionised water) to 1 mL of plasmolyticum, from which 100 µL was added to the pellets with *Mingxian169* and *Y2155a* protoplasts, prior to precipitating them as above. Under UV light, protoplasts stained with FDA fluoresced yellow-green while those stained with Rhodamine B fluoresced red. Density and viability were evaluated as described previously.

Factors and Levels

The optimized system for protoplast fusogen and washing solution were worked out with an orthogonal experimental design. The experimental factor levels for fusion agent (PEG6000 content), osmoticum, osmoticum content, pH and $CaCl_2$ (washing solution), are presented in Table 1.

This experiment had five factors, each at five levels. To screen and develop the optimized system of protoplast fusion condition, an orthogonal experimental design, L25 (5^5), was used (Table 2), where L=orthogonal table, 5=factors, 5=five levels of each, and 25=experimental number.

Protoplast fusion

Small volumes (about 100 µL with 10^6 protoplasts) of stained protoplasts of each partner were dispensed in the centre of culture wells and, after 20 min, were mixed (1:1) with the fusing solution (Table 1); 15 min later, the fusion solution was replaced by a 2:1 volume of washing solution prepared by adding $CaCl_2$ of different strengths to CPW 13 M (Table 1). After 10 min, repeat the last step one time. After 10 min, this solution was removed and all wells were filled to 1 mL with culture medium. The percentage of heterokaryons formed was determined by counting the protoplasts that fluoresced both green and red under UV light.

RESULTS AND DISCUSSION

Protoplast isolation

With the isolation protocol used, more than 90% viable protoplasts were obtained for two *Mingxian169* and *Y2155a* (Figures 1 and 2). Such a high viability is a prerequisite for the subsequent fusion successfully. It is sufficient for culture at the initial plating density of 1×10^5 protoplasts mL^{-1} for the density of $6.8\pm0.2\times10^6$ protoplasts g^{-1} FW of digested tissues to be consistently obtained.

This is a report of the successful isolation of large numbers of highly viable protoplasts from tissues of *Y2155a*. Also, for the first time the fusion of protoplasts of *Mingxian169* and *Y2155a* has been completed successfully. Russian wildrye (*Psathyrostachys juncea (fisch.) Nevski*) and wheat grass (*Agropyron elongatum (host) Nevski*) had been studied previously (Xia et al., 1996).

The results diluted allowed optimal fusion density of 10^5 protoplast per mL, but some improvement can be made by the adjustment of various parameters, such as plasmolysis, enzyme concentration and the time of incubation aiming at a larger yield and coupled with an improved initial culture response (Li, 2002).

Table 2. Orthogonal design, L25 (5^5): effect of fusogen and washing solution on protoplast fusion rate.

S/N	PEG6000 content (w/v)	Osmoticum	Osmoticum content (M)	pH	Washing solution $CaCl_2$ (mM)	Blank	Percentage of heterokaryon (%)
1	1	1	1	1	1	1	15.6
2	1	2	2	2	2	2	16.9
3	1	3	3	3	3	3	16.7
4	1	4	4	4	4	4	16.6
5	1	5	5	5	5	5	15.0
6	2	1	2	3	4	5	15.9
7	2	2	3	4	5	1	15.6
8	2	3	4	5	1	2	16.2
9	2	4	5	1	2	3	14.2
10	2	5	1	2	3	4	12.5
11	3	1	3	5	2	4	14.9
12	3	2	4	1	3	5	16.6
13	3	3	5	2	4	1	13.2
14	3	4	1	3	5	2	13.7
15	3	5	2	4	1	3	11.2
16	4	1	4	2	5	3	13.5
17	4	2	5	3	1	4	13.4
18	4	3	1	4	2	5	16.3
19	4	4	2	5	3	1	14.2
20	4	5	3	1	4	2	13.8
21	5	1	5	4	3	2	16.9
22	5	2	1	5	4	3	15.2
23	5	3	2	1	5	4	13.3
24	5	4	3	2	1	5	13.8
25	5	5	4	3	2	1	11.7

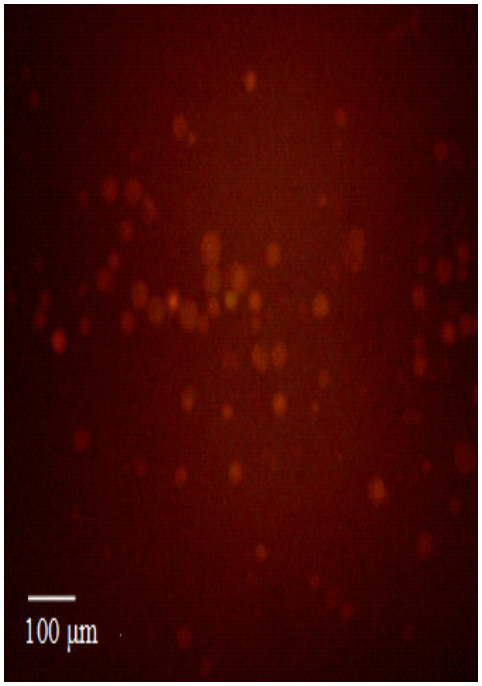

Figure 1. Freshly isolated *Y2155a* protoplasts under UV light. Protoplasts fluoresce in red.

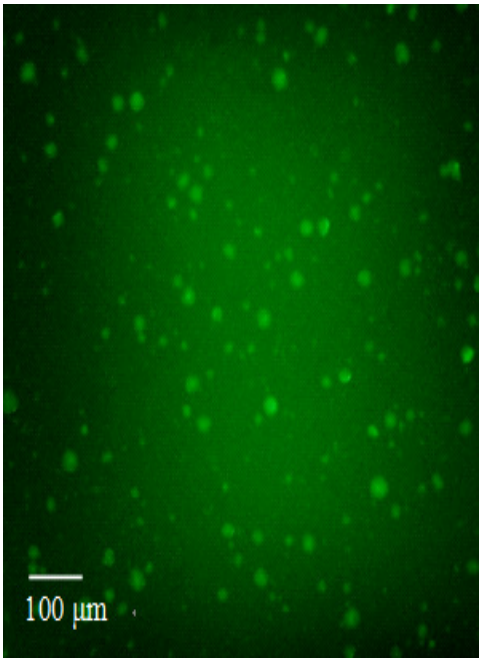

Figure 2. Freshly isolated *Mingxian169* protoplasts under UV light. Protoplasts fluoresce in green.

Table 3. Summary of orthogonal experiment analysis for effect of protoplasts fusion.

Factor	PEG6000 content (w/v)	Osmoticum	Osmoticum content (M)	pH	Washing solution $CaCl_2$ (mM)	Blank
Level1 average	16.16	15.36	14.66	14.70	14.04	14.06
Level2 average	14.88	15.54	14.30	13.98	14.80	15.50
Level3 average	13.92	15.14	14.96	14.28	15.38	14.16
Level4 average	14.24	14.50	14.92	15.32	14.94	14.14
Level5 average	14.18	12.84	14.54	15.10	14.22	15.52
Max	16.16	15.54	14.96	15.32	15.38	15.52
Min	13.92	12.84	14.30	13.98	14.04	14.06
Range	2.24	2.70	0.66	1.34	1.34	1.46
Modulation R'	2.0035	2.415	0.5903	1.1985	1.1985	0.3059

Protoplast fusion

Effect of fusogen and washing solution on protoplast fusion rate

The orthogonal design is a mathematical method used for planning multifactor tests. It is characterized as a balanced arrangement of pairs or groups and applied broadly to optimize test designs. In this study, we opted to select the above mentioned parameters as investigation targets while maintaining all other factors. Each of the above five factors could be changed at five levels, and the orthogonal array was used to arrange the tests. Effects of these five factors were then examined. Details of the five levels for each factor and Percentage of heterokaryons in 25 experiments are shown in Table 2.

The optimized protoplast fusion system was found at PEG6000 content (w/v) 25%, 0.50 M sucrose, pH 6.0 and 5.0 mM $CaCl_2$ (Table 3). Followed by the influence of descending is osmoticum, PEG6000 content (w/v), pH, washing solution $CaCl_2$ (mM), osmoticum content (M).

PEG 6000 content was the key of the PEG fusion. When the PEG 6000 content is less, it was not getting closer for the majority of protoplasts and the opportunity of fusion was reduced. And on the other hand, the protoplasts massed into a dense cluster to reduce the probability of hybridization. In addition, PEG has toxicity to protoplasts, so the higher of the PEG content the more harmful to the heterokaryons. So 25% PEG6000 was optimized content for the fusion, with about 16.16% of heterokaryons produced (Table 3).

Five different osmoticums were used for adjusting osmosis pressure (mannitol, sucrose, glucose, proline, KCl; see Table 1). Fusion was possible with all five osmoticums (Table 3), but KCl was the least efficient statistically, with about 12.84% of heterokaryons produced. Osmoticum with sucrose (0.50 M) was most efficient, with about 15.54% of heterokaryons produced. Maybe sucrose was the main hydrates form of transportation *in vivo*. Statistically, mannitol, glucose and proline could also play an important role as osmoticum, but the effect was good as sucrose (Table 3).

The extreme pH can cause membrane lipid peroxidation level increased. The membrane was damaged and membrane permeability increased. Soluble proteins including some metabolic enzymes and protective enzymes content decreased. The decrease of enzyme bound to affect cell metabolism and protection, thereby affect the growth and differentiation of the protoplast.

The Ca^{2+} had an obvious influence on the cytomixis. When the density of Ca^{2+} was too low, the intercellular channel was too small to fuse. If the Ca^{2+} density is higher, the protoplast may break down easily. So we must choose the appropriate Ca^{2+} density. In this study, 5.0 mM $CaCl_2$ was the best.

Protoplast fusion with the best combination

The heterokaryons to be formed by PEG6000 fusion techniques are shown in Figures 3 and 4. Because of the absence of centrifugation, which consistently decreased their density and damaged the protoplasts, precipitation could be more suitable technically. The isolation protocols for each species were identical during all successive experiments, but small differences (age of material, exact duration of digestion, time between two precipitation, etc.) still exist and cannot be entirely eliminated. These factors could influence protoplast quality and percentage of heterokaryons. Fusion agents could be other chemical fusogen such as PEG 4000 (2% glucose, 1.5% Ca $(NO_3)_2$, 40% PEG4000, pH 7.0) or other electrical parameters such as voltage and pulse duration. They can also obtain good fusion effects (Xia, 1996; Cai, 2007; Ge, 2006).

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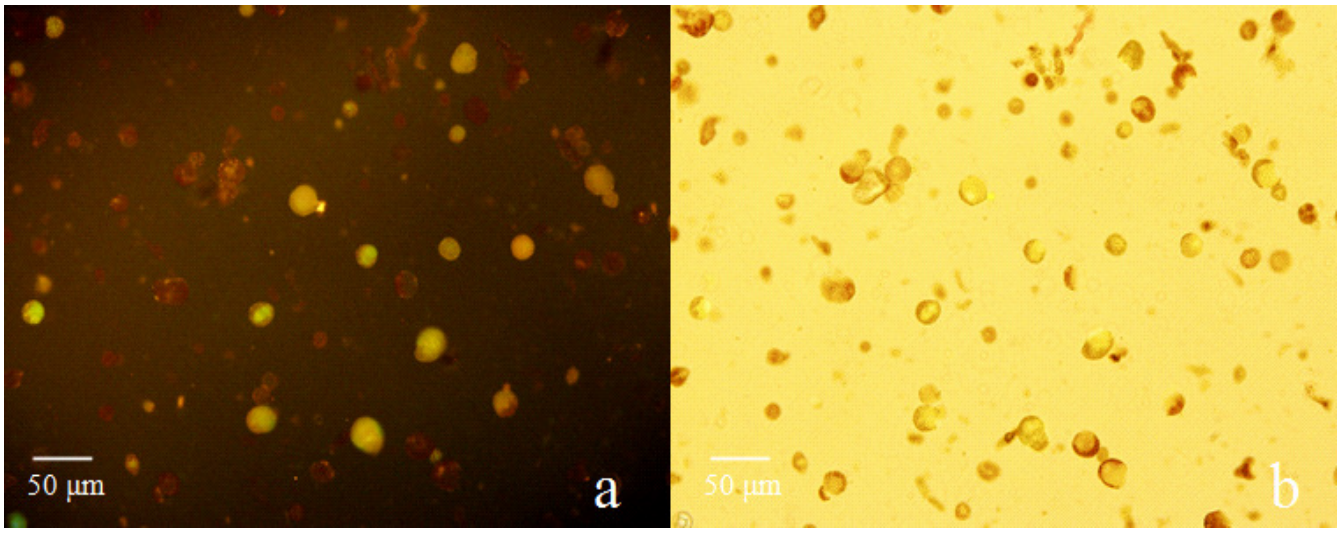

Figure 3. Typical microscopic field after a PEG6000 fusion observed under UV (a) and direct light (b). Heterokaryons fluoresce in red/green.

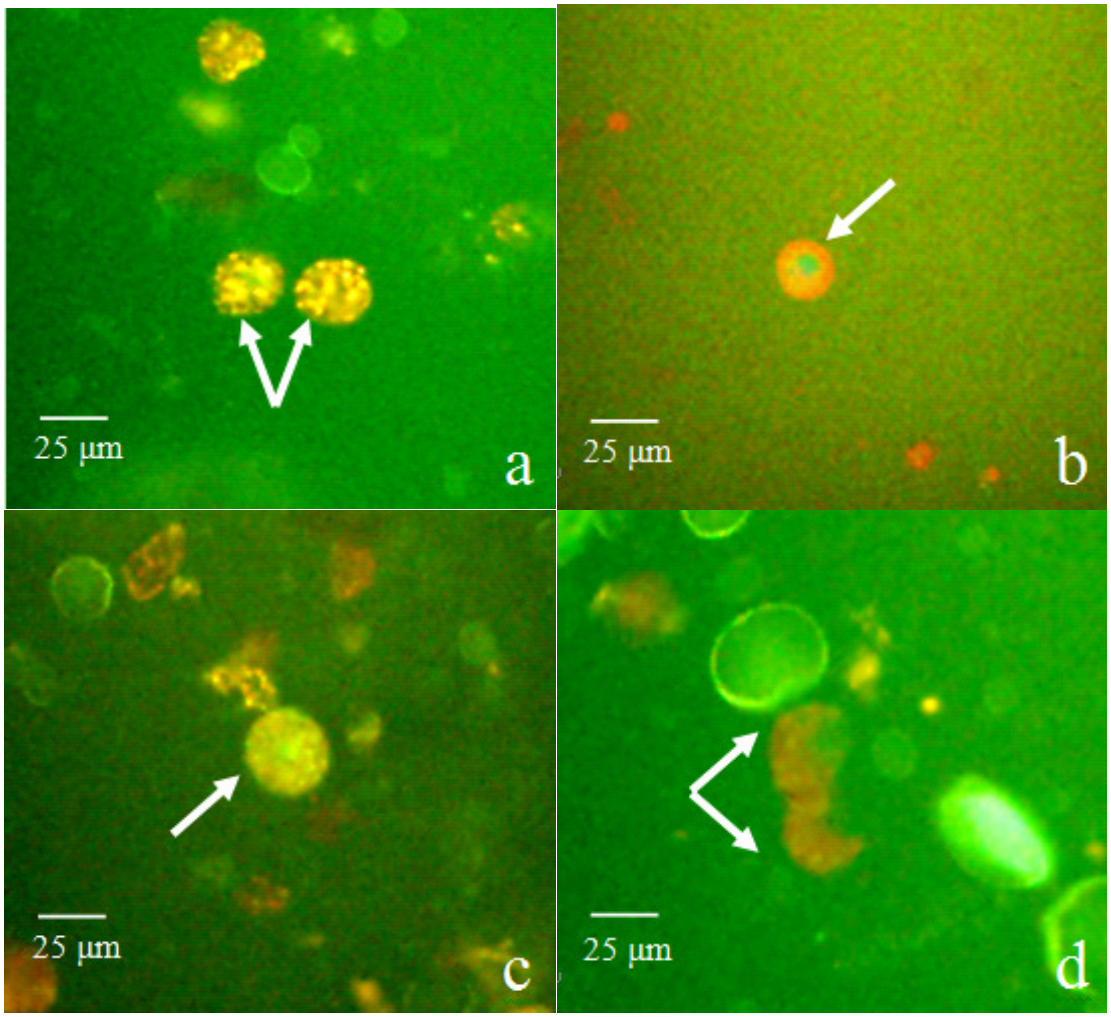

Figure 4. Heterokaryons from a *Mingxian169+ Y2155a* fusion showing dual labeling by the differential flourochromes (arrowed).

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