Stage-specific appearance of cytoplasmic microtubules around the surviving nuclei during the third prezygotic division of *Paramecium*

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Abstract: There are six micronuclear divisions during conjugation of *Paramecium caudatum*: three prezygotic and three postzygotic divisions. Four haploid nuclei are formed during the first two meiotic prezygotic divisions. Usually only one meiotic product is located in the paroral cone (PC) region at the completion of meiosis, which survives and divides mitotically to complete the third prezygotic division to yield a stationary and a migratory pronucleus. The remaining three located outside of the PC degenerate. The migratory pronuclei are then exchanged between two conjugants and fuse with the stationary pronuclei to form synkarya, which undergo three successive divisions (postzygotic divisions). However, little is known about the surviving mechanism of the PC nuclei. In the current study, stage-specific appearance of cytoplasmic microtubules (cMTs) was indicated during the third prezygotic division by immunofluorescence labeling with anti-alpha tubulin antibodies surrounding the surviving nuclei, including the PC nuclei and the two types of prospective pronuclei. This suggested that cMTs were involved in the formation of a physical barrier, whose function may relate to sequestering and protecting the surviving nuclei from the major cytoplasm, where degeneration of extra-meiotic products occurs, another important nuclear event during the third prezygotic division.

Keywords: *Paramecium*; Conjugation; Meiosis; Paroral cone region; Cytoplasmic microtubules

Ciliates are a group of unicellular eukaryotes with nuclear dualism, possessing both polygenomic somatic macronuclei and diploid germinal micronuclei derived from synkaryon (fertilized nucleus) division products through conjugation, one kind of sexual reproduction (Orias et al, 2011). *Paramecium caudatum* is a globally distributed ciliate with one micronucleus and one macronucleus, and six micronuclear divisions during conjugation: three prezygotic and three postzygotic. The first two meiotic prezygotic divisions form four haploid nuclei,among which one is located and survives in the paroral cone region (PC, the area around the degenerated oral apparatus) of each conjugant (each cell of a conjugating pair), with the remaining three degenerating outside the PC. The surviving PC nucleus undergoes a third prezygotic division yielding two gametic nuclei: a stationary pronucleus (StP) and a migratory pronucleus (MiP), corresponding to a female and a male gamete of multicellular organisms, respectively. The MiPs then exchange reciprocally between two conjugants and fuse with the StPs to form synkarya, which divide three times successively (postzygotic divisions) to form eight synkaryon products (Calkins & Cull, 1907; Wichterman, 1986).

In our previous studies, immunofluorescence labeling and protargol staining techniques indicated stage-specific spindle microtubular behavior at the telophase of the third prezygotic division (Gao et al, 2011a, 2011b). Concerning cytoplasmic microtubules (cMTs), it has been suggested they play a role in pronuclear exchange (Nakajima et al, 2001). In the current study, immunofluorescence labeling with monoclonal antibodies of anti-alpha tubulin indicated stage-specific behavior of cytoplasmic microtubules (cMTs) during the third prezygotic division in *P. caudatum*, which might be related with the surviving mechanism of meiotic products and two gametic pronuclei.

MATERIAL AND METHODS

Chemicals

Immunofluorescence staining-related chemicals

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including monoclonal antibodies of mouse anti-α tubulin, FITC-conjugated goat anti-mouse Ig G, propidium iodide (PI), Triton-X 100, 0.5 mol/L EGTA (pH 8.0), 4% paraformaldehyde, and RNase A were purchased from the Beyotime Institute of Biotechnology (Haimen Jiangsu, China). Other chemicals were provided by the Hangzhou Dafang Chemical Reagent Inc (China).

Cell culture and induction of conjugation

Two complementary mating types of *P. caudatum* were collected from East Lake Campus of Zhejiang A & F University (China). Cell culture and conjugation induction followed previous studies (Hiwatashi, 1968). Conjugating pairs were isolated by iron-dextran particles (Sun et al, 2010; Yang & Takahashi, 1999).

Immunofluorescence staining

Immunofluorescence staining with monoclonal antibodies of anti-α tubulin followed previous studies (Gao et al, 2011b; Yang & Takahashi, 2002). 1) Cells were fixed in 2% paraformaldehyde diluted in 2 mmol/L phosphate buffer (pH 7.0) containing 25 mmol/L KCl (PBS). 2) Fixed cells were rinsed three times with washing buffer (PBS containing 5 mmol/L MgSO4, 2 mmol/L EGTA, and 0.05% Triton-X 100). 3) Cells were blocked with 1% BSA dissolved in 5 mmol/L NH4Cl. 4). Cells were incubated overnight in 1 000× diluted monoclonal antibodies of anti-α tubulin containing 10 μg/mL RNase A. 5) After three rinses, cells were incubated for 2 h in 500× diluted FITC-conjugated goat anti-mouse Ig G containing 2.5 μg/mL PI. 6) After a brief rinse, the stained cells were observed under a fluorescence Olympus DX60 microscope. All experiments were performed at room temperature (25 °C).

RESULTS

It is well-known that microtubules are involved in pronuclear transfer in both *Paramecium* (Jurand, 1976; Nakajima et al, 2001) and *Tetrahymena* (Orias et al, 1983). In 2001, cytoplasmic microtubules (cMTs) and intranuclear microtubules (nMTs) were reported to play important roles in reciprocal nuclear exchange in *P. caudatum* (Nakajima et al, 2001). To determine if any cytoplasmic microtubules played any roles in the surviving mechanism of post-meiotic nuclei, immunofluorescence labeling with anti-alpha tubulin antibodies was performed on the prezygotic conjugating pairs.

No definite orientation of spindle extension during the first prezygotic division

At the telophase of the first prezygotic division, anti-α tubulin antibodies recognized long and slender spindles and two meiotic products, but no macronuclei were detected (Figure 1A). The PI staining recognized two meiotic products and the macronuclei (Figure 1A′). Neither definite orientation of spindle extension nor definite localization of the first meiotic products was observed. As a result, the two meiotic products were randomly distributed in the cytoplasm (Figure 1A"), as reported previously (Gao et al, 2011b).

One telophase nucleus located in PC during the second prezygotic division

During the second prezygotic division, the anti-α tubulin antibodies also recognized long and slender spindles and four telophase division products. There was no definite orientation of spindle extension, but one or more telophase nuclei were already located in the PC (Figure 1B, B′, B"), as observed previously (Gao et al, 2011b, 2011c). However, during the observation of ten conjugants in the earlier telophase, the spindles showed a tendency of extending towards the PC area directly (Figure 1C, D, E).

Microtubular behavior soon after meiosis

At the completion of meiosis, one meiotic nucleus was already located in the PC and nMTs were observed in all four meiotic products showing the same immunostaining pattern regardless of their locations inside or outside the PC (compare yellow and white arrows in Figure 2A, A′, A"). With the third prezygotic division, more anti-α tubulin antibodies recognized tiny dots, the cytoplasmic microtubules (cMTs) appeared and accumulated around the PC areas of two conjugants (Figure 2B, B′, B"), and at the metaphase such cMTs formed upside down heart shapes (Figure 2C, C′, C"). However, these cMTs never appeared around the meiotic nuclei outside the PC and even the nMTs faded away from them (compare yellow and white arrows in Figure 2C, C"). More than 50 conjugants were observed in each case and 100% of cells showed the same characteristics.

Microtubular behavior around the telophase of the third prezygotic division

At the telophase of the third prezygotic division, many tiny cMTs dots still existed and were mainly observed around both the prospective StP and MiP (blue and red arrows in Figure 3A, A" and B, B", respectively). At the stage of pronuclear exchange, however, almost no cMTs were observed around the two pronuclei, but nMTs still existed (Figure 3C, C"). More than 50 conjugants were observed in each case and 100% of cells showed the same characteristics.

DISCUSSION

Previous studies on immunofluorescence labeling with anti-α tubulin antibodies have indicated a

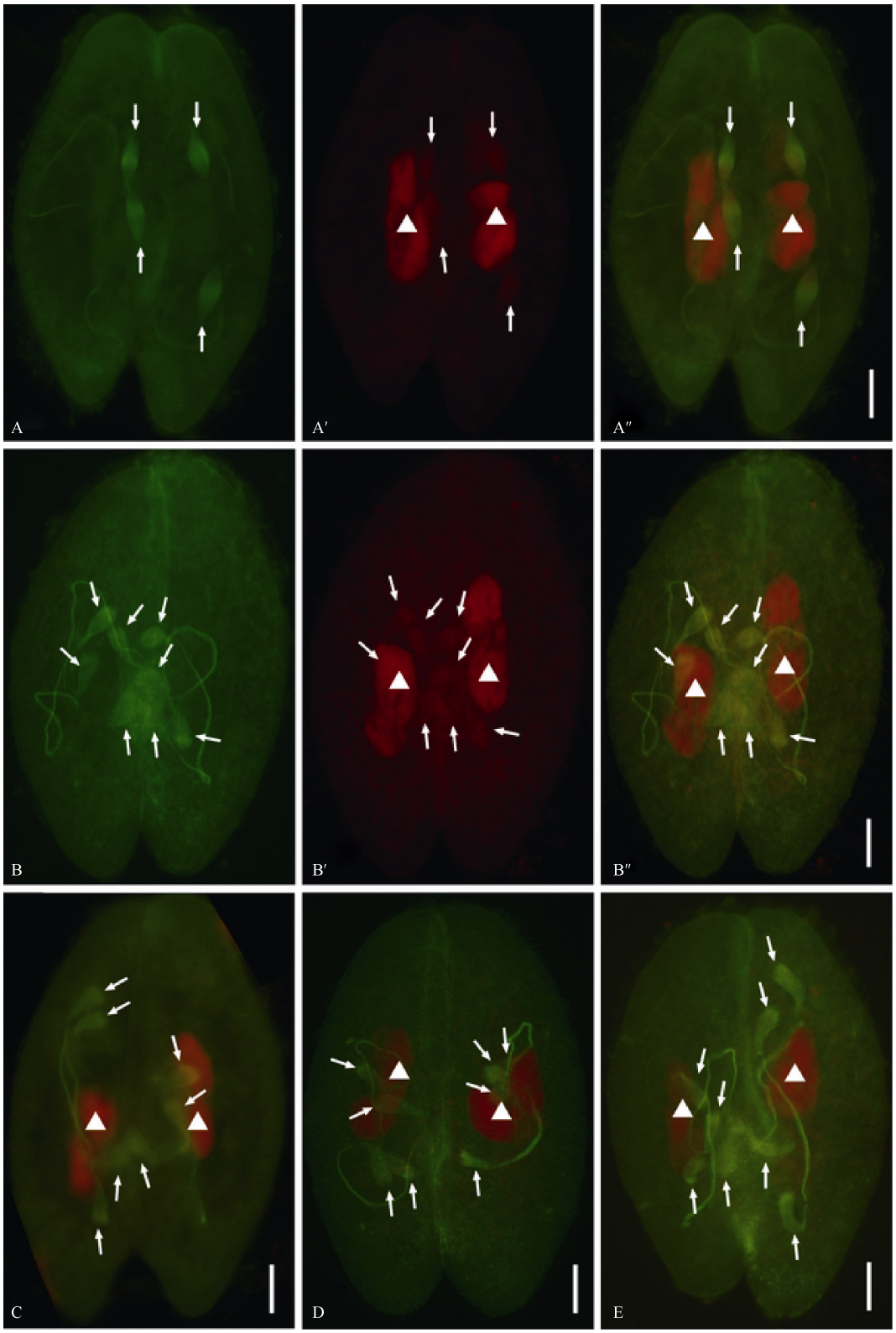

Figure 1 Nuclear behavior during the first and the second prezygotic divisions of *P. caudatum* indicated by FITC and PI doubled-staining

A: Telophase of the first prezygotic division; B: Telophase of the second prezygotic division; C, D, E: Earlier telophase of the second prezygotic division, one meiotic product entered the PC region. A and B are FITC images; A′ and B′ are PI images; A″, B″, C, D and E are merged images of FITC and PI; A, A′ , A″ and B, B′ , B″ are the same cell in each row; Triangles: macronuclei; Bars=20 μm.

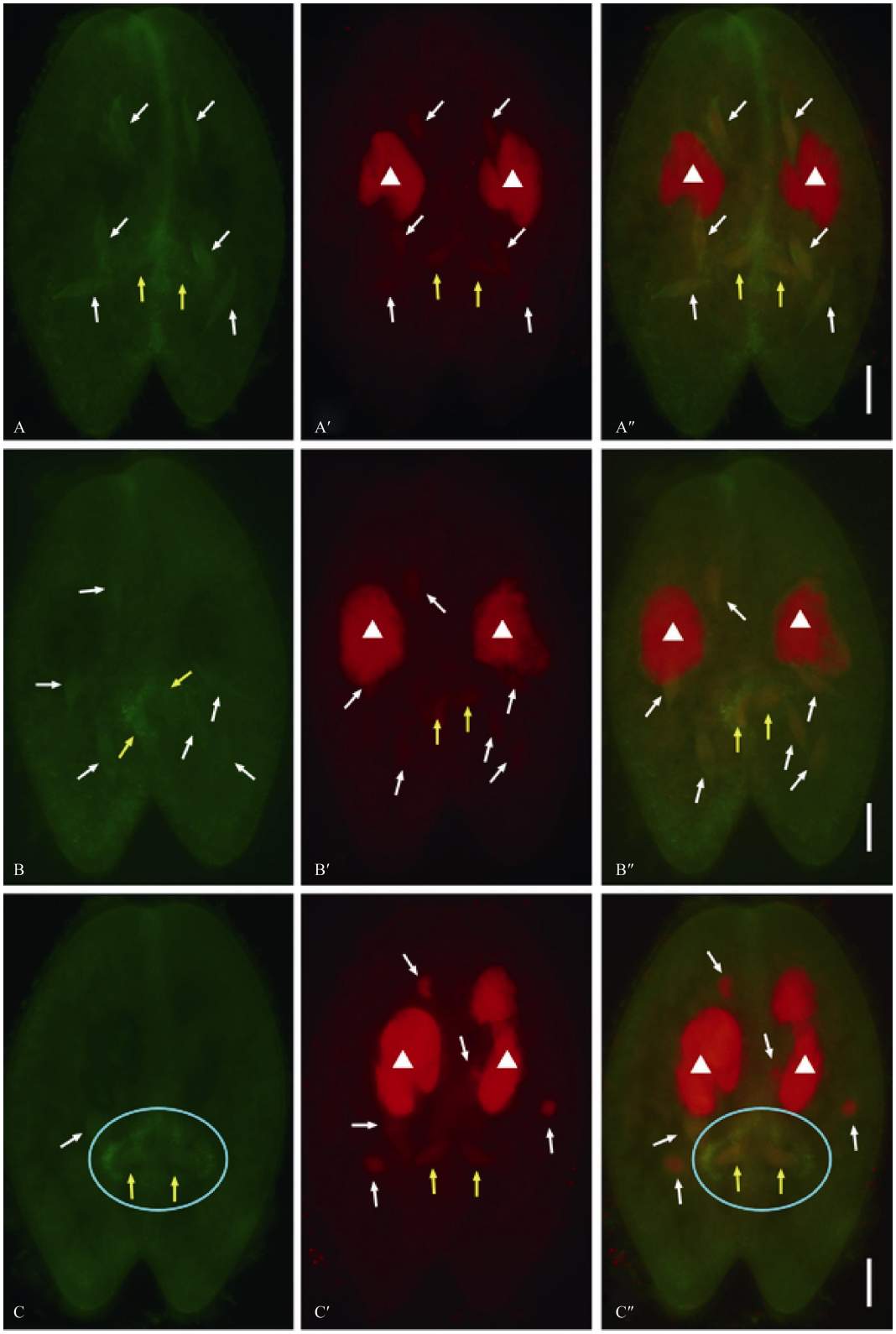

Figure 2 Appearance of cytoplasmic microtubules (cMTs) soon after meiosis of *P. caudatum* indicated by FITC and PI doubled-staining

A, B: Soon after meiosis; C: At the beginning of the third pre-zygotic division. A, B and C are FITC images; A′, B′ and C′ are PI images; A″, B″ and C″ are merged images of FITC and PI; A, A′, A″ and B, B′, B″ and C, C′, C″ are the same cell in each row; Yellow arrows: meiotic products in the PC; White arrows: meiotic products outside the PC; Triangle: old macronuclei; Oval area: the area of cMT appearance; Bars=20 μm.

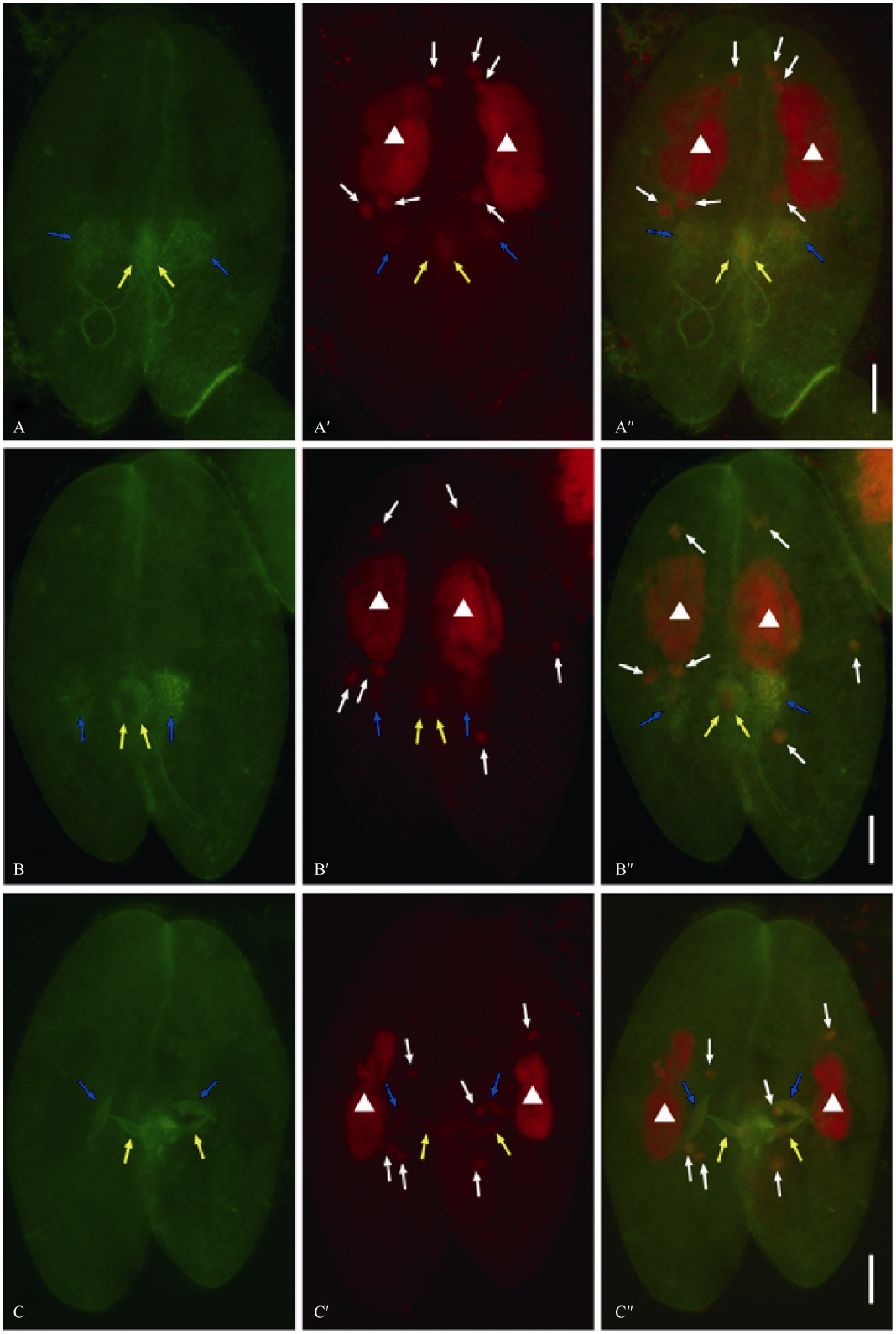

Figure 3 Distribution of cMTs before and after the completion of the third prezygotic division of *P. caudatum* indicated by FITC and PI doubled-staining

A, B: Late telophase of the third prezygotic division; C: Reciprocal migratory pronuclear exchange. A, B and C are FITC images; A′, B′ and C′ are PI images; A″, B″ and C″ are merged images of FITC and PI; A, A′, A″ and B, B′, B″ and C, C′, C″ are the same cell in each row; Yellow arrows: (prospective) migratory pronuclei; Blue arrows: (prospective) stationary pronuclei; White arrows: meiotic products outside PC; Triangle: old macronuclei; Bars=20 μm.

microtubular function in at least two aspects. The first involves a role during reciprocal pronuclear exchange in *P. caudatum* (Nakajima et al, 2001), which is supported by different experimental techniques in *Tetrahymena* (Orias et al, 1983) and other *Paramecium* species (Jurand, 1976; Yang & Shi, 2007). The second is guiding the nuclei to the destined locations including the PC entrance at the completion of meiosis and anterior and posterior localization at the telophase of the third postzygotic division (Gao et al, 2011a, 2011b, 2011c; Yang & Takahashi, 2002).

In the current study, stage- and space-specific cMTs were observed by immunofluorescence labeling with anti-alpha tubulin antibodies. They appeared during the third prezygotic division and were distributed around the surviving nuclei including meiotic products in the PC (Figure 2) and two prospective StPs and MiPs (Figure 3). However, such cMTs appeared neither around the extra-degenerating meiotic products (Figure 2C", 3A", B") nor the nuclear products of the first prezygotic division (Figure 1C", 3C") and the three postzygotic divisions (Yang & Takahashi, 2002). These results might indicate cMT involvement in the surviving mechanism of the post-meiotic nuclei.

In fact, intranuclear microtubules (nMTs) exist in all normal functional micronuclei including vegetative micronuclei, two meiotic division products, the selected meiotic products in the PC, two types of pronuclei, the products of three postzygotic divisions (Gao et al, 2011b, 2011c; Ishida et al, 1999; Nakajima et al, 2001, 2002; Yang & Takahashi, 2002), and the presumptive micronuclei in exconjugants (Ishida et al, 1999; Taka et al, 2006). But such nMTs are never kept in the degenerating meiotic products or in the differentiated macronuclear anlagen (Ishida et al, 1999). In the current study, the cMTs were observed around the PC nuclei, and these nuclei also kept their nMTs during the third prezygotic division (Figure 3). No such cMTs appeared around the meiotic products outside the PC, and even the nMTs faded away from them completely (Figure 3). In other words, the behavior of both cMTs and nMTs was consistent during the third prezygotic nuclear division. During the third prezygotic division, degeneration of the extra meiotic products outside the PC occurs (Gao et al, 2010; Yang et al, 2007). Combining all the observations obtained in the current study with previous studies, it is suggested that both nMTs and cMTs are indispensable during the third prezygotic division, whose function might relate to the survival of PC nuclei and two prospective pronuclei, which needs further investigation.

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