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EFFECTS OF COLCHICINE TREATMENT ON THE MICROTUBULE CYTOSKELETON AND TOTAL PROTEIN DURING MICROSPOROGENESIS IN *GINKGO BILOBA* L.

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Abstract

The purpose of this study was to examine the effects of colchicine treatment on the microtubule cytoskeleton and the expression of proteins during microsporogenesis in *G. biloba*, as observed by immunofluorescence and 2-DE analysis in microsporangia treated with colchicine. The results showed the microtubule structures were affected by the colchicine in *Ginkgo biloba*, but the treatment effect of the colchicine had certain limitation in *G. biloba*. The percentage of microsporocytes whose microtubule structures were affected by the colchicine treatment was less than that observed in other plant species, not higher than 10 %. It was also found that the expression level of several endogenous proteins were changed in *G. biloba* when the microsporangia were treated with colchicine. Although we only tested colchicines was only tested in the present study, *G. biloba* appeared to possess factors that restricted the effect of such chemical agents. Our observations led us to speculate that the endogenous proteins are possibly responsible for the reduced effects of colchicine treatment in *G. biloba.*

Keywords: *Ginkgo biloba* L., Colchicine treatment; Microtubules; Proteins.

Introduction

Ginkgo biloba L. is an ancient gymnosperm with great economic benefits (Franklin, 1959; Hori *et al.*, 1997; Yang *et al.*, 2013). However, it is very difficult to induce polyploidy into *G. biloba*, and the production of a 2n gamete was an important step toward breeding the triploid plant (Einspahr, 1984; Wang & Kang, 2009). Between, 2005 and 2007, our research group used 4 different types of chemical agents (propyzamide, oryzalin, 6-DMAP and colchicine) to induce the formation of 2n male gametes in *G. biloba* (Hu, 2008), but 2n male gametes were found only in *G. biloba* treated with colchicine. In addition, the yield of 2n male gametes was lower (7 %) than that of other tree species, such as Eucommia (49.5 %) (Gao *et al.*, 2004), white poplar (88 %) (Kang *et al.*, 1999), and black poplar (90.25%) (Zhang, 2006). This lower yield of 2n male gametcs (7%) in *G. biloba* is insufficient to meet the needs of triploid breeding in this species.

Microtubules play an important role in the production of 2n gametes (Higuchi & Uhlmann, 2005), and the polymerization of microtubules was affected by colchicine treatment (Vandecandelaere *et al.*, 1997; Muhammad *et al.*, 2012); however, certain proteins (e.g., metallothionein) are activated by colchicine and decrease the effects of colchicine treatment in animal cells (Itoh *et al.*, 1997; Qiu *et al.*, 2005). Accordingly, the main purpose of the present study is to ascertain the effects of colchicine treatment on the microtubule cytoskeleton and the expression of proteins during microsporogenesis in *G. biloba* and to verify whether the microtubule cytoskeleton is destroyed by the treatment in microsporangia. These results can help to verify further the mechanism of colchicine in the production 2n male gametes in *G. biloba* and also help in the preliminarily determination of the low yield of 2n male gametes. These results can also lay the groundwork for future follow-up studies to increase the yield rate of 2n male gametes with colchicine treatment of *G. biloba* microsporangia.

Materials and Methods

Plant material culture: Male floral branches of *G. biloba* (approximately 30 years old) were collected from a Beijing Forestry University plantation (40.06787 N, 116.08134 E) before meiosis began in the spring season. The branches were cultured in a greenhouse (20-30°C) to induce meiosis.

Colchicine treatment: The microsporangia of *G. biloba* were treated with a colchicine solution while they were developing into the pachytene stage using a cotton soaking method (Fig. 1), with a colchicine concentration of 0.6% (6 mg/ml) (Cheng *et al.*, 2006); the control samples were treated with distilled water. The treatment period was two days (48 hours). During the treatment process, the treated and control samples were collected and fixed every six hours for indirect immunofluorescence staining. When the treatment was finished, both the treated and control samples were collected and stored at -80°C for the two-dimensional electrophoresis (2-DE) analysis of total protein.

Fig. 1. The cotton-soaking method.

Indirect immunofluorescence staining: *G. biloba* microsporangia were fixed for 40 min at 25°C in a solution containing 10% DMSO, 0.01% MBS (3-maleimidobenzoic acid N-hydroxysuccinimide ester), and 4% paraformaldehyde prepared in PEMS buffer (50 mM PIPES, 5 mM EGTA, 2 mM $MgSO_4$, and 4% sucrose, pH 6.9). The samples were rinsed with PEMS buffer three times for approximately 5 min per rinse, then rinsed three times with distilled water. After rinsing, the samples were dehydrated in a graded series of ethanol (volume/volume, V/V) (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%) for 30 min at each concentration, and the dehydrated samples were then infiltrated with a polyethylene glycol (PEG) mixture and embedded following the protocol of Xu *et al.* (2001). The embedded samples were sliced into 40 µm thick sections using razor blades and a rotational microtome (Leica RM2235). The section thickness was chosen with the objective of including all of the structures of the microsporangium in most of the sections. The sample sections were incubated with a monoclonal anti-α-tubulin antibody (Sigma T-9026), diluted 1:100 with PBS, for 60 min. After washing, the samples were incubated with FITC-conjugated anti-mouse IgG (Sigma F-0257), diluted 1:200 with PBS, for 60 min. The nuclei were stained with PI (propidium iodide, Sigma). The chromosomes and microtubules were observed using a Leica TCS-SP2 laser-scanning confocal microscope, and FITC and PI were observed at two different excitation wavelengths (494 nm and 535 nm). The sections containing all of the structures of the cell were identified (Liu *et al.*, 1996) and serially scanned at different levels (or layers) of approximately 2 µm in thickness. The optical sections were obtained and reconstructed into a complete 3-D structure of the cell with computer graphics.

Two-dimensional electrophoresis analysis of total protein: The samples were combined with 10% polyvinylpolypyrrolidone (PVPP) and a few high-purity quartz sand grains (particle sizes of 0.1 - 0.01 mm) and then ground to a fine powder in liquid nitrogen. Ten volumes of ice-cold (-20°C) 10% (weight/volume, W/V) trichloroacetic acid (TCA) and 0.07% (V/V) β-mercaptoethanol in acetone were added to the fine powder of the sample, mixed and incubated overnight at -20°C to precipitate the proteins. The sample was then centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was discarded, and a fourfold volume of ice-cold (-20°C) 0.07% β-mercaptoethanol in acetone was added to wash the sample. The sample was centrifuged as above, and two additional acetone washes and centrifugations were performed to remove all traces of the TCA. The acetone mixture containing the crude protein was kept at -20°C until the final traces of acetone had evaporated. The samples of crude protein were diluted for 2 h in rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 4% [W/V] CHAPS, 65 mM/L DTT, and 0.2% [W/V] Bio-Lyte, pH range 3-10). Next, the diluted sample was centrifuged at 13,000 rpm for 30 min at 20°C, and the resulting supernatant was centrifuged again under the same conditions. The protein concentration was determined by the Bradford assay (Bradford, 1976).

Two-dimensional gel electrophoresis was carried out according to the principal methods outlined by Gorg *et al.,* (1988). First, 17 cm non-linear pH 3-10 immobilized pH gradient (IPG) strips (Bio-Rad) were rehydrated overnight at 20°C with 1500 µg of the samples diluted to 300 µL with buffer G (7 mol/L urea, 2 mol/L thiourea, 4% [W/V] CHAPS, 65 mM/L DTT, 0.2% [W/V] Bio-Lyte, pH range 3-10, and 0.001% bromophenol blue). The strips were loaded into a Protean IEF cell system (Bio-Rad) and focused under the following conditions (Table 1).

Table 1. IPG dry strips IEF parameters for Protean IEF cell isoelectric focusing.

Step	Voltage	Time (h)	Voltage change mode	Function
0	50	14		Passive hydration
1	100	5	Slow	Salt elimination
2	250	3	Slow	Salt elimination
3	500	2	Slow	Salt elimination
4	1000	2	Slow	Salt elimination
5	10000	6	Linear	Boostin voltage
6	10000	10	Fast	Focusing
7	500	Arbitrary	Fast	Storage

The IPG strips were incubated at 20°C in equilibrium solution A (6 M urea, 20% glycerol, 2% SDS, 2% DTT and 1.5 M Tris–HCl, pH 8.8), followed by solution B (identical to solution A except that the DTT was replaced with 2.5% iodoacetamide), for 15 min each. The second dimension separation was performed using a 25% SDS–PAGE resolving gel overlaid with a 1% low melting-temperature agarose. The second dimension electrophoresis was carried out using a Protean II xi cell system (Bio-Rad) with a two-step program (30 min at 10 W followed by 4 W constant power) until the dye front was within 1 cm of the bottom edge of the gel. The chamber temperature was held between 25 and 32°C for the duration of the electrophoresis. The resulting gels were fixed for 1 h in a mixture of 50% methanol and 10% acetic acid and were stained overnight in Coomassie blue staining solution (0.12% Coomassie blue, 50% methanol, and 10% acetic acid) with agitation (30 rpm). Lastly, the destaining solution (25% methanol and 8% acetic acid) was applied until the desired level of destaining was obtained.

The gels were scanned using a Model GS-800 Calibrated Imaging Densitometer (Bio-Rad), and the PDQuest 8.0.1 software system (Bio-Rad) was used to analyze the images.

Results

Immunofluorescence: The microtubule cytoskeleton and chromosomes were clearly observable in the control *G. biloba* during meiosis using immunofluorescence. An unusually large nucleus occupied a central position in the sporocyte (Fig. 2, 1-c) and that the microtubules were arranged in a reticulate pattern over the nucleus and throughout the cytoplasm in early meiosis (Fig. 2, 1-a, b). From the leptotene stage to the diakinesis stage, the chromosomes gradually became coarse and short (Fig. 2, 4-c), the microtubules became more concentrated around the chromosomes, and the early multipolar spindles were formed by the microtubules (Fig. 2, 4-a and 4-b). In metaphase I, the mature metaphase spindle gradually consolidated into two pointed polar regions (Fig. 3, 1-a and 1-b). In anaphase I, many of the microtubules radiated from the sister groups of chromosomes, and the chromosomes formed two caps on the distal surfaces (Fig. 3, 2-a and 2-b). The phragmoplast was formed by the spindles in the interzonal region of the two sister nuclei during telophase I, and at the same time, a distinct organelle band was formed in the spindle midzone (Fig. 3, 4-a and 4-b). At metaphase Ⅱ, the chromosomes had moved to the midzones of the second division, the microtubules could barely be observed in the distinct organelle band, and the second-division spindles had appeared simultaneously on either side of the organelle band (Fig. 4, 1-a). The separations of the chromosomes and spindle at anaphase Ⅱ (Fig. 4, 2-a) were similar to those of the first division. The phragmoplasts were observed among the four nuclei in telophase Ⅱ (Fig. 4, 3-c). The tetrad spores were generated in the final phase of meiosis (Fig. 4, 4-c) and were located in the distal region of the original microsporocyte. The microtubules were arranged in a reticulate pattern over the nuclei of the four young spores (Fig. 4, 4-a).

Compared with the meioses of the control *G. biloba*, significant differences were observed during the meioses in the colchicine-treated *G. biloba*. We found that the α-tubulin of the colchicine-treated *G. biloba* had a more dispersed distribution than the control in all of the stages of meiosis (Fig. 5). Some of the microsporocytes of the colchicine-treated *G. biloba* had fewer filamentous microtubules than the control during early meiosis (Fig. 5, 3-a). The microtubules of many microsporocytes did not become concentrated around the chromosomes in the diakinesis stage of the colchicine-treated *G. biloba* (Fig. 5, 4-a). In addition, there were no mature metaphase spindles observed at metaphase I (Fig. 6, 1-a), and there were a few remaining univalent chromosomes on one side of the microsporocyte (Fig. 6, 2-a) when the rest of the chromosomes had moved to the midzone during metaphase I. The phragmoplast was indistinct in the interzonal region of the two sister nuclei in telophase I (Fig. 6, 4-a). From telophase I to anaphase Ⅱ, the observation of the microtubule cytoskeleton was very difficult, and we could only observe the fluorescence of α-tubulin (Fig. 7, 1-a and 2-a). We also found that the number of these germ cells whose microtubule structure was changed and affected by the colchicine treatment was less than in other plant species, with an occurrence rate of no higher than 10%: the total number of observed microsporocytes was 2607, and the number of affected microsporocytes was 225; thus, the rate was approximately 8.6%.

Results of the 2-DE analysis of total protein: Through our analysis using the PDQuest 8.0.1 software system, it was found that the protein spots of *G. biloba* microsporangia were relatively concentrated in the pH range of 5-9 and that there were some differences in the types of protein between the control and colchicine-treated microsporangia (Fig. 8). After the microsporangia were treated with colchicine for 48 h, two new protein spots were observed in the gel images of the colchicine-treated microsporangia (Fig. 9, spots 1 and 2) that were not found in any of the gel images of the control microsporangia (Fig. 9, spot 3). At the same time, a protein spot that was observed in the gel images of the control microsporangia was absent from all of the gel images of the colchicine-treated microsporangia.

The expression level of the proteins in the microsporangia was also changed by the colchicine treatment. The sizes of the protein spots were analyzed using the PDQuest 8.0.1 software system, and it was found that the expression levels of three protein spots had distinct differences between the control microsporangia and the colchicine-treated microsporangia. The expression of one protein spot was higher in the colchicine-treated microsporangia than in the control microsporangia (Fig. 9, spot 4), whereas the expression of two protein spots was less in the colchicine-treated microsporangia than in the control microsporangia (Fig. 9, spots 5 and 6).

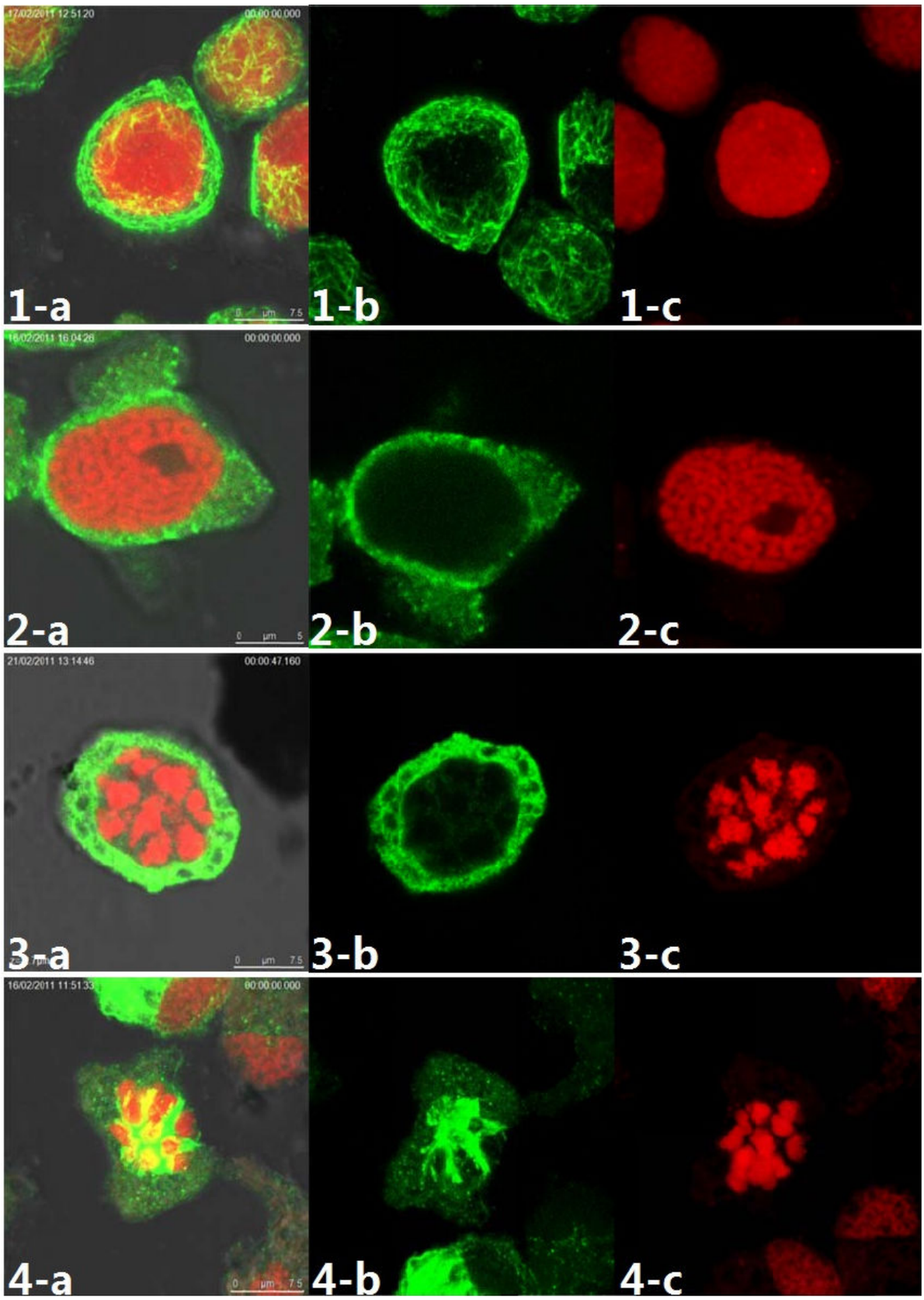

Fig. 2. The changes in the microtubule cytoskeleton and chromosomes during microsporogenesis in control microsporangia of *G. biloba* (Part 1). a, Merged image; b, Tubulin (FITC) image; c, Chromosome (PI) image. 1, Prophase; 2, Leptotene; 3, Pachytene; 4, Diakinesis.

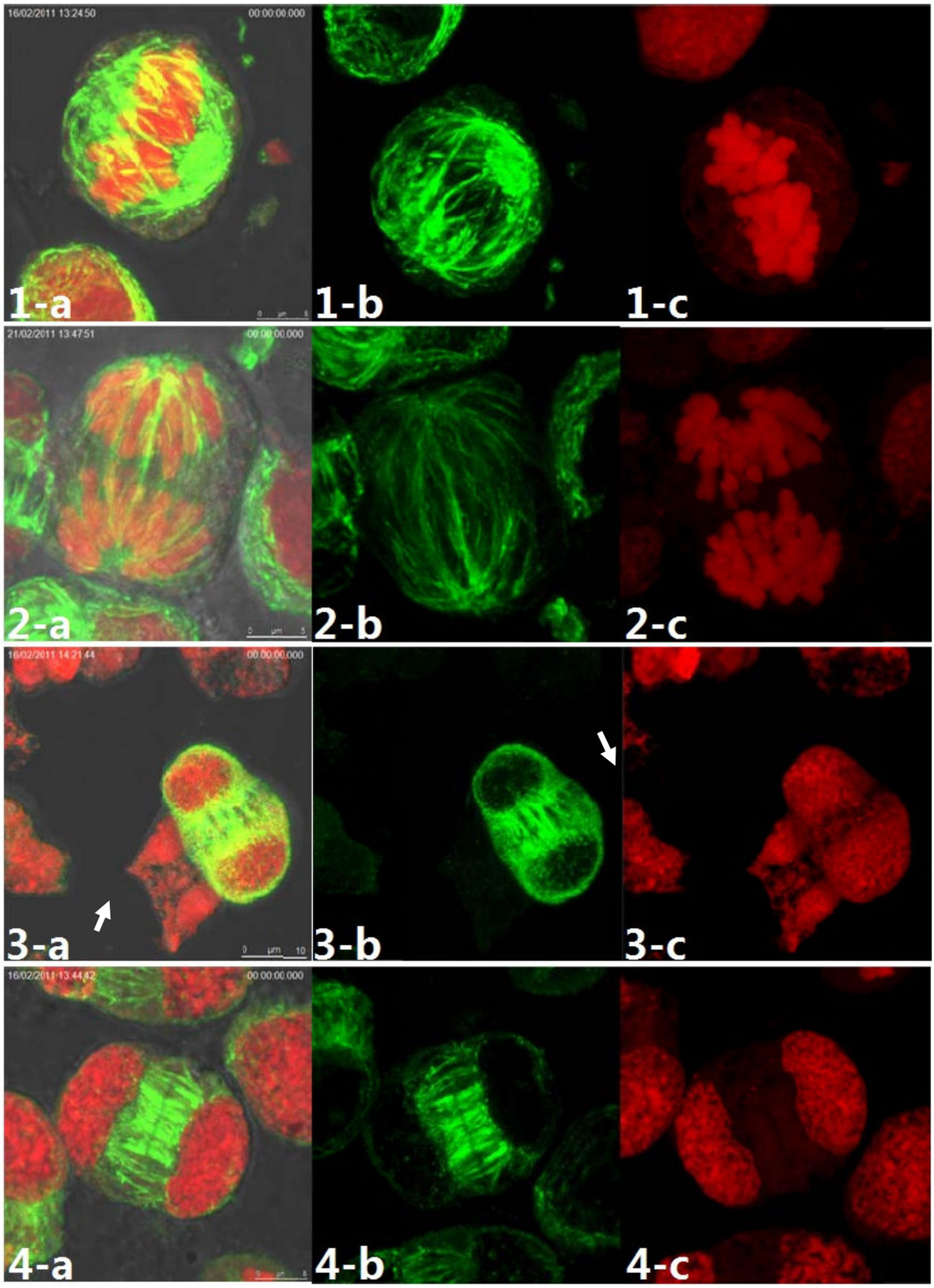

Fig. 3. The changes in the microtubule cytoskeleton and chromosomes during microsporogenesis in control microsporangia of *G. biloba* (Part 2). a, Merged image; b, Tubulin (FITC) image; c, Chromosome (PI) image. 1, Metaphase I; 2, Anaphase I; 3, Telophase I; 4, Phragmoplast (arrow).

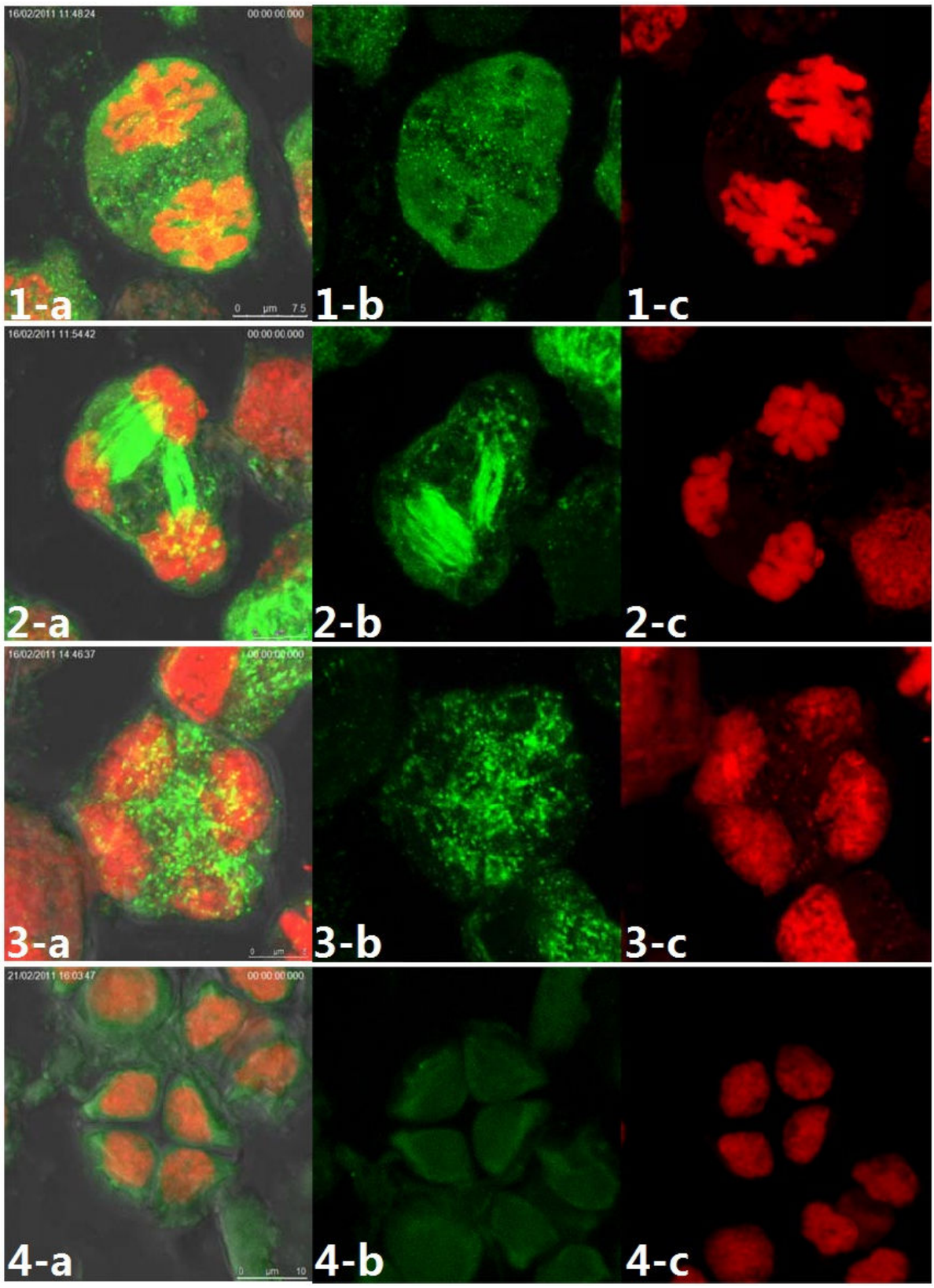

Fig. 4. The changes in the microtubule cytoskeleton and chromosomes during microsporogenesis in control microsporangia of *G. biloba* (Part 3). a, Merged image; b, Tubulin (FITC) image; c, Chromosome (PI) image. 1, Metaphase II; 2, Anaphase II 3, Telophase II; 4, Four young spores.

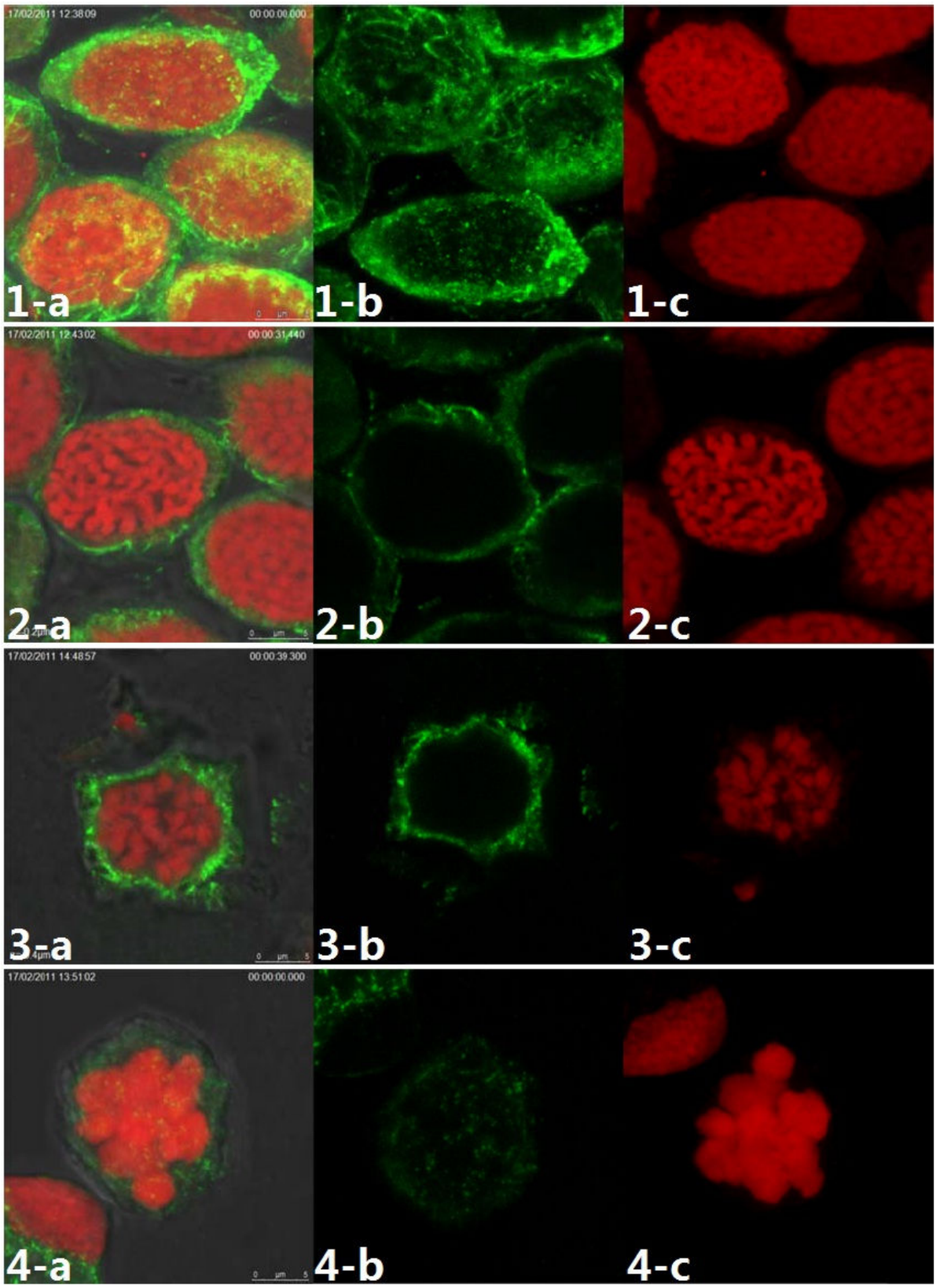

Fig. 5. The changes in the microtubule cytoskeleton and chromosomes during microsporogenesis in colchicine-treated microsporangia of *G. biloba* (Part 1). a, Merged image; b, Tubulin (FITC) image; c, Chromosome (PI) image. 1, Prophase; 2, Leptotene; 3, Pachytene; 4, Diakinesis.

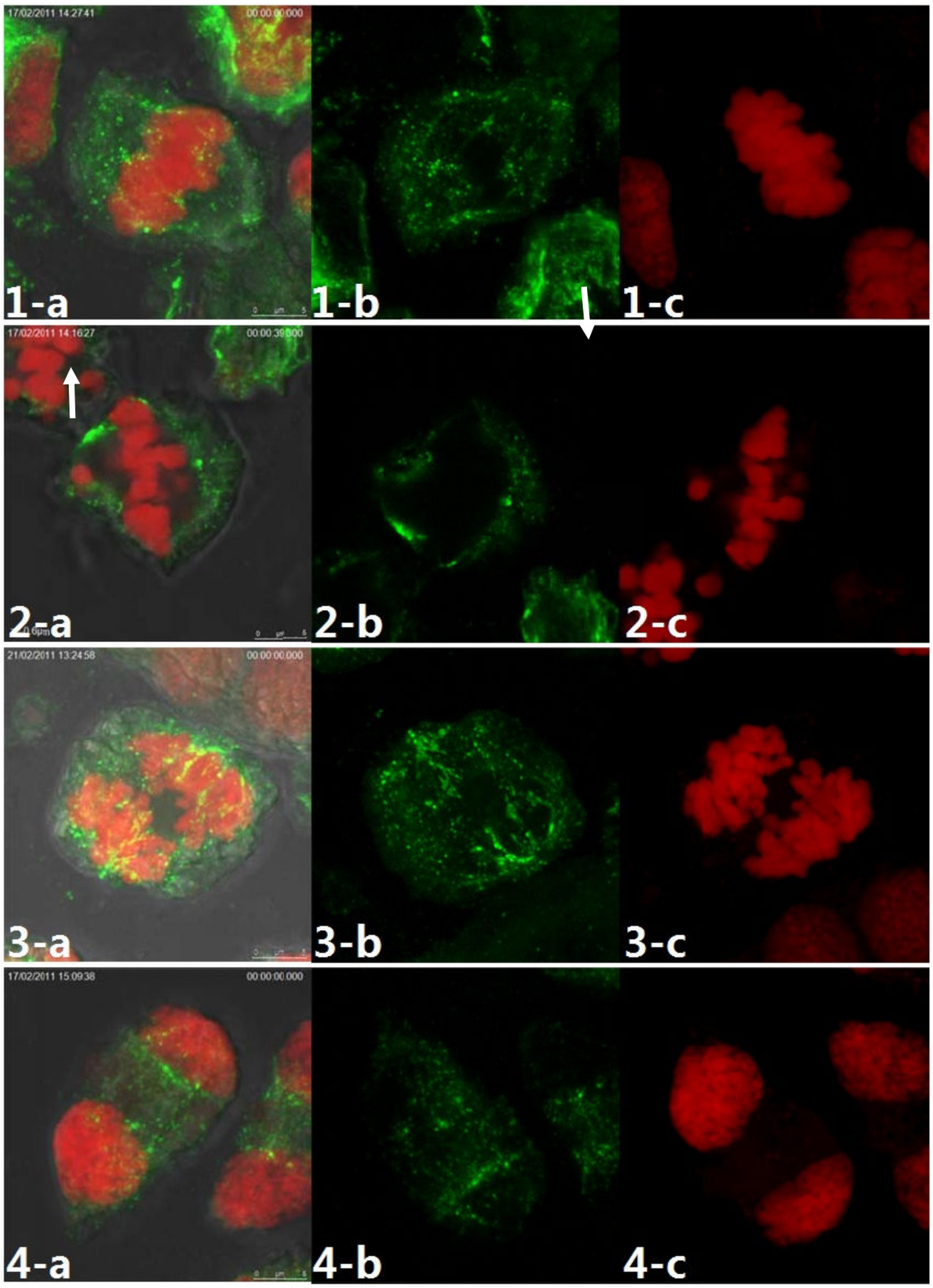

Fig. 6. The changes in the microtubule cytoskeleton and chromosomes during microsporogenesis in colchicine-treated microsporangia of *G. biloba* (Part 2). a, Merged image; b, Tubulin (FITC) image; c, Chromosome (PI) image. 1, Metaphase I; 2, Univalent (arrow); 3, Anaphase I; 4, Telophase I.

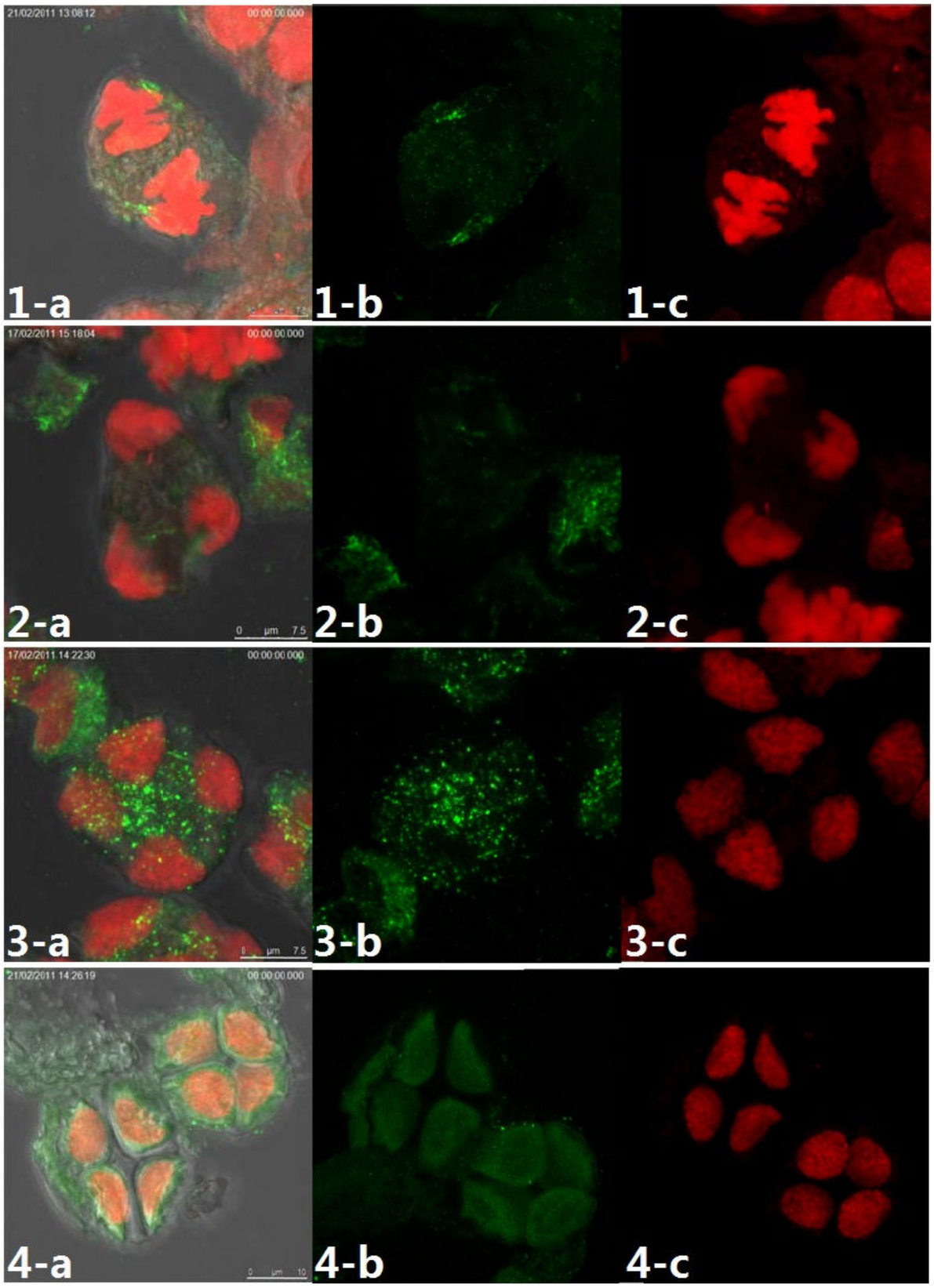

Fig. 7. The changes in the microtubule cytoskeleton and chromosomes during microsporogenesis in colchicine-treated microsporangia of *G. biloba* (Part 3). a, Merged image; b, Tubulin (FITC) image; c, Chromosome (PI) image. 1, Metaphase II; 2, Anaphase II; 3, Telophase II; 4, Four young spores.

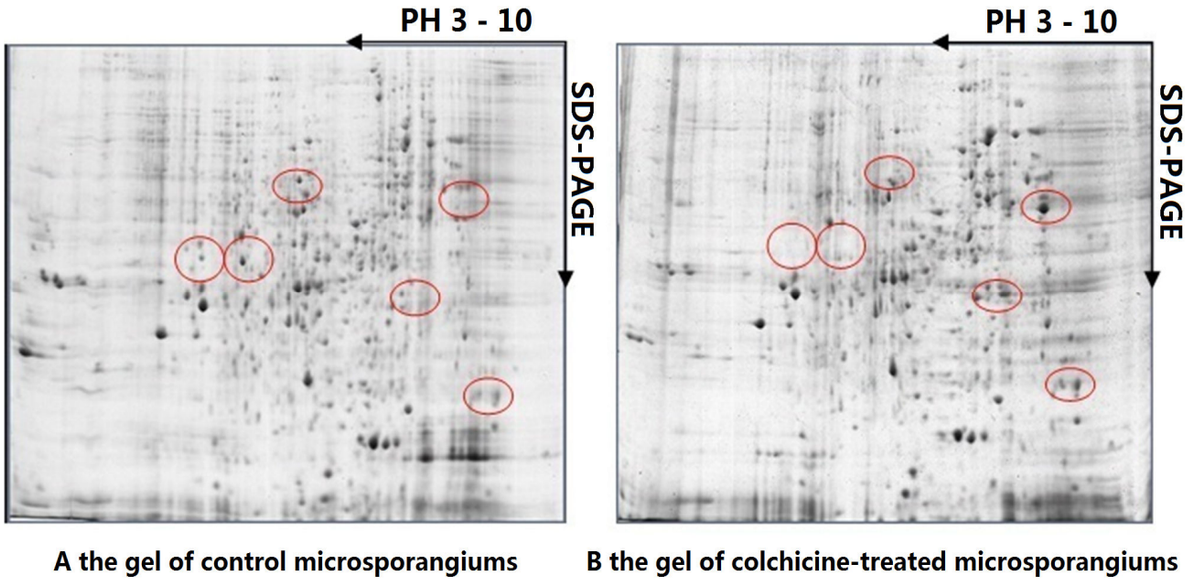

Fig. 8. Total protein 2-D gel images. Left, the control; Right, the colchicine treated.

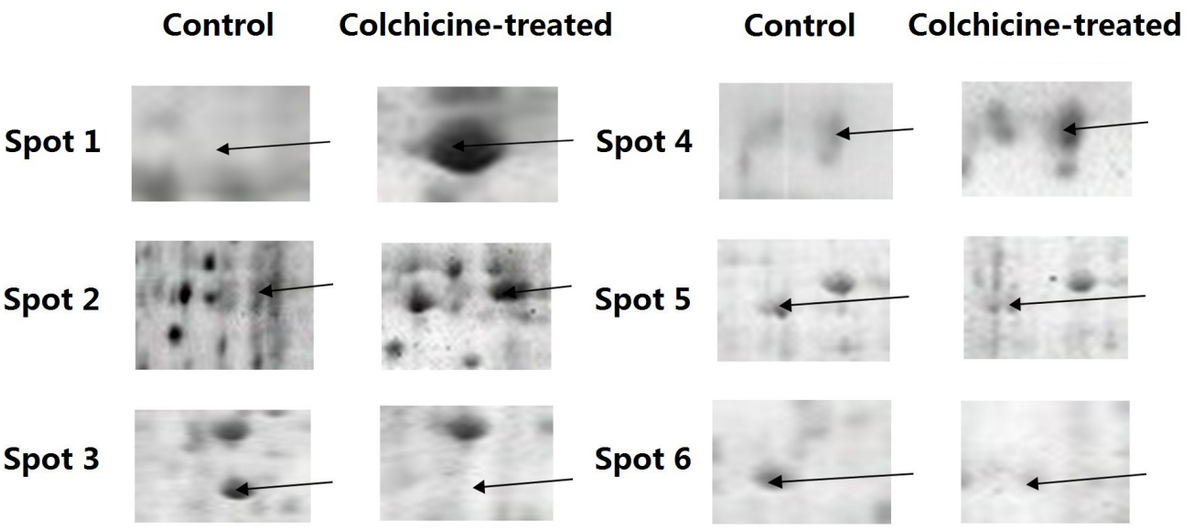

Fig. 9. The different protein spots.

Discussion

The production of 2n male gametes has great significance for the breeding of polyploid *G. biloba*, and researchers have worked on improving the useful characteristics of *G. biloba* for economic purposes for some time. These research efforts have mainly been concentrated on faster growth of *G. biloba*, a higher leaf yield, and a higher content of secondary metabolites. However, with the exception of polyploid breeding, the existing breeding measures only improved these aspects to a limited extent (Hu, 2008). Because triploid plants grow fast, their organs are significantly larger, and their content of secondary metabolites is also high (Johnsson, 1953; Weisgerber *et al.*, 1980). The urgent need to meet the market demands of this plant's products may be realized in a short period of time if new triploids of *G. biloba* are bred. Until now, there have been no reports describing the successful breeding of triploid plants in *G. biloba*, and a 2n male gamete is required to breed triploid plants quickly (Einspahr, 1984; Kang, 2003; Gao *et al.*, 2004).

The results of our study showed that 2n male gametes were indeed induced by colchicine in the germ cells of *G. biloba.* Colchicine binds to tubulin, blocking polymerization, and microtubules depolymerize at high colchicine concentrations (Vandecandelaere *et al.*, 1997; Yasar *et al.*, 2013). In this study, univalent chromosomes and the deletion of metaphase spindles were observed in the colchicine-treated microsporangia. Combined with the results of our earlier study, we found that the structural change in the microtubules was caused by the colchicine treatment; the chromosome segregation

abnormalities were further deduced by the changes in meiosis (Sun *et al.*, 2011). Other studies showed that the movement of chromosomes and the formation of the cell wall in meiosis were affected by microtubules (Brown & Lemmon, 2005) and that these abnormalities of chromosome segregation could ultimately affect the production of 2n gametes (Wang & Kang, 2009). Even though 2n male gametes were only successfully induced by colchicine treatment in the germ cells of *G. biloba*, the observed abnormalities in chromosome segregation and the yield of 2n male gametes were both reduced in comparison with other plants (Cheng *et al.*, 2006; Hu, 2008; Sun *et al.*, 2011). It is rather difficult to breed a 2n germ cell, and the chromosome doubling of somatic cells in *G. biloba* was always unsuccessful (Chen, 1996). Fortunately, the occurrence rate of affected microsporocytes of this study was similar to the abnormality rate of chromosome segregation, as was the yield rate of 2n male gametes in our earlier studies (Cheng *et al.*, 2006; Sun *et al.*, 2011).

Sun *et al*. (2011) found that the microsporangium and microsporocyte wall are not the major factors hindering the process of colchicine induction of diploid male gametes in *G. biloba*. Some studies show that, when colchicine is introduced, cells can produce one type of metallothionein and increase the expression of metallothionein, which helps to preserve cell tissue and prevent colchicine-induced damage (Itoh *et al.*, 1997; Qiu *et al.*, 2005). In our study, it was found that the expression of one protein increased and that there were two new protein spots when the microsporangia were treated with colchicine. We speculate that the spot(s) may correspond to metallothionein or endogenous protective protein that it is similar to metallothionein in *G. biloba* that can disturb actions of colchicine; of course, this hypothesis requires further experiments for verification.

Our 2-DE analysis revealed that there were several proteins affected when the microsporangia were treated with colchicine. Indeed, meiosis is regulated by many proteins. Moreover, the literature shows that there are other colchicine-binding proteins in addition to tubulin and metallothionein in cells (James & Dinkar, 1973). However, it is currently unknown whether these colchicine-binding proteins are involved in or disturb the regulation of meiosis. If other colchicine-binding proteins can affect the regulation of meiosis in *G. biloba*, some of these proteins may also be activated by the colchicine treatment and might, thus, affect the yield rate of 2n male gametes in *G. biloba*.

Conclusion

In this study, we preliminarily concluded that the lower yield rate of 2n male gametes in *G. biloba* treated with colchicine was due to endogenous proteins. If we can confirm the significant proteins and find the mechanism of lower mutation rate of diploid male gamete when the microsporangium of *G. biloba* was induced by colchicine, we can try to optimize the ploidy breeding method in *G. biloba*.

Acknowledgement

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