Proteomic Analysis of the Low Mutation Rate of Diploid Male Gametes Induced by Colchicine in *Ginkgo biloba* L.

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**Abstract**

Colchicine treatment of *G. biloba* microsporocytes results in a low mutation rate in the diploid (2n) male gamete. The mutation rate is significantly lower as compared to other tree species and impedes the breeding of new economic varieties. Proteomic analysis was done to identify the proteins that influence the process of 2n gamete formation in *G. biloba*. The microsporangia of *G. biloba* were treated with colchicine solution for 48 h and the proteins were analyzed using 2-D gel electrophoresis and compared to protein profiles of untreated microsporangia. A total of 66 proteins showed difference in expression levels. Twenty-seven of these proteins were identified by mass spectrometry. Among the 27 proteins, 14 were found to be up-regulated and the rest 13 were down-regulated. The identified proteins belonged to five different functional classes: ATP generation, transport and carbohydrate metabolism; protein metabolism; ROS scavenging and detoxifying enzymes; cell wall remodeling and metabolism; transcription, cell cycle and signal transduction. The identification of these differentially expressed proteins and their function could help in analysing the mechanism of lower mutation rate of diploid male gamete when the microsporangium of *G. biloba* was induced by colchicine.

**Introduction**

*Ginkgo biloba* L. is a living fossil tree with no close living relatives. It is cultivated because of its importance in traditional medicine and food value; and as an ornamental tree for its attractive shape and foliage. Recent studies have also implicated a use for Gingko extract in prevention of Alzheimer’s disease [1] and dementia [2], thus creating a high market demand for this tree species. However, it has a tedious and inefficient growth process, and although effective breeding methods are emerging, it is still a challenge to meet the increased production requirement. Gingko extract contains secondary metabolites that have medicinal value; therefore, breeding methods that help in enhancing the quantity of these secondary metabolites as compared to 1n and 2n, ploidy breeding can be a useful tool for breeding new economic varieties of *G. biloba*.

Triploid plants are produced when diploid male gametes are induced and fertilized with normal female gametes. Colchicine is a wide-ranging and effective triploid-inducer used in studies of polyploidy breeding. It hinders the formation of spindle fibers in the metaphase of cell division and plays a minor role in affecting the structure of chromosomes [3].

Previous studies have shown that diploid pollen could be produced from the microsporocytes treated with colchicine, however, the rate of mutation was only 7 per cent [4] as compared to other trees such as, white *Populus* 88% [5], *Eucommia* 49.5% [6], and black *Populus* 90.25% [7]. The low mutation rate in diploid male gametes of *G. biloba* is insufficient for its application in ploidy breeding.

Sun et al. [8] showed that the microsporangium and microsporocytes wall are not the major factors hindering the process of colchicine induction of diploid male gametes in *G. biloba*. Several other studies have indicated that the proteome is an influencing factor in the formation process of male gametes. The events of meiosis are controlled by a protein enzyme complex known as the “maturation promoting factor.” These enzymes interact with one another as well as with other cell organelles to cause the breakdown and reconstruction of the nuclear membrane, the formation of the spindle fibers, and the cell division process [9–12].

We hypothesize that perhaps there are some proteins that do not destroy the chemical structure of colchicine; however, they can interfere with the process in which tubulin combines with colchicine. Therefore, it is necessary to determine differences between protein function in colchicine-treated and untreated microsporocytes during meiosis. Since colchicine stress has not yet been extensively examined at the proteome level in plant breeding studies, no data on this topic are currently available in the literature. Therefore, this study provided a framework to investigate the cause of low-level diploid male gametes formation in *G. biloba* treated with colchicine.
Materials and Methods

Chemicals and Reagents

- IPG strip (17 cm, pH 4–7), 2-DE marker, Biolyte (pH 4–6 and pH 5–7), mineral oil, Dithiothreitol (DTT) and iodoacetamide were obtained from Bio-Rad Laboratories (Hercules, CA). Tris-base, ammonium persulfate (AP), agarose, sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylethylenediamine (TEMED), glycine, Acrylamide, N,N'-methylenebisacrylamide, bromophenol blue, Coomassie brilliant blue (CBB) G-250, thiourea, 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS), urea, glycerol, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Trypsin was obtained from Roche (city, state of manufacturer); trisfluoroacetic acid (TFA) and acetonitrile were from JT Baker (Phillipsburg, NJ).

Plant materials

The male floral branches of *G. biloba* were collected in the spring season from the Teaching and Experimental Forestry Center of Beijing Forestry University (40.04060 N, 116.05256E). The branches were placed in cold storage (4°C) for 24 h before the microsporangiums of *G. biloba* began to develop in meiosis. The branches were cultured in the greenhouse (25°C) in order to induce meiosis. The microsporangia of *G. biloba* were treated with colchicine solution as soon as most of them turned green, were full of microsporangiums, and then dried under vacuum for 10 min at 4°C.

Protein Extraction

Total protein extracts were prepared according to the method described by Wang et al. [13] with minor modifications. The powder of the microsporangia of *G. biloba* was suspended in 1 mL cold extraction solution (10% TCA in acetone containing 1 mM PMSF and 0.07% β-mercaptoethanol) and then incubated at –20°C for 12 h. The samples were then centrifuged at 12,000 g for 30 min at 4°C. The gel strips were equilibrated for 15 min with two equilibration buffers: (i) reducing buffer containing 0.8% (w/v) colchicine solution [4]; (ii) alkylating buffer containing 0.8% (w/v) cold extraction solution (10% TCA in acetone containing 1 mM PMSF, 0.07% β-mercaptoethanol, and then incubated at –20°C for 12 h. The samples were then centrifuged at 12,000 g for 30 min at 4°C, and then dried under vacuum for 10 min at 4°C.

Protein identification by MALDI-TOF/TOF-MS

Before obtaining the mass spectra of the peptide mixture, the digested peptides were desalted and cleaned with ZipTip C18 pipette tips (Millipore Corporation, Bedford, MA, USA) according to the manufacturer’s instructions. All analyses were performed using a Bruker Daltonics Autoflex (Bruker Daltonics Billerica, MA, USA) operated in the delayed extraction of 190 ns and reflector mode with an accelerating voltage of 20 kV. The peptide mixture was analyzed using a saturated solution of R-cyano-4-hydroxycinnamic acid (CHCA Bruker Daltonics Billerica, MA, USA) in 50% acetonitrile/0.1% trisfluoroacetic acid. External calibration was performed with a peptide calibration standard (Bruker Daltonics Billerica, MA, USA, Part No.: 206195) and internal calibration with trypsin autoproteolytic fragments. The samples were analyzed on a MALDI-TOF/TOF-MS 4800 proteomics analyzer (Aglient, USA) and data were analyzed using GPS explorer software (Applied Biosystems, Foster City, Calif.) and MASCOT software (Matrix Science, London, UK).
Results

Comparative protein profiles of colchicine treated and control microsporangia

Approximately, 1000 microsporangia proteins were detected on coomassie brilliant blue-stained gels (Figure 1B). To accurately and quantitatively analyze proteomic changes, spot volume differences of more than 2-fold between two identical spots were defined as significant. The spots that revealed changing their abundance significantly after quantitative image analysis were randomly selected to check again. A total of sixty-six proteins were differentially expressed in response to colchicine (Figure 1A, 1B). It was noted that some protein spots demonstrated qualitative changes in intensity. The spots 7704, 8104 and 3109 were absent in the colchicine-treated gels (Figure 1).

MOLDI-TOF/TOF-MS identification and classification of colchicine-responsive proteins

Thirty-nine of these differentially expressed spots were arbitrarily selected and excised from the gels and subjected to MOLDI-TOF/TOF-MS. Twenty-seven proteins were successfully identified and are listed in Table 1.

The identified proteins were associated with a wide variety of cellular processes. They were classified into several categories according to their function (Figure 2), including the ATP generation, transport and carbohydrate metabolism, protein metabolism, ROS scavenging and detoxifying enzymes, cell wall remodeling and metabolism, transcription, cell cycle, and signal transduction.

The identified spots corresponded to proteins that were related to energy, carbohydrate metabolism and photosynthesis. In our study, nine proteins were in this category. Four of them were found to be up-regulated, and three of them were changed in the opposite way. The rest of them evenly has been disappeared after colchicine treated. Several chaperones involved in protein processing, folding and degrading were also identified as being responsive to colchicine treatment. These included two HSP (spot3605 and spot3611) and two EF-Tu (spot4312 and spot5313) proteins. In this study, the levels of protein (3605), which was analogous to HSP-70 (3611) and heat shock protein 70, were found to be elevated (Figure 3).

The SE-wap41 (spot 2207) was found to be down-regulated. 2-dehydro-3-deoxyphospho-octonate aldolase (spot 7110), a protein involved in cell wall remodeling and metabolism, was found to be up regulated. Both of them indicating that colchicines might perhaps have interfered with cell wall formation. The three spots, 4607, 4801 and 8305 were related to transcription, cell cycle, and signal transduction. Spot 4607 was found to be a response factor of a plant growth hormone and was up-regulated by colchicine treatment. Spot 4801 was found to be the homolog of CDC48 and spot 8305 was classified as a putative polyprotein; and expression levels of both proteins were elevated in this study.

APX (spot 6008) and GST (spot 5004) were found to be a part of the antioxidant system employed by plants [15]. Previous microarray results demonstrated that these proteins were responsive to various stresses including osmotic, drought, and cold stresses [16–18]. We found that the abundance of GST was three times higher post colchicine treatment. However, the abundance of APX was drastically down regulated (half of the level in control).

Discussion

The protein profiles of microsporangia treated with colchicine enabled us to classify the individual response proteins on the basis of their function.

1. Cell wall remodeling and metabolism

The decline in the abundance of se-wap41 after 48 h of colchicine treatment corroborated the results of Chen et al. [19]. Se-wap41, a salt-extractable 41-kD wall-associated protein that has been reported to label plasmodesmata and the Golgi was found to be a class 1 reversibly glycosylated polypeptide (C1 RGP) [9]. Using immunogold labeling, Dhugga et al. [20] showed that RGP1 was specifically localized to Golgi stacks, where it was likely to be involved in xyloglucan biosynthesis [21]. This suggests that colchicine can interfere with the cell wall remodeling. Hogetsu and Shibaoka [22] also demonstrated that colchicine could inhibit cell
division and disturb the microfibril arrangement in *Closterium acerosum*. 2-dehydro-3-deoxyphosphooctonate aldolase (spot 7110) is known to play an important role in the LPS biosynthetic pathways. Lipopolysaccharide (LPS) serves as a selectively permeable

**Table 1.** Obs. pl and Mr, observed pl, Mr calculated from the 2D-gels with PDQuest 8.0.1 software according to standard marker proteins.

| Ssp         | Name                                                                 | Accession No | Protein Score | Mr Theor./obs. | pl Theor./obs. | Expression pattern |
|-------------|----------------------------------------------------------------------|--------------|---------------|----------------|----------------|--------------------|
|             | **Proteins involved in ATP generation, transport and carbohydrate metabolism**                                           |              |               |                |                |                    |
| 3407        | ATP synthase beta subunit [Triticum aestivum]                         | gi|525291         | 211            | 59.21/71.50    | 5.56/5.58         | up                 |
| 4018        | thiazole biosynthetic enzyme [Pseudotusga menziesii]                  | gi|56481847       | 118            | 37.30/34.22    | 5.85/5.44         | up                 |
| 7204        | glutamate-1-semialdehyde 2,1-amminotase [Nicotiana tabacum]          | gi|19875          | 118            | 50.98/55.18    | 7.05/6.47         | up                 |
| 7304        | isocitrate dehydrogenase (NADP+) [Arabidopsis thaliana]              | gi|15221788       | 85             | 47.20/65.31    | 7.58/6.37         | up                 |
| 5305        | enolase2 [Zea mays]                                                  | gi|162460735      | 182            | 48.13/68.08    | 5.70/6.03         | down               |
| 3113        | Cytochrome c [Ginkgo biloba]                                         | gi|117987         | 54             | 123.5/48.10    | 9.76/5.14         | down               |
| 3109        | 33 kDa oxygen-evolving protein [Arabidopsis thaliana]                | gi|22571          | 142            | 35.11/35.08    | 5.68/5.31         | disappear          |
| 7704        | putative transketolase [Oryza sativa Japonica Group]                 | gi|28190676       | 103            | 79.98/94.68    | 6.12/6.30         | disappear          |
| 8104        | NADPH thioredoxin reductase [Arabidopsis thaliana]                   | gi|468524         | 73             | 32.42/38.43    | 5.78/6.60         | disappear          |
|             | **Protein metabolism**                                               |              |               |                |                |                    |
| 3605        | heat shock protein 70 like protein [Arabidopsis thaliana]            | gi|4467097        | 84             | 71.13/88.74    | 5.31/5.54         | up                 |
| 3611        | heat shock protein 70 [Cucumis sativus]                              | gi|6911553        | 123            | 70.00/92.07    | 5.29/5.63         | up                 |
| 8408        | 26S proteasome ATPase subunit [Pismum sativum]                       | gi|49175787       | 214            | 23.56/68.35    | 7.16/6.69         | up                 |
| 4207        | Protein disulfide-isomerase [Medicago truncatula]                    | gi|357442333      | 80             | 40.47/55.98    | 5.54/5.81         | down               |
| 4312        | Elongation factor Tu [Chlamydomonas reinhardtii]                     | gi|41179007       | 182            | 45.71/61.41    | 5.90/5.89         | down               |
| 5313        | Elongation factor Tu [Arabidopsis thaliana]                          | gi|15236220       | 112            | 49.38/62.11    | 6.25/6.18         | down               |
|             | **ROS scavenging and detoxifying enzymes**                            |              |               |                |                |                    |
| 5004        | GST [Ginkgo biloba]                                                  | gi|66736578       | 421            | 25.77/26.64    | 6.24/5.71         | up                 |
| 6008        | ascorbate peroxidase [Ginkgo biloba]                                 | gi|220898265      | 296            | 27.51/30.31    | 5.81/6.29         | down               |
| 9111        | ferredoxin-NADP + reductase [Arabidopsis thaliana]                   | gi|162459168      | 98             | 39.30/40.13    | 8.53/8.66         | up                 |
|             | **Wall remodeling and metabolism**                                   |              |               |                |                |                    |
| 7110        | 2-dehydro-3-deoxyphosphoconionate aldolase [Arabidopsis thaliana]   | gi|13620976       | 65             | 31.60/37.81    | 6.33/6.38         | up                 |
| 2207        | golgi associated protein se-Zea mays                                 | gi|162463414      | 124            | 41.18/57.79    | 5.75/5.38         | down               |
|             | **Transcription, cell cycle and signal transduction**                |              |               |                |                |                    |
| 4607        | ARF-L1 protein [Ginkgo biloba]                                       | gi|291196881      | 40             | 110.7/89.10    | 5.64/5.45         | up                 |
| 4801        | Cell division cycle protein 48 homolog                              | gi|1705678       | 69             | 89.77/100.8    | 5.18/5.72         | up                 |
| 8505        | putative polyprotein [Nicotiana tabacum]                             | gi|15963359       | 65             | 61.40/85.59    | 9.36/6.51         | up                 |
|             | **Unclassified proteins**                                            |              |               |                |                |                    |
| 3206        | Os02g0698000 [Oryza sativa (japonica cultivar-group)]                | gi|115448091      | 75             | 44.84/54.23    | 5.68/5.14         | down               |
| 5012        | putative protein [Arabidopsis thaliana]                              | gi|7573371        | 98             | 34.75/47.97    | 6.19/6.00         | down               |
| 6409        | Os02g0125100 [Oryza sativa (japonica cultivar-group)]                 | gi|115443927      | 73             | 55.50/75.83    | 7.05/6.15         | up                 |
| 6512        | Sequence 4 from patent US 6946283                                   | gi|77372606       | 40             | 99.47/77.53    | 5.53/6.36         | down               |

ER: endoplasmic reticulum, PM: Plasma Membrane, M: Mitochondrion.
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membrane for organic molecules, increases the negative charge of the cell wall and stabilizes the overall membrane structure. Wang et al. [23] found that 2-dehydro-3-deoxyphosphooctonate aldolase was upregulated in boron deficient *Brassica napus* roots. It is speculated that plants synthesize LPS to resist injury when cell membranes suffer the colchicine stress.

2. Proteins involved in ATP generation, transport and carbohydrate metabolism

Respiration is critical to metabolism in higher plants. In addition to its link with carbon metabolism, respiration releases energy stored in carbon-based compounds in a controlled manner for cellular use. It also generates many carbon precursors for biosynthesis. Carbon metabolism enzymes appear to be major targets for oxidative modification and breakdown in *vitro* and *in vivo* [24]. In our study, 48% of the proteins were found to be involved in ATP generation, transport and carbohydrate metabolism. Most of them responded in a manner similar to other abiotic stress conditions. For example, it was noted that enolase (spot 5303), which catalyzes the formation of high-energy phosphoenol pyruvate from 2-phosphoglycerate in the glycolytic pathway, decreased after 48 h of colchicine stress. Previous reports showed that a mutation in the enolase locus results in the repression of cold-responsive genes and therefore, it acts as a positive regulator of cold-responsive genes [25]. The decreased abundance of enolase protein was similar to the response of *Arabidopsis* roots to NaCl stress [26]. Other energy-related proteins, including the two ATP synthases (spot 8408 and 3407) were found to be elevated in response to colchicine treatment. This response was similar to that observed in other stress conditions such as salt or cold stress [27–29].

Thiazole biosynthetic enzyme (spot 4018) also showed higher abundance in response to colchicine treatment treated. Tunc-Ozdemir et al. [30] indicated that when *Arabidopsis* (*Arabidopsis thaliana*) plants showed an elevated expression of transcripts encoding thiamin biosynthetic enzymes in response to abiotic stress conditions, such as high light, cold, osmotic stress, salinity, and oxidative treatments.

The glutamate 1-semialdehyde 2,1-aminomutase (spot 7204), which is involved in the C5 pathway for synthesis of δ-aminolevulinic acid (ALA) [31]. δ-Aminolevulinic acid (ALA) is the first intermediate in the synthesis of chlorophylls [32].

3. ROS scavenging and detoxifying enzymes

Abiotic stresses induce the production of ROS, which can cause damage to cellular components and act as a signaling molecule for stress responses [15]. In recent years, additional reports have indicated that GSTs in plants play major roles in herbicide detoxification [33–40]. GSTs also conjugate natural products such as anthocyanins, serving as binding proteins or ligandins for plant hormones, or catalyze GSH-dependent peroxidase and isomerase reactions [34,41]. Herbicide safeners are chemicals used to minimize the effect of the herbicides on crop plants. They protect cereal crops from herbicide injury by increasing the expression of GSTs as well as other herbicide-detoxifying enzymes and proteins such as cytochrome P-450 s, glucosyl transferases, and tonoplast transporters that can detoxify herbicides and transport them into the vacuole [39]. In this study, colchicine treatment resulted in elevated levels of GSTs, perhaps to reduce colchicine toxicity. An increased detoxification of compounds mediated by high levels of glutathione (GSH) and glutathione transferase (GST) has been found in cells resistant to cothicine [42]. Ruiz-Gomez et al. [42] suggested that GSTs may play an important role in main multidrug resistance (MDR) pumps in cells that express high levels of P-glycoprotein (P-gly).

APX in plants utilizes ascorbate as an electron donor to catalyze H₂O₂ conversion into H₂O. It is a multigenic enzyme localized in different cellular compartments, which complements and coordinates antioxidant defenses [43]. In rice it was induced under conditions like ozone stress, drought stress, chitosan, and in blast lesion mimic mutants [44–47]. Studies on the possible interference of colchicine and H₂O₂ in *Arabidopsis thaliana* cv. Col-0 suggested that APX was the most sensitive enzyme to colchicine and H₂O₂ [48].

FNR (spot 9111) is a widespread family of FAD-containing enzymes. In different tissues and organisms these flavoproteins catalyze the reversible electron transfer between two molecules of the obligatory one-electron carrier Fd and a single molecule of NADP (H) [49]. The expression of a cloned plant FNR gene in a mutant *E. coli* strain did restore the oxidative tolerance to wild-type levels, indicating that the eukaryotic flavoenzyme behaves as a toxic radical scavenger in the bacterial host [50]. In our study, the FNR (spot 9111] was found to increase under colchicine stress. This indicates the toxic radical scavenger function of FNR in gymnosperms.

4. Protein metabolism

The proteolytic system, consuming metabolic energy, plays an essential role in the regulation of cellular functions by catalyzing rapid and irreversible reactions for various important biological processes, such as the cell cycle, apoptosis, signal transduction, protein processing, and immune and stress responses [51]. We detected three proteins that promote the proper folding of proteins.
and proteolysis and found that their abundance increased following colchicine treatment. One of them was the 26 s proteasome ATPase subunit. G. N. DeMartino and his colleagues [52] attempted the molecular cloning of cDNAs encoding the human 26S proteasome regulatory subunits. These subunits are divided into two distinct sub-groups, ATPase and non-ATPase subunits. The 26S proteasomal regulatory complex has intrinsic ATPase activity that seems to play an essential role in its function [52,53]. The 26S proteasome attacks poly- or multi-Ub chain to act as a degradation signal for proteolysis. Furthermore, research has shown that different kinds of stresses can result in degradation of the Ub chain. Treatment with HgCl2 increases the expression of

Figure 3. Comparison of the differential proteins expression level in two groups. CK: the control, samples treated with cotton balls soaked distilled water only. T: the test, samples treated with cotton balls soaked 0.8% (8 mg/mL) colchicine solution. doi:10.1371/journal.pone.0076088.g003
ubiquitin genes in Nostoc; and a strong accumulation of a ubiquitin conjugating enzyme (Ubc1) was observed in tomato after exposure to CdCl₂ [54,55]. The expression of polyubiquitin gene is affected by darkness, UV radiation, starvation, and an enhanced level of ozone [56–59]. A significant increase in the level of de novo biosynthesis of ubiquinone and plastoquinone (PQ) in plant tissues was observed as a result of colchicine treatment [60].

One of the first described chaperone regulators of Tau is the Hsp70 chaperone family. Increased levels of Hsp70 promote tau solubility and tau binding to microtubules; the tau protein is normally expressed in the cytoplasm of cell bodies and axons, where it binds to and stabilizes microtubules [61]. The greatly increased abundance of HSP 70 s in our study suggested that the G. biloba microsporangia were building resistance to colchicine. This powerful resistance helped in maintaining the stability of microtubule when the microtubule of G. biloba was treated by colchicine.

Elongation factor-1α (EF-1α), a highly conserved protein named for its role in protein translation, is also a microtubule-associated protein (MAP) [62] and binds to the microtubule lattice [63–65]. This protein can also modulate microtubule dynamics in vitro and in vivo [66–71]. In our study this protein was found to be down regulated. Therefore, G. biloba might have a strong self-protection system to defend the colchicine invasion, which hinders the induction of diploid gametes.

5. Transcription, cell cycle and signal transduction

Auxin plays a crucial role in diverse aspects of plant growth and development, including troic responses, apical dominance in the shoot, lateral root formation and differentiation of the vascular system. In this study, the increased ARF-L1 protein indicated that colchicine may stimulate the differentiation of microsporangiums.

In particular, studies of cell division cycle protein 48 (Cdc48p) in Saccharomyces cerevisiae have uncovered roles in many cellular processes, including membrane fusion [10], ERAD [11] and spindle disassembly [12].

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Conclusion

In this study, G. biloba microsporangia were subjected to 48 h of colchicine treatment followed by proteomic analysis of treated and control samples. A total of 27 colchicine-responsive proteins were identified (Table 1) by 2-D gel electrophoresis. Complementary strategies to protein electrophoresis can be employed at the transcript and metabolite levels in future to gain insight into the intricate network of plant responses to colchicine. Proteins are the most direct reflection of colchicine response. And genes are the most fundamental evidences. Through above analysis, we can see the possible roles the twenty-seven proteins played in defending colchicine invasion. So a new dimension to our work is pointed out. The differently expressed proteins should be the most important part of future study. The real reason for the low mutation rate in diploid male gametes of G. biloba might be found out among them. Much work still to be done about the twenty-seven proteins, such as comparing the twenty-seven genes with colchicine responsive genes in other species to see if any of them are specific to G. biloba. 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.

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Author Contributions

Conceived and designed the experiments: NY YS Yu Li. Perfomred the experiments: NY YS. Analyzed the data: NY YW CL. Contributed reagents/materials/analysis tools: Yingyu Li. Wrote the paper: NY YS.
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