A Simplified Method to Isolate Rice Mitochondria

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Abstract

Background: Mitochondria play critical roles in plant growth, development and stress tolerance. Numerous researchers participate in the studies of plant mitochondrial genome structure, mitochondrial metabolism and nuclear-cytoplasmic interactions. However, traditional plant mitochondria extraction methods are time-consuming and complicated operation of ultra-centrifuge with the expensive reagent. To develop a more rapid and convenient method for isolation of plant mitochondria, in this study we established a simplified method to isolate rice mitochondria efficiently for further study.

Results: To isolate rice mitochondria, the cell wall was first dispelled by enzymolysis to obtain the protoplast which is similar to the animal cell. Then the rice mitochondria were isolated with a modified method basing on the animal mitochondria isolation protocol. The extracted mitochondria were next detected on DNA level and protein level to rule out the contamination of nucleus and chloroplasts. Furthermore, we examined the physiological status and characters of the isolated mitochondria, including the integrity of mitochondria, mitochondrial membrane potential, and the activity of inner membrane complexes. Our results demonstrated that the extracted mitochondria were remained intact for further studies.

Conclusion: The combination of plant protoplasts isolation and animal mitochondria extraction methods facilitates the extraction of plant mitochondria without ultracentrifugation. Consequently, this improved method is cheap and time-saving with good operability, and can be broadly applied in the researches on plant mitochondria.

Background

Mitochondrion was first found in 1850 and it was considered to evolve from engulfment of an α-proteobacterium by a precursor of the modern eukaryotic cell [1]. The difference between plant mitochondria and animal mitochondria is little as to its size and function since both of them are evolved from the same microorganism [2, 3], while plant mitochondrial genome can be 100 times larger than those of animals [4, 5].

Mitochondria produce more than 90% of the cellular energy (ATP) required for an organism's growth, reproduction, and maintenance [6]. In plant, dysfunction of mitochondria always causes the retardation of plant growth, hypersensitive to disease, embryo lethal, the pollen abortion and so on [7–9].

Mitochondrion is also vital to the mammal as the deficiency of the mitochondrial function resulted in many serious diseases, such as Alzheimer's, Parkinson's, and Huntington's disease [10–12]. Nowadays a growing number of researchers focus on the studies of mitochondria to explore an efficient therapy approach to deal with the existing and emerging mitochondrial disease [13, 14]. Massive studies have revealed that the reason for the mitochondria deficiency might be the incompatibility between mitochondria and nucleus, or the mutation of the mitochondria genome.

Generally, those dysfunctions resulted from the disorders on the transcriptional, post-transcriptional and translational regulations. Many groups have devoted to the plant mitochondrial omics studies, and the interaction between nuclear and mitochondrion has contributed to the speciation and cytoplasmic male sterility which could be applied to explore the crop hybrid vigor [15–22].

The classic method of rice mitochondria isolation has been reported a dozen years ago, which was broadly applied in many labs, including ours [23]. Nevertheless, there are still some flaws in this method. Firstly, it needed enormous plant materials since the great loss during the extraction process. Secondly, it also needed heavy labors to grind the materials. Thirdly, the preparation of percoll gradient solution was also laborious and time-consuming and it is an expensive reagent. More importantly, the high-speed centrifuge and ultra-speed centrifuge were dispensable. All these requirements restrain most of the labs from isolating plant mitochondria and conducting mitochondria-associated researches.

In this study, we established an improved method to extract the rice mitochondria. In this procedure, we excluded the heavy labor, the expensive equipment and reagents, and reduced the requirement for the enormous material. To confirm the purity of the isolated plant mitochondria, we detected the extracts on DNA level and protein level, respectively. The results of proteinase digestion assay and electron scan microscopy observation also confirmed the intact structure of mitochondria. The mitochondrial membrane potential and functional activity of inner membrane electron transport chain (ETC) complex activity were checked to assure further studies.

Results

Workflow of mitochondria isolation

In this study, we improved mitochondria extraction method by combining the traditional methods of plant protoplast isolation and the mammal mitochondria extraction protocol with slight modifications [24, 25]. (Fig. 1)

Firstly, the rice seeds were cultured in the 1/2 MS medium at 25℃ under the dark environment for 10 days to avoid the contamination from bacteria. Next the 10-days young seedlings were cut into debris with ~ 0.5 mm and dipped in the hyper-osmotic buffer for 10 min, which were further incubated with enzymolysis buffer for 5 hours at 28℃ or overnight at 25℃ [26]. Results suggested the treatment overnight at 25℃ could yield more viable protoplasts (Table 1). Subsequently, the protoplasts were further collected by centrifugation at 600 g for 5 min, and resuspended gently in mitochondrial isolation buffer (MIB). The suspension was incubated at 4℃ for 1 hour, and the protein inhibitor PMSF was added to the final concentration 1 mM to prevent the degradation of the mitochondria by cytoplasmic proteinase. To obtain the highest yield of the mitochondria without destroying its integrity, researcher needs to explore the optimal homogenization times. Finally, centrifuged the sample at 600 g for 10 min, decant the supernatant into another tube and collected the mitochondria by centrifugation at 11,000 g for 10 min. By this means, we got about 10 mg mitochondria from 12 g seedlings.

Table 1
The yields of protoplasts and mitochondria from seedlings treated with enzymolysis buffer under different incubation temperatures and times.

Dataset	25℃			28℃		
	5 hours	8 hours	12 hours	5 hours	8 hours	12 hours
Protoplasts (Number)	3.75 ⊆10^7(0.75)	7.55⊆ 10^7(1.65)	5.10⊆ 10^7(0.40)	5.40 ⊆10^7(0.54)	6.00 ⊆ 10^7(1.20)	1.65⊆ 10^7 (0.54)
Mitochondria (mg)	7.00(3.12)	12.00(6.54)	8.50(5.68)	9.00(3.00)	9.00 (0.00)	4.85 (3.76)

The numerical values in each cell represent the mean and standard deviation (in parentheses).

The purity of mitochondria

To detect the purity of our isolated mitochondria, we first employed PCR assay to detect on DNA level. *COX III* and *Actin* were selected as the mitochondria and nucleus marker gene, respectively. No *actin* PCR products were observed even after 35 cycles amplification, indicating that the genomes of isolated mitochondria were free from the nucleus genome (Fig. 2a).

Furthermore, several specific commercial antibodies, anti-HISTONE, anti-RUBISCO, anti-CATALASE and anti-VDAC were applied to estimate the contamination from the nucleus, chloroplast, cytoplasm and mitochondrion, respectively. Three types of components were further analyzed, the supernatants which contained the cytoplasmic substance in step 10 (S10, mentioned in materials and methods section) were collected, and the precipitates in step 9 (S9, mentioned in materials and methods section) which contained the cell lysates and the intact protoplasts were retained. Results showed that all of the cell organelle maker proteins could be detected in the total cells, indicating the intact of the protoplast (Fig. 2b). While in the mitochondria fraction, only the marker protein voltage-dependent anion channels (VDAC) can be detected, indicating that the mitochondria were solidly enriched. No peroxisome, rubisco and histone were detected in the mitochondria fraction, suggesting most of organelles were removed (Fig. 2). All these results demonstrated that the isolated mitochondria were clean with high purity.

Determination of the mitochondrial integrity

To detect the intact of the extracted mitochondria, we examined the integrity of the mitochondrial membrane via proteinase K treatment. As the inner membrane proteins are more resistant against the proteinase K than those located on the outer membrane in the intact mitochondrion. While in the fractured mitochondrion, the two types of proteins show identical degradation patterns when exposed to the proteinase K. The result showed that the outer membrane protein VDAC was degraded under the proteinase treatment, while no degradation occurred for the inner membrane protein NADH dehydrogenase subunit 3 (NAD3) with the increasing concentration of proteinase K (Fig. 3a). In contrast,

both VDAC and NAD3 exhibited identical degradation patterns when the mitochondria were treated with proteinase K after resuspended in full lysis buffer (FLB), suggesting the members were destroyed completely (Fig. 3b). We further used thermolysis to detect the integrity of mitochondrial membrane. In chloroplast, thermolysin can digest the outer membrane peptide, while it can't penetrate into the intermembrane space [27]. Therefore, we proposed that thermolysin has a similar biological mechanism to mitochondrial outer membrane. Under the treatment with increasing thermolysin concentration, we observed the continuous degradation of VDAC, whereas the inner membrane marker protein COXII remained stable (Fig. S2). This distinct degradation pattern implied that our extracted mitochondria were intact.

To further observed the morphology of mitochondria, the mitochondria were also examined by transmission electron microscopy (Fig. 4). And we observed numerous mitochondria that contained the high electron density, indicating that the isolated mitochondria preserved high viable activity [28]. All of these results suggested that the mitochondria were well enriched and the structure remained intact for further research, which can be used for further research.

The mitochondrial membrane potential assay

Previous study showed JC-1 could specifically aggregate in the viable mitochondria and emitted the red fluorescence signals, while in the apoptosis mitochondria, JC-1 existed in the monomer state which emitted the green fluorescence [29]. To analyze the mitochondrial membrane potential, the fluorescence substrate JC-1 was then used. Rice protoplasts were incubated with JC-1 according to the protocol of mitochondrial membrane potential detection kit (JC-1). We also treated the protoplasts with the Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which is a mitochondrial membrane potential inhibitor, before staining with JC-1. Results showed that the protoplasts treated with CCCP exhibited stronger green signals than the untreated protoplasts (Fig. 5a), suggesting that more apoptosis mitochondria were generated when the protoplasts were treated with the mitochondrial membrane potential inhibitor.

Moreover, we applied the Multimode Reader Cytation 3 to detect the fluorescence signal emitted by JC-1 aggregates or monomer, and the ratio of red and green fluorescence is calculated to indicate the mitochondrial membrane potential. As a control, results demonstrated that CCCP treated protoplasts exhibited significantly decreased mitochondrial membrane potential compared to the untreated protoplasts. Then we further detected the membrane potential of isolated mitochondria, results showed the membrane potential was significantly declined, indicating that the isolated mitochondria kept its potential to some degree. (Fig. 5b)

The inner membrane complex activity of mitochondria

The activities of ETC complex are quite essential for the organism. Therefore, the activity assay of the mitochondrial inner membrane complex also needs to be performed in this study even though the integrity of mitochondria was confirmed.

We investigated the activity of isolated mitochondria using three different mitochondria inhibitors, rotenone (0 μg/ml, 3 μg/ml ,6 μg/ml, 9 μg/ml), which inhibits at complex I, malonate (0 mg/ml, 0.5 mg/ml, 2.5 mg/ml, 5 mg/ml), which inhibits at complex II, and sodium azide (0, 0.1%, 1%, 2%), which acts at complex IV [30–32]. All of these three inhibitors were incubated with the extracted protoplasts for 30 min at 25℃ before isolating the mitochondria. The complex activity was assayed according to the manufacturer's instructions of mitochondrial complex activity with slight modification. Results showed that the activities of mitochondria inner membrane ETC complexes were significantly declined under different inhibitor treatments (Fig. 6). And the complex activities were severely decreased with the increased concentration of inhibitor, suggested that the isolated mitochondria could be applied to further researches.

Discussion

The simplified mitochondria isolation procedure

Plant mitochondria are difficult to isolate due to the presence of the cell wall which is absent in the animal cell. The basic idea of our method was to get the protoplasts that were similar to the mammalian cells, and then the mitochondria could be extracted easily according to the slightly modified mammal mitochondrial isolation methods. We chose the 10 days-old rice seedlings to isolate protoplasts. Since the plant cell underwent active mitosis at this young stage, and the cell wall can be easily degraded by the cellulose and macerozyme. The cell structure of extracted protoplast is similar to the animal cell, so it is practicable to isolate the plant cell mitochondria by applying the homogenizer to burst cell membrane in the MIB. By this way, mitochondria can be easily isolated from seedlings. Besides, we also successfully obtained abundant rice protoplasts from callus (Fig. S3), suggesting that the callus can be used to isolate the rice mitochondria in this method.

Compared to the traditional rice mitochondria extraction methods which need strenuous labors to prepare the percoll density gradients solution and multiple differential centrifugations, this improved method simplified the solution preparation and reduced the labors since most of the experiment time was spend in the enzyme solution incubation period. The exposure of mitochondria is not only important for the morphology and integrity of mitochondria, but also essential for the activities and function. In this method, we greatly reduced the operation times to keep it intact. In addition, the expenditure was also decreased, since the expensive reagent, percoll, and equipment, ultra-speed centrifuge, is not necessary, which were not always affordable in many labs.

Tips for the high yield of mitochondria

Here, we think that the successful acquisition of plant protoplasts is the guarantee for extraction. To harvest more mitochondria, the high yield of protoplasts is the key step. Although the chopped rice seedlings were suggested to incubate with enzymolysis buffer for 5 hours at 28℃ or overnight at 25℃, we recommended treating the young seedlings for 8 hours at 25℃ (Table 1). For the calli, the incubation time can be extended longer to avoid the incomplete cell wall degradation. Generally, the shorter the

incubation time, the higher the incubated temperature is needed for the enzyme reaction. To yield more protoplasts from different tissues or species, various temperatures or times should be tested. The protoplasts are then lysed in the MIB, the incubation time is changeable when combined with the homogenized time. The longer MIB incubation time, the less homogenized times. Otherwise, both over incubation and over homogenized can result in the broken mitochondria and inefficient mitochondria yields (Fig. S4).

The extracted mitochondria remained active

Since the isolated mitochondria are vulnerable to damage, and the fractured mitochondria structure greatly affects the results of the experiment. In this study, we performed the proteinase digestion experiments to examine the integrity of mitochondrial structure. The mitochondrial marker proteins in the inner membrane and matrix were protected from the digestion of proteinase K and thermolysin, which established that the isolated mitochondria were intact and the outer membrane is completeness. Furthermore, both of the CCCP treated protoplasts and isolated mitochondria exhibited significantly declined mitochondrial membrane potential, which demonstrated that the mitochondria still kept its membrane potential in our extraction procedure.

We also examined the activity of ETC in the inner membrane mitochondria. All of the tested mitochondrial ETC complexes in the inner membrane revealed declined activity, which exhibited a negative relationship with the increasing concentration of toxicant, indicating that the function of mitochondrial ETC complexes was blocked by the toxicant. All of the results demonstrated that the isolated mitochondria could be applied for biochemical analyses.

Conclusions

In summary, this simplified method provides researchers an alternative way to isolate rice mitochondria. As the protoplasts of different plants are accessible [33–35], the mitochondria extraction method can be applied to other plants without preparing abundant plant materials. Compared to the traditional mitochondria extraction methods, this mitochondria isolation method does not include the expensive reagents, instruments and complicated steps. Moreover, the improved mitochondria extraction method can greatly shorten the time and save the labors. All these improvements demonstrated that this simplified method has wide potential applications in plant mitochondria extraction for further research.

Materials And Methods

Plant materials

Preparing 200 rice seeds, dehull the seeds with sheller (ZHEJIANG CHERRING SEWING machine, China), and sterilized with 75% ethanol for 1–2 min. Rinsing the rice with sterilized water, followed 0.15% $HgCl_2$ treated for 15 min. Cultivating the rice on the sterilized rooting medium for sprouting or induction medium for calli generation in the dark condition at 25℃ for 10 days and 20 days, respectively.

Protocol for the mitochondria isolation

The improved plant mitochondria isolation method was based on the protoplast extracted and lysate, and the mitochondria were isolated further through differential centrifugate, the protocol consists of the following 10 steps:

S1. Collect the etiolated seedlings and remove the seeds away, dip the seedlings in the balance buffer (0.6 M mannitol). For the callus, the new generated calli were chosen, and dipped in the balance buffer.

S2. Select the middle region, and chop the seedlings into 0.5 mm pieces with the razor blade, and put the chopped seedlings in the balance buffer for at least 10 min.

S3. Filter the chopped seedlings or calli with nylon net (300 mesh) and transfer it into enzyme buffer (10 mM MES, pH 5.7, 0.6 M mannitol, 0.1% BSA, 1.5% (w/v) cellulase R10 (YakultHon sha, Tokyo, Japan), 0.75% (w/v) macerozyme R10 (Yakult Honsha), 1 mM $CaCl_2$, 1 mM β-mercaptoethanol, and 50 μg/ml carbenicillin) which needs to recover to room temperature after water bath for 10 min at 55℃.

S4. Shake at 80 rpm for 5 hours at 28℃ under the dark environment.

S5. Filter the chopped seedlings or calli and collect the filtrate in the 7 ml EP tube, centrifuge at 600 g for 3 min at 25℃.

S6. Resuspend the pellet with W5 solution (2 mM MES, pH 5.8, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 5 mM glucose), centrifuge at 600 g for 3 min at 25℃. Discard the supernatant, and repeat this step.

S7. Resuspend the pellet with MIB (70 mM sucrose, 210 mM mannitol, 10 mM HEPES, 1 mM EDTA, 1 mM PMSF, pH 7.5), and shake for 1 hour at 4℃ gently.

S8. The suspension is homogenized using a Teflon dounce homogenizer, it is needed to explore the homogenized time for the sufficient lysis of the cell, because of the over-lysis results in the burst of mitochondria. Empirically, 20 times homogenized is sufficient to burst the protoplast.

S9. Centrifuge at 600 g for 10 min at 4℃, pipet the supernatant into new EP tube, and centrifuge at 11,000 g for 10 min at 4℃.

S10. Discard the supernatant and resuspend the mitochondria with storage solution (0.4 M Mannitol, 50 mM Tris, 1 mM EDTA, 5 mM KCl, 5% DMSO, pH 7.4)

Mitochondrial purity determination

Preparing different components of cell, including total protoplast, nucleus, mitochondria and the cytoplasmic, resuspended in the FLB (100 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA,10 mM DTT, and 0.5% [v/v] Triton X-10010). Samples were mixed with loading buffer (250 mM Tris-HCL, 10% SDS, 0.5% BPB, 50% glycerol, 5% 2-ME), and were denatured at 98℃ for 10 min. The proteins

were separated by 10% SDS-page gel. For the western blot, the protein was transferred onto the PVDF membrane (Bio-Rad, Hercules, CA, USA). Western blot was conducted by using the primary antibody in the following dilution: anti-RUBISCO 1:5000, anti-VDAC 1:2000, anti-Histone 1:5000, anti-COX II 1:1000, and anti-CATALASE 1:1000, followed the corresponding secondary antibody incubate at 1:5000 dilution. Signals were visualized by ChemiDoc XRS (Bio-Rad).

For the genome purity detection, the protoplast and mitochondria were resuspended in the CTAB buffer (2% CTAB, 20 mM Na_2.EDTA, 100 mM Tris-HCl, 1.4 M NaCl, pH 8.0) at 60℃ for 1 hour. The DNA was extracted according to the standard protocol. DNA concentration was measured by nanodrop 2000c and diluted to the 15 ng/ul. PCR reaction was performed with 2 × Taq plus master mix (Vazyme) according to the manufacturer's instructions. The forward primer *actin* -F (5'-AACTGAAACCCCCATGTCCC-3') and reverse primer *actin* -R (5'-TGCAGAACGGAAAAGTCCCA-3') were used for the amplification of the *actin* domain (*actin*, *LOC_Os03g61970*). The forward primer *cox III* -F (5'-GACAAATGGGAATAACCGAA-3') and reverse primer *cox III* -R (5'-GGGGAAGGAAAAACGAGCAG-3') were used for the amplification of the *cox III* domain (*cox III*, *LOC_Osm1g00110.1*). PCR conditions were as follow: 95 °C for 5 min, 95 °C for 15 s, 55 °C for 15 s, 72 °C for 30 s, 35 cycles from step 2 to step 4 were set. PCR products were separated in the 2% agarose gel and visualized by gel imaging system (GE).

Intact mitochondria examination

The extracted mitochondria were incubated with proteinase K (0 μg/ml, 5 μg/ml, 20 μg/ml, 50 μg/ml), thermolysin (0 μg/ml, 10 μg/ml, 50 μg/ml, 100 μg/ml) at 4℃ for 20 min. Adding $CaCl_2$ to the thermolysin to the final concentration 0.5 μM. All of the reactions were quenched by adding the EDTA to the final concentration of 5 mM, then performed the SDS-page electrophoresis and conducted the western blot as described above.

Transmission electron microscopy observation

Sample preparation of mitochondria for transmission electron microscopy was described as follows: (i) the isolated mitochondria were fixed in the 2% glutaraldehyde (2.5% glutaraldehyde, 100 mM NaH_2PO_4.H_2O, 80 mM Na_2HPO_4.$12H_2O$) overnight, and washed in phosphate buffer (100 mM NaH_2PO_4.H_2O, 80 mM Na_2HPO_4.$12H_2O$) for 5 times with 20 min each; (ii) the sample was then fixed with 1% osmic acid (1% osmic acid, 100 mM NaH_2PO_4.H_2O, 80 mM Na_2HPO_4.$12H_2O$) for 2 hours, 5 washings in phosphate buffer with 30 min each; (iii) dehydration in ethanol with increasing concentration of 15%, 30%,50%, 70%, 80%, 85%, 90%, 95% successively, 20 min each; (iv) dehydration in ethanol for 2 times with 45 min each; (v) sample was treated with the mix of ethanol and Propylene oxide (the ratio was 1:1) for 30 min; (vi) followed the treatment of Propylene oxide and spur resin (ratio was 3:1, 1:1, 1:3) with each different ratio for 12 hours in order; (vii) The sample was embed by spur resin, and incubation in oven for 8 hours at 40℃, followed 60℃ f or 1–2 days; (viii) the sample was chopped with ultrathin microtome, followed staining, washing, and drying; (ix) the nickel net which loaded the sample was observed with Transmission Electron Microscope JEM-1400 plus.

Mitochondrial membrane potential analysis

For the protoplasts, centrifuged the suspension at 600 g for 3 min, the pellets were resuspended in W5 solution, and diluted to appropriate concentration (no cell stacking in the light microscopy). Incubating the protoplast with 1X JC-1 staining solution (6,6'-tetrachloro-1, 1', 3,3'-tetraethylbenzimidazole-carbocyanide iodine) (1:50) (Beyotime) for 20 min, following 20 min CCCP (carbonyl cyanide m-chlorophenylhydrazone) treatment. The dual emission fluorescence was detected by the fluorescence microscopy (FV1000 confocal system). Briefly, the JC-1 monomer can be detected by 514 nm excitation wavelengths and 529 nm emission wavelengths. JC-1 aggregates can be detected by the 525 nm excitation wavelengths and 590 nm emission wavelengths. As to the fluorescent enzyme labeling detection, each sample was set three replicates, and loaded into black enzyme plate (costar), the dual fluorescence was detected by mono-photometric (multimode reader Cytation3, PengDe).

For the mitochondria, the pellets were suspended in storage buffer, and treated with CCCP for 5 min, followed incubation with 0.1X JC-1 staining solution for 5 min. The dual fluorescence was detected by mono-photometric as the same as protoplasts.

Mitochondrial complex activity detection

The activity of mitochondrial complex I, complex II, and complex IV was detected by the nanodrop 2000c using the MRC (mitochondrial respiration chain) Complex I, complex II and complex IV activity assay kit (Solarbio Life Sciences, Beijing, China). Briefly, the protoplasts were treated with rotenone (0 μg/ml, 3 μg/ml ,6 μg/ml, 9 μg/ml), malonate (0 mg/ml, 0.5 mg/ml, 2.5 mg/ml, 5 mg/ml) and sodium azide (0, 0.1%, 1%, 2%) for 30 min at 25℃, respectively. Then followed the mitochondria isolation procedure as mentioned above. All of the assays were performed according to the manufacturer's instructions.

Abbreviations

ETC: electron transfer chain; MIB: mitochondrial isolation buffer; FLB: full lysis buffer; VDAC: voltage-dependent anion channels; COX II: cytochrome C oxidase II; NAD3: NADH dehydrogenase subunit 3; CCCP: Carbonyl cyanide 3-chlorophenylhydrazone.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and materials

All the data generated or analyzed during this study are included within this article.

Competing interests

The authors declare that they have no competing interests.

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Authors contributions

JH and YHX conceived and designed the experiment, YHX, XYL, JSH, LLP, DHL, QNZ performed the experiments, YHX wrote the manuscript, HJX, ZWD, FY and JH revised the manuscript. All authors read and approved the manuscript.

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Figures

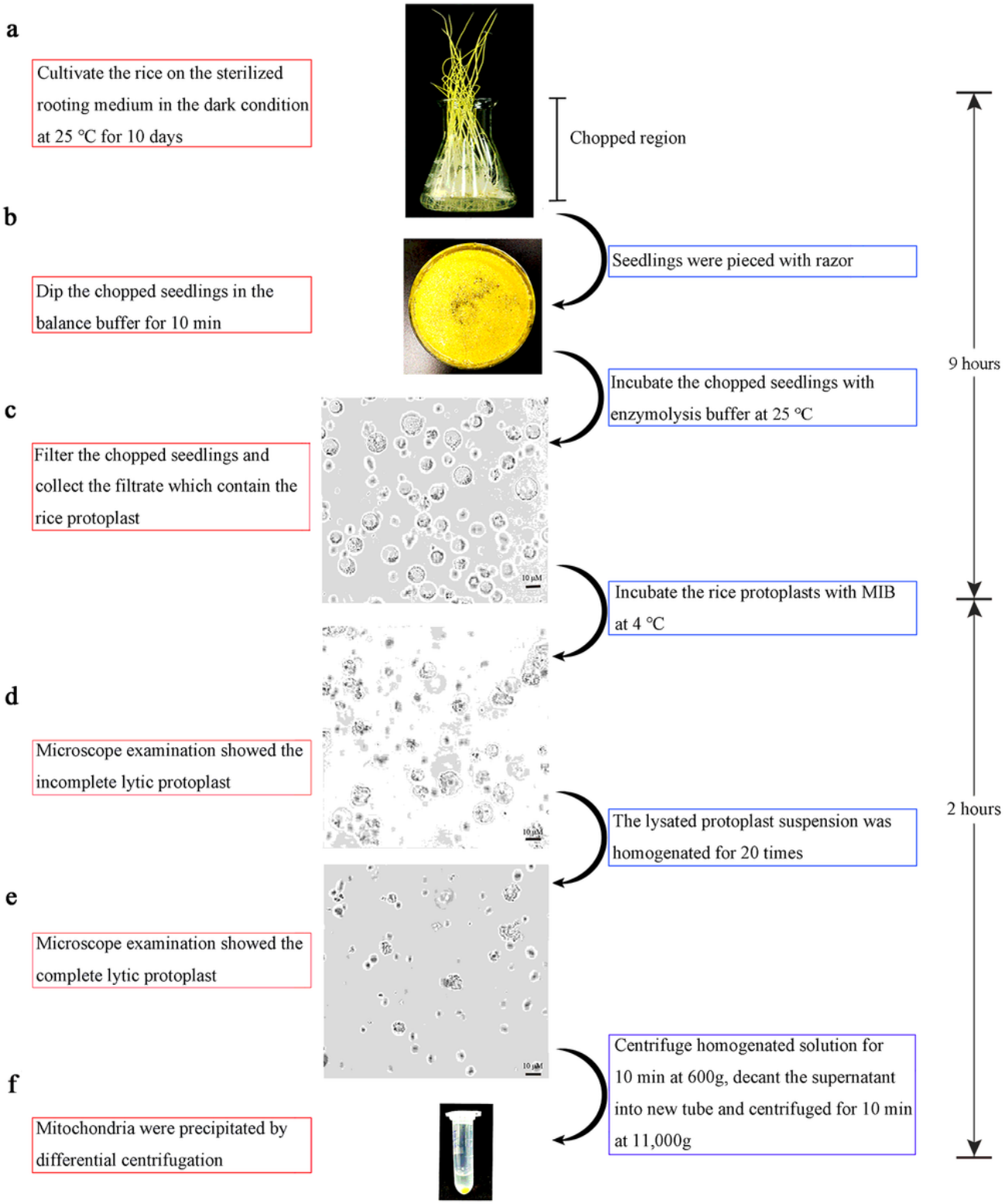

Figure 1

Procedure of mitochondria isolation from the rice seedlings. The procedure contains two parts: the protoplasts extraction (a-c) and the mitochondria isolation from protoplasts (d-f). The reverse double arrows show the times consumed by each part, MIB: mitochondria isolation buffer. Bars, 10 μm.

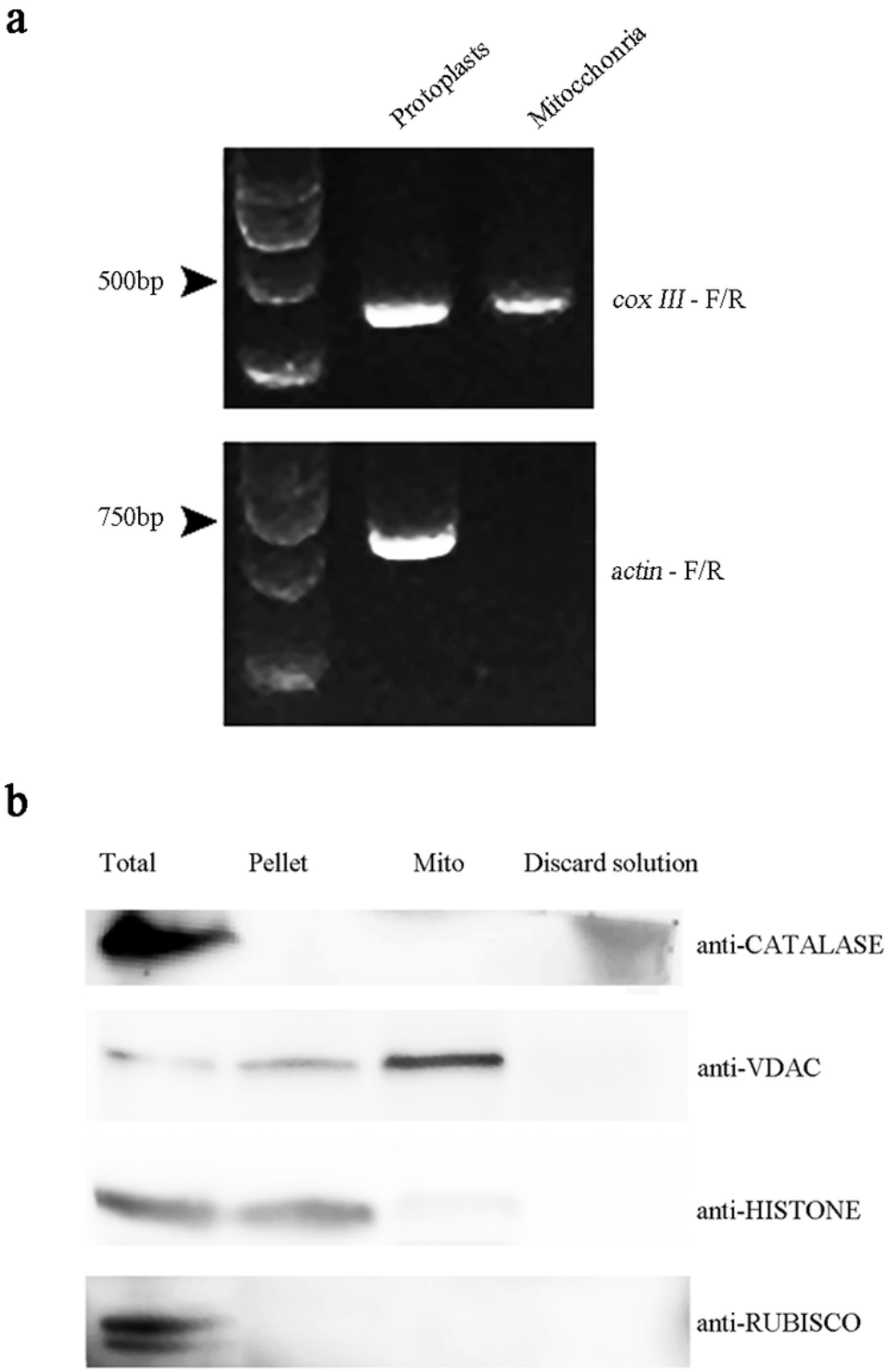

Figure 2

Mitochondria purity determination. a The existence of nucleus genome and mitochondrial genome was examined by actin and cox III, respectively. M, DNA 2000 marker. b Four components of cells were collected to the experiment. Total lane indicates the protoplast; pellet lane indicates lysates in the S9 (as mentioned in material and methods part); mito lane indicates the mitochondria and discard solution indicates the cytoplasmic which derives from the supernatant in S10 (as mentioned in material and

methods part). RUBISCO, HISTON, VDAC and CATLASE were examined as the marker protein of chloroplast, nucleus, mitochondria and peroxisome, respectively.

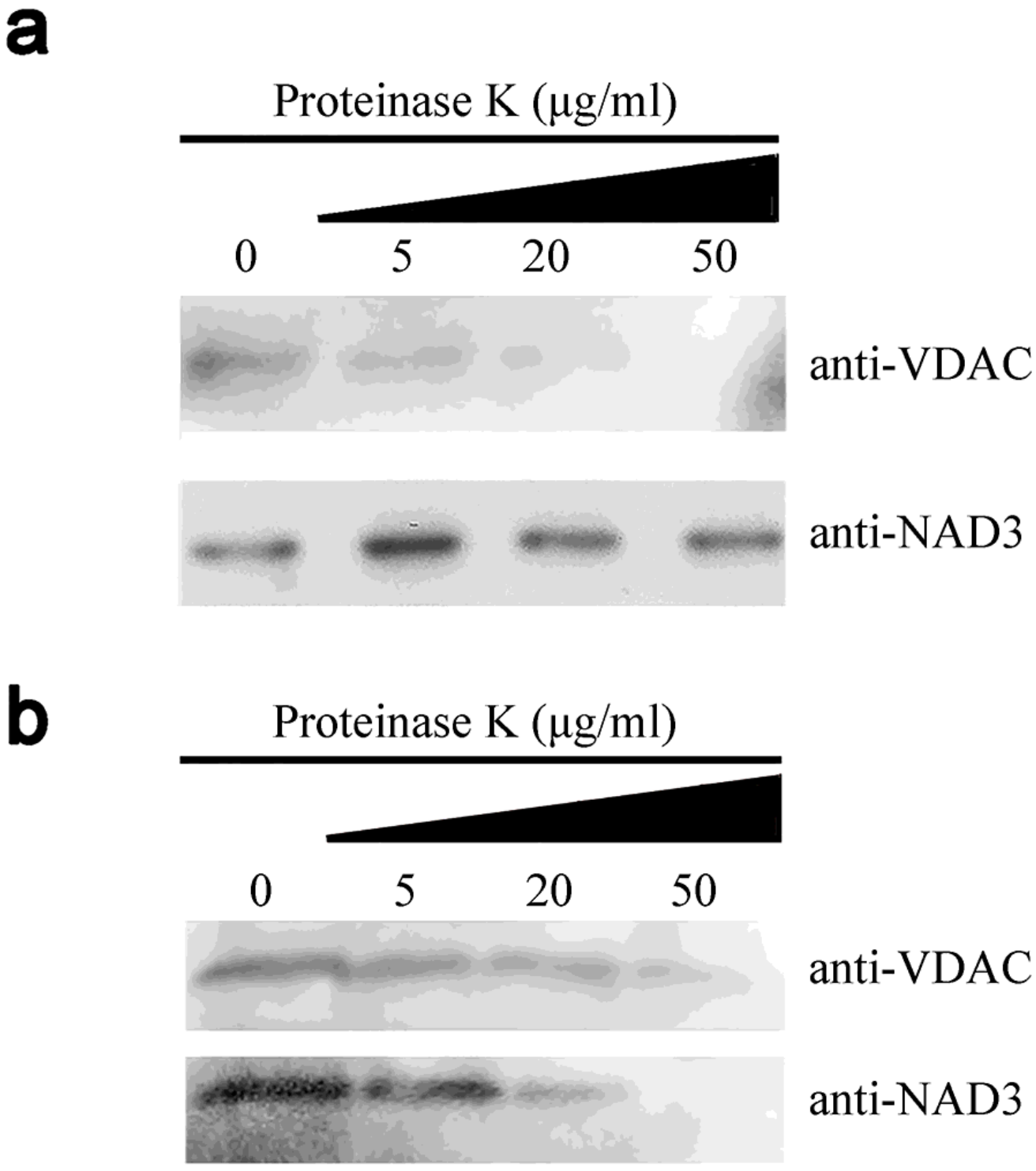

Figure 3

Mitochondria intact examination. Proteinase K was applied to digest the membrane protein of untreated mitochondrial in panel a and lysis buffer treated mitochondria in panel b. Antibody VDAC and NAD3 represent the marker proteins of mitochondrial outer membrane and inner membrane, respectively.

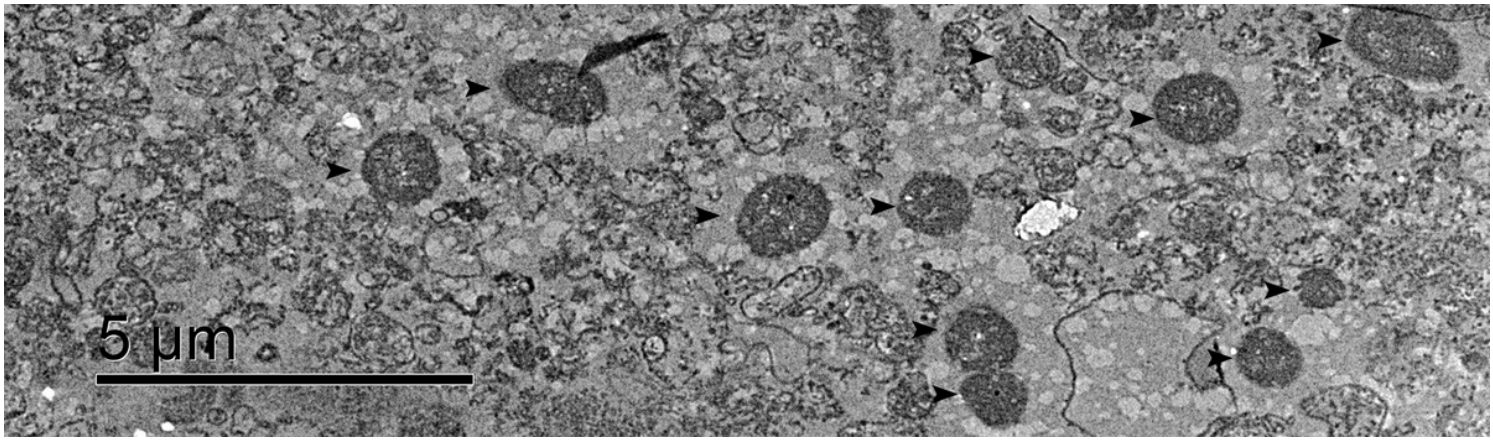

Figure 4

Mitochondria observation with the transmission electron microscopy. Isolated mitochondria were observed enriched, black arrows indicate the intact mitochondria with high electron-dense in matrix. Bars, 5μm.

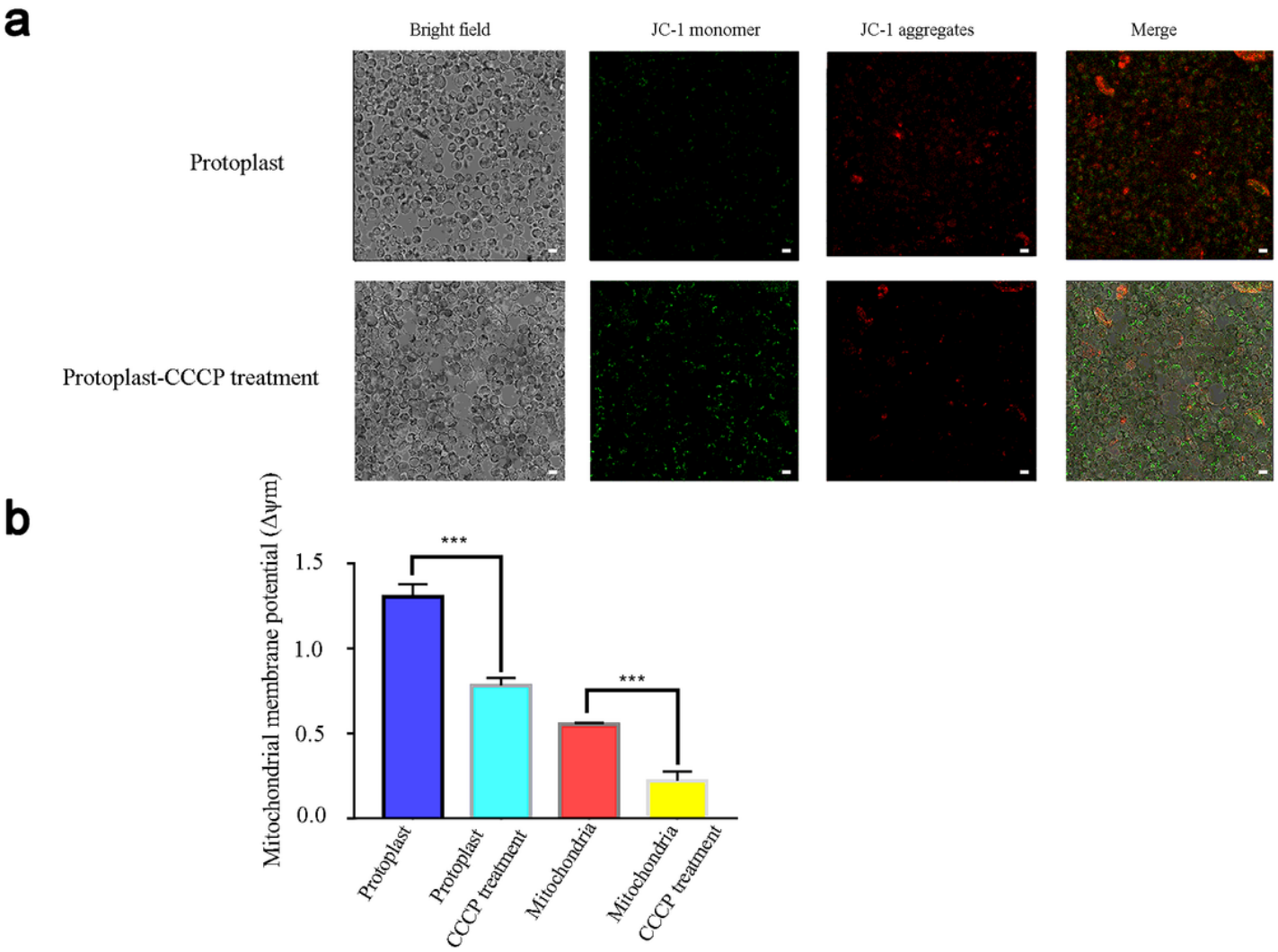

Figure 5

Mitochondrial membrane potential examination. a Mitochondrial membrane potential examination in protoplast. JC-1 monomer exhibited green signal, which indicated the inactive mitochondria. JC-1 aggregates exhibited red signal, which indicated the viable mitochondria. bars, 10μM. b The isolated mitochondrial membrane potential examination. JC-1 monomer was detected by the wavelength 514nm/529nm, JC-1 aggregates were detected by the wavelength 525nm/590nm. Error bars indicate the SDs of the means (n = 3). Significant differences were determined with student's t-test (***p<0.005).

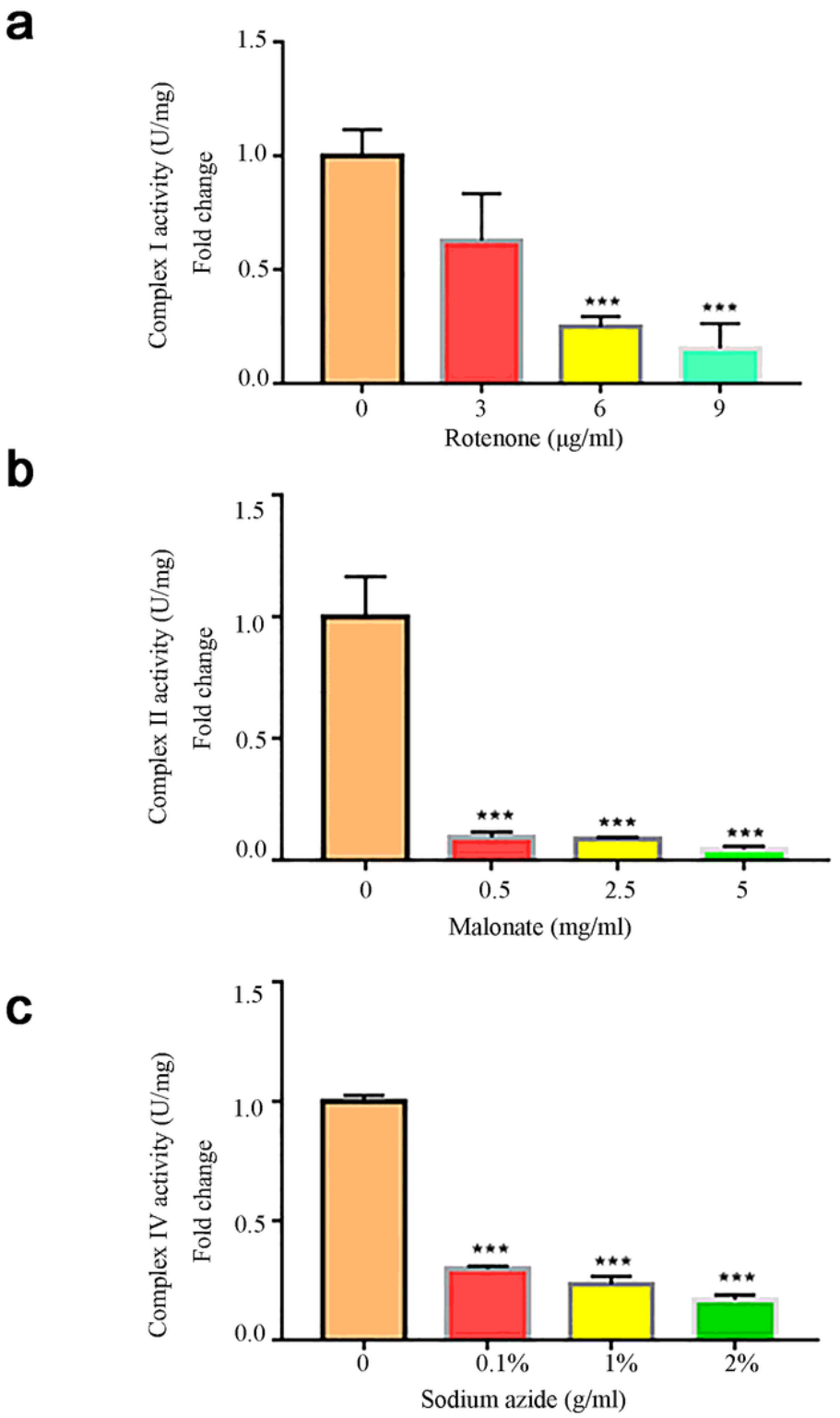

Figure 6

Mitochondrial inner membrane complex activity assay. The activities of complex I in panel a, complex II in panel b, and complex IV in panel c were tested under the treatment of the inhibitor rotenone, malonate, sodium azide, respectively. Error bars indicate the SDs of the means (n = 3), Significant differences were determined with student's t-test (***p<0.005).

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