The Differential Responses of Chlorophyll Fluorescence Parameters and Lipid Metabolism to Low Temperature between Cycas Bifida and C. panzhihuaensis

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Abstract

Background: Our previous work showed that freezing tolerance of *Cycas panzhihuaensis* was higher than that of *C. bifida*. However, the mechanisms underlying the differential freezing tolerance of the two species is not clear. Photosynthesis is one of the most temperature-sensitive processes. Lipids play important roles in membrane structure, signal transduction and energy storage which are closely related to stress response of plants. Hence, the chlorophyll fluorescence parameters and lipid profiles of the two species were characterized to explore the dynamic changes of photosynthetic activity and lipid metabolism following low temperature and subsequent recovery.

Results: The photosynthetic activity decreased significantly with the decrease of temperatures in *C. bifida*, reaching to zero after recovery, which however, was little affected in *C. panzhihuaensis*. Lipid composition of *C. bifida* was more affected by cold and freezing treatments than *C. panzhihuaensis*. Compared to the control, the proportions of all the lipid categories recovered to the original level for *C. panzhihuaensis* but those of most lipid categories changed significantly for *C. bifida* after 3 d of recovery. Particularly, the glycerophospholipids and prenol lipids of *C. bifida* degraded severely during recovery period for *C. bifida*. The changes of acyl chain length and double bond index (DBI) occurred in more lipid classes immediately after low temperatures in *C. panzhihuaensis* than those in *C. bifida*. DBI of the total main membrane lipids of *C. panzhihuaensis* was significantly higher than that of *C. bifida* following all the treatments.

Conclusions: The results of chlorophyll fluorescence parameters confirmed that the freezing tolerance of *C. panzhihuaensis* was higher than that of *C. bifida*. The lipid metabolism of the two species had differential responses to low temperatures. The homeostasis and plastic adjustment of lipid metabolism and the higher level of DBI of the main membrane lipids might contribute to the higher tolerance of *C. panzhihuaensis* to low temperature.

Background

Low temperature is a major threat to plants whose geographical distribution and development are limited. It has been shown that membrane systems are particularly sensitive to low temperature [1]. Freezing induced extracellular ice formation could lead to the membrane rupture due to the mechanical stress and dehydration of the living cells [2]. Thylakoids possess the most abundant membranes of plant leaves wherein the light-dependent reactions of photosynthesis occur. Under low temperature, the balances between light harvesting and light utilization for assimilation are liable to be broken [3], and the excessive absorbed energy can lead to oxidative stress by overproducing reactive oxygen species (ROS) [4]. The chloroplast is proven to be the main site of ROS production and ROS attack under stress [5]. Therefore, photosynthesis is extremely sensitive to cold/freezing stress in plants [6]. However, many plant species have evolved various adaptive mechanisms to minimize the negative effects of freezing temperatures [2]. Observations have demonstrated that some of the molecular, metabolic and physiological characteristics are modulated to enhance plant freezing tolerance [2, 7].

Lipids have important functions in membrane structure, signal transduction and energy storage. Lipid composition varies among species, tissues and membranes which is affected by developmental stages and environmental conditions [8, 9]. Therefore, lipid metabolism is closely related to the development and stress response of plants. Membrane is the primary site of low-temperature-induced injury in plants [10, 11]. Therefore,
the membrane properties such as integrity and temperature-compatible fluidity are crucial to maintain plant function and survival under varying temperatures [12]. Glycerophospholipids and saccharolipids are the main membrane lipids, the unsaturation level and acyl chain length of which affect the membrane fluidity [13]. To cope with the adverse effects of low temperature, lipid compositions are modulated to increase the membrane fluidity and the amount of bilayer-stabilizing lipids such as phosphatidylcholine (PC) and digalactosyldiacylglycerol (DGDG) is also increased [13, 14]. Besides membrane lipids, glycerolipids are also involved in the tolerance of plants to low temperature due to their key functions in intracellular homeostasis and energy balance [15, 16]. Studies show that the conversion of diacylglycerol (DAG) to triacylglycerol (TAG) contributes to freezing tolerance of some plant species [1].

Cycads are long considered the living fossils. Considering the primitiveness and persistence of cycads, their study is of great interest in terms of evolution and ecological adaptation of plants and global environmental changes. However, 62% of the known species of cycads are now in danger of extinction [17]. Cycas is the oldest genus of cycads which are restricted to the tropical and subtropical areas of Asia, Eastern Africa and Madagascar islands and Australia Pacific islands [18, 19]. More than 20 species of Cycas are distributed in China and all of them has been proposed as first-ranked plants for national protection in China [20]. The Cycas species are generally considered to be sensitive to low temperature. However, few studies have focused on the adaptation of these species to cold and freezing temperatures.

Cycas bifida is one of the most endangered cycads in China and the distribution of the species is restricted to some areas of Yunnan and Guangxi provinces. C. panzhihuaensis is endemic to the dry-hot valleys of the Jinsha River in southwest China and its natural distribution is in the northernmost limit areas and at the highest altitude among the Cycas species. The natural distribution of C. bifida and C. panzhihuaensis in China is restricted to subtropical zone, our previous work showed that the freezing tolerance of C. panzhihuaensis is higher than that of C. bifida [21]. However, their responses to low temperature and subsequent recovery conditions are not clear. The aim of this study was to dissect the effects of cold, freezing and subsequent recovery on photosynthetic activities and lipid metabolism of C. bifida and C. multipinnata. The results can provide theoretical bases for the freezing sensitivity and introduction of the two species.

Results

Changes of chlorophyll fluorescence parameters

Chlorophyll fluorescence is a non-invasive and highly sensitive probe in monitoring the effects of environmental stresses on photosynthesis. Fv/Fm, Y(II) and ETR of C. bifida decreased significantly with the decrease of temperature, reaching the lowest level (near zero) after 3 d of recovery (Table 1). However, Y(NO) of C. bifida presented the opposite change trend, increasing by 323.21% for the recovered seedlings compared to the control. Y(NPQ) of C. bifida decreased significantly for seedlings subjected to freezing and recovery treatments, by 75.87% and 83.49%, respectively. qP and qN of C. bifida decreased significantly for seedlings subjected to freezing treatment, then qP increased to the control level and qN increased significantly following recovery. All these suggested that cold diminished the photosynthetic activity but freezing severely damaged the photosynthetic apparatus of C. bifida. Fv/Fm of C. panzhihuaensis decreased significantly for seedlings subjected to cold, freezing and recovery treatments, by 4.96%, 5.54% and 5.07%, respectively. Y(NO) increased significantly but qN decreased significantly for seedlings subjected to freezing treatment. Except for Fv/Fm,
Y(NO) and qN, all other parameters maintained unchanged following low temperature treatments. Both Y(II) and ETR of *C. panzhihuaensis* decreased significantly but Y(NPQ) increased significantly for recovered seedlings. Fv/Fm, Y(II) and ETR of *C. panzhihuaensis* were significantly higher than those of *C. bifida* after various treatments. These results demonstrated that the photosynthesis of *C. panzhihuaensis* was slightly affected by cold and the photosynthetic apparatus was damaged under freezing to a much lesser extent than that of *C. bifida*.

**Table 1**
Effects of low temperature treatments on chlorophyll fluorescence parameters of *Cycas bifida* and *C. panzhihuaensis*

| Parameters | Plant species       | Treatment       |       |       |       |
|------------|---------------------|-----------------|-------|-------|-------|
|            |                     | Control         | Cold  | Freezing | Recovery |
| Fv/Fm      | *C. bifida*         | 0.826±0.006a    | 0.782±0.015b | 0.717±0.046c | 0.001±0.002d |
|            | *C. panzhihuaensis* | 0.857±0.010a*   | 0.824±0.004b* | 0.819±0.007b* | 0.823±0.009b* |
| Y(II)      | *C. bifida*         | 0.462±0.068a    | 0.325±0.052b | 0.152±0.040c | 0±0d |
|            | *C. panzhihuaensis* | 0.465±0.050a    | 0.421±0.044ab* | 0.427±0.018ab* | 0.383±0.026b* |
| Y(NPQ)     | *C. bifida*         | 0.315±0.084a    | 0.321±0.045a | 0.076±0.057b | 0.052±0.005b |
|            | *C. panzhihuaensis* | 0.260±0.046b    | 0.288±0.034ab | 0.226±0.030b* | 0.313±0.034a* |
| Y(NO)      | *C. bifida*         | 0.224±0.016d    | 0.354±0.014c | 0.776±0.104b | 0.948±0.005a |
|            | *C. panzhihuaensis* | 0.275±0.016b*   | 0.291±0.039b* | 0.348±0.023a* | 0.304±0.013b* |
| qp         | *C. bifida*         | 0.744±0.044a    | 0.533±0.061ab | 0.241±0.054b | 0.8±0.0447a |
|            | *C. panzhihuaensis* | 0.635±0.048a*   | 0.644±0.070a* | 0.634±0.019a* | 0.588±0.028a* |
| qN         | *C. bifida*         | 0.682±0.106b    | 0.596±0.043b | 0.134±0.100c | 0.987±0.029a |
|            | *C. panzhihuaensis* | 0.561±0.050a*   | 0.602±0.054a | 0.490±0.051b* | 0.612±0.043a* |
| ETR        | *C. bifida*         | 120.4±17.501a   | 83.6±13.390b | 38±9.925c | 0±0d |
|            | *C. panzhihuaensis* | 117.4±12.740a   | 108.6±11.127a* | 108.6±4.393a* | 94.8±6.686b* |

Different letters in the same row indicate significant differences between treatments within species and * indicates significant difference between species within treatment (P<0.05). Data are mean±standard deviation (n=4)

**Lipids profiling in the leaves of *C. bifida* and *C. panzhihuaensis***

26 lipid classes including 613 lipid species were identified from the leaves of *C. bifida* and *C. panzhihuaensis* (Additional file 1). The lipids contained two neutral glycerolipid classes, eight classes of glycerophospholipids (excluding lysophospholipids here), four of lysophospholipids, four of saccharolipids, four of sphingolipids, two of sterol lipids, one of prenol lipids (coenzyme Q) and one of fatty acyls (wax esters). The total lipid content of *C. bifida* was not significantly different from that of *C. panzhihuaensis*. Neutral glycerolipids, glycerophospholipids and saccharolipids were the main lipid categories for both the species, accounting for more than 90% of the total lipids.
Lipids (Table 2). The absolute content of neutral glycerolipids, glycerophospholipids and saccharolipids did not differ but the proportions of glycerophospholipids and saccharolipids differed significantly between the two species (Fig 1; Table 2). The ratio of saccharolipids to phospholipids was significantly higher in C. panzhihuaensis (Additional file 2). Phosphatidic acid (PA), PC, phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylinositol (PI) were the main glycerophospholipids. Monogalactosyldiacylglycerol (MGDG), DGDG and sulfoquinovosyldiacylglycerol (SQDG) were the main saccharolipids (Table 3).

Table 2
The proportion of each lipid category in Cycas bifida and C. panzhihuaensis treated with low temperatures

| Lipid class   | Plant species        | Treatment          |    |    |    |    |
|---------------|----------------------|--------------------|----|----|----|----|
|               |                      | Control            | Cold | Freezing | Recovery |
| Neutral glycerolipids | C. bifida           | 15.50±6.63b        | 7.11±1.78c | 8.76±2.04c | 23.86±1.16a |
|               | C. panzhihuaensis    | 16.72±6.93a        | 9.35±1.89b | 17.81±4.63a* | 15.12±0.80a* |
| Glycerophospholipids | C. bifida           | 31.09±5.19b        | 40.77±3.67a | 35.90±6.46ab | 21.87±2.38c |
|               | C. panzhihuaensis    | 21.99±1.03b*       | 20.58±1.84b* | 24.58±1.64a* | 21.11±1.32b |
| Saccharolipids | C. bifida           | 44.69±4.48a        | 44.02±5.24a | 46.47±4.19a | 41.47±2.81a |
|               | C. panzhihuaensis    | 54.86±7.14b*       | 65.25±1.91a* | 50.55±5.31b | 55.87±2.26b* |
| Lysophospholipids | C. bifida           | 1.92±0.94a         | 3.06±0.92a | 3.06±0.68a | 2.15±0.28a |
|               | C. panzhihuaensis    | 0.51±0.28a*        | 0.59±0.14a* | 0.43±0.29a* | 0.76±0.15a* |
| Sphingolipids  | C. bifida           | 2.89±0.79b         | 2.25±1.05b | 2.27±0.45b | 6.03±1.19a |
|               | C. panzhihuaensis    | 2.17±0.50a         | 1.60±0.24a | 2.25±0.59a | 2.27±0.61a* |
| Sterol lipids  | C. bifida           | 1.51±0.39b         | 1.30±0.39b | 1.85±0.22b | 3.27±0.73a |
|               | C. panzhihuaensis    | 1.48±0.52a         | 1.29±0.75a | 1.72±0.47a | 2.30±0.43a* |
| Prenol lipids  | C. bifida           | 1.97±0.64a         | 1.44±0.34a | 1.53±0.25a | 0.81±0.18b |
|               | C. panzhihuaensis    | 2.08±0.45a         | 1.17±0.35b | 2.17±0.53a* | 2.05±0.18a* |
| Fatty acyls    | C. bifida           | 0.40±0.10a         | 0.04±0.02b | 0.17±0.04b | 0.55±0.24a |
|               | C. panzhihuaensis    | 0.19±0.08b*        | 0.17±0.03b* | 0.51±0.20a* | 0.15±0.04b* |

Different letters in the same row indicate significant differences between treatments within species and * indicates significant difference between species within treatment (P<0.05). Data are mean±standard deviation (n=5)
Changes of the composition of the lipid categories

For *C. bifida*, the content of total lipid, glycerophospholipids, lysophospholipids, saccharolipids, prenol lipids and sterol lipids increased after cold and freezing treatments. However, glycerophospholipids and prenol lipids decreased significantly and sterol lipids increased significantly after 3 d of recovery compared to those of the control (Fig. 1). The content of fatty acyls decreased significantly after cold treatment but recovered to the original level after freezing and recovery treatments. The ratio of saccharolipids to glycerophospholipids of *C. bifida* increased significantly after recovery treatments which was mainly due to the severe degradation of glycerophospholipids (Additional file 2). It also suggested that glycerophospholipids of *C. bifida* were more easily affected by freezing temperature than saccharolipids. Compared to those of the control in *C. bifida*, the relative contents of all the lipid categories except saccharolipids, lysophospholipids and fatty acyls changed significantly after recovery (Table 2). This showed that the lipid metabolic balances of *C. bifida* were disturbed by low temperatures. For *C. panzhihuaensis*, the content of total lipids and saccharolipids increased significantly after cold treatment and fatty acyls increased significantly after freezing treatment (Fig. 1). The ratio of saccharolipids to glycerophospholipids of *C. panzhihuaensis* increased significantly after cold treatment which was mainly due to the accumulation of saccharolipids (Additional file 2). Although the relative contents of neutral glycerolipids, glycerophospholipids, saccharolipids, prenol lipids and fatty acyls changed significantly following cold or freezing treatment, they all returned to the control level after recovery (Table 2). This showed that the lipid metabolism of *C. panzhihuaensis* could positively respond to low temperature stress and recover to the normal state after recovery. It can also be seen that freezing and subsequent thawing did not disturb the lipid metabolic system.

Compared between the two species, the contents of glycerophospholipids and lysophospholipids were significantly lower and those of saccharolipids and fatty acyls were significantly higher in *C. panzhihuaensis* after cold treatment. The contents of total lipids, glycerophospholipids, lysophospholipids, sphingolipids and sterol lipids were significantly lower and those of neutral glycerolipids and fatty acyls were significantly higher in *C. panzhihuaensis* following freezing treatment. After recovery treatment, the contents of total lipids, glycerophospholipids, saccharolipids and prenol lipids were significantly higher and those of lysophospholipids and sphingolipids were significantly lower in *C. panzhihuaensis*.

Changes of the composition of main lipid classes and lipid species of glycerophospholipids and saccharolipids

Phosphatidylinositol (PIP) and cardiolipin (CL) were also detected but their contents were extremely low (Additional file 3). Therefore, the two lipid classes were not analyzed separately but included in the analysis of total glycerophospholipids. PA, PC, PE, PG, PI and PS contents of *C. bifida* increased significantly after cold and/or freezing treatments (Table 3). However, PA, PC, PG and PI contents recovered to the original level and PE and PS contents decreased significantly after 3 d of recovery. Compared to those of the control, PC and PE contents of *C. panzhihuaensis* increased significantly after cold treatment (Table 3). PG content decreased significantly but PI content increased significantly after freezing treatment. After recovery, PG content increased significantly compared to that of the freezing-treated seedlings but did not reach the original level. PA, PG and PI contents of *C. panzhihuaensis* were significantly lower than those of *C. bifida* after cold. PA, PC, PE and PG contents of *C. panzhihuaensis* were significantly lower than those of *C. bifida* after freezing treatment. After
recovery, PG, PI and PS contents of *C. panzhihuaensis* were significantly higher than those of *C. bifida*. Among the main lipid species of the several lipid classes, there were significantly different responses of the two species to various treatments in all the PA species except 32:0, 34:1, 34:2, 34:3 and 43:2; all the PC species except 35:3, 35:4, 37:3, 37:4 and 37:5; all the PE species except 36:4 and 47:4; all the PG species except 30:2, 34:5, 36:3, 36:4, 44:1 and 46:1; PI species 33:2, 34:1, 36:3, 49:3, 50:2, 50:3 and 51:4; PS species 33:0, 39:4 and 40:8 (Fig. 2).

### Table 3
The content of each main membrane lipid class in *Cycas bifida* and *C. panzhihuaensis* treated with low temperatures

| Lipid class | Plant species | Control     | Cold          | Freezing      | Recovery     |
|-------------|---------------|-------------|---------------|---------------|--------------|
| PA          | *C. bifida*   | 0.74±0.41b  | 1.81±0.59a    | 1.27±0.30ab   | 0.26±0.06b   |
|             | *C. panzhihuaensis* | 0.30±0.16a | 0.42±0.06a*   | 0.36±0.05a*   | 0.35±0.07a   |
| PC          | *C. bifida*   | 0.21±0.09b  | 0.46±0.17a    | 0.42±0.08a    | 0.13±0.02b   |
|             | *C. panzhihuaensis* | 0.26±0.15b | 0.41±0.01a    | 0.15±0.02b*   | 0.22±0.12b   |
| PE          | *C. bifida*   | 0.10±0.01c  | 0.22±0.04a    | 0.17±0.02b    | 0.06±0.00d   |
|             | *C. panzhihuaensis* | 0.12±0.06b | 0.19±0.04a    | 0.09±0.02b*   | 0.10±0.04b   |
| PG          | *C. bifida*   | 0.27±0.09b  | 0.52±0.17a    | 0.49±0.05a    | 0.18±0.02b   |
|             | *C. panzhihuaensis* | 0.32±0.04a | 0.28±0.04ab*  | 0.19±0.03c*   | 0.25±0.02b*   |
| PI          | *C. bifida*   | 0.45±0.15b  | 0.85±0.21ab   | 1.06±0.04a    | 0.23±0.05b   |
|             | *C. panzhihuaensis* | 0.47±0.10b | 0.57±0.08ab*  | 0.78±0.30a    | 0.37±0.08b*   |
| PS          | *C. bifida*   | 0.03±0.01b  | 0.04±0.01a    | 0.04±0.00ab   | 0.02±0.00c   |
|             | *C. panzhihuaensis* | 0.03±0.01a | 0.04±0.00a    | 0.04±0.01a    | 0.04±0.00a*   |
| MGMG        | *C. bifida*   | 0.04±0.01b  | 0.07±0.02a    | 0.07±0.02a    | 0.04±0.01b   |
|             | *C. panzhihuaensis* | 0.08±0.05b | 0.17±0.05a*   | 0.07±0.02b    | 0.08±0.02b*   |
| MGDG        | *C. bifida*   | 0.50±0.10b  | 1.15±0.57a    | 1.03±0.22a    | 0.55±0.10b   |
|             | *C. panzhihuaensis* | 1.25±0.76b | 2.64±0.35a*   | 0.65±0.21b*   | 1.02±0.46b   |
| DGDG        | *C. bifida*   | 0.56±0.07c  | 1.13±0.23b    | 1.36±0.11a    | 0.09±0.01d   |
|             | *C. panzhihuaensis* | 0.80±0.24b | 1.39±0.17a    | 0.84±0.45b    | 0.84±0.41b*   |
| SQDG        | *C. bifida*   | 1.53±0.36b  | 1.99±0.36a    | 2.00±0.20a    | 1.04±0.13c   |
|             | *C. panzhihuaensis* | 1.72±0.39a | 1.98±0.17a    | 1.91±0.50a    | 1.69±0.41a*   |
| Total       | *C. bifida*   | 4.46±0.99b  | 8.25±2.34a    | 7.94±0.90a    | 2.63±0.28b   |
|             | *C. panzhihuaensis* | 5.37±1.86b | 8.12±0.54a    | 5.14±1.61b*   | 5.00±0.76b*   |
Different letters in the same row indicate significant differences between treatments within species and * indicates significant difference between species within treatment (P<0.05). Data are mean±standard deviation (n=5)

Compared to those of the control, the content of each class of saccharolipids in *C. bifida* increased significantly after cold and freezing treatments (Table 3). Monogalactosylmonoacylglycerol (MGMG) and MGDG then returned to the original level but DGDG and SQDG decreased by 83.93% and 32.03% respectively after 3 d of recovery compared to the control. The results showed that the equilibrium of saccharolipid metabolism of *C. bifida* was broken due to the degradation of DGDG and SQDG at the recovery stage. For *C. panzhihuaensis*, SQDG content did not vary among the control and different treatments (Table 3). MGMG, MGDG and DGDG only responded to cold treatment, increasing by 112.5%, 111.2%, 73.75%, respectively. Compared to those of *C. bifida*, both MGMG and MGDG contents of *C. panzhihuaensis* were significantly higher for the cold-treated seedlings; MGDG content was significantly lower for the freezing-treated seedlings; MGMG, DGDG and SQDG content were significantly higher for the recovered seedlings. Among the main lipid species of the several lipid classes, there were significantly different responses of the two species to various treatments in MGMG species 18:3; MGDG species 34:2, 34:3, 34:4, 34:5, 34:6, 35:3, 36:2, 36:3, 36:4, 36:6 and 38:6; all the DGDG species except 31:3, 35:3, 36:5, 40:0 and 42:1; SQDG species 36:6, 38:7, 38:9, 41:8 and 44:8 (Fig. 3).

**Changes of the ACL and DBI of glycerophospholipids and saccharolipids**

The ACL of total glycerophospholipids, PE and PI of *C. bifida* were not affected by various treatments (Table 4). Although the ACL of PA and PC was not affected by low temperature but increased significantly after recovery. The ACL of PG decreased significantly after low temperature treatments but then increased to the control level after recovery. The ACL of PS increased significantly after low temperature and recovery treatments. *C. panzhihuaensis* showed significantly different responses to various treatments with *C. bifida* in the ACL of PC, PE, PI and PS (Table 4). The ACL of PA and PE decreased and that of PS increased significantly after cold treatments. The ACL increased significantly for PC, PE, PI and total glycerophospholipids and decreased for PA and PS after freezing treatments. Compared to those in *C. bifida*, the ACL of most glycerophospholipid classes in *C. panzhihuaensis* were higher following cold or/and freezing treatments but that of PS was lower following freezing. The ACL of total glycerophospholipids of *C. panzhihuaensis* was significantly higher than that of *C. bifida* after low temperature treatments (Table 4).
Table 4
Acyl chain length (ACL) of each main membrane lipid class in *Cycas bifida* and *C. panzhihuaensis* treated with low temperatures

| Lipid class/category | Plant species                        | Control       | Cold          | Freezing       | Recovery       |
|----------------------|--------------------------------------|---------------|---------------|----------------|----------------|
| PA                   | *C. bifida*                          | 35.26±0.67b   | 35.03±0.10b   | 35.08±0.08b   | 35.91±0.51a   |
|                      | *C. panzhihuaensis*                   | 36.36±0.62a*  | 35.73±0.16b*  | 35.70±0.21b*  | 36.00±0.32ab  |
| PC                   | *C. bifida*                          | 34.99±0.27b   | 34.89±0.11b   | 34.99±0.06b   | 35.50±0.09a   |
|                      | *C. panzhihuaensis*                   | 35.13±0.07b   | 35.11±0.04b*  | 35.33±0.10a*  | 35.12±0.17b*  |
| PE                   | *C. bifida*                          | 39.20±4.61a   | 36.26±0.37a   | 36.37±0.16a   | 37.61±0.98a   |
|                      | *C. panzhihuaensis*                   | 39.33±2.02b   | 37.07±0.81c   | 44.68±1.23a*  | 40.04±1.75b*  |
| PG                   | *C. bifida*                          | 34.73±0.78a   | 33.64±0.19b   | 33.77±0.07b   | 35.28±0.55a   |
|                      | *C. panzhihuaensis*                   | 36.20±1.43a   | 35.06±0.58a*  | 35.93±0.22a*  | 36.02±0.65a   |
| PI                   | *C. bifida*                          | 35.85±1.97ab  | 34.41±0.40b   | 34.96±0.63ab  | 36.51±0.25a   |
|                      | *C. panzhihuaensis*                   | 35.21±0.83b   | 36.02±0.54b*  | 38.27±0.47a*  | 36.06±0.72b   |
| PS                   | *C. bifida*                          | 33.11±0.02b   | 33.40±0.11a   | 33.40±0.18a   | 33.32±0.04a   |
|                      | *C. panzhihuaensis*                   | 33.44±0.28b   | 33.82±0.15a*  | 33.10±0.03c*  | 33.29±0.25bc  |
| Phospholipids        | *C. bifida*                          | 36.04±1.83a   | 34.81±0.12a   | 35.03±0.22a   | 36.43±0.48a   |
|                      | *C. panzhihuaensis*                   | 36.25±0.10b   | 36.03±0.24b*  | 38.06±0.52a*  | 36.49±0.47b   |
| MGMG                 | *C. bifida*                          | 17.23±0.34a   | 17.41±0.42a   | 17.50±0.22a   | 17.15±0.08a   |
|                      | *C. panzhihuaensis*                   | 17.41±0.12b   | 17.70±0.14a   | 17.49±0.12ab  | 17.64±0.24a   |
| MGDG                 | *C. bifida*                          | 35.22±0.53a   | 35.19±0.16a   | 35.30±0.07a   | 35.04±0.07a   |
|                      | *C. panzhihuaensis*                   | 35.41±0.25b   | 35.26±0.05b   | 35.78±0.03a*  | 35.66±0.07a*  |
| DGDG                 | *C. bifida*                          | 34.54±0.23a   | 34.43±0.24a   | 34.63±0.18a   | 33.57±0.46b   |
|                      | *C. panzhihuaensis*                   | 34.71±0.07c   | 34.96±0.05a*  | 34.87±0.02b*  | 34.92±0.10ab* |
| SQDG                 | *C. bifida*                          | 39.68±0.36a   | 39.69±0.30a   | 39.61±0.04a   | 39.93±0.01a   |
|                      | *C. panzhihuaensis*                   | 39.71±0.11a   | 39.77±0.11a   | 39.29±0.30b   | 39.76±0.06a*  |
ACL = (∑ [n × mol % lipid]) / 100, where n is the number of acyl carbons in each lipid molecule. Different letters in the same row indicate significant differences between treatments within species and * indicates significant difference between species within treatment (P<0.05). Data are mean±standard deviation (n=5).

Except for decreasing significantly in DGDG after recovery, the ACL of other classes of saccharolipids in *C. bifida* did not vary with the treatments (Table 4). The ACL of total saccharolipids of *C. bifida* showed no significant change after various treatments. For *C. panzhihuaensis*, the ACL of MGMG, MGDG and DGDG increased significantly after cold or/and freezing treatment and that of SQDG decreased significantly after freezing treatment (Table 4). Except for SQDG, the ACL of all the classes of saccharolipids increased significantly after 3 d of recovery, compared to those of the control. The ACL of total saccharolipids in *C. panzhihuaensis* showed significant decrease after cold treatment, which was significantly lower than that of *C. bifida*. The ACL of the total main membrane lipids (glycerophospholipids and saccharolipids) decreased significantly after low temperature treatments in *C. bifida* and increased significantly after freezing treatment in *C. panzhihuaensis*, being significantly higher than that of *C. bifida*.

Except for increasing significantly in PS after low temperature treatments, DBI of every glycerophospholipid class and total glycerophospholipids in *C. bifida* did not change (Table 5). For *C. panzhihuaensis*, DBI of all the glycerophospholipid classes increased significantly after cold treatment and those of PG and PI also increased significantly after freezing and recovery treatments but the unsaturation level of PS decreased significantly after freezing treatment. The DBI of total glycerophospholipids in *C. panzhihuaensis* increased significantly after cold treatment. Compared to those of *C. bifida*, the DBI of all the glycerophospholipid classes except PG of *C. panzhihuaensis* were significantly higher in cold-treated seedlings, which of PA, PE, PG and PI were significantly higher in freezing-treated and recovered seedlings (Table 5). The DBI and DBI/ACL of total glycerophospholipids of *C. panzhihuaensis* was significantly higher than that of *C. bifida* after various treatments (Table 5; Additional file 3).
Table 5
Double bond index (DBI) of each main membrane lipid class in *Cycas bifida* and *C. panzhihuaensis* treated with low temperatures

| Lipid class/category | Plant species         | Control | Cold     | Freezing | Recovery |
|----------------------|-----------------------|---------|----------|----------|----------|
| PA                   | *C. bifida*           | 1.57±0.40a | 1.48±0.09a | 1.53±0.05a | 1.60±0.23a |
|                      | *C. panzhihuaensis*   | 1.87±0.12b | 2.02±0.13a* | 1.81±0.07b* | 1.95±0.11ab* |
| PC                   | *C. bifida*           | 2.03±0.30a | 2.26±0.32a | 2.19±0.21a | 2.70±0.35a |
|                      | *C. panzhihuaensis*   | 2.84±0.37b* | 3.69±0.14a* | 2.43±0.16b | 2.55±0.49b |
| PE                   | *C. bifida*           | 2.43±0.38a | 2.40±0.22a | 2.34±0.08a | 2.57±0.09a |
|                      | *C. panzhihuaensis*   | 3.08±0.23b* | 3.53±0.22a* | 2.92±0.10b* | 2.98±0.29b* |
| PG                   | *C. bifida*           | 1.74±0.23a | 1.75±0.19a | 1.82±0.10a | 1.71±0.06a |
|                      | *C. panzhihuaensis*   | 1.69±0.18c | 1.97±0.09ab | 2.02±0.03a* | 1.85±0.06b* |
| PI                   | *C. bifida*           | 1.65±0.33a | 1.51±0.08a | 1.59±0.06a | 1.48±0.06a |
|                      | *C. panzhihuaensis*   | 1.94±0.08b | 2.05±0.08a* | 2.09±0.06a* | 2.06±0.04a* |
| PS                   | *C. bifida*           | 0.10±0.02b | 0.37±0.13a | 0.36±0.16a | 0.29±0.04ab |
|                      | *C. panzhihuaensis*   | 0.39±0.25b | 0.80±0.18a* | 0.10±0.04c* | 0.25±0.20bc |
| Phospholipids        | *C. bifida*           | 1.70±0.35a | 1.67±0.12a | 1.72±0.10a | 1.80±0.06a |
|                      | *C. panzhihuaensis*   | 2.10±0.19b | 2.50±0.14a* | 2.06±0.05b* | 2.13±0.20b* |
| MGMG                 | *C. bifida*           | 1.58±0.49a | 1.92±0.67a | 2.03±0.39a | 1.44±0.13a |
|                      | *C. panzhihuaensis*   | 1.93±0.18b | 2.44±0.22a | 2.02±0.23b | 2.29±0.40ab* |
| MGDG                 | *C. bifida*           | 4.53±1.07a | 4.70±0.41a | 4.80±0.26a | 4.08±0.14a |
|                      | *C. panzhihuaensis*   | 5.27±0.13c | 5.46±0.08b* | 5.77±0.08a* | 5.51±0.13b* |
| DGDG                 | *C. bifida*           | 2.93±0.44b | 2.82±0.26b | 3.11±0.33b | 3.69±0.12a |
|                      | *C. panzhihuaensis*   | 3.35±0.18c | 3.88±0.18a* | 3.49±0.06bc* | 3.56±0.13b |
| SQDG                 | *C. bifida*           | 6.96±0.03a | 6.95±0.03a | 6.95±0.01a | 6.97±0.00a |
|                      | *C. panzhihuaensis*   | 6.89±0.09a | 6.95±0.03a | 6.94±0.01a | 6.95±0.01a* |
| Saccharolipids       | *C. bifida*           | 5.56±0.34ab | 5.32±0.24b | 5.22±0.06b | 5.75±0.09a |
|                      | *C. panzhihuaensis*   | 5.55±0.12a | 5.51±0.02a | 5.84±0.17a* | 5.70±0.36a |
| Total                | *C. bifida*           | 3.99±0.46ab | 3.55±0.16b | 3.69±0.30b | 4.39±0.17a |
|                      | *C. panzhihuaensis*   | 4.55±0.07b | 4.79±0.09a* | 4.59±0.06b* | 4.72±0.20ab* |
DBI=$\Sigma(N \times \text{mol\% lipid})/100$, where $N$ is the number of double bonds in each lipid molecule. Different letters in the same row indicate significant differences between treatments within species and * indicates significant difference between species within treatment ($P<0.05$). Data are mean±standard deviation ($n=5$).

Except that DBI of DGDG increased significantly after recovery, the DBI of every classes of saccharolipids and total saccharolipids in *C. bifida* were not affected by low temperatures. For *C. panzhihuaensis*, the DBI of MGDG in low-temperature-treated seedlings and those of MGMG and DGDG in cold-treated seedlings increased significantly compared to the control. Compared to those of *C. bifida*, the DBI of MGDG and DGDG in low-temperature-treated seedlings of *C. panzhihuaensis* and that of total saccharolipids in freezing-treated seedlings were significantly higher. The DBI and DBI/ACL of total main membrane lipids was significantly higher in *C. panzhihuaensis* than that in *C. bifida* following low temperature and recovery treatments (Table 5; Additional file 3).

**Discussion**

Low temperature is one of the key factors limiting the introduction of tropical and subtropical plants including cycads to the areas at higher latitude and altitude. Our previous study showed that the freezing tolerance of *C. panzhihuaensis* was higher than that of *C. bifida* [21]. However, there is still a lack of systematic and in-depth study on the adaptation of the two species to low temperature. Lipids play key roles in diverse cellular processes and lipid metabolism is closely related to freezing tolerance of some plants [1, 22]. However, how they adjust under low temperature to regulate the tolerance of *C. panzhihuaensis* and *C. bifida* to low temperature stress are poorly understood. Photosynthesis is one of the most temperature-sensitive processes [6]. Therefore, it is often used to reflect the adaptability of plants to temperature change. In the present study, the chlorophyll fluorescence parameters and lipid profiles of the two species subjected to cold, freezing and subsequent recovery were characterized.

**The reduction and loss of photosynthetic activities**

Chlorophyll fluorescence could sensitively reflect the physiological status of plants. The significant decrease of Fv/Fm, Y(II) and ETR and significant increase of Y(NO) with the decrease of temperatures in *C. bifida* demonstrated that photosynthetic activities of *C. bifida* were affected, particularly severely by freezing treatment. That Fv/Fm, Y(II) and ETR reached to zero after 3 d of recovery suggested the photosynthetic apparatuses of *C. bifida* were severely damaged and cannot recover. For *C. panzhihuaensis*, only Fv/Fm of the cold-treated seedlings decreased by 3.85% and Fv/Fm, Y(II) and ETR of the recovered seedlings decreased only by 3.97%, 17.63% and 19.25% respectively, compared to those of the control. These showed that *C. panzhihuaensis* were relatively little affected by cold and freezing temperatures in comparison with *C. bifida*. The damaging effects of freezing on plant morphology might not appear immediately after treatments, which however, can be more obvious after a period of recovery. According to our observations, leaves of *C. bifida* gradually became yellow and dry but those of *C. panzhihuaensis* maintained green after 10 d of recovery. These results confirmed that *C. bifida* was more sensitive to low temperatures than *C. panzhihuaensis*.

**The changes of composition of the lipid categories**
The metabolism of neutral glycerolipids are affected by low temperature which are related to the tolerance of plants to low temperature [22]. For example, DAG and TAG accumulated and DAG/TAG ratio decreased under freezing in Arabidopsis [14, 22]. It has been reported that the accumulation of TAG due to the conversion of DAG contributes to the freezing tolerance of plants [1, 22]. However, the neutral glycerolipid content maintained unchanged and the DAG-TAG ratios of the treated seedlings were not significantly different from the control for both the species (Fig. 1; Additional file 3). These showed that the freezing sensitivity of the two species had little relation with the neutral glycerolipid metabolism under freezing. Glycerophospholipids and saccharolipids are the main extraplastidic and plastidic membrane lipids respectively in plants. Some findings suggested that the two categories of lipids degraded under low temperatures [13, 23]. However, the glycerophospholipids and saccharolipids contents increased significantly after cold and freezing treatments in C. bifida and saccharolipids increased after cold treatment in C. panzhihuaensis. The sources of these accumulated lipids under low temperatures were not clear. As the metabolic pathways of carbohydrates and lipids undergo cross talk to regulate energy homeostasis [24], whether the increased lipids are ascribed to the conversion of the stored carbohydrates needs to be verified. For all the treatments, the saccharolipids/glycerophospholipids ratio of C. panzhihuaensis was always significantly higher than that of C. bifida (Additional file 2). Whether this was related to the higher freezing tolerance of C. panzhihuaensis was not clear.

Except that saccharolipid and fatty acyl content increased significantly following cold and freezing treatment respectively, the absolute contents of all the lipid categories of C. panzhihuaensis did not change after various treatments (Fig. 1). For C. bifida, the absolute contents of all the lipid categories except neutral glycerolipids and sphingolipids varied with the treatments to different extent (Fig. 1). The results suggested that lipid metabolism of C. bifida was more affected by cold and freezing treatments than C. panzhihuaensis. The proportion of some lipid categories changed after low temperature treatments for both C. bifida and C. panzhihuaensis (Table 2). However, the proportions of all the lipid categories recovered to the original level after 3 d of recovery for C. panzhihuaensis, which of most lipid categories changed significantly for C. bifida in comparison with the control. This showed the plastic adjustment of lipid metabolism in C. panzhihuaensis which might be related to the more tolerance of the species to low temperature. Phospholipids are major structural components of cell membranes and play roles in signal transduction and energy storage [25]. Prenol lipids (coenzyme Q here) are essential for energy metabolism in the electron transport system and also function as antioxidants within membrane systems [26]. The disorders of lipid metabolism after recovery such as the degradation of phospholipids and prenol lipids might contribute to the ultimate death of aboveground parts of C. bifida seedlings.

Lysophospholipids, sphingolipids and sterols are not only the structural components of membranes but also important signaling moleculars involved in plant development and environmetal responses [27-29]. It has been reported that the accumulation of lysophospholipids and sphingolipids under stresses might be detrimental to the cells [30, 31]. Besides sterol contents, the contents of lysophospholipids and sphingolipids of C. bifida were significantly higher than those of C. panzhihuaensis. Whether the differential tolerance of the two species to freezing is related to their different contents and change patterns of these lipids needs to be explored. Cuticular waxes are the primary structures of the cuticle and play crucial roles in plant defense against biotic and abiotic stress including drought and frost [32]. The significantly higher content of wax esters of C. panzhihuaensis following cold and freezing treatments might contribute to its higher freezing tolerance than C. bifida.
The changes of composition of main lipid classes and lipid species of glycerophospholipids and saccharolipids

Increasing evidence suggests that PA can form nonbilayer lipid structure with MGDG or DAG during low temperature, disrupting the integrity of cell membrane [33, 34]. Some studies showed that PA content increased dramatically under stresses including freezing [13, 33]. PA content maintained unchanged in C. panzhihuaensis after various treatments but increased by 144.59% and 71.62% in C. bifida after cold and freezing treatments, respectively (Table 3). The maintenance of PA content in C. panzhihuaensis was conducive to keep the membrane stability but the increase of PA in C. bifida after low temperature treatments might pose potential threat to membrane integrity. Meanwhile, the significantly lower level of PA after cold and freezing treatments might confer higher freezing tolerance to C. panzhihuaensis.

Some studies showed that glycerophospholipid composition of various plants presented different responses to stresses [9, 13, 35]. For C. bifida, the content of each glycerophospholipid class increased significantly after cold or freezing treatment but the PE, PS and total glycerophospholipid contents decreased significantly after 3 d of recovery (Fig. 1; Table 3). These results suggested that glycerophospholipid metabolism of C. bifida was dramatically affected by low temperature and the membrane was severely damaged following freezing treatment. For C. panzhihuaensis, different classes of glycerophospholipids showed different change trends following low temperature treatments. However, all the classes except for PG recovered to the original level. It demonstrated that PG was more sensitive to freezing temperature than other phospholipids in C. panzhihuaensis. Studies have suggested that the level of high-melting-point PG moleculars such as 32:0 and 32:1 are related to the sensitivity of plants to low temperatures [36, 37]. The high-melting-point PG moleculars were much lower in C. panzhihuaensis and showed decrease and increase after low temperature for C. panzhihuaensis and C. bifida, respectively (Fig. 2). This might be an important factor underlying the difference of freezing tolerance between the two species.

Saccharolipids are the main lipids of chloroplast envelope and thylakoid membrane which play key roles in the photosynthetic process [38]. Studies have suggested that these lipids are likely to tend to be degraded under some stresses [9, 39]. However, all the classes of saccharolipids in C. bifida increased significantly after low temperature treatments and all with the exception of DGDG and SQDG recovered to the control level after recovery (Table 3). The increase of saccharolipids after low temperatures might imply that seedlings of C. bifida positively resist the adverse effects of cold and freezing stresses on photosynthetic apparatus by stabilizing plastidic membranes. High DGDG/MGDG is proven to be more conducive to maintain bilayer membrane structure [14, 38]. The substantial degradation of DGDG during post-freezing recovery suggested that the membrane integrity of C. bifida was damaged following freezing temperature. This was consistent with the results of chlorophyll fluorescence parameters which showed the severe loss of photosynthetic activity of C. bifida. By contrast, the saccharolipids of C. panzhihuaensis including MGMG, MGDG and DGDG only responded to cold treatment. This might be the adaptive mechanisms of C. panzhihuaensis to cold temperature but the relevance of these findings with the freezing tolerance of the species remained to be further explored. MGDG and DGDG are the main components of saccharolipids in most plants [8, 40]. However, SQDG contents of C. bifida and C. panzhihuaensis accounted for 58.21% and 44.69% of the total saccharolipids respectively, surpassing the MGDG and DGDG contents (Fig. 1; Table 3). Similar phenomenon was found in some algae and lichens [41, 42]. As SQDG were also found in cyanobacteria and non-photosynthetic bacteria [41]. The so high content of SQDG in
the two *Cycas* species might be related to the highly diverse endophytic microbiome such as cyanobacteria and actinomycetes [43, 44]. It might also be the long-term adaptation of these species to nutrient-poor environments as P-starvation could promote SQDG accumulation [45].

The changes of ACL and DBI of glycerophospholipids and saccharolipids

ACL and DBI are two important determinants of membrane fluidity which are related to the development and environmental adaptability of plants [13]. The decrease of ACL and increase of DBI under low temperature enable the membranes to be more fluid which contribute to the acquirement of plant tolerance to low temperatures [13]. The ACL and DBI of different classes of glycerophospholipids and saccharolipids showed different change trends following various treatments for either species. Among glycerophospholipids, PG is the major component in chloroplast membranes. ACL of PG in *C. bifida* decreased significantly and DBI of PG in *C. panzhihuaensis* increased significantly after low temperature treatments. It suggested that the two species adopted different strategies to increase the membrane fluidity. Our previous work suggested that ACL of PS was related to the plant lifespan which can be accelerated by promoted senescence but ceased to increase in plants being on the verge of death [46]. In the present study, ACL of PS increased significantly after low temperatures and did not return to the control level after 3 d of recovery in *C. bifida*. ACL of PS varied with the decrease of temperature but recovered to the control level subsequently in *C. panzhihuaensis*. This implied that freezing-treated seedlings of *C. bifida* might gradually lose viability, which of *C. panzhihuaensis*, however, were not severely damaged.

In general, the ACL and DBI changes occurred in more lipid classes immediately after low temperatures in *C. panzhihuaensis* than those in *C. bifida* (Table 4 and 5). The ACL and DBI of glycerophospholipids and saccharolipids maintained unchanged in *C. bifida*. However, ACL and DBI of glycerophospholipids and ACL of saccharolipids responded to cold or freezing treatment and they all recovered to the original level subsequently in *C. panzhihuaensis* (Table 4 and 5). The results showed that *C. panzhihuaensis* could better adjust the membrane fluidity to respond to the decreasing temperature. Based on the higher level of DBI/ACL (Additional file 3), the higher level of DBI of total glycerophospholipids could maintain higher fluidity of extraplastidic membranes under low temperature in *C. panzhihuaensis*, although the ACL being significantly higher. For total saccharolipids, the ACL of *C. panzhihuaensis* was shorter after cold temperature and DBI was higher after freezing temperature compared to those of *C. bifida*. These could enable the seedlings of *C. panzhihuaensis* to obtain more fluidity of plastidic membranes which are apt to be damaged under low temperature. The results were consistent with the chlorophyll fluorescence parameters that photosynthetic activity following treatments severely lost in *C. bifida* but little changed in *C. panzhihuaensis*. The DBI of the total main membrane lipids of *C. panzhihuaensis* was significantly higher than that of *C. bifida* following all the treatments. The higher level of DBI after low temperature treatments might contribute to the higher freezing tolerance of *C. panzhihuaensis*.

Conclusions

The photosynthetic activity of *C. bifida* was more severely affected by low temperature than that of *C. panzhihuaensis*. The differential effects of freezing temperature were more obvious after 3 d of recovery that
seedlings of *C. bifida* almost lost photosynthetic capacity but *C. panzhihuaensis* were little affected. The results confirmed our previous work that the freezing tolerance of *C. panzhihuaensis* was higher than that of *C. bifida*. Lipid composition of *C. bifida* was more affected by cold and freezing treatments than *C. panzhihuaensis*. The proportions of all the lipid categories recovered to the original level for *C. panzhihuaensis* but those of most lipid categories changed significantly for *C. bifida* after 3 d of recovery. The homeostasis and plastic adjustment of lipid metabolism in *C. panzhihuaensis* might be related to the more tolerance of the species to low temperature than *C. bifida*. However, the severe degradation of glycerophospholipids and prenol lipids might be an important determinant of seedling death during recovery period for *C. bifida*. The changes of ACL and DBI occurred in more lipid classes immediately after low temperatures in *C. panzhihuaensis* than those in *C. bifida*. The higher level of DBI of the main membrane lipids following low temperature treatments might contribute to the higher freezing tolerance of *C. panzhihuaensis*. It can be seen that the lipid metabolism underwent different changes between seedlings of *C. panzhihuaensis* and *C. bifida* which might lead to the differential tolerance of the two species to low temperature.

**Methods**

**Plant materials and treatments**

Seeds of *C. panzhihuaensis* were collected in Panzhihua which was approved by Administration Bureau of Panzhihua Cycas National Nature Reserve, Sichuan province. Seeds of *C. bifida* were collected from horticultural sources in Gejiu, Yunnan province which was permitted by the private land owners. The germinated four-year-old seedlings of *C. bifida* and *C. panzhihuaensis* grown in a greenhouse in Southwest Forestry University were used to conduct the experiments. The seedlings of the two species were identified based on the morphological characteristics by Shuangzhi Li of Southwest Forestry University, taxonomist expert. The voucher specimens of *C. panzhihuaensis* (No. ZYL-001) and *C. bifida* (No. ZYL-002) were prepared and deposited in the Southwest Forestry University. The average temperature of the greenhouse was about 30±1 °C and the daytime photosynthetic photon flux density was about 250-300 μmol m⁻² s⁻¹. All the seedlings were planted in plastic pots containing humus and laterite soil (1:1 v/v). For cold treatment, seedlings were transferred to artificial chambers at constant temperature of 4 °C for 3 d. The procedure of freezing and thaw treatments were referred to Zhang et al (2016) [47] and Arisz et al (2018) [1] with some modifications. For freezing treatment, the cold-treated seedlings were put in a programmable temperature incubator set at 0 °C. Freezing was initiated by spraying ice cold water, and the temperature of the chamber was lowered at a rate of 2 °C h⁻¹ until -5 °C was reached. They were then maintained for 1.5 h at -5 °C, after which the temperature was increased up to 4 °C at a rate of 2 °C h⁻¹. The seedlings were thawed at 4 °C for 12 h and then recovered for 3 d at 30 °C. The seedlings which are not subjected to cold and freezing treatments are as the control. There were 16 seedlings for each treatment.

**The chlorophyll fluorescence parameters**

Four seedlings were selected from each treatment, and one leaf was sampled from each seedling. Fluorescence parameters were tested at indoor temperature using a chlorophyll fluorometer (PAM-2500, Walz, Germany). Seedlings were dark-adapted for 30 min before measurements were conducted. The maximum quantum yield of photosystem II (PSII) (Fv/Fm), effective quantum yield of PS II (Y(II)), photochemical quenching coefficient (qP),
non-photochemical quenching coefficient (NPQ), non-regulated (Y(NO)) and regulated (Y(NPQ)) non-photochemical energy loss in PS II as well as electron transport rate (ETR) were measured.

Sample preparation and lipid extraction

Lipids were extracted according to MTBE method. Briefly, samples were first spiked with appropriate amount of internal lipid standards and then homogenized with 200 µL water and 240 µL methanol. After that, 800 µL of MTBE was added and the mixture was ultrasound 20 min at 4 °C followed by sitting still for 30 min at room temperature. The solution was centrifuged at 14000 g for 15 min at 10 °C and the upper organic solvent layer was obtained and dried under nitrogen. Five seedlings were selected from each treatment, and one leaf was sampled from each seedling for lipid extraction.

LC-MS/MS method for lipid analysis

Reverse phase chromatography was selected for LC separation using CSH C18 column (1.7 µm, 2.1 mm × 100 mm, Waters). The lipid extracts were re-dissolved in 200 µL 90% isopropanol/ acetonitrile, centrifuged at 14000 g for 15 min, finally 3 µL of sample was injected. Solvent A was acetonitrile–water (6:4, v/v) with 0.1% formic acid and 0.1 Mm ammonium formate and solvent B was acetonitrile–isopropanol (1:9, v/v) with 0.1% formic acid and 0.1 Mm ammonium formate. The initial mobile phase was 30% solvent B at a flow rate of 300 µL/min. It was held for 2 min, and then linearly increased to 100% solvent B in 23 min, followed by equilibrating at 5% solvent B for 10 min.

Mass spectra was acquired by Q-Exactive Plus in positive and negative mode, respectively. ESI parameters were optimized and preset for all measurements as follows: Source temperature, 300 °C; Capillary Temp, 350 °C, the ion spray voltage was set at 3000 V, S-Lens RF Level was set at 50% and the scan range of the instruments was set at m/z 200–1800.

Identification by Lipid Search

“Lipid Search” is a search engine for the identification of lipid species based on MS/MS math. Lipid Search contains more than 30 lipid classes and more than 1500000 fragment ions in the database. Both mass tolerance for precursor and fragment were set to 5 ppm.

Calculation of lipid double bond index (DBI) and acyl chain length (ACL)

\[
ACL = \frac{\sum [n \times \text{mol} \% \text{ lipid}]}{100}, \text{ where } n \text{ is the number of acyl carbons in each lipid molecule; DBI} = \frac{\sum [N \times \text{mol} \% \text{ lipid}]}{100}, \text{ where } N \text{ is the number of double bonds in each lipid molecule} \ [13].
\]

Statistical analysis
The data were subjected to one-way analysis of variance (ANOVA) with SPSS 15.0. Statistical significance was tested by Fisher's least significant difference (LSD) method. Comparisons between two species were evaluated by T-test.

**Abbreviations**

ACL: Acyl chain length; CL: Cardiolipin; DAG: Diacylglycerol; DBI: Double bond index; DGDG: Digalactosyldiacylglycerol; ETR: Electron transport rate; Fv/Fm: The maximum quantum yield of photosystem II (PSII); MGDG: Monogalactosyldiacylglycerol; MGMG: monogalactosylmonoacylglycerol; NPQ: Non-photochemical quenching coefficient; PA: Phosphatidic acid; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PG: Phosphatidylglycerol; PI: Phosphatidylinositol; PIP: Phosphatidylinositol; PS: Phosphatidylserine; qP: Photochemical quenching coefficient; SQDG: Sulphoquinovosyldiacylglycerol; TAG: Triacylglycerol; Y(II): Effective quantum yield of PS II; Y(NO): Non-regulated non-photochemical energy loss in PS II; Y(NPQ): Regulated non-photochemical energy loss in PS II

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**
YZ, JW and ZY conceived and designed the research. YZ performed the experiments and wrote the manuscript. YY, MW and SH provided assistance in data analysis. JW and ZY made helpful comments on the manuscript. All authors read and approved the final manuscript.

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Figure 1

The content of each lipid category in Cycas bida and C. panzhihuaensis treated with low temperatures. Different letters indicate significant differences between treatments within species and * indicates significant difference between species within treatment (P<0.05). Data are mean±standard deviation (n=5).
Figure 2

Changes in lipid molecular species of glycerophospholipids in Cycas bifida and C. panzhiahuensis treated with low temperatures. Data are mean±standard deviation (n=5)
Figure 3

Changes in lipid molecular species of saccharolipids in Cycas bifida and C. panzhihuaensis treated with low temperatures. Data are mean±standard deviation (n=5)

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