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Induction and quantitative proteomic analysis of cell dedifferentiation during callus formation of lotus (*Nelumbo nucifera* Gaertn.spp. *baijianlian*)

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ABSTRACT

Lotus is an aquatic plant with high nutritional, ornamental and medical values. Its callus formation is crucial for germplasm innovation by genetic transformation. In this study, embryogenic callus was successfully induced on appropriate medium using cotyledons at 12 days after pollination as explants. To dissect cellular dedifferentiation and callus formation processes at the proteome level, cotyledons before and tissues from 10 to 20 days after induction were sampled for shotgun proteomic analysis. By applying multivariate statistics 91 proteins were detected as differentially regulated, and sorted into 6 functional groups according to MapMan ontology analysis. Most of these proteins were implicated in various metabolisms, demonstrating that plant cells underwent metabolism reprogramming during callus induction. 14.3% proteins were associated with stress and redox, indicating that the detached explants were subjected to a variety of stresses; 13.2% were cell and cell wall-related proteins, suggesting that these proteins played important roles in rapid cell division and proliferation. Some proteins were further evaluated at the mRNA levels by quantitative reverse transcription PCR analysis. In conclusion, the results contributed to further deciphering of molecular processes of cellular dedifferentiation and callus formation, and provided a reference data set for the establishment of transgenic transformation in lotus.

1. Introduction

Lotus (*Nelumbo nucifera* Gaertn.) is a perennial aquatic plant of economic, ecological, cultural and religious importance with a wide distribution in East Asia. In addition to its high ornamental value, it is also widespread cultivated as a food crop for its seeds and rhizomes, and as a traditional herbal medicine for its seeds, fruit and leaves [1–3]. According to its agricultural purpose and morphological difference, *N. nucifera* has been divided into three types corresponding with three important organs, separately named as flower lotus, seed lotus and rhizome lotus, which has also been confirmed by random amplification polymorphic DNA analysis [1,4]. These species can be propagated by both sexual (seeds) and asexual (rhizomes) methods. Among them, rhizomes reproduction is the predominant reproduction mode used in horticultural applications. However, it is mother rhizome-consuming, low reproduction coefficient, difficult storage and transportation, and can cause the degeneration of good species. In vivo tissue culture acting as a good alternative method may overcome these obstacles to some degree. Although a remarkable success in lotus regeneration by de novo shoot organogenesis has been achieved [5–6], regeneration via callus phase is still a long unsolved question due to difficult embryogenic callus induction [7–9]. In recent years, whole genome of lotus was successfully sequenced [10–11], which will accelerate the study of gene function and then molecular breeding, thereby promoting enhancement of germplasm resources with high quality and high yield by transgenic technology. However, the establishment of highly efficient genetic transformation systems was restricted to embryogenic callus induction in lotus.

Callus is a group of unorganized cell masses with totipotency. Under certain condition, callus obtained by in vitro culture is able to regenerate the whole plant body through organogenesis or somatic embryogenesis [12]. Besides the highly frequent usage in the propagation of economically important plants, callus acting as the most suitable acceptor materials of genetic transformation has been extensively used in molecular genetic research. In general, callus can be induced artificially in vitro by an appropriate ratio of auxin and cytokinin application in various plants [13]. Additionally, success in culturing embryogenic callus is also affected by many other factors such as plant genotypes and the concentrations of various supplied substances. However, how these factors regulated callus formation have long been obscured. Moreover, it has been demonstrated that differentiated plant tissues or cells can recover cell division ability and then generate callus on a suitable callus inducing medium. Which metabolism changed and how metabolism processes

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changed during cellular dedifferentiation and callus formation were also unclear. Although molecular genetic analyses of *Arabidopsis* mutants impaired in callus formation [14], and proteome or transcriptome profiling of callus formation in nodal segment of *Vanilla planifolia* [15], *Arabidopsis* hypocotyls [16], *Arabidopsis* multiple organs [17] and *Dimocarpus longan* [18] greatly advanced our knowledge of molecular mechanisms underlying callus formation in recent, the molecular mechanisms that lead to callus formation of lotus cotyledons still remain unknown.

Proteomics is a high throughput and powerful approach to identify and characterize a wide range of differentially expressed proteins, and has been used to dissect the molecular mechanisms underlying callus formation [16,19]. In the present study, embryogenic callus was successfully induced using developmental cotyledons at 12 days after pollination (DAP) as explants. Subsequently, we employed a label-free shotgun proteomic approach (GEL-LC-Orbitrap-MS) to compare the proteome of cotyledons before and tissue from 10 to 20 days after induction. The proteomic results were also further verified at the mRNA level through qRT-PCR analysis. The findings may contribute to further deciphering of molecular mechanisms that lead to callus formation of lotus and other plants as well.

2. Materials and methods

2.1. Plant materials and callus induction

Plants of *N. nucifera* 'baijianlian' were grown in lotus research base located in Wuhan Botanical Garden, Chinese Academy of Sciences. Developmental seeds of 10, 12 and 15 DAP were harvested, shelled and surface sterilized in 70% ethanol for 30 s and 2% sodium hypochlorite for 3 min. After five times of washing with sterile water, immature cotyledons were aseptically detached from the developmental seeds with a scalpel and then cultured in vitro. Callus-inducing medium MS supplemented with 3% sucrose, 2.5% gelrite, 100 mg/L casein hydrolysate and different hormone combinations was used to induce callus. The pH of MS medium was adjusted to 5.8 mol/L and then autoclaved for 15 min. Immature cotyledons were transferred on one 10 cm petri dish containing callus-inducing medium in ultraclean bench. Subsequently, the explants were cultured at 26 °C in dark condition until the harvest of tissues from 10 to 20 days after induction.

2.2. Harvest of samples for proteomic analysis

Before callus induction, about 0.5 cm width of cotyledons located near the micropylar region was cut out, pooled and frozen for further use. Additionally, tissues from 10 days to 20 days after callus-induction were also collected, respectively. The samples for proteomic analysis were harvested during July to September.

2.3. Histological observation

After harvest, the samples were immediately immersed in fixative of formalin-acetic acid-alcohol. Fixed samples were then vacuum-dried for 1 h, successively dehydrated with 30%–100% ethanol, transparented, embedded into the paraffin, dewaxed and stained with aniline blue. The embedded samples were cut into 6–10 μm slices using LEICA2150 rotary microtome (Leica, German). Olympus BX-61 upright metallurgical microscope (Olympus, Japan) was employed to observe histological slices.

2.4. Protein extraction

Proteins were extracted from about 1.0 g cotyledons before and tissues from 10 to 20 days after callus induction using the phenol method according to previously described protocol [20]. Briefly, the samples were ground into fine power with liquid nitrogen, homogenized in pre-cooled homogenization buffer used by Yang et al. [21] followed by centrifugation at 12,000 *g* for 20 min at 4 °C. Supernatants were collected, an equal volume of Tris–phenol (pH > 8.0) was then added, vortexed and centrifuged at 12,000 *g* for 20 min at 4 °C. The top phenol phase was carefully transferred to a new tube. Subsequently, homogenization buffer was added again, thoroughly mixed and centrifuged at 12,000 *g* for 20 min at 4 °C. The phenol phase was transferred to another new tube followed by addition 5 volumes of 0.1 M methanolic ammonium acetate in 100% methanol and incubation overnight at −20 °C. The precipitated proteins were washed one time with 0.1 M methanolic ammonium acetate and two times with acetone. The protein pellets were vacuum-dried and then stored at −80 °C for further use.

2.5. One-dimensional gel electrophoresis (1-DE)

Prior to 1-DE, protein samples were redissolved in lysis buffer (7 M urea, 2 M thiourea, 4% *w*/*v* CHAPS, and 65 mM DTT) and quantified using the Bradford method [22]. 25 μg of total proteins was loaded onto 12.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and run at 50 V for 30 min and 150 V for 1 h. After running, the gels were stained with Coomassie Brilliant Blue (CBB R-250) solution for 4 h, and then destained until protein bands were clear, scanned with the scanner (EPSON PERFECTION™ V700) (Supplementary Fig. 1).

2.6. Quantitative proteome analysis and database searching

Each lane of the gels was divided into four fractions using the sterile scalpel. Gel pieces were destained, equilibrated, digested with trypsin, sequentially as described by Valledor and Weckwerth [23]. Afterwards, tryptic peptides from a lane were dissolved in 4% acetonitrile and 0.1% formic acid, thoroughly mixed, desalted and then subjected to analysis using nanoHPLC coupled to LTQ-Orbitrap-MS according to previously published report [24]. In brief, 10 μg of digested peptides was injected into nano-flow LC–MS/MS system equipped with a pre-column (Eksigent, Germany) following by peptides elution using a Ascentis® Express ES-C18 HPLC column (SUPELCO Analytical, USA) during a 80 min gradient from 5% to 50% acetonitrile and 0.1% formic acid. MS analysis was performed on an Orbitrap LTQ XL mass spectrometer (Thermo, Germany) with a controlled flow rate of 500 nL/min. Specific tune was set to 1.8 kV for spray voltage and 180 °C for temperature of the heated transfer capillary.

MS raw data were searched by SEQUEST algorithm of Proteome Discoverer version 1.3 (Thermo, Germany) according to previous report [15]. Briefly, the following filter criteria were set: (i) 1% false discovery rate (FDR); (ii) a variable modification of acetylation of N-terminus and oxidation of methionine; and (iii) mass tolerance of 10 ppm for parent ion and 0.8 Da for fragment ion.

The newly annotated genome database of lotus (http://lotus-db.wbgcas.cn) was employed to identify proteins. The assembled genome of lotus was 804 Mb, which represented 86.5% of the estimated 929 Mb lotus genome. Following repeat-masking and annotation, 26,685 unigenes were inferred, 82% of them have similarity to proteins in SwissProt [11]. Peptides were matched against lotus database plus decoys with user-defined searching parameters as follows: (i) medium or high peptide confidence; (ii) minimum Xcorr of 1 for +1, 2 for +2 and 3 for +3 charged peptides, etc. Subsequently, a label-free approach based on spectral counts (SpCs) was used to quantify the identified proteins followed by a normalized spectral abundance factor (NSAF) normalization strategy [25]. The NSAF was calculated as follows

$$NSAF_i = (SpC_i/L_i)/\sum_{i=1}^{N}(SpC/L)_i.$$

In this equation, the NSAF for a protein is the number of spectral counts (SpCs) divided by protein length (L) and then divided by the sum of SpC/L.

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