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Identification and analysis of differentially expressed proteins during cotyledon embryo stage in longan

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ABSTRACT

The cotyledon stage is a crucial developmental stage during longan embryo development. Two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption ionization-time of flight-tandem mass spectrometry (MALDI-TOF/TOF-MS) were conducted to separate and identify proteins expressed in the longan cotyledon embryos at different development stages. A total of 28 proteins that exhibited regulated expression were successfully identified with a protein identification success rate of 72.2%. The 28 proteins were assigned to six functional classes based on their putative biological functions: energy and metabolism (21%), secondary metabolism (18%), protein metabolism (21%), cell division (11%), antioxidation (4%), nucleic acid metabolism (4%), hormonal regulation (4%) and unknown proteins (18%). Interestingly, three enzymes involved in flavonoid biosynthesis, whose expression in embryos had not been observed previously were clearly up-regulated during the cotyledon stage of embryo development in longan. Identification and analysis of the 28 proteins would shed new lights on further understanding of the biochemical and physiological processes of the embryo development at cotyledon stage in longan.

1. Introduction

Although most plants can be propagated asexually, embryo development is an important event in seeded plants, from which a sexually derived new generation arises. Embryo development is a complex process orchestrated by the coordinated expression of many genes (Chandler et al., 2008). In angiosperm, embryogenesis initiates from a single-cell zygote, which subsequently divides into two asymmetrical cells each with a different developmental fate. In dicots, the upper cell of two-celled embryos develop through a series of stages that have been defined morphologically as preglobular, globular, heart-shaped, torpedo-shaped and bent- or mature-cotyledon (Steeves and Sussex, 1989).

In traditional comparative embryology, morphological changes of embryonic development in different plant species have been described in detail by observation and microanatomy (West and Harada, 1993). More recently, proteomic approaches have been applied to investigate the protein changes during embryo development in several plant species: carrot (Dodeman and Ducreux, 1996), *Cupressus sempervirens* (Sallandrouze et al., 1999), maize (Campo et al., 2004), *Picea glauca* (Lippert et al., 2005), *Medicago truncatula* (Imin et al., 2005), *Lycopersicon esculentum* (Sheoran et al., 2005), rapeseed (Joosen et al., 2007), *Manihot esculenta* (Baba et al., 2008), *Vitis vinifera* (Marsoni et al., 2008) and Valencia sweet orange (Pan et al., 2009). Majority of these studies (Dodeman and Ducreux, 1996; Sallandrouze et al., 1999; Imin et al., 2005; Lippert et al., 2005; Baba et al., 2008; Marsoni et al., 2008) are focused on somatic embryogenesis (SE). Although somatic embryogenesis has emerged as a powerful experimental tool for investigating the mechanisms of plant embryogenesis, important differences exist between somatic and zygotic embryogenesis. These differences include the lack of endosperm, suspensor, and seed coat surrounding a somatic embryo.

Prior to desiccation, two major phases of embryo development, establishment of organization as an embryo and accumulation of storage substances in the embryo, can be conceptualized (West and

Abbreviations: SE, somatic embryogenesis; DAA, days after anthesis; 2-DE, two-dimensional gel electrophoresis; MALDI-TOF-MS/MS, matrix-assisted laser desorption ionization-time of flight-tandem mass spectrometry; IEF, isoelectric focusing; IPG, immobilized pH gradient; pI, isoelectric point; ROS, reactive oxygen species; DOX, dioxygenases.

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Harada, 1993). Being the last stage of embryo development, the cotyledon embryo has accumulated large amount of storage substances. These storage substances later serve as a nutrient source for the growing seedling. Therefore, the cotyledon embryo is a crucial stage in embryo development that affects the success of the next generation. Until now, there had been few reports focused on the development of zygotic cotyledon embryos, especially in economically important crops and woody plants (Goldraij and Polacco, 1999; Campalans et al., 2000). Longan (*Dimocarpus longan* Lour.) is an economically important fruit tree widely distributed in southern China and southern Asia. Some researches were performed on longan explants in the 1980s, such as cotyledons and immature embryos. Wei and Yang (1981) first reported plant regeneration via somatic embryogenesis from callus derived from cotyledon culture. Yang and Chen (1984) obtained monoploid plant regeneration via somatic embryogenesis. Litz (1988) reported that embryogenic callus was initiated from leaflets of new flushes of mature 30-year-old longan trees. However, few works involved in protein change during embryo development, especially cotyledon stage. The objective of this study was to discover and identify the proteins differentially expressed at the cotyledon stage of embryo development in longan. The identities of these proteins have helped us to assign their putative biological functions in the development of longan embryo.

2. Materials and methods

2.1. Plant material

Longan (*D. longan* Lour.) cultivar 'Qingkebaoyuan' with normal embryo development was grown in Fruit Research Institute at Fujian Academy of Agricultural Sciences, Fuzhou, Fujian, China. Longan clusters at similar developmental stage were selected. The mature-cotyledon embryo developmental stages were defined arbitrarily as 38, 45, 52 and 59 days after anthesis (DDA) (Fig. 1). Random samples of four longan clusters were selected for each stage. Cotyledon embryos were excised from the young fruits in the laboratory and immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Determination of total flavonoid content

The embryos were collected and dried for 48 h using a hot air oven at 60 °C and powered. The experiments were carried out in a domestic microwave oven (Set power: 480 W, Galanz Co., Guangdong, China) following Wei et al. (2009). Flavonoid content was determined by $NaNO_2$–$Al(NO_3)_3$–NaOH colorimetric assay with Rutin as reference substance, and absorbance readings were taken at 510 nm (Jiang et al., 2009).

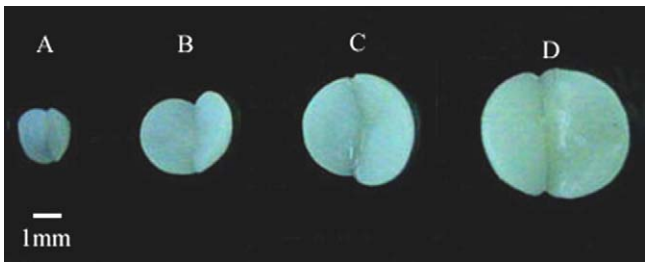

Fig. 1. Developing cotyledon embryos excised from longan at different development stages: (A) 38 DAA; (B) 45 DAA; (C) 52 DAA; (D) 59 DAA.

2.3. Protein extraction

Total protein extracts were prepared using the protocol of Carpentier et al. (2005). Proteins were extracted using a phenol extraction procedure. Longan cotyledon embryos (1 g) were ground into fine powder in liquid nitrogen, and homogenized on ice for 10 min with 4 mL pre-cooled extraction buffer [100 mM Tris, 50 mM L-ascorbic acid, 100 mM KCl, 50 mM disodium tetraborate decahydrate, 1% Triton X-100, 2% β-mercaptoethanol]. The homogenate was shifted into a 50 mL centrifuge tube and an equal volume of ice-cold Tris-saturated phenol (pH 8.0) was added. After mixing, centrifuged at 10,000 × *g* for 15 min at 4 °C. The phenol phase was shifted into anther 50 mL centrifuge tube and 5 vol of 0.1 M ammonium acetate in methanol was added in, and incubated at −20 °C overnight. The specimen was centrifuged at 15,000 × *g* for 15 min at 4 °C and then the supernates were discarded. The precipitate was washed three times with methanol and once with acetone, centrifuged and natural-dried at −20 °C.

2.4. Two-dimensional gel electrophoresis

Two-dimensional electrophoresis was performed using IPG strips (24 cm long, pH 4–7 linear) in the first dimension. The proteins concentration was determined by Bradford method (Bradford, 1976). Dry IPG strips were rehydrated for 12 h in 450 μL rehydration buffer containing 1.2 mg protein samples. Isoelectric focusing was conducted at 20 °C with an Ettan IPGphor system (GE Healthcare Amersham Bioscience). Focusing was performed in four steps: 1 h each at 200, 500, 1000 and 8000 V for 6 h. Focused strips were then equilibrated during the first incubation step in an equilibration solution [6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris–HCl, pH 8.8, and 20 mM DTT] for 15 min, followed by incubating in 4% (w/v) iodoacetamide in the same equilibration solution for 15 min. For the second dimension, the proteins were separated on 12.5% SDS polyacrylamide gels. To ensure data reliability, sample preparation and 2-DE were performed in triplicate.

2.5. Gel staining and image analysis

The 2-DE gels were stained by Coomassie Brilliant Blue (CBB) R-250. Gel image was scanned at 300 dpi with image scanner (EPSON PERFECTION 2480 PHOTO) and analyzed with PDQuest™ software package (Version 7.3.0; BioRad). Spots were detected, matched, and normalized on the basis of total density of the gel with the parameter of percent volume according to the software guide.

2.6. In-gel digestion and MALDI-TOF-MS/MS analysis

Protein spots with differential expression patterns were manually excised from gels and washed with ddH_2O three times. Then, each spot was destained in a destaining buffer (25 mM ammonium bicarbonate, 50% (v/v) ACN), dehydrated by ACN and spun dry. Samples were then rehydrated for 30 min at 4 °C in a digestion buffer containing 25 mM ammonium bicarbonate and 12.5 ng/mL trypsin. After digestion at 37 °C for at least 12 h, peptides were extracted twice using 0.1% TFA in 50% acetonitrile before being spotted on the target plate.

Samples were allowed to air dry and analyzed by 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). The UV laser was operated at a 200 Hz repetition rate with wavelength of 355 nm. The accelerated voltage was operated at 20 kV. Myoglobin digested by trypsin was used to calibrate the mass instrument in internal calibration mode. All acquired spectra of samples were processed using 4700 Explore™ software (Applied Biosystems, Framingham, MA, USA) in a default mode. Combined MS and MS/MS spectra were submitted to MASCOT (V2.1, Matrix