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Expression of the moss *PpLEA4-20* gene in rice enhances membrane protection and client proteins stability

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ABSTRACT

Green vegetative tissues of the moss *Physcomitrella patens* possess a powerful ability to tolerate severe drought stress. Proteomics analysis have revealed that a large number of late embryogenesis abundant (LEA) proteins were key players in the drought tolerance of the photosynthetic tissues. PpLEA4-20, a member of the moss LEA protein family, was selected for further function study using an ectopic expression method in rice. Through molecular identification via PCR, southern blotting and TAIL-PCR, we demonstrated that the *PpLEA4-20* gene was transformed and inserted into a non-encoded region in chromosome 4 of rice and expressed stably in transgenic rice. Unexpectedly, PpLEA4-20 protein emerged as two high-expressed spots on 2-D gels generated from transgenic rice, suggesting that PpLEA4-20 proteins are complete compatible and might be modified in rice. Both growth and physiological analysis showed that seedlings of transgenic *PpLEA4-20* rice displayed altered phenotypes and tolerance to salt. In addition, electrolyte leakage was reduced in transgenic *PpLEA4-20* compared to wild type under stress conditions. Anti-aggregation analysis found that the PpLEA4-20 protein expressed in rice remained soluble at high temperature and in addition to some native proteins from transgenic *PpLEA4-20* rice. Based on Nano LC MS/MS analysis, we identified several proteins from transgenic *PpLEA4-20* rice of increased heat-stability. Our results provide evidence for a role of PpLEA4-20 in salt tolerance and stabilization of client proteins.

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1. Introduction

During the late stage of plant embryo development, seeds produce late embryogenesis abundant (LEA) proteins prior to dehydration, which are thought to be associated with the establishment of desiccation tolerance in seeds [1]. Subsequently, LEA proteins are found accumulated in vegetative organs, especially when subjected to various stresses such as cold, drought or high salinity [1,2]. Although numerous studies support the viewpoint that LEA proteins have a broad impact in the abiotic stress response in plants [3,4], their precise biological functions are elusive.

According to data from LEAPdb [5], approximately 700 LEA proteins have been discovered in various plant species [6], and in desiccation tolerant bacteria and invertebrates [7]. In general, *LEA* genes are highly represented in plant genomes, including 51 genes in Arabidopsis [4,8]; 35 genes in rice, 33 genes in poplar, 10 genes in green algae [8] and 77 genes in moss *Physcomitrella patens* [9]. Using Pfam nomenclature based on sequence motifs, Hundertmark and Hincha [8] developed a new system for the classification of LEA proteins, and the 51 *LEA* genes from Arabidopsis were classified into nine distinct groups. The encoded LEA proteins have experimentally been localized to various subcellular compartments including the cytosol, nucleus, plastids, mitochondria, endoplasmic reticulum, vacuolar and pexophagosome, in addition to being secreted [4]. The diversity and compartmentalization of LEA proteins in plant tissues further raises questions about their physiological function and modes of action. Although LEA proteins might function as protectants of biomolecules and membranes, in addition to

Abbreviations: IPG, immobilized pH gradient; LEA, late embryogenesis abundance; LC, liquid chromatograph; MS, mass spectrometer; MALDI, matrix-assisted laser desorption/ionization; PEG, polyethylene glycol; TOF, time of flight.

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sequestering ions and reactive oxygen species (ROS) [3], their accurate function is still necessary to be characterized one by one.

Physcomitrella patens is a moss with high tolerance to extreme environments such as cold, salt and drought [10]. With the release of its genome sequence, the moss is positioned to become an excellent model system for the study of various stress processes [11,12]. Using a proteomic approach, we previously observed that a number of LEA proteins (n = 11) were strongly expressed and accumulated in green vegetative tissues of the moss during periods of dehydration [12]. This might imply an essential role for LEA proteins in mediating drought tolerance in vegetative tissues, especially in photosynthetic cells. Thus, it would be of great interest to identify the biomolecules protected by moss LEA proteins and probe the molecular mechanisms evolved in photosynthetic cells to cope with drought stress.

In the present study, we investigated the function of PpLEA4-20, a member of the moss LEA protein family, using an ectopic expression approach in rice, in which transgenic lines were generated and subjected to different stress conditions. Using two-dimensional (2-DE) electrophoresis analysis, expression of PpLEA4-20 protein was detected in transgenic rice leaves. Notably, we found that *PpLEA4-20* expression in rice conferred salt tolerance and we identified a group of rice proteins with increased stabilization in transgenic *PpLEA4-20* rice. To our knowledge, this is the first report identifying putative protein targets stabilized by LEA proteins.

2. Materials and methods

2.1. Plant materials and stress treatment

Leafy-shoots of 20 day old *Physcomitrella patens* were obtained as described previously [12]. To recovery more RNA transcripts of PpLEA4-20 for full-length cDNA cloning, leafy-shoots were subjected to dehydration stress for 3 days.

Transgenic seedlings of rice (*Oryza sativa* L. Japonica) were cultured in hydroponic condition with Hoagland solution [13] and placed a growth chamber at 28 ± 1 °C with a 16-h light/8-h dark cycle for two weeks. Hoagland solution was renewed every 2 days for nutrition. To avoid damage caused by rapid stress, a progressive osmotic stress treatment was performed. Rice seedlings were first transferred into a low concentration of PEG-8000 (15% w/v) or NaCl (100 mM) for 2 days, and then a high concentration of PEG-8000 (30%) or NaCl (200 mM) for 10 days. For control experiments, wild type rice was used. After 6 days of recovery, plant survival rates were calculated.

2.2. Gene cloning, vector construction, and rice transformation

Full-length cDNA encoding PpLEA4-20 was cloned from dried leafy-shoots of *Physcomitrella patens* using the primers 5'-GGGGTACCCCTTCCCACTAAATACCGT-3' and 5'-GGACTAGTTCCAGAGCCAATGCTTG-3'. Using pCAMBIA1390 vector (Cambia, Queensland, Australia) containing the maize ubiquitin promoter and hygromycin B resistance gene, *PpLEA4-20* (829 bp) was inserted at BamHI and SpeI sites (Fig. 1). The *Ubi::PpLEA4-20* construct was introduced into rice callus derived from mature embryos, by *Agrobacterium tumefaciens* (LBA4404)-mediated transformation [14]. Transgenic *PpLEA4-20* rice candidates were selected for analysis based on hygromycin resistance (30 mg/L).

2.3. Detection of PpLEA4-20 transgene in rice

Genomic DNA was extracted from transgenic rice leaf tissues by the CTAB method [15]. The inserted moss *PpLEA4-20* fragment was amplified using a forward primer: 5'-CCTTCCCACTAAATACCGT-3' and a reverse primer: 5'-TCCAGAGCCAATGCTTG-3' in T1, T2 and T3 generation of rice plants. PCR analysis was performed using the following cycling parameters: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min; an initial denaturation step of 5 min and a final extension time of 10 min; 30 cycles. The PCR product was analyzed on a 0.8% agarose gel.

Insertion number of the *PpLEA4-20* in transgenic rice was analyzed by southern blotting according to the standard procedures. The probe for hygromycin was amplified using the primers: 5'- GCGCTTCTGCGGGCGATTTG-3' and 5'- CGCGCTACTTCGAGCGGAGG-3' and the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Germany) by PCR. Genomic DNA from transgenic rice and wild type was extracted and digested with HindIII. Digested DNA was separated on 0.8% agarose gel and transferred to nylon membrane (Hybond-N+, Millipore). The membrane was hybridized with DIG-labeled probes according to the manufacturer's instruction. For signal detection, the Image Quant LAS 4000 mini system (GE Healthcare, USA) was used.

Localization of *PpLEA4-20* in the genome of transgenic rice was analyzed by thermal asymmetric interlaced (TAIL) – PCR [16]. Three special primers (sp) based on hygromycin and one arbitrary degenerate primer (ad) were used for primary, secondary, and tertiary TAIL-PCR reactions. These primers were sp1: 5'-GTTGGCTTGTATGGAGCAGCAG-3', sp2: 5'- CGTCCGAGGGCAAAGAAATAGAG-3', sp3: 5'- AATAATGTGTGAGTAGTTCCCAGAT-3' and ad: 5'- NTCGASTWTSGWGTT-3'. Products of each reaction were analyzed on a 1% agarose gel and distinct DNA bands were recovered. Bands were sequenced and resulting data was aligned to the rice genome released to NCBI.

2.4. Protein expression analysis

Two-dimensional electrophoresis (2-DE) was used to detect PpLEA4-20 expression in transgenic plants. Total proteins were extracted from rice leaves using fractionation method [12] and was measured using a modified Bradford method [17]. Protein extracts from wild type rice and transgenic rice were loaded on separate 24 cm IPG pH4-7 strips (GE healthcare, Uppsala) for isoelectric focusing (IEF) and resolved by SDS-PAGE. Gel images were analyzed using the PDQuest software v8.0 (Bio-Rad, USA). Spots of interest were excised and subjected to MALDI TOF/TOF MS identification using an established method [12].

2.5. Anti-aggregation assay

Soluble fraction of proteins was extracted from rice leaves according to established protocol [18]. Fractions of soluble proteins were heated individually at different temperatures (25 °C, 55 °C, 65 °C and 75 °C) for 10 min. Insoluble pellets were removed by centrifugation at 15000 rpm for 10 min to recover heat-soluble proteins in the supernatant. Heat-soluble proteins were separated by SDS-PAGE on 12.5% (w/v) acrylamide gels and stained with Coomassie Brilliant Blue. The bands of interest were excised and subjected to in-gel trypsin digestion followed by Nano LC MS/MS analysis.

2.6. Nano LC-MS/MS

Both desalting and MS identification of digested peptides was carried out as previously described [19]. In brief, desalted peptides were analyzed using a TripleTOF 5600^{+} mass spectrometer (AB Sciex, Canada) coupled to an Ekspert™ nanoLC 425 system (Eksigent, OH). Five microliters of each sample was loaded onto a trap column (ChromXp C18, 350 μm × 0.5 mm, 5 μm, 120 Å, Eksigent,

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