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Polyclonal antibody preparation and immunolocalization of maize (*Zea mays*) seed protein EMB564

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Abstract

Maize protein EMB564 is a member of group 1 LEA (late embryogenesis abundant) proteins. Currently, there is accumulating information available concerning the biological functions and subcellular localization of group 1 LEA proteins. We recently found that EMB564 protein is associated with maize seed viability. In order to elucidate the function of EMB564, its tissue and subcellular localization in mature seeds was analyzed by immunochemical analysis. A peptide corresponding to a 20-mer consensus (TVVPGGTGGKSVEAQEHLAE) of EMB564 was chemically synthesized and used to immunize rabbits for polyclonal antibody preparation. This antibody reacted strongly with EMB564. Immunoblot analysis revealed that EMB564 existed in highest abundance in the plumule, followed by the radicle and scutellum in the maize embryo. Immunoelectron microscopy demonstrated the preferential nuclear localization of EMB564, associated with chromatin, in the embryonic cells. This subcellular localization is consistent with the previous bioinformatic prediction. This is the first immunoelectron microscopy evidence of subcellular localization of the group 1 LEA proteins. This work would help to understand the mechanism underlying the function of the EMB564 in seed vigor maintenance.

Keywords: LEA protein EMB564; immunoblot analysis; subcellular immunolocalization; nucleus; maize embryo.
Abbreviations: 2-DE_Two-Dimensional Electrophoresis; LEA_Late Embryogenesis Abundant; BSA_Bovine Serum Albumin; DTT_Dithiothreitol; CBB_Coomassie Brilliant Blue; GFP_Green Fluorescent Protein, IEF_Isoelectric focusing.

Introduction

Late embryogenesis abundant (LEA) proteins were first described more than 30 years ago as proteins that accumulate during late maturation stage of cotton seed development (Dure et al., 1981). They were subsequently found in other seeds and pollen during maturation and in vegetative tissues in responses to abiotic stress (Battaglia et al., 2008; Bray, 1993). LEA proteins are widely recognized to play a role in cellular dehydration tolerance and in controlling water uptake during imbibition (Bray, 1993; Tolleter et al., 2007; Tunnacliffe and Wise, 2007). However, the molecular functions and mechanisms of most LEA proteins remain largely unknown (Battaglia et al., 2008). LEA proteins have been classified into several groups on the basis of amino acid sequences and conserved motifs (Wise, 2003; Battaglia et al., 2008; Bies-Ethève et al., 2008). Group 1 LEA proteins (Pfam 00477) contain at least one copy of a 20-mer motif (TRKEQ[L/M]G[T/E]EGY[Q/K]EMGRKGG[L/E]). They accumulate during embryo development, especially in dry seeds (Battaglia et al., 2008; Hundertmark and Hincha, 2008; Manfre et al., 2009) responding to abscisic acid (Williams and Tsang, 1991, 1994) and/or water-limiting conditions (Chakrabortee et al., 2007; Pouchkina-Stantcheva et al., 2007; Huang et al., 2012). Based on consensus protein or oligonucleotide probability profile analysis, group 1 is subdivided as la and 1b, and group 1a is hypothesized to match with chromosomal and nuclear proteins with a possible DNA binding function (Wise and Tunnacliffe, 2004**)**. However, no further experimental data supports this hypothesis. Maize seed protein EMB564 is a member of group 1 LEA proteins. At present, there is limited information concerning the putative function of EMB564. Moreover, there are no clear annotations for group 1 LEA proteins (including EMB564) relating to their molecular functions and subcellular locations in the Universal Protein Resource KnowledgeBase (http:// www.uniprot.org). Amara et al. (2012) reported that EMB564-green fluorescent protein (GFP) fusions were expressed in the cytosol and nucleus in agroinfiltrated leaves of *Nicotiana bentamiana*. By bioinformatic analysis, we suggested that EMB564 is most likely to function within the nucleus by binding DNA or RNA (Wu et al., 2013). We found that EMB564 is associated with seed vigor (Wu et al., 2011), and is highly thermal stable (Wang et al., 2012). However, it is unclear how EMB564 functions in seed vigor maintenance. In this study, a polyclonal antibody was raised using a synthetic 20-mer peptide of EMB564 sequence. Using this antibody, we examined the tissue distribution and subcellular immunolocalization of EMB564 protein. The localization information at the tissue and subcellular levels is important for elucidating the function of EMB564 protein.

Results

The antibody reacts specifically with EMB564

The use of a synthetic peptide as an immunogen is applicable when a complete protein is not available in sufficient quantities

to carry out an adequate immunization response. Generally, a 15 to 20 amino acid peptide is of a sufficient length to elicit an effective immunization response (Disis et al., 1996). Sequence regions with β-strand or amphipathic helix character have been found to be antigenic (Parker and Hodges, 1991). In our experiment, the 20-mer motif TVVPGGTGGKSVEAQEHLAE of EMB564 contains a β-strand (TVVP) between two irregular coil fragments by secondary structure analysis (Wu et al., 2011). This sequence scored 3.4 (excellent) as analyzed by the antigen profiler peptide tool (http://www.pierce-antibodies.com/custom-antibodies/peptide-design-antigen-profiler.cfm).

The immunoreactivity of the polyclonal antibody (anti-Pep20) was examined using SDS-PAGE, two-dimensional electrophoresis (2-DE) and immunoblot analyses (Fig. 1). The immunoblot analysis showed that anti-Pep20 was high specificity for the synthetic 20-mer peptide and a 12 kDa protein, with a strong reactivity even at the dilution 1:5,000. The preimmune rabbit serum was not reactive (data not shown). The 2-DE immunoblot analysis confirmed that the antibody only detected one spot. This spot was identified as EMB564 with mass spectrometry (Wu et al., 2011). Thus, the antibody had highly specific to EMB564, which is important for subcellular immunolocalization.

EMB564 accumulates differentially in embryonic tissues

The expression of EMB564 in different tissues was examined by the immunoblot analysis. On an equal protein basis, EMB564 protein existed in plumule in highest abundance, followed by radicle and scutellum (Fig. 2). No bands were detected by anti-Pep20 in maize endosperm extract (result not shown). Obviously, the expression of EMB564 on the protein level differs among tissues in maize seed.

EMB564 localizes preferentially in the nucleus

In order to investigate the subcellular localization of EMB564 in maize embryonic cells, we performed immunogold labeling for the binding of anti-Pep20 at the electron microscope level, (Fig. 3). The ultrastructural localization of EMB564 was performed on sections prepared from imbedded plumule. In tissue sections of plumules cells, the gold- labelled particles were found in the nucleus, associated with chromatin. Gold labelled particles were dispersed unevenly in the nucleus (10 nuclei examined), and sometimes grouped gold particles were found (Fig. 3), but none was observed in the nucleolus. Occasionally, a few golden particles were observed in the cytoplasm (data not shown), which may be the residual of EMB564 because it is synthesized in the cytoplasm and then transferred to the nucleus. Cell walls, cytoplasmic vacuoles and other organelles of every cell examined were devoid of gold particles (data not shown). In the negative control, no gold particles were found in areas where EMB564 was localized.

Discussion

To our knowledge, only 26 plant LEA proteins have been experimentally localized in subcellular compartments to date. They exist in the nucleus (Iusem et al., 1993, Farias-Soares et al., 2013, Amara et al. 2012), cytosol (Roberts et al., 1993), mitochondria (Grelet et al., 2005), chloroplast (Ferro et al., 2010), vacuoles (Egerton-Warburton et al., 1997), plasma membrane (Marmagne et al., 2007) and its vicinity (Danyluk et al., 1998), cell wall (Franz et al., 1989) and plasmodesma (Fernandez-Calvino et al., 2011).After synthesis they are transported to cell organelles and membranes, where they stabilize cell structures and molecules. The partition of LEA proteins into various cell compartments determines their

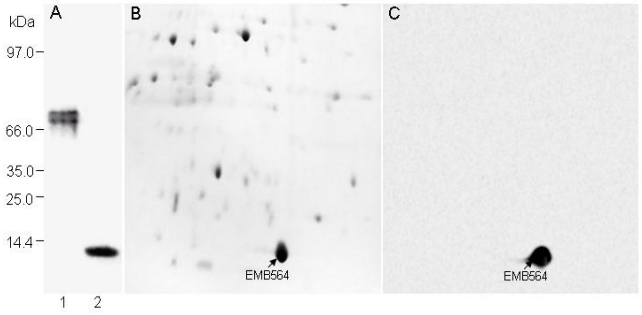

Fig 1. Specificity of the antibody anti-Pep20. (A) Immunoblotting analysis. Blots were probed with anti-Pep20 (1:5000 dilution). Lane 1 is the synthetic 20 peptide coupled with BSA (2 μg); lane 2 is EMB564 from maize embryos (30 μg). (B) 2-DE analysis of maize embryo proteins (300 μg, CBB-stained). (C) 2-DE immunoblotting analysis of maize embryo proteins (150 μg). (B, C) Maize embryo proteins were resolved by IEF using 11 cm pH 4–7 IPG dryStrip. Secondary SDS-PAGE was carried out on a 12.5% gel. The arrow indicates EMB564.

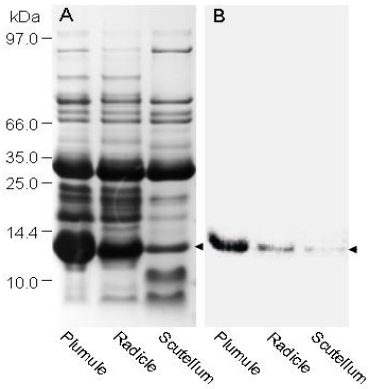

Fig 2. Expression and tissue distribution of EMB564 in maize embryos. Proteins were extracted from maize plumule, radicle and scutellum, respectively. Protein (30 μg/lane) was resolved by 12.5% Tricine-SDS-PAGE. (A) CBB stained gel. (B) Blot probed with anti-Pep20 (1:5,000 dilution). The arrowhead indicates EMB564 protein.

possible function in the protection or regulation of essential biochemical processes. However, to date, there is only limited information (Amara et al. 2012) on experimentally verified subcellular locations of group 1 LEA proteins. In this work, we confirmed that EMB564 protein is present in the nuclear of maize embryonic cells using immunogold electron microscopy. It also raises the question of how this protein is targeted to the nucleus after synthesis. EMB564, similar to the dehydrins in *Araucaria angustifolia* (Farias-Soares et al., 2013), does not contain any known nuclear localization signals (Cokol et al., 2000). The EMB564 protein is devoid of the serine-rich cluster found in Rab proteins which is believed to be involved in nuclear import (Houde et al., 1995). Probably, EMB564 is co-transported to the nucleus through protein-protein interactions. It is also possible that EMB564 may diffuse through the nuclear pores into the nucleus due to its small size (Zaltsman et al., 2007). Furthermore, computer analysis showed that the glycine-rich repeat has weak homology with several viral nuclear proteins. Amara et al. (2012) suggested that post-translational modifications in EMB564 might be critical

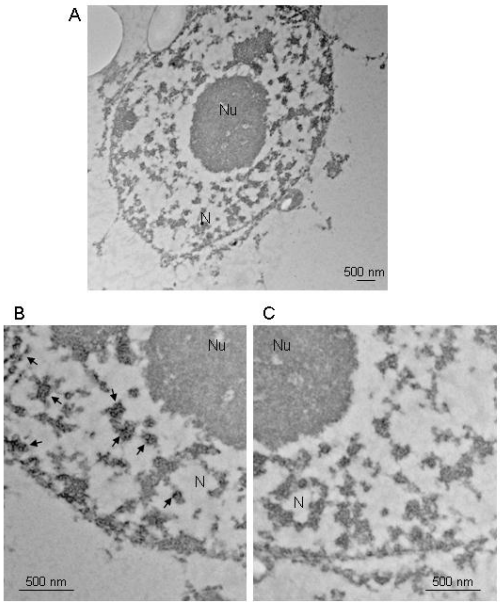

Fig 3. Subcellular localization of EMB564 in maize embryonic cells by immunogold electron microscopy. Sections of maize plumules were probed with anti-Pep20 (A, whole nucleus; B, part of nucleus) or preimmune rabbit serum (negative control, C), and incubated with 10-nm gold-conjugated anti-rabbit IgG. EMB564 protein is localized in the nucleus. This picture is the representative on among 10 nuclei examined. The arrow indicates golden particle. N, nucleus; nu, nucleolus. Bar length corresponds to 500 nm.

factors for its localization. Using the POPP program, additional possible functions of group 1 LEA proteins have been proposed (Wise and Tunnaclife, 2004), i.e. they may have enzymatic or chaperone activity and act as nucleus proteins that unwind or repair DNA, regulate transcription, and might be associated with chromatin or the cytoskeleton. A function assignment of two group 1 LEA proteins from mung bean implies a possible DNA binding function (Rajesh and Manickam, 2006). The function and action mechanism of group I LEA proteins were reviewed by Tunnacliffe and Wise (2007). We predicted that EMB564 would have nuclear localization by five different predictors and EMB564 protein may function within the cell nucleus (Wu et al., 2013). EMB564 is remotely homologous with DNA/RNA helicases and single-stranded DNA-binding proteins, contains similar DNA/RNA binding sites, and structurally resembles a variety of nuclear and DNA/RNA-binding proteins (Wu et al., 2013). We demonstrated here that EMB564 preferentially exists in the nucleus. Amara et al. (2012) reported that EMB564 exists mainly in the cytosol, followed by the nucleus. This discrepancy may be due to the different experimental methods used. We examined the *in situ* expression (localization) of EMB564 in the maize seeds using immunoelectron microscopy, whereas Amara et al. (2012) analyzed the transient expression (localization) of EMB564-GFP fusions in agroinfiltrated leaves of *Nicotiana bentamiana*.Reactive oxygen species (ROS) can attack the nucleic acid and lead it to oxidative damage, which is the primary cause of seed vigor loss (Vanderauwera et al., 2011). DNA needs protections from nuclear inside and outside to reduce oxidative damage. We found an association of EMB564 with maize seed viability (Wu et al., 2011). EMB564 accumulates in abundance in maize embryo meristem and its nuclear location suggests that it may play a general protective role. The possibility of EMB564 as a DNA-binding protein needs to be further confirmed experimentally.

Materials and methods

Plant material

Maize (*Zea mays* L. cv. Zhengdan 958) seeds were bought from local market. Seeds were surface-sterilized, soaked in water for 20 h (25 ºC), and then the scutellum, plumule and radicle were excised and frozen in liquid nitrogen and stored at -70 ℃ until use.

Antibody production

A 20 amino acid sequence (TVVPGGTGGKSVEAQEHLAE) corresponding to the conserved motif (20–39 amino acid residues) of that EMB564, was chosen for antibody production. The 20-amino acid peptide was chemically synthesized by a solid phase method using an Fmoc strategy peptide synthesizer (PSSM-8, Shimadzu, Kyoto, Japan). The synthetic 20-mer peptide was purified using high performance liquid chromatography (HPLC). Its sequence was checked by MASS SPECTRUM. The synthetic peptide was coupled with bovine serum albumin (BSA). About 500 μg of the synthetic peptide in 0.5 ml of phosphate buffer (pH 7.4) was mixed with 1.0 ml of Freund's complete adjuvant. The mixture was injected into a 4-month-old New Zealand white female rabbit to produce polyclonal antibodies. This operation was repeated three times at 20-day intervals using 250 μg of synthetic peptide. After the final booster, the rabbit was bled and the anti-serum was separated by keeping the blood at 4℃ overnight. The anti-serum containing polyclonal antibodies was passed through a kromasil C18-5 column and used in the experiment. The antibody is designed as anti-Pep20.

Extraction of protein from embryos and the separated embryo parts

Protein was extracted by a phenol-based extraction protocol as described previously (Wang et al., 2003, 2006). Maize embryos including plumule, scutellum and radical were ground to fine powders in liquid N_2 with a mortar and pestle. Tissue samples were homogenized with cold acetone containing 10 mM dithiothreitol (DTT) in the mortar, then transferred to a centrifuge tube and centrifuged at 15000×g for 5 min (4℃). The resultant pellet was washed 3 times with cold acetone with 10 mM DTT and air-dried. The dry powder was resuspended in extraction buffer containing 0.1 M Tris-HCl, pH 7.8, 1% SDS, 20 mM DTT and 2 mM phenylmethanesulfonyl fluoride. The crude extract was centrifuged at 15000×g for 15 min at room temperature. The supernatant was mixed with equal volume of buffered phenol (pH 8.0, Sigma). Phases were separated by centrifugation at 15000×g for 5 min. The phenol phase was precipitated with 5 volumes of cold methanol containing 0.1 M ammonium acetate overnight (-20℃). Precipitated proteins were recovered by centrifugation at 15000×g for 5 min, after which the pellet was washed twice with ice-cold acetone containing 20 mM DTT, centrifuged, and then air-dried. dissolved in SDS containing buffer for SDS-PAGE or 2-DE rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (v/v) IPG buffer, 20 mM DTT and a trace amount of bromophenol blue). Protein extract was clarified by centrifugation prior to 2-DE. Protein was quantified by the Bio-Rad protein assay with BSA as a standard.

Electrophoresis and immunoblot

SDS-PAGE was performed in a Laemmli gel system (4.75% stacking gel and 12.5% resolving gel) (Laemmli, 1970). Tricine-SDS-PAGE was performed as Schägger (2006).

For 2-DE, first dimension isoelectric focusing (IEF) separation was performed using gel strips forming an immobilized linear pH gradient from 4 to 7 (Immobiline DryStrip, 11 cm, Bio-Rad). The strips were loaded with protein samples (500 µg in 220 µl) and rehydrated for 12 h at 20 ºC on a PROTEAN IEF CELL system (Bio-Rad). IEF and secondary SDS-PAGE was performed as described previously (Wu et al., 2011).

After electrophoresis, proteins in gels were visualized with colloidal CBB R350 or electroblotted onto polyvinylidene difluoride membrane (Hybond-P, GE healthcare) in a transfer buffer (20% v/v methanol, 50 mM Tris, 40 mM glycine).

For immunoblot analysis, protein blots were soaked in TBST buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween-20) containing 5% low fat milk powder and gently shaken for 2 h at room temperature. The blot was then incubated with anti-Pep20 (1: 5000 dilution) for 1 h. After washing with TBST, the blot was incubated in peroxidase-conjugated goat anti-rabbit IgG (1: 2000 dilution) at room temperature for 1 h. The blot was visualized with 0.08% 3,3'-diaminobenzidine tetrahydrochloride, 0.05% H_2O_2, 0.1 M Tris-HCl, pH 7.5.

Immunoelectron localization

The plumules of maize seeds were used for immunoelectron localization. The samples were fixed in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 Mcacodylate buffer, pH 7.3, for 8 h at room temperature, and then infiltrated in LR White Resin (London Resin Company Ltd., Reading, UK) according to Ross et al (2000). 0.8 mm-thick sections were cut using an ultramicrotome. They were incubated with anti-Pep20 (1:100) for 1 h at room temperature, and then reacted with anti-rabbit IgG conjugated with 10 nm diameter gold particles (Amersham, Uppsala, Sweden) for 1 h at room temperature (Lancelle and Hepler, 1991). After immunolabelling, the sections were stained with uranyl acetate and were observed using transmission electron microscopy. As a negative control, specimens were incubated with preimmune rabbit serum, diluted 1:100, in place of the primary antisera.

Conclusions

Our results showed that EMB564 accumulates differentially in embryonic tissues and localizes preferentially in the nuclear of the embryonic cells. This is the first immunoelectron microscopy evidence of subcellular localization of group 1 LEA proteins. This paper provides new data to support the hypothesis by Wise and Tunnaclife (2004) that group 1a LEA proteins may act as nucleus proteins that unwind or repair DNA and regulate transcription. It will help understand the mechanism underlying the biological function of group 1 LEA proteins, especially the role of the EMB564 protein in seed vigor maintenance.

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