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Expression of Recombinant EARLI1, a Hybrid Proline-rich Protein of Arabidopsis, in *Escherichia coli* and Its Inhibition Effect to the Growth of Fungal Pathogens and *Saccharomyces cerevisiae*

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Abstract

EARLI1 is an Arabidopsis gene with pleiotropic effects previously shown to have auxiliary functions in protecting plants against freezing-induced cellular damage and promoting germinability under low-temperature and salinity stresses. Here we determined whether recombinant EARLI1 protein has anti-fungal activity. Recombinant EARLI1 protein lacking its signal peptide was produced in *Escherichia coli* BL21(DE3) using isopropyl β-d-1-thiogalactopyranoside (IPTG) induction and the prokaryotic expression vector pET28a. Expression of EARLI1 was analyzed by Western blotting and the protein was purified using affinity chromatography. Recombinant EARLI1 protein was applied to fungal cultures of *Saccharomyces cerevisiae*, *Botrytis cinerea* and *Fusarium oxysporum*, and membrane permeability was determined using SYTOX green. Full-length *EARLI1* was expressed in *S. cerevisiae* from the *GAL1* promoter using 2% galactose and yeast cell viability was compared to control cells. Our results indicated that application of recombinant EARLI1 protein to *B. cinerea* and *F. oxysporum* could inhibit the growth of the necrotrophic fungi. Besides, addition of the recombinant protein to liquid cultures of *S. cerevisiae* significantly suppressed yeast growth and cell viability by increasing membrane permeability, and *in vivo* expression of the secreted form of EARLI1 in *S. cerevisiae* also had a remarkable inhibition effect on the growth of yeast cells.

Abbreviations

8CM eight-cysteine motif
6 × His 6 × histidine
BCIP 5-bromo-4-chloro-3-indolyl phosphate
BLAST basic local alignment search tool
HyPRP hybrid proline-rich protein
IPTG isopropyl β-d-1-thiogalactopyranoside
MCS multiple cloning site
NBT nitroblue tetrazolium
NCBI national center for biotechnology information
NMR nuclear magnetic resonance
nsLTP non specific lipid transfer protein
ORF open reading frame
PRD proline-rich domain
PRP proline-rich protein
PVDF polyvinylidene fluoride
SAR systemic acquired resistance

TAIR the arabidopsis information resource

Keywords

Antifungal activity, *Arabidopsis thaliana, Botrytis cinerea, EARLI1, Fusarium oxysporum, Saccharomyces cerevisiae*

1. Introduction

Non specific lipid transfer proteins (nsLTPs) are found in a variety of organisms, including fungi, animals and plants, and play important roles in stress defense. *In vitro* experiments indicated that nsLTPs bind lipids and are able to transfer them between membranes (Kader, 1996). In plants, nsLTPs are classified into two groups: nsLTP1, with a molecular mass of about 9 kDa; and nsLTP2, with a molecular mass of about 7 kDa (Carvalho and Gomes, 2007). nsLTPs have eight cysteine residues that form four disulfide bonds. The positions of the eight cysteines are conserved, such that the third and fourth cysteines are consecutive in the polypeptide chain, and the fifth and sixth cysteines are separated by only one residue. This special structure has been named the eight-cysteine motif (8CM), which appears to be a scaffold of conserved helical regions connected by variable loops (Zhang and Schläppi, 2007). However, the pattern of disulfide bonds between nsLTP1 and nsLTP2 is different. In nsLTP1, the first and sixth cysteine residues, and the fifth and eighth cysteine residues, respectively, form disulfide bridges. In nsLTP2, the first and fifth cysteine residues, and the sixth and eighth cysteine residues, respectively, form disulfide bridges (Douliez et al., 2001). Analyses of three-dimensional structures with nuclear magnetic resonance (NMR), infrared, and Raman spectroscopy demonstrated that the polypeptide backbone of nsLTPs was composed of four α-helices connected by disulfide bridges (Gincel et al., 1994, Heinemann et al., 1996). The crystal structure of a maize LTP confirmed that four α-helices are involved in formation of a hydrophobic cavity that has acyl chain binding and transfer activities (Shin et al., 1995).

nsLTPs have putative signal peptides at their N-termini and are generally thought to be extracellular proteins. However, several reports showed that certain nsLTPs can be also found intracellularly. AlLTPs from *Allium* species are localized in endomembrane compartments (Yi et al., 2009), while a LTP in germinating sunflower seeds was found in both intracellular and extracellular localizations (Pagnussat et al., 2009). Moreover, a LTP from castor bean (*Ricinus communis*) was localized in the glyoxysome matrix (Tsuboi et al., 1992), and a LTP from *Vigna unguiculata* seeds in the protein storage vacuoles (Carvalho et al., 2004). Another exception is the apparent chloroplast localization of RlemLTP, a rough lemon nsLTP with antifungal activity against *Alternaria alternata* and *Fusarium oxysporum*. Although RlemLTP has a putative signal peptide at its N-terminus, recombinant RlemLTP fused to the green fluorescence protein was shown to localize to chloroplasts (Nishimura et al., 2008).

LTP genes are mainly expressed in the aerial portions of plants including leaves, stems, and shoot meristems, and their transcripts were detected at high levels in peripheral cell layers such as the epidermis (Reina-Pinto and Yephremov, 2009) and vascular tissues (García-Olmedo et al., 1995). Previous data suggested that LTPs may be involved in generating a special hydrophobic surface such as with cutin in the wall of epidermal cells, or with suberin layers, which could prevent the incursion of water and the germination of fungal spores (Pyee et al., 1994). Consistent with this, LTPs are concentrated in areas where surface wax is deposited, particularly in young leaves containing cutin depositions. LTPs were thus considered to play a role in the secretion or deposition of extracellular lipophilic materials by transferring of wax or cutin monomers (DeBono et al., 2009). When leaves of tree tobacco were dried at periodic intervals, cuticular wax accumulation and LTP expression increased simultaneously, and overexpression of LTP genes increased the synthesis of wax in tree tobacco (Cameron et al., 2006). This suggested that the high expression levels of LTP genes in peripheral cell layers surrounding aerial

organs are associated with the establishment of the cell wall and the cuticle of epidermal cells (Salcedo et al., 2007).

Other experiments revealed that LTPs might promote the transfer of lipids to the cell wall, which would increase the thickness of the cuticle and enhance the resistance of plants to pathogens (Jiang et al., 2010). DIR1, an Arabidopsis nsLTP, appears to be involved in systemic acquired resistance (SAR) and responsible for long-distance signaling throughout the plant, probably by interacting with oxylipins (Maldonado et al., 2002). In addition, nsLTPs from different plant species had *in vitro* activity against phytopathogenic bacteria and fungi, and the variable activities of them to different strains and species of pathogens indicated that each nsLTP might have specificity for different pathogens (Ge et al., 2003). Because of their protective effects against bacteria, fungi, and viruses, nsLTPs are regarded as pathogenesis-related proteins and were included in the PR-14 family (Van Loon and Van Strien, 1999). At present, it is generally believed that nsLTP genes are induced by phytopathogens and function in biotic resistance pathways such as SAR (Suzuki et al., 2004). All these results suggest that nsLTPs belong to potential components that link signal molecules to plant defense.

EARLI1 from Arabidopsis has been characterized as a hybrid proline-rich protein (HyPRP), because it consists of 168 amino acids with a putative signal peptide of 25 amino acids at the N-terminus, a hydrophilic proline-rich domain (PRD) in the middle, and a hydrophobic C-terminus (Wilkosz and Schläppi, 2000, Zhang and Schläppi, 2007). Based on the high similarity of its 83-amino-acid-long C-terminal 8CM to plant nsLTPs, EARLI1 is also classified as a putative LTP. It has been thought to be involved in maintenance of membrane or cell wall stability (Bubier and Schläppi, 2004). Most nsLTPs have a signal peptide which can lead to cell wall or membrane localization *via* the secretory pathway. Consistent with this, EARLI1 was shown to localize to the plasma membrane or to the cell wall (Zhang and Schläppi, 2007). As a putative member of the LTP family, EARLI1 might participate in cutin formation, surface wax formation, embryogenesis, defense reactions against phytopathogens, symbiosis, and in the adaptation of plants to various environmental conditions (Kader, 1996).

LTPs have been purified from many plants. However, their *in vivo* physiological functions and the mechanism of their inhibitory effects against microbes are poorly understood. Since the discovery of LTPs as potential anti-phytopathogenic peptides, it has been speculated that this activity could result from the interaction of LTPs with biological membranes of pathogens, possibly leading to loss of membrane integrity and/or membrane permeabilization (Regente et al., 2005). In the present work, recombinant EARLI1 expressed in *E. coli* was used to analyze the activity of this protein against the necrotrophic fungus *Botrytis cinerea*, *F. oxysporum* and the non-pathogenic yeast *Saccharomyces cerevisiae*. These *in vitro* studies and an *in vivo* approach of induced gene expression in yeast cells showed that EARLI1 can inhibit the growth of fungal species.

2. Materials and methods

2.1. In silico analysis and molecular modeling

The encoding sequence of *EARLI1* (AT4G12480, GenBank accession number NM_117318) was downloaded from the TAIR database (http://www.arabidopsis.org). Amino acid sequence, isoelectric point and molecular weight were deduced using EditSeq of DNAStar (http://www.dnastar.com). Homology search was conducted using the BLAST program of NCBI (http://www.ncbi.nlm.nih.gov). The software Clustal W (1.82) from the European Biotechnology Information was used for multiple sequence alignments of amino acid sequences of LTPs retrieved from GenBank. Putative signal peptides and hydrophobicity were analyzed using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) and ProtScale (http://expasy.org/tools/protparam.html), respectively. Protein modeling was done using SWISS-MODEL, a fully automated protein structure homology-modeling server (http://swissmodel.expasy.org).

2.2. Bacterial strains, plasmids and fungal cultures

Escherichia coli strain DH5α was used to clone the encoding sequence of PRD-8CM of EARLI1. *E. coli* strain BL21 (DE3) was adopted as host in the expression of recombinant EARLI1 (rEARLI1) lacking the signal peptide. pET28a (Novagen) was used as prokaryotic expression vector. *E. coli* cells with plasmids were grown aerobically in LB medium or on LB agar plates at 37 °C supplemented with 30 μg/mL kanamycin. *B. cinerea* Pers. ex Fr. of Garlic Sprout, *F. oxysporum* f. sp. *vasinfectum* and *S. cerevisiae* strain W303-1A *MATα* (*leu2-3*,*112*; *trp1-1*; *can1-100*; *ura3-1*; *ade2-1*; *his3-11*,*15*) were used in *in vitro* antifungal test. *S. cerevisiae* strain W303-1A *MATα* also was used in *in vivo* test of the antifungal activity of EARLI1.

2.3. Construction of prokaryotic expression vector

The *EARLI1* ORF lacking the putative signal peptide sequence was amplified from Arabidopsis ecotype Col-0 using PCR primers 5′-CGCGGATCCACAGATTGTGGTTGC-3′ (*Bam*HI site is underlined) and 5′-CGCCTCGAGTCAAGCACATTGGAA-3′ (*Xho*I site is underlined) (Zhang and Schläppi, 2007). PCR conditions included 10 min denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 65 °C, 1 min elongation at 72 °C, and a final 10 min elongation at 72 °C. The PCR product was purified, double digested with *Bam*HI and *Xho*I, ligated into *Bam*HI and *Xho*I digested pET28a, and transformed into competent cells of *E. coli* DH5α to construct the recombinant expression plasmid, pET28a-EARLI1. Positive clones carrying the recombinant plasmid were identified by PCR and sequenced by Sangon Biotech Company (Shanghai, China). PCR reagents, T4 DNA ligase and restriction endonuclease were purchased from Takara (Dalian, China).

2.4. Inducible expression of EARLI1 in *E. coli*

Validated pET28a-EARLI1 plasmids and the pET28a empty vector were transformed into competent BL21(DE3) *E. coli* cells using the calcium chloride method. Single colonies were grown at 37 °C in 4 mL LB containing 30 μg/mL kanamycin with a shaking speed of 200 rpm. When the absorbance of *E. coli* cultures reached 0.5–1.0 at 600 nm, EARLI1 expression was induced by addition of IPTG to produce mRNAs encoding a N-terminally tagged 6 × His-EARLI1 recombinant protein. The effects of IPTG concentration, temperature, and induction time on the expression level of His-EARLI1 were studied to determine the optimal induction conditions. Bacterial cultures were initially induced for 6 h at 37 °C with 0.2, 0.4, 0.6, 0.8, 1.0, or 1.2 mM IPTG to select the optimal IPTG concentration. Induction was then performed at the optimal IPTG concentration for 1 to 7 h at 37 °C to determine the optimal induction time. Bacterial cells were harvested by centrifugation at 20,000 *g* for 1 min, then resuspended in 50 μL 1 × SDS loading buffer and frozen at − 20 °C until use. Samples were boiled for 5 min at 95 °C and were centrifugated at 20,000 *g* for 10 min. The expression level of 6 × His tagged EARLI1 was initially estimated by 12% (w/v) SDS-PAGE and staining with coomassie brilliant blue R250. Unstained Protein Molecular Weight Marker SM0431 (Fermentas China, Shenzhen) was used as molecular mass marker, and the molecular mass of each component from big to small is 116.0, 66.2, 45.0, 35.0, 25.0, 18.4 and 14.4 kDa, respectively.

2.5. Solubility analysis of recombinant EARLI1 expressed in *E. coli*

To test the solubility of recombinant EARLI1 expressed in *E. coli*, bacteria were harvested by centrifugation at 4 °C and 4000 *g* for 10 min, and resuspended in cell lysis buffer (50 mM Tris, 50 mM NaCl, pH 8.0) containing protease inhibitor (Complete, Mini, EDTA-free, MBI) and lysozyme. Cells were sonicated at 40 W for 10 cycles (10 s working, 15 s free) in an ice-water bath. After centrifugation at 20,000 *g* and 4 °C for 10 min, the supernatant and the precipitate were collected and analyzed by SDS-PAGE. The supernatant contained the soluble cellular substances and the pellet contained insoluble substances and cell debris.

After electrophoretic separation, proteins were transferred to a PVDF membrane using a semi-dry transfer system. Membrane was rinsed with TBS-T (1 × TBS + Tween-20) and incubated for 3 h at room temperature in

blocking solution (TBS containing 5% skim milk). Recombinant EARLI1 was visualized by incubating of the PVDF membranes with rabbit anti-His-tag polyclonal antibody (Abcam; 1:1000 dilution) and alkaline phosphatase conjugated goat-anti-rabbit IgG (CWBIO, Beijing; 1:2000) in blocking solution. NBT and BCIP were used as substrates for color development.

2.6. Large-scale expression and purification of EARLI1 fusion protein

Large-scale expression was carried out under conditions established during small expression trials. After induction, bacteria were harvested in 50 mL tubes by centrifugation at 4 °C and 4000 *g* for 10 min. Approximately 2.0 g of pellet from 1 L bacterial cultures was resuspended in 8 mL of lysis buffer (50 mM NaH_2PO_4, 0.3 M NaCl, pH 8.0) and transferred to ice. After addition of protease inhibitor (Complete, Mini, EDTA-free, MBI) and lysozyme, cells were disrupted by sonication for 10 cycles (10 s working, 15 s free) in an ice-water bath. Because EARLI1 was mainly found in inclusion bodies, the sonicated material was centrifuged at 20,000 *g* and 4 °C for 10 min, and the precipitate was gently collected for subsequent manipulations. The precipitate was rinsed twice with washing buffer (50 mM Tris–HCl, 50 mM NaCl, 1% TritonX-100, pH 8.0) and inclusion bodies were harvested by centrifugation at 4 °C and 20,000 *g* for 10 min and dissolved in 20 mM Tris–HCl, 50 mM NaCl, and 8 M urea (pH 8.0) for 2–6 h at room temperature. After centrifugation at 20,000 *g* and 4 °C for 10 min, the supernatant was dialyzed against 20 mM Tris–HCl, 50 mM NaCl (pH 9.0) containing different concentrations of urea (6 M, 4 M, 2 M, 0 M) for 8–10 h at each gradient. After centrifugation at 4 °C and 20,000 *g* for 10 min, the supernatant containing soluble fusion proteins was filtered through a 0.45 μm filter membrane and applied to a Ni^{2+} high-affinity resin column pre-equilibrated with 50 mM NaH_2PO_4, 0.3 M NaCl (pH 8.0) at a flow rate of 0.5–1 mL/min, because the recombinant EARLI1 lacking the signal peptide was expressed with an additional N-terminal 6 × His tag, which facilitated further purification by IMAC (Immobilized metal affinity chromatography). After washing with eight volumes of the same buffer, the column was further washed with 50 mM NaH_2PO_4, 0.3 M NaCl, 10 mM imidazole (pH 8.0) at a flow rate of 1 mL/min until A_{280} was stable. The polyhistidine-tagged protein was eluted with five to ten volumes of 50 mM NaH_2PO_4, 0.3 M NaCl, and 250 mM imidazole (pH 8.0). The elute was collected and dialyzed against 20 mM Tris–HCl, 50 mM NaCl (pH 9.0). Recombinant EARLI1 protein was further purified with the protein extraction kit BSP062 (Sangon, Shanghai). The protein concentration was determined by the Bradford method (Bradford, 1976). The purified protein was analyzed by 12% (w/v) SDS-PAGE.

2.7. *In vitro* test of the antifungal activity of EARLI1

B. cinerea Pers. ex Fr. of Garlic Sprout and *F. oxysporum* f. sp. *vasinfectum* were grown on 1 × PDA (potato/dextrose/agar) medium for 3 days at 28 °C. Conidial suspensions were collected by adding sterile water to the surface of the mycelium. Antifungal activity assays with recombinant EARLI1 against *B. cinerea* and *F. oxysporum* were carried out in 10 × 15 mm petri plates containing 10 mL of PDA. Mixtures of 10 μL conidial suspensions (5×10^4 conidia/mL) and 20 μL of 100, 200, or 300 μg/mL recombinant EARLI1 dissolved in PBS (pH 7.4) were added to sterile filter paper discs (0.625 cm in diameter) placed on the medium in advance. PBS buffer without EARLI1 was used as control. Plates were incubated at 28 °C and observed within 72 h.

Cell cultures of *S. cerevisiae* were prepared by inoculation of a single colony of W303-1A *MATa* strain into YPAD Broth (0.0075% l-adenine hemisulfate salt, 1% yeast extract, 2% Bacto® peptone, 2% dextrose) and growing at 28 °C and 200 rpm for 2 days. To assay the effect of EARLI1 on the growth of yeast, cell cultures were diluted to an OD_{600} of 0.1 with YPAD liquid medium and incubated at 28 °C and 200 rpm for 20 h in the presence of 200–600 μg/mL of the recombinant PRD-8CM of EARLI1. Yeast cultures were diluted, spread onto YPAD medium, and the number of colonies formed on the medium from a specified volume was counted to compare the viability. Colony numbers without addition of the recombinant EARLI1 protein were determined as controls. Data of three replicates were statistically analyzed using the Student's *t* test.

2.8. Plasma membrane permeabilization

Plasma membrane permeabilization was measured by SYTOX green uptake as described previously by Diz et al. (2006) with some modifications. Aliquots of 100 µL *S. cerevisiae* cells grown for 20 h in the presence of 200 µg/mL rEARLI1 were incubated with 0.2 µM SYTOX green for 30 min at 25 °C with periodic agitation, followed by observation using an Olympus Fluoview FV1000 laser scanning confocal microscope equipped with a fluorescence filter set for fluorescein detection (excitation wavelengths, 450 to 490 nm; emission wavelength, 500 nm). Cell mortality was determined by counting the number of cells showing strong SYTOX green fluorescence under the confocal microscope. BSA (150 µg/mL) and nystatin (1000 U) were used as negative and positive controls, respectively.

2.9. *In vivo* analysis of the effect of EARLI1 on the growth of yeast cells

To transform *S. cerevisiae* with *EARLI1*, the full-length coding sequence of *EARLI1* was inserted in front of the c-myc epitope (EQKLISEEDL) sequence located downstream of the galactose inducible *GAL1* promoter in pESC-URA (Stratagene, La Jolla, CA, USA) to generate C-terminal tagged protein (Zhang and Schläppi, 2007). The plasmid was transformed into *S. cerevisiae* host strain W303-1A *MATa*. Yeast cells were grown on agar solidified SC-URA medium (1.45 g/L yeast nitrogen base without amino acid, 5 g/L ammonium sulfate, 2 g/L yeast dropout mix without uracil) at 28 °C for 2–3 days. Single colonies transformed with the pESC-URA empty vector or pESC-EARLI1 were inoculated into SC-URA liquid medium and shaken at 200 rpm under the same conditions for 2–3 days. Two microliters of yeast cells was used in PCR reactions to verify the presence of *EARLI1* using *GAL1* promoter *CYC1* terminator specific primers flanking the *EARLI1* insert in pESC-URA. Cell cultures were then diluted to an OD_{600} of 0.1 with SC-URA supplemented with 2% sucrose and grown for 24 h. Cell cultures grown for 24 h were diluted to 10^{-1}, 10^{-2} or 10^{-3} with SC-URA medium containing 2% sucrose. Aliquots of 5 µL were spotted onto solidified SC-URA medium containing 2% sucrose or SC-URA medium containing 2% galactose respectively and grown for 72 h to evaluate the influence of EARLI1 expression on the growth of yeast cells.

Yeast cells growing in SC-URA medium containing 2% sucrose with an OD_{600} of 0.2 were precipitated and resuspended in SC-URA medium containing 2% galactose. Analysis of plasma membrane permeabilization was carried out by addition of SYTOX green with a final concentration of 0.2 µM after 1 h of galactose induction.

2.10. Western blot analysis of yeast cells transformed with EARLI1

Yeast cells were grown on agar-solidified SC-URA medium. Single colonies transformed with empty vector or *EARLI1* were inoculated into SC-URA liquid medium containing 2% sucrose and cultured at 28 °C for 12–24 h by shaking. Cell cultures were diluted to an OD_{600} of 0.1 with SC-URA liquid medium containing 2% sucrose and grown overnight. Then the cell cultures were diluted to an OD_{600} of 0.2 with SC-URA liquid medium containing 2% galactose to induce *EARLI1* from the *GAL1* promoter and grown for 24 h. Yeast cells were collected by centrifugation from 3 mL suspension cultures. After rinsing with autoclaved water, yeast cells were resuspended in 0.5 mL 1 × TBS buffer. To isolate total proteins, yeast cells were broken with acid-washed glass beads by vortexing for about seven times for 30 s alternating with 30 s incubation on ice. After centrifugation, supernatants were transferred to new Eppendorf tubes and 125 µL of 10% TCA was added to precipitate proteins at 4 °C for 10 min. Precipitated proteins were collected by centrifugation at 20,000 *g* for 5 min and washed twice with cold acetone. After drying at 95 °C for 5 min, protein samples were resuspended in 20 µL 1 × loading buffer and separated by 15% SDS-PAGE. For Western blotting, proteins were electrotransferred to PVDF membranes. Membranes were blocked in TBS buffer supplemented with 5% skim milk according to standard procedures, followed by incubation for 3 h at room temperature with 1:1000 diluted rabbit anti-myc antibodies (GenScript, China). Monoclonal goat anti-rabbit IgG alkaline phosphatase conjugate (Beijing Biosynthesis Biotechnology CO., LTD.), 1:5000 diluted, was used to visualize antibody-bound c-myc portions on membrane

with BCIP and NBT. PageRuler™ Prestained Protein Ladder RDM604 (Runde, Xi'an) was used as molecular mass marker, and the molecular mass of each component from big to small is 170, 130, 100, 70, 55, 40, 35, 25, 15 and 10 kDa respectively.

3. Results

3.1. Sequence analysis and protein modeling

The full-length open reading frame of *EARLI1* contains 507 base pairs and encodes a HyPRP protein of 168 amino acids with a calculated molecular mass of 17.3 kDa and a pI of 8.96. The deduced amino acid sequence of EARLI1 (Fig. 1) starts with a putative signal peptide consisting of 25 amino acids. A hydrophilic PRD in the middle shares high similarity with polypeptides found in the cell wall, such as proline-rich proteins (PRPs) and extensins. The C-terminal hydrophobic 8CM from amino acids 85 to 168 of EARLI1 contains eight conserved cysteine residues similar to the arrangement found in plant LTPs, including AtLTP1 of *Arabidopsis thaliana* (NM_129411), HvLTP1 of *Hordeum vulgare* (X59253), Zm9C2 of *Zea mays* (J04176), and TdpTd4.9 of *Triticum durum* (X63669). Amino acid sequence analysis of the 8CM of EARLI1 revealed typical features of plant LTPs, that is, absence of tryptophan and phenylalanine residues, and conservation of the positions of the eight cysteine residues that could form a network of disulfide bridges necessary for the maintenance of the tertiary structure of the molecule together with the central helical core, while several variable loops might provide sequence specificity of the protein (Fig. 2). However, in contrast to LTPs, the 8CM of EARLI1 was shown to be insoluble (Zhang and Schläppi, 2007).

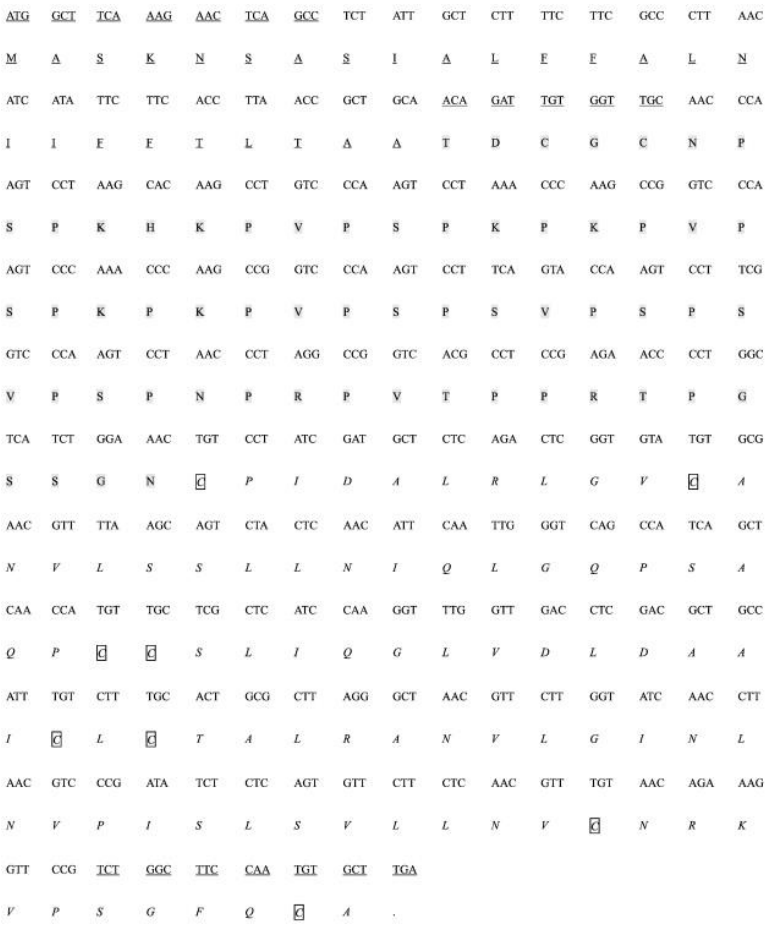

Fig. 1. Nucleotide sequence (AT4G12480, NM_117318) and deduced amino acid sequence (NP_192985) of *EARLI1*. Forward primer and reverse primer used in PCR are underlined. A dot marks the stop codon. The 25

N-terminal amino acids of the signal peptide are underlined. PRD is shadowed. Eight conserved cysteines are framed. 8CM is shown in italics.

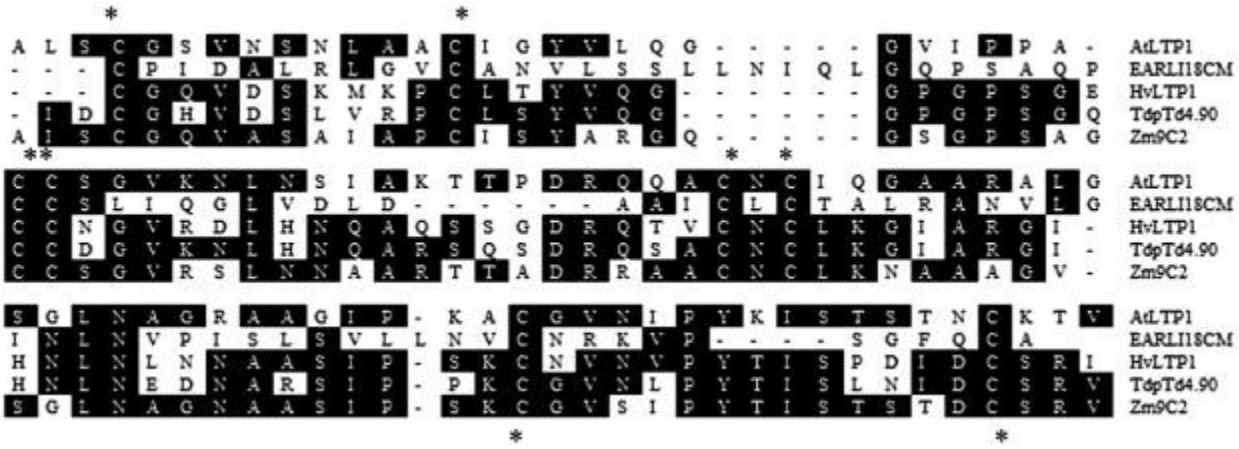

Fig. 2. Alignment of the deduced amino acid sequence of EARLI1 8CM with that of typical lipid transfer proteins (LTPs). AtLTP1, amino acids (aa) 26–117 of *Arabidopsis thaliana* LTP1 (NM_129411). EARLI18CM, aa 85–168 of EARLI1 (NM_117318). HvLTP1, aa 29–116 of *Hordeum vulgare* LTP1 (X59253). TdpTd4.9, aa 24–113 of *Triticum durum* pTd4.90 (X63669). Zm9C2, aa 28–119 of *Zea mays* 9C2 (J04176). Positions of identical amino acid are shown in black. Eight conservative cysteines are tagged with stars. Gaps (−) were introduced to optimize the alignment.

Three-dimensional structure of the 8CM of EARLI1 was simulated *in silico* using the crystal structure of a hydrophobic protein (1hypA) from soybean (*Glycine max*) as template (Fig. 3) (Arnold et al., 2006). The structure of the 8.3 kDa hydrophobic protein from soybean was previously determined by X-ray diffraction methods at the 1.8 Å resolution. The molecule is made up of four helices together with the connecting loops and a twisted beta-strand. Its crystal packing is dominated by hydrophobic interactions and 70% apolar atoms distributed around the surface (Baud et al., 1993). Based on the high similarity between the 8CM of EARLI1 and plant LTPs, it can be speculated that the global fold of the 8CM of EARLI1 involves four helical fragments connected by three loops and a C-terminal tail, and that the structure is stabilized by four disulfide bridges presumably formed by the eight conserved cysteine residues. The striking feature of this structure is the existence of an internal hydrophobic cavity running through the whole molecule (Heinemann et al., 1996).

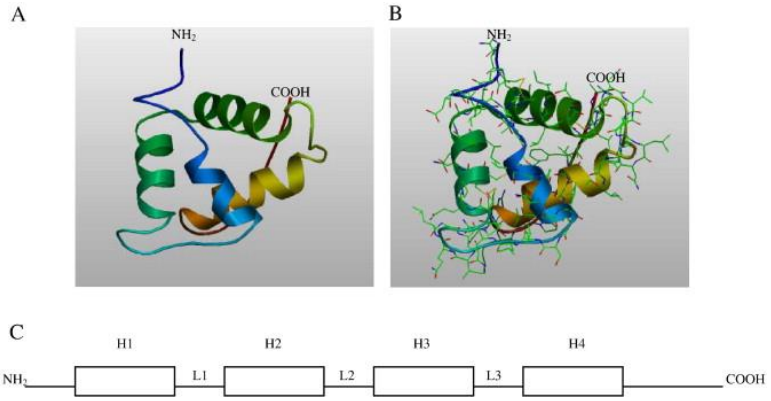

Fig. 3. Structure of the 8CM of EARLI1. (A) Tertiary structure of the 8CM of EARLI1 simulated with the soybean 1hypA as template by homology modeling using the SWISS-MODEL server (http://swissmodel.expasy.org/). (B) Tertiary structure of the 8CM of EARLI1 with side chains of amino acids. C, Diagram of the secondary structure of the 8CM of EARLI1. H, helix; L, loop.

3.2. Characterization of antifungal activity of recombinant EARLI1 protein

SDS-PAGE analysis revealed a 18 and a 36 kDa protein band corresponding to monomers and dimers of the 6 × His-EARLI1 fusion protein lacking the signal peptide (Fig. 4A). Western blotting analyses indicated that the recombinant EARLI1 expressed in *E. coli* mainly existed in the insoluble fraction (Fig. 4B). In addition, long time incubation with urea reduced the amounts of EARLI1 dimers (Fig. 4C). Recombinant His-EARLI1 purified by metal affinity chromatography and the protein extraction kit BSP062 (Sangon, Shanghai) mainly existed as dimmers (Fig. 4D). Ni^{2+} high-affinity resin column can be used specially to purify the recombinant protein carrying His tag. The protein bands of high molecular mass came from oligomerization of rEARLI1 by means of hydrophobic interaction, because EARLI1 contains a C-terminal 8CM with strong hydrophobicity (Zhang and Schläppi, 2007).

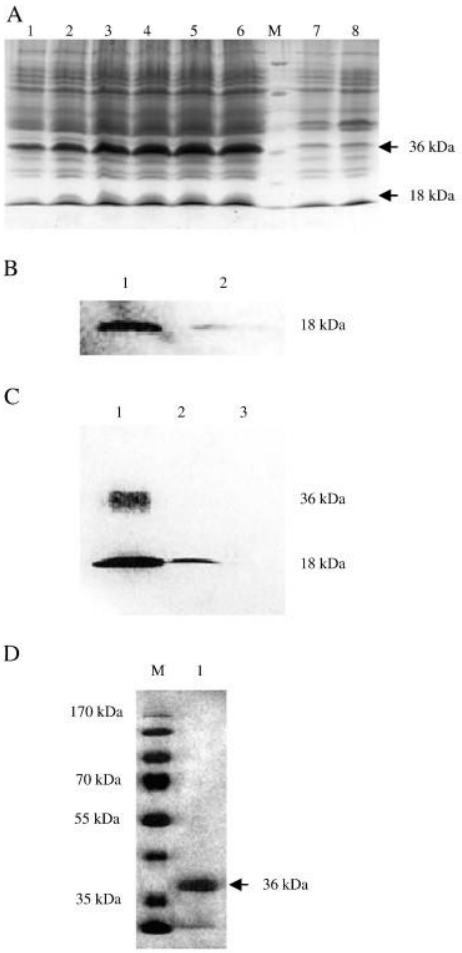

Fig. 4. Expression analyses of EARLI1 lacking the signal peptide in *E. coli*. (A) Optimization of induction conditions. Bacterial suspensions with an OD_{600} of 0.6 were induced with 1 mM IPTG at 37 °C. Cells were harvested at different time points. 1–6, BL21(DE3) clones containing pET28a-EARLI1 were induced with IPTG for 1 to 6 h; M, Unstained Protein Molecular Weight Marker SM0431; 7, BL21(DE3) clone containing pET28a-EARLI1 not induced with IPTG; 8, BL21(DE3) containing the empty pET28a vector was induced with IPTG. The arrows show the dimer (36.4 kDa) and monomer (18.2 kDa) of recombinant His-EARLI1 protein lacking the signal peptide. (B) Solubility analysis of the recombinant His-EARLI1 fusion protein by Western blotting. Lane 1, insoluble fraction of IPTG-induced *E. coli* BL21(DE3) containing pET28a-EARLI1; lane 2, soluble fraction of IPTG-induced *E. coli* BL21(DE3) containing pET28a-EARLI1. (C) Western blotting analysis of recombinant EARLI1 protein in inclusion bodies after urea solubilization. Lane 1, inclusion bodies after 2 h urea treatment; lane 2, inclusion bodies after 6 h urea treatment; lane 3, total protein from *E. coli* transformed with empty pET28a. (D) SDS-PAGE analysis of the His-tagged EARLI1 protein purified by immobilized metal affinity chromatography with

Ni-NTA resin and the protein extraction kit BSP062 (Sangon, Shanghai). M, PageRuler™ Prestained Protein Ladder RDM604; 1, purified recombinant His-EARLI1 protein lacking the signal peptide, the arrow shows the dimer (36.4 kDa).

The inhibitory effect of rEARLI1 against *S. cerevisiae* was first determined by treatment of yeast cultures with different concentrations of purified fusion protein and measuring a reduction in the number of surviving cells. Compared to untreated controls, addition of 200, 400 and 600 μg/mL rEARLI1 reduced the number of viable yeast cells by 12%, 33%, and 41%, respectively (Fig. 5). Antifungal activity of the rEARLI1 against *B. cinerea* was determined on PDA medium. Addition of 300 μg/mL of the rEARLI1 protein to a conidial suspension completely inhibited conidial germination and hypha growth (Figs. 6A–C). Similarly, addition of 200 μg/mL of the rEARLI1 protein to a conidial suspension could completely repress conidial germination and hypha growth of *F. oxysporum* (Fig. 6D).

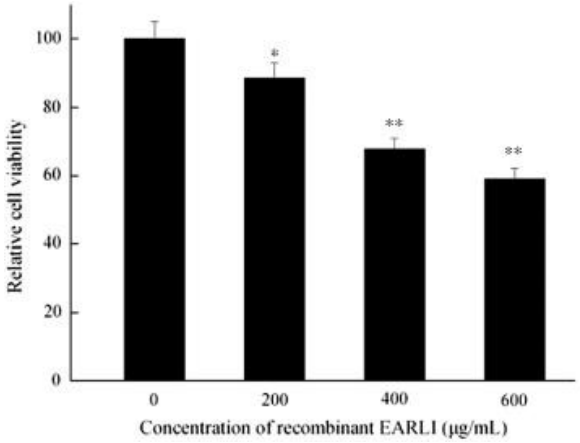

Fig. 5. Inhibition of *S. cerevisiae* growth by recombinant EARLI1 protein lacking the signal peptide. Cell viability after treatment with 200–600 μg/mL of EARLI1 recombinant protein for 20 h was determined by counting the number of colonies formed on agar solidified medium containing 2% sucrose. The viability of the control without addition of EARLI1 recombinant protein was set to 100. All values are means (± SD) of triplicates. * indicates significant differences at $P < 0.05$, ** indicates significant differences at $P < 0.01$.

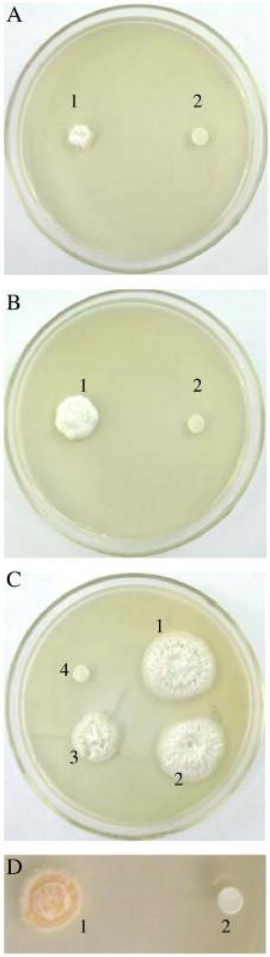

Fig. 6. Antifungal activity of recombinant EARLI1 protein against *B. cinerea* and *F. oxysporum*. 10 µL conidial suspension was mixed with 20 µL PBS buffer (pH 7.4) containing recombinant (r) EARLI1 protein and added to filter paper disc, then incubated on agar solidified medium at 28 °C. (A) 24 h incubation of *B. cinerea*. 1, PBS without rEARLI1; 2, PBS containing 300 µg/mL rEARLI1. (B) 48 h incubation of *B. cinerea*. 1, PBS without rEARLI1; 2, PBS containing 300 µg/mL rEARLI1. (C) 72 h incubation of *B. cinerea*. 1, PBS without rEARLI1; 2, PBS containing 100 µg/mL rEARLI1; 3, PBS containing 200 µg/mL rEARLI1; 4, PBS containing 300 µg/mL rEARLI1. (D) 72 h incubation of *F. oxysporum*. 1, PBS without rEARLI1; 2, PBS containing 200 µg/mL rEARLI1.

To investigate the potential mechanism of antifungal activity, we determined whether rEARLI1 permeabilized the plasma membrane of *S. cerevisiae* cells. SYTOX green penetration into permeabilized cells was assessed in the presence of 200 µg/mL rEARLI1 30 min after the addition of the fluorescent dye using a confocal microscope. Compared to negative control cells grown in the presence of BSA, many *S. cerevisiae* cells showed strong SYTOX green fluorescence either in the presence of nystatin (positive control) or rEARLI1 (Fig. 7, Fig. 8), indicating treatment with rEARLI1 would lead to an increase of the plasmalemma permeability of yeast cells.

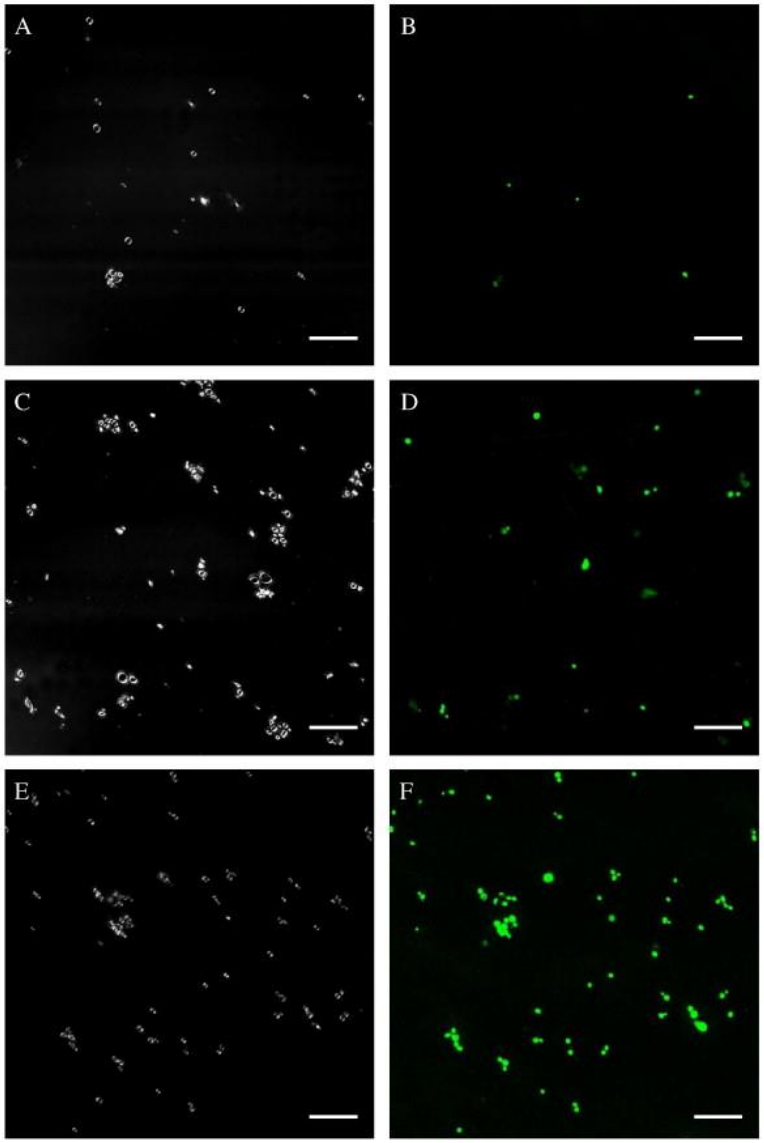

Fig. 7. Confocal microscopy of *S. cerevisiae* cells treated with different proteins for 20 h and incubated with SYTOX green for 30 min. (A) negative control cells treated with 150 μg/mL BSA under dark field; (B) negative control cells treated with BSA viewed by fluorescence; (C) cells treated with 200 μg/mL rEARLI1 under dark field; (D) cells treated with rEARLI1 viewed by fluorescence; (E) positive control cells treated with 1000 U nystatin under dark field; (F) positive control cells treated with nystatin viewed by fluorescence. Bar = 50 μm.

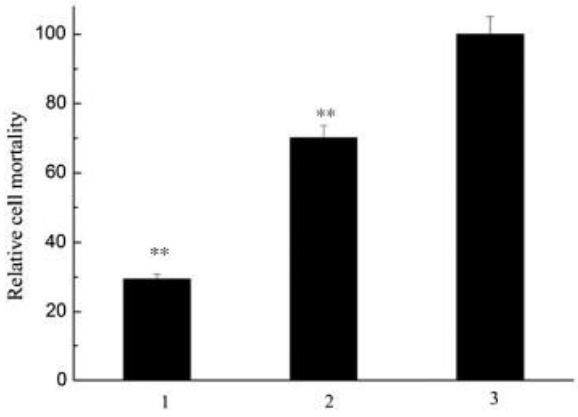

Fig. 8. Quantification of plasmalemma permeability of *S*. *cerevisiae* after treatment with recombinant EARLI1 protein lacking the signal peptide. 1, 150 μg/mL BSA; 2, 200 μg/mL of recombinant EARLI1 protein; 3, 1000 U nystatin. The value for the positive control (treated with 1000 U nystatin) was set to 100. All values are means ± SD of triplicates. ** indicates significant differences at $P < 0.01$.

3.3. Influence of *in vivo* expression of EARLI1 on the growth of yeast cells

To further assess the antifungal activity observed in *in vitro* experiments using rEARLI1 lacking its signal peptide, the intact open reading frame of *EARLI1* was ligated into the yeast expression vector pESC-URA and introduced into *S. cerevisiae*. Individual colonies were examined to determine the presence of pESC-EARLI1 with forward primer 5′-ATTTTCGGTTTGTATTACTTC-3′ and reverse primer 5′-GTTCTTAATACTAACATAACT-3′ by PCR. Because the forward and reverse primers are located on both sides of the MCS, the expected size of the PCR product should be 288 bp plus the size of *EARLI1* open reading frame (507 bp). As shown in Fig. 9A, the size of PCR product of pESC-EARLI1 transformed yeast clone is 795 bp, while the size of PCR product of yeast clone transformed by empty pESC-URA is 288 bp.

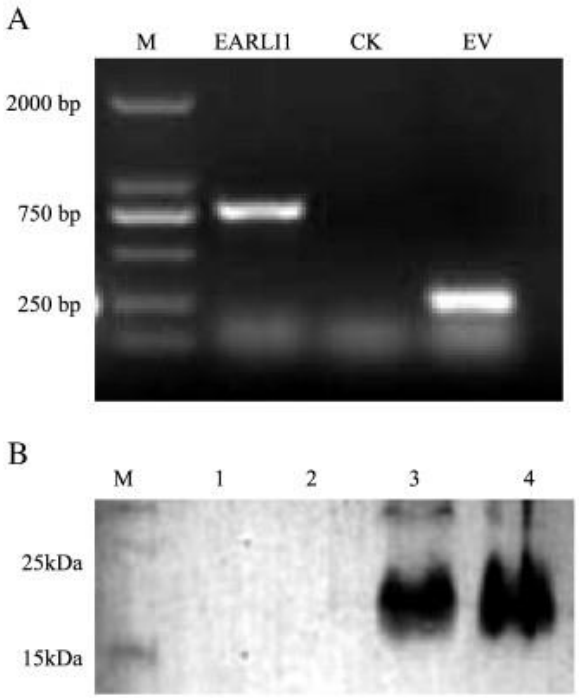

Fig. 9. Analysis of EARLI1 expression in yeast cells transformed with pESC-EARLI1 by PCR and Western blotting analyses. (A) Colony PCR of pESC-EARLI1 transformed yeast cells; M, DL2000 DNA Ladder (Sangon, Shanghai), the size of the bands from top to bottom was 2000, 1000, 750, 500, 250 and 100 bp respectively; EARLI1, yeast clone transformed by pESC-EARLI1; CK, negative control, no template; EV, yeast clone transformed by empty pESC-URA. (B) Western blotting analysis of EARLI1 expression in yeast cells; M, PageRuler™ Prestained Protein Ladder RDM604; 1–2, yeast cells transformed with pESC-URA empty vector; 3–4, yeast cells transformed with pESC-EARLI1.

To determine whether the c-myc epitope tagged EARLI1 fusion protein was expressed efficiently in yeast cells after galactose induction, Western blotting was carried out using anti-myc antibodies. The results indicated that the EARLI1-myc fusion protein was translated efficiently in yeast cells transformed with pESC-EARLI1, while no signal was observed in control samples transformed with the empty vector (Fig. 9B).

To measure the effect of EARLI1-myc induction during growth of yeast cells, overnight yeast cultures grown in SC-URA medium under non-inducing conditions were diluted to 10^{-1}, 10^{-2}, 10^{-3} and spotted onto agar-solidified

SC-URA medium supplemented with 2% sucrose (*EARLI1* non-inducing) or 2% galactose (*EARLI1*-inducing). The growth state of the yeast colonies were evaluated after 48 h and 72 h of incubation at 28 °C. The results indicated that compared to control yeast cells transformed with the empty vector, cells harboring *EARLI1* containing plasmids showed a significant decrease in growth on *EARLI1*-inducing SC-URA medium containing 2% galactose, but not on control medium containing 2% sucrose (Fig. 10). In general, yeast cells transformed with pESC-URA harboring a foreign gene will form visible colonies after 72 h growth on SC-URA medium containing galactose even though the expression of the foreign gene could repress the propagation rate of yeast cells. However, when the dilution ratio reached to 1/1000, the expression of EARLI1 induced by galactose can inhibit the growth of yeast cells completely and no colony could be observed on SC-URA medium containing galactose.

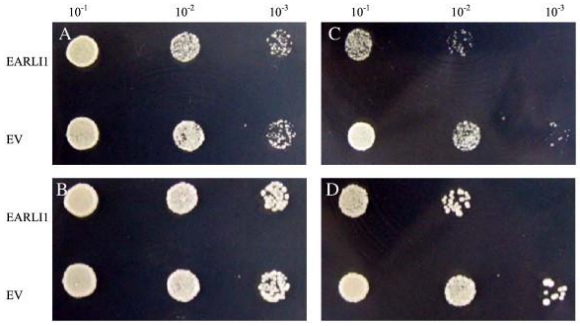

Fig. 10. Inhibition of the growth of yeast cells by *in vivo* expression of *EARLI1*. (A) 48 h after growth on SC-URA medium containing 2% sucrose; (B) 72 h after growth on SC-URA medium containing 2% sucrose; (C) 48 h after growth on SC-URA medium supplemented with 2% galactose; (D) 72 h after growth on SC-URA medium supplemented with 2% galactose. EARLI1, pESC-EARLI1 transformed yeast cells; EV, empty vector pESC-URA transformed yeast cells. Cell cultures with an OD_{600} of 0.1 were grown for 24 h in SC-URA medium supplemented with 2% sucrose and were diluted to 10^{-1}, 10^{-2} or 10^{-3} with the same medium.

The influence of EARLI1 to the plasma membrane of yeast cells after a brief period of *EARLI1* overexpression in SC-URA medium containing galactose also was measured. When SYTOX green was added 1 h after galactose induction, 15.3% of yeast cells transformed by pESC-EARLI1 were permeable to SYTOX green. In contrast, in noninducible SC-URA medium containing sucrose, only 3.1% of yeast cells transformed by pESC-EARLI1 were permeable to SYTOX green. Yeast has no homologous protein of EARLI1, *in vivo* experiment further confirmed that EARLI1 could inhibit the growth of yeast cells by increasing the permeability of plasma membrane (Fig. 11).

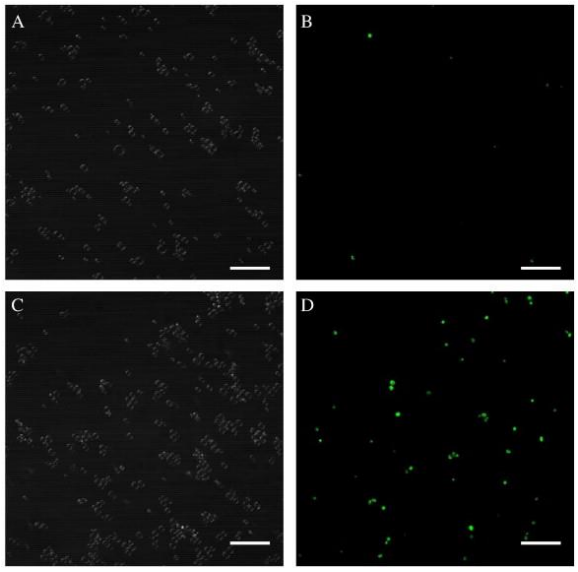

Fig. 11. Confocal microscopy of the effect of *in vivo* expression of *EARLI1* on plasma membrane permeability of *S. cerevisiae* cells. (A) Uninduced yeast cells transformed with pESC-EARLI1 under dark field; (B) Uninduced yeast cells transformed with pESC-EARLI1 viewed by fluorescence; (C) Yeast cells transformed with pESC-EARLI1 after 1 h induction by galactose under dark field; (D) Yeast cells transformed with pESC-EARLI1 after 1 h induction by galactose viewed by fluorescence. Bar = 50 μm.

4. Discussion

EARLI1 from Arabidopsis consists of 168 amino acids, its 83-amino-acid-long C-terminal 8CM shares high similarity with plant nsLTPs (Xu et al., 2011a, Zhang and Schläppi, 2007). Increasing evidences confirmed that LTPs play an important role in the protection of plants against microbial infection (Zottich et al., 2011). In the present work, the encoding sequence of EARLI1 lacking the signal peptide was amplified by PCR and expressed in BL21(DE3) strain of *E. coli*. Purified recombinant EARLI1 was adopted to analyze its antifungal activity. At the same time, the intact encoding sequence of EARLI1 was inserted into pESC-URA to evaluate the influence of its inducible expression under the control of *GAL1* promoter on the growth of *S. cerevisiae*. Sequence analysis and protein modeling indicated that the C-terminus from 85 to 168 amino acids of EARLI1 contains eight conserved cysteine residues similar to plant LTPs. *In vitro* experiments indicated that recombinant EARLI1 lacking the signal peptide could repress the growth of *B. cinerea*, *F. oxysporum* and *S. cerevisiae* significantly. SYTOX green uptake experiments confirmed that treatments with the recombinant EARLI1 lacking the signal peptide could increase the plasmalemma permeability of yeast cells. *In vivo* expression of intact EARLI1 after induction with galactose also showed remarkably inhibitory effect on the growth of *S. cerevisiae* cells. It suggests that recombinant EARLI1 lacking the signal peptide possesses remarkable antifungal activity and *in vivo* expression of intact EARLI1 can repress the division of yeast cells. Our results demonstrated that as a protein localized to cell surface (Zhang and Schläppi, 2007), EARLI1 was involved in stress defense of *A. thaliana* just like LTPs.

EARLI1 belongs to the plant specific HyPRP family because it possesses a hydrosoluble PRD and a water-insoluble 8CM. Due to the presence of N-terminal signal peptides, HyPRPs were found to be located on the surface of plant cells and might be involved in defense reactions against phytopathogens and the adaptation of plants to various environmental conditions (Zhang and Schläppi, 2007). EARLI1-GFP fluorescence in transgenic Arabidopsis roots and immunoblot analyses using protoplasts indicated that EARLI1 is localized to the cell wall (Zhang and Schläppi, 2007). It suggests that EARLI1 might have a bimodular architecture in which the PRD interacts with the cell wall and the 8CM domain with the plasma membrane. SDS-PAGE analyses in the present work exhibited that recombinant EARLI1 lacking signal peptide could form higher order complexes possibly *via* its hydrophobic C-terminus.

The existence of 8CM implies EARLI1 shares similarity in function with LTP. It was previously demonstrated that LTPs might have antimicrobial activities. Transgenic rice containing a nsLTP gene of *Allium cepa* had enhanced resistance against *Magnaporthe grisea*, *Rhizoctonia solani*, and *Xanthomonas oryzae* pv. *oryzae* (Patkar and Chattoo, 2006). Transgenic wheat harboring a nsLTP gene showed enhanced antifungal activity against *Blumeria graminis* f. sp. *tritici* (Roy-Barman et al., 2006). The tobacco NtLTP1 was shown to complex with jasmonic acid (JA) and to interact with a plasma membrane-located elicitin receptor involved in the hypersensitive response, and exogenous application of the NtLTP1-JA complex to tobacco plants conferred protection against infection of *Phytophthora parasitica* (Buhot et al., 2004). Exogenous application of a VvLTP4-JA complex to grapevine plantlets also induced a high level of tolerance against *B. cinerea* (Girault et al., 2008). The recombinant wheat LTP 3F1 exhibited broad-spectrum antifungal activity against *Alternaria* sp., *Rhizoctonia solani*, *Curvularia lunata*, *Bipolaris oryzae*, *Cylindrocladium scoparium*, *B. cinerea*, and *Sarocladium oryzae* (Kirubakaran et al., 2008). LTPs were also reported to be involved in long-distance signaling during systemically acquired resistance in Arabidopsis and in cell wall loosening during pollen-pistil interactions (Nieuwland et al., 2005), and the anti-

phytopathogenic activity of LTPs probably involves membrane permeabilization of target cells (Regente et al., 2005).

To determine whether a LTP related plant specific HyPRP had antifungal activity, we purified recombinant EARLI1 protein lacking its signal peptide from *E. coli*. One disadvantage of bacterial expression systems is the frequently observed misfolding of target proteins, formation of insoluble aggregates such as inclusion bodies, and/or oligomerization, which can lead to a biologically inactive protein (Cardamone et al., 1995). SDS-PAGE analyses showed that the recombinant His-EARLI1 fusion protein mainly existed in the insoluble fraction. In subsequent experiments, the recombinant His-EARLI1 protein was solubilized from inclusion bodies using urea and purified by immobilized metal affinity chromatography (Janknecht et al., 1991). *In vitro* experiments then showed that the recombinant EARLI1 protein inhibited growth of the three different fungal species *S. cerevisiae*, *F. oxysporum* and *B. cinerea*. In the present work, the antimicrobial activity of EARLI1 also was confirmed in *in vivo* experiments by expression of EARLI1 without 6 × His tag in yeast cells. It suggested that 6 × His tag located in the N-terminus and in front of the PRD was not interfering with the function of EARLI1.

To corroborate the antifungal activity observed in *in vitro* experiments, full-length *EARLI1* was introduced into yeast cells under the control of *GAL1* promoter and induced by galactose. Once the yeast cells were pipetted onto agar-solidified medium containing 2% galactose, their growth was inhibited significantly compared to yeast cells containing the empty vector only. In contrast to this, the growth of yeast cells transformed with either the empty pESC-URA vector or pESC-EARLI1 was similar on medium supplemented with 2% sucrose, a sugar that does not activate the *GAL1* promoter. The observed inhibition of yeast growth in the presence of induced EARLI1 was consistent with the results of the *in vitro* experiments, further confirming that EARLI1 had a role in inhibiting fungal growth. Our results thus demonstrated that *S. cerevisiae* can be used as an efficient system to assay antifungal activities of plant genes. In Arabidopsis, EARLI1 has been proven to be localized to cell surface (Zhang and Schläppi, 2007). That suggests that EARLI1 is a membrane binding protein rather than a protein secreted out of the cell. AZI1 is a paralog closely related with EARLI1 in structure, both contain a signal peptide. Our previous work indicated that AZI1 expressed in yeast cells existed both in proteins from protoplast and proteins from the cell wall, indicating that AZI1 is also a protein localized to the cell surface, it probably binds the plasma membrane with the C-terminal 8CM and the cell wall with the hydrophilic PRD (Xu et al., 2011b). These evidences showed that the signal peptide of a plant protein could be recognized by yeast cells and EARLI1 expressed in yeast cells was most likely to be secreted to the cell surface. On the other hand, it is possible that EARLI1 was expressed in the cytoplasm of yeast cells where it could interact with intracellular target and functioned as an antimicrobial peptide.

Compared to BSA, plasmalemma permeability of yeast cell after 20 h treatment with recombinant EARLI1 protein increased. In addition, a brief period of *EARLI1* expression driven by the *GAL1* promoter in SC-URA medium containing galactose also had obvious effect on plasmalemma permeability of yeast cells. It suggests that EARLI1 is cytotoxic to yeast, it may kill cells through increased cell membrane permeability, and as a consequence the dead cells have become permeable to SYTOX green. Intact *EARLI1* under the control of galactose-inducible *GAL1* promoter encodes a protein containing a signal peptide in N-terminus. After translation in the endoplasmic reticulum, *in vivo* expressed EARLI1 might be localized to cell surface. Our results indicated that EARLI1 expressed by *S. cerevisiae* also showed inhibition effect on the growth of yeast cells by increasing the permeability of the plasma membrane. On the other side, the 8CM of EARLI1 has homology to the protease inhibitor/seed storage protein/lipid transfer protein family of proteins. Therefore, a potential protease inhibitor activity of EARLI1 might interfere, directly or indirectly, with the proteolytic cleavage of regulatory proteins bound to plasma membrane. In this scenario, overexpression of EARLI1 protein results in increased protease inhibitor activity, which possibly affects signal transmission needed by normal growth of yeast cells.

In the present work, we also adopted other pathogens and found that the antimicrobial activity of EARLI1 was microorganism and fungus dependent. This property of EARLI1 is consistent with other plant peptides with antimicrobial activity, including rice LTP110 (Ge et al., 2003), onion Ace-AMP1 (Cammue et al., 1995), BSD1 from Chinese cabbage (Park et al., 2002) and basic cysteine-rich proteins from Brassicaceae species (Terras et al., 1993). Previous researches showed that EARLI1 had a protective effect to yeast cells under stress conditions, for example, yeast cells were frozen at − 20 °C (Xu et al., 2011b, Zhang and Schläppi, 2007) and subjected to 20% PEG, 1 M NaCl, 100 mM LiCl or 42 °C treatments (Priyanka et al., 2010). In contrast to this, the growth of yeast cells after galactose induction was analyzed under normal conditions in the present work. Our results indicated that EARLI1 could inhibit the growth of yeast cells by increasing the permeability of the plasma membrane. This is not conflicting with the results present in previous researches, because it is possible that the increase of plasma membrane permeability have a protective effect to yeast cells under extreme stress conditions instead (Dumont et al., 2004). Extreme stress conditions such as freezing always lead to osmotic stress, expression of EARLI1 could promote the survival rates in these situations probably because it could change the permeability of the membrane. Under normal circumstances, EARLI1 might repress the reproduction of *S. cerevisiae* by influencing the formation of the plasma membrane and the cell wall during asexual budding process (Diz et al., 2006, Regente et al., 2005, Zottich et al., 2011).

Arabidopsis nsLTP1 was identified as a calmodulin (CaM)-binding protein, suggesting that the function of nsLTPs might be mediated by CaM signaling (Wang et al., 2005). Consistent with this, the calcium channel blocker lanthanum chloride or the calcium chelator EGTA repressed the cold-induced expression of *EARLI1*, whereas the calcium ionophore Bay K8644 resulted in cold-independent activation of *EARLI1*, indicating that calcium flux affects the expression of this gene in plants (Bubier and Schläppi, 2004). Our results demonstrated that EARLI1 was involved in the process of pathogen defense, which might also involve calcium signaling. Multiple sequence alignments and molecular modeling showed that the C-terminal 82 amino acids of EARLI1 might form a four-helix bundle with an internal cavity homologous to plant LTPs. Because plant LTPs are thought to be involved in the deposition of extracellular lipophilic molecules such as cutin or wax into the cell wall, it is possible that EARLI1 plays an important role in modification of the Arabidopsis cell wall or membrane composition, or in signal transduction in response to fungal pathogen attack.

5. Conclusions

The present work expressed the Arabidopsis gene *EARLI1* in *E. coli* and demonstrated the antifungal activity of purified recombinant EARLI1 protein against *B. cinerea*, *F. oxysporum* and *S. cerevisiae*. Treatment with recombinant EARLI1 protein increases the plasmalemma permeability of yeast cells and expression of full-length *EARLI1* in *S. cerevisiae* represses the growth of yeast cells. It suggests that recombinant EARLI1 has antifungal activity and expression of the secreted form of the protein in *S. cerevisiae* inhibits yeast growth. EARLI1 most likely affects fungal membrane permeability. *EARLI1* has thus additional pleiotropic effects and plays auxiliary roles in protecting plants against both abiotic and biotic stresses.

Acknowledgments

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