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Inhibitory effects of Arabidopsis *EARLI1* against *Botrytis cinerea* and *Bradysia difformis*

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Abstract

The aim of this study is to understand the function of *EARLI1* in plants subjected to different biotic stresses using *EARLI1* overexpressing (OX) and T-DNA knockout (KO) transgenic Arabidopsis lines. Higher levels of expression of *EARLI1* in OX lines were confirmed by RT-PCR and Northern blot analysis. The full-length *EARLI1* mRNA could not be detected by RT-PCR in KO lines, while only a shorter transcript could be found by RNA gel blotting. In wild-type Col-0 plants (Wt), *EARLI1* could be induced by *Botrytis cinerea* and H_2O_2, indicating this gene might be involved in plant defense system against pathogens. Trypan blue staining of the infected leaves showed that overexpression of *EARLI1* could inhibit the growth of *B. cinerea* and disruption of *EARLI1* in KO lines led to vigorous propagation of the necrotrophic fungus. In addition, KO plants were attacked earlier and more frequently than the wild-type Col-0 plants by fungus gnat (*Bradysia difformis*). In vivo expression in *Saccharomyces cerevisiae* demonstrated that the secreted form of EARLI1 could suppress the cell viability by increasing the permeability of the plasma membrane. As a protein localized to cell wall, EARLI1 might play as a component of a receptor and function in resistant response of plants to biotic stresses by sensing environment changes and delivering the signals to intracellular regulation network.

Introduction

EARLI1 (*EARLY ARABIDOPSIS ALUMINIUM-INDUCED GENE1*, AT4G12480) encodes a HyPRP (hybrid proline-rich protein) protein containing a N-terminal signal peptide, a middle proline-rich domain (PRD) and a C-terminal eight-cysteine-motif (8CM) (Richards and Gardner 1995; Dvořáková et al. 2007). Screening of GENEVESTIGATOR database, a publicly available online resource of microarray analyses (http://www.genevestigator.ethz.ch), indicated *EARLI1* and other three HyPRP genes (*AZI1*, AT4G12490 and AT4G12500) had a significant degree of co-expression, they could be induced by environmental stress factors such as low temperature, salt and drought. Because the nucleotide sequences of these four genes and the aminoacid sequences they encoded share high similarity, *EARLI1*, *AZI1* (AT4G12470), AT4G12490 and AT4G12500 had been classified as EARLI1 subfamily of the HyPRP family (Zhang and Schläppi 2007). In EARLI1 subfamily, *EARLI1* and *AZI1* are more closely related paralogs in comparison with AT4G12490 and AT4G12500.

The inducible expression of *EARLI1* by low temperature had been investigated comprehensively. *EARLI1* could be stably activated by vernalization, and the high transcription level could be maintained when the Arabidopsis

plants subjected to cold treatment were transferred to room temperature (Wilkosz and Schläppi 2000). Overexpression of *EARLI1* in transgenic Arabidopsis plants resulted in reduced electrolyte leakage during freezing damage (Bubier and Schläppi 2004). Moreover, *EARLI1* showed a similar expression profile with *DEA1* of tomato under cold environmental conditions, these two closely related HyPRP genes from distantly related species appeared to have a conserved function (Weyman et al. 2006).

The EARLI1 protein belongs to the lipid transfer protein (LTP) family too. LTPs were considered to be associated with the deposition of cutin and play important roles in plant resistance to pathogen infection (Arondel et al. 2000). Enhanced resistance to *B. cinerea* was observed in Arabidopsis plants overexpressing *EARLI1*, *AZI1* and AT4G12490 (Chassot et al. 2007). In the present work, overexpressing and T-DNA knockout lines of Arabidopsis were utilized to investigate the resistance of *EARLI1* against the infection of *B. cinerea* and infestation of fungus gnat, our results comfirmed that *EARLI1* functioned in defense system of plants under biotic stresses.

Materials and methods

Plant materials and culture conditions

Seeds of Arabidopsis ecotype Columbia-0 (Col-0) were adopted as initial materials. *EARLI1* overexpressing plants were prepared using floral dip method (Bechtold et al. 1993; Clough and Bent 1998). Homozygous T-DNA knockout plants of *EARLI1* were screened from T4 generation of SAIL_86_A06 line (Nottingham Arabidopsis Stock Center, England).

As *EARLI1* has no introns, its coding sequence was amplified directly from genomic DNA of wild-type Col-0 plants by PCR. A *Bam*H I site and an *Eco*R I site were introduced into the 5′ end of the forward primer 5′-GGATCCTTAAAACAAACTTTTG-3′ and the reverse primer 5′-GAATTCCTTCAAGCACATTGGAAG-3′, respectively. To construct an effective plant expression vector, digested PCR product was inserted behind the *Mac* promoter and in front of the *mas* (mannopine synthase gene) terminator in pPZP211 (Gleave 1992). *Mac* is a chimeric promoter combining CaMV (cauliflower mosaic virus) 35S and *mas* elements, including the *mas* region from −301 to +65 and the 35S enhancer region from −941 to −90, and giving a transcription activity of 3–5 times in comparison with double 35S promoter (Comai et al. 1990). *Agrobacterium* strain ABI harbouring pPZP211-EARLI1 were used to transform wild-type Col-0 plants by floral dip method (Clough and Bent 1998). T1 seeds were selected on 1/2 MS (Murashige and Skoog 1962) medium containing 50 mg/L kanamycin (Kan). Kan resistant seedlings were transplanted into humus/vermiculite/perlite (2:1:1) and grown to maturation under long-day photoperiods (16 h day/8 h night). Homozygous transgenic lines were screened by Kan selection and PCR in T3 generation. Col-0 plants transformed with empty vector were used as control. Three independent overexpressing lines were analyzed in subsequent experiments.

Seeds of T-DNA knockout line SAIL_86_A06 in Col-0 background were provided by Nottingham Arabidopsis Stock Center. SAIL_86_A06 possesses Basta resistance because pCSA110 used in preparation of T-DNA lines contains a *bar* gene encoding phosphinothricin N-acetyl transferase. The insertion site of T-DNA in SAIL_86_A06 line is located at 7,406,602 bp of the fourth chromosome of Arabidopsis and the coding sequence of *EARLI1* was disrupted. In the present work, homozygous mutants were screened from seedlings of the T4 generation by two parallel PCR, one PCR contained left border primer of T-DNA and the forward primer of *EARLI1* flanking the insertion site, the other contained the forward primer and the reverse primer of *EARLI1* flanking the insertion site. The sequences of these primers are 5′-TAGCATCTGAATTTCATAACC-3′, 5′-TTTCTTCGCCCTTAACATCA-3′ and 5′-AAGCCAGACGGAACCTTTC-3′, respectively. With the forward primer and the reverse primer of *EARLI1*, a 462 bp fragment should be amplified from genomic DNA of heterozygous T-DNA lines and wild-type plants, but not from genomic DNA of homozygous mutants because *EARLI1* in two homologous chromosomes were all disrupted by T-DNA. With the forward primer of *EARLI1* and left border primer of T-DNA, an expected band of

300 bp should be amplified from homozygous and heterozygous plants, wild-type plants could not produce this product owing to the absence of the T-DNA insert. Genomic DNA was extracted with CTAB (cetyltrimethyl ammonium bromide) method (Stewart and Via 1993).

Expression analyses of *EARLI1*

RT-PCR (reverse transcription-PCR) and RNA gel blot were used in expression analyses of *EARLI1* in wild-type Col-0, *EARLI1* overexpressing and *EARLI1* T-DNA knockout plants. Seeds of different materials were sown on 1/2 MS medium to germinate. After 2 weeks, total RNA was extracted with E.Z.N.A. Total RNA Kit II (Omega Bio-Tek Inc., Doraville, GA, USA), DNA was eliminated with RNase-Free DNase I. The first strands of cDNAs were synthesized with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA). The forward primer and the reverse primer of *EARLI1* used in RT-PCR was 5′-TTTCTTCGCCCTTAACATCA-3′ and 5′-AAGCCAGACGGAACCTTTC-3′, respectively, and the expected PCR product was 462 bp in length. A 428 bp fragment of Arabidopsis housekeeping gene *ACT8* was amplified simultaneously as internal reference with primer pairs of 5′-ATGAAGATTAAGGTCGTGGCA-3′ and 5′-TCCGAGTTTGAAGAGGCTAC-3′. In Northern blot analysis, the above-mentioned PCR products were used as templates in probe preparation with DIG (digoxigenin) High Prime DNA Labeling and Detection Starter Kit II (Roche, Swissland). RNA was fractionated in 1.2 % formaldehyde gel. Hybridization signals were detected with substrates of alkaline phosphatase, NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate).

Response of transgenic lines to infection with *B. cinerea*

Seeds of wild-type Col-0, *EARLI1* overexpressing and *EARLI1* T-DNA knockout lines were sterilized with 0.1 % $HgCl_2$ for 7 min, rinsed with autoclaved distilled water for 5 times and sown on plates containing 1/2 MS medium supplemented with 1 % sucrose and 0.8 % agar. Plates were placed at 4 °C for 2 days to break seed dormancy. After that, the seeds were grown for 2 weeks at 22 °C and 12 h day/12 h night photoperiod in illumination incubator. Two-week-old seedlings were transplanted into vermiculite: perlite (3:1) and grown under the same conditions. Three-week-old plants were used in inoculation with *B. cinerea*.

Botrytis cinerea Pers.ex of Garlic Sprout was grown on 1 × PDA (potato/dextrose/agar) medium. Conidia were harvested in water, filtered through glass wool to remove hyphae, and diluted to 5×10^4 conidia/mL with 1/4 PDB (potato/dextrose) liquid medium (Chassot et al. 2007). Leaves of wild-type Col-0, *EARLI1* overexpressing and *EARLI1* T-DNA knockout plants were inoculated with 5 μL droplets of conidial suspension. The infected plants were covered with a transparent lid to keep 90 % humidity. The presence of lesions on leaf surface was monitored after 1 week.

48 h after *B. cinerea* infection, leaves were stained with 0.4 % trypan blue for 2–3 min in boiling water. The samples were allowed to sit at room temperature for 1 h, and then the chlorophyll was removed with 80 % ethanol. The stained materials were immersed in fresh ethanol and mounted with 20 % glycerol. Growth of hyphae was observed under light microscope. In Northern blot analysis, total RNA was extracted from systemic leaves distal to the localized infection leaf of wild-type Col-0 plants 2 days after inoculation with *B. cinerea* or at different time point after H_2O_2 treatment.

Response of transgenic lines to Infestation with *Bradysia difformis*

Seeds of wild-type Col-0, *EARLI1* overexpressing and *EARLI1* T-DNA knockout lines were sown in pots filled with vermiculite and watered with nutrient solution in 1 week intervals. Three-week-old plants were transferred to illumination incubator infested by fungus gnat, and the injury situation was checked after 1 week. Data of three replicates were statistically analyzed using the Student's *t* test. Damaged and healthy leaves were fixed in FAA fixative containing 3.7 % formaldehyde (v/v), 5 % acetic acid (v/v) and 50 % ethanol, rinsed with water,

dehydrated in an alcohol series, critical-point dried in CO_2, mounted on aluminum stubs, sputter-coated with gold and checked with a Hitachi S2570 scanning electron microscope.

The effect of *EARLI1* on plasma membrane permeability of yeast cells

Saccharomyces cerevisiae strain W303-1A *MATa* (*leu2-3,112; trp1-1; can1-100; ura3-1; ade2-1; his3-11,15*) was used in analysis of the effect of EARLI1 on plasma membrane permeability. The full-length open reading frame of *EARLI1* was amplified by PCR from genomic DNA of wild-type Col-0 and ligated into pESC-URA contains a galactose inducible *GAL1* promoter (Stratagene, La Jolla, CA, USA). *EARLI1* transformed 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 3 h of galactose induction. The fluorescence of SYTOX Green was observed under OLYMPUS FLUOVIEW FV1000 laser scanning confocal microscope (excitation wavelengths, 450–490 nm; emission wavelength, 500 nm). Yeast cells transformed with empty pESC-URA was used as control.

Results

Identification of homozygous *EARLI1* overexpressing and T-DNA knockout plants

EARLI1 overexpressing plants could grow vigorously on 1/2 MS medium containing 50 mg/L Kan because they harboured a bacterial neomycin phosphotransferase gene *NPTII*. In contrast, the cotyledons of the wild-type Col-0 seedlings would turn white and the growth of their roots would be restrained on medium supplemented with 50 mg/L Kan. In the present work, seeds of different T2 plants were sown on 1/2 MS medium containing 50 mg/L Kan, the segregation ratio of Kan resistance in T3 generation was statistically analyzed to discriminate homozygous and heterozygous transgenic plants. All of the offsprings of homozygous transgenic plants were resistant to Kan (Fig. 1).

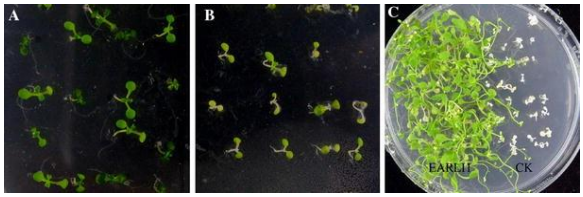

Fig. 1 Screening for homozygous overexpressing lines of *EARLI1*. **a** Homozygous overexpressing line grown on 1/2 MS medium containing 50 mg/L Kan. **b** Wild-type Col-0 plants grown on 1/2 MS medium containing 50 mg/L Kan. **c** Homozygous overexpressing line and wild-type Col-0 plants grown on the same plate containing 50 mg/L Kan. EARLI1, homozygous *EARLI1* overexpressing line; CK, wild-type Col-0 plants

Homozygous *EARLI1* knockout seedlings were identified by PCR from progenies of SAIL_86_A06 line in T3 generation. As shown in Fig. 2a, only a 462 bp fragment could be amplified from genomic DNA of plant 1 with the forward primer and the reverse primer of *EARLI1*, it suggested that this plant was a wild-type plant. In plant 3, a 462 bp band and a 300 bp band could be amplified with two primer combinations respectively, it suggested that this plant was a heterozygous mutant. In plant 2, no PCR product could be amplified with the forward primer and the reverse primer of *EARLI1*, but the 300 bp fragment could be amplified with the forward primer of *EARLI1* and left-border primer of T-DNA, this plant was verified as a homozygous mutant of *EARLI1* (Fig. 2a). Seeds of homozygous T-DNA knockout lines were used in analysis of the mutation effect of *EARLI1* on resistant response of Arabidopsis against biotic stresses subsequently.

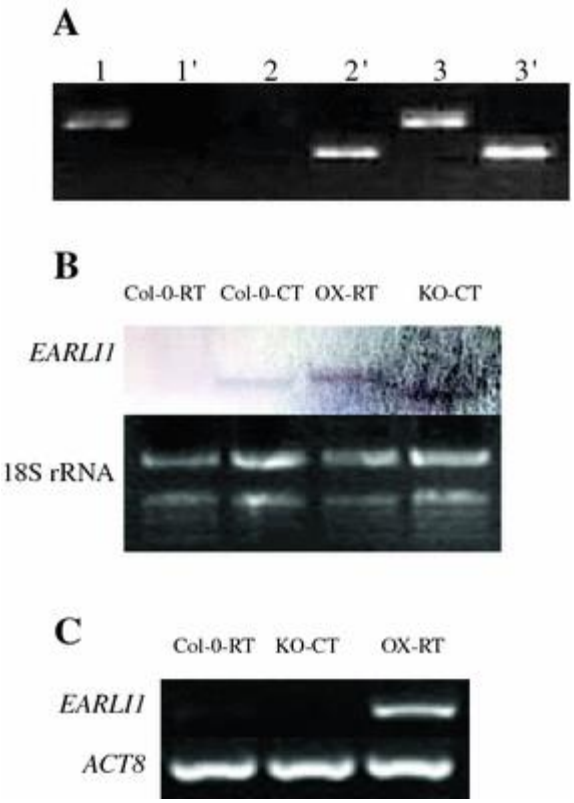

Fig. 2 Expression analysis of *EARLI1* in wild-type Col-0, overexpressing and T-DNA knockout plants. **a** Identification of homozygous T-DNA knockout mutant. *Lanes 1, 2* and *3*, PCR with *EARLI1* upstream and downstream primers; *Lanes 1', 2'* and *3'*, PCR with *EARLI1* upstream primer and T-DNA left border primer. **b** RNA gel blot analysis of *EARLI1*. **c** RT-PCR analysis of *EARLI1*. Col-0-RT, wild-type Col-0 plants grown at room temperature; Col-0-CT, wild-type Col-0 plants grown at 4 °C for 3 h; OX-RT, overexpressing transgenic Arabidopsis plants of *EARLI1* grown at room temperature. KO-CT, homozygous T-DNA knockout plants of *EARLI1* grown at 4 °C for 3 h

Expression analysis of *EARLI1* in homozygous overexpressing and T-DNA knockout plants

RNA gel blotting was used in expression analysis of *EARLI1* in wild-type Col-0, homozygous overexpressing and homozygous T-DNA knockout plants. As shown in Fig. 2b and c, transcription of *EARLI1* was undetectable in wild-type Col-0 plants grown at room temperature. However, *EARLI1* expression could be induced if the wild-type Col-0 plants were incubated at 4 °C for 3 h. In homozygous overexpressing plants grown at room temperature, *EARLI1* was expressed constitutively and the hybridization band was slightly higher than that of the wild-type Col-0 plants subjected to cold treatment, because the transcript of *EARLI1* overexpressing construct was different from that of native *EARLI1* in length. In homozygous T-DNA knockout lines, a smaller band in comparison with the transcript of *EARLI1* in wild-type Col-0 plants could be detected after cold treatment, it represented the incomplete transcript of *EARLI1* owing to interruption by T-DNA. RT-PCR analysis also showed that the transcription level of *EARLI1* was very weak in wild-type Col-0 plants, and no product could be amplified from homozygous T-DNA knockout lines because the primers used in PCR were located in upstream and downstream of T-DNA insertion site respectively (Fig. 2b, c).

Expression of *EARLI1* could be induced by *B. cinerea* infection and H_2O_2 treatment

Wild-type Col-0 plants were infected with *B. cinerea* or treated with 100 mmol/L H_2O_2, RNA was isolated at 0, 24 and 48 h after inoculation with *B. cinerea* or 0, 1, 2, 4, 8, 12 and 24 h after treatment with H_2O_2. As shown in Fig. 3, *EARLI1* could be induced effectively by *B. cinerea*. In treatment with H_2O_2, transcription level

of *EARLI1* was gradually increased from 1 h and peaked in 4 h. After that, the abundance of *EARLI1* mRNA decreased abruptly and returned to normal level from 8 to 24 h (Fig. 3). It suggested that H_2O_2 could activate the expression of *EARLI1* in a short time, but the upregulation of *EARLI1* by H_2O_2 could not be maintained for more than 8 h.

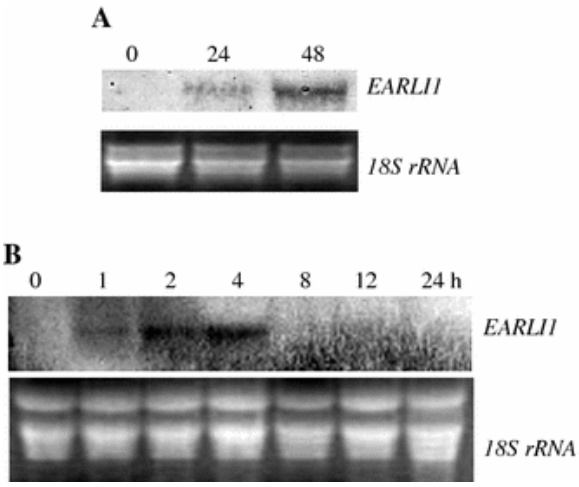

Fig. 3 Inducible expression of *EARLI1* by *B. cinerea* and H_2O_2 in wild-type Arabidopsis plants. **a** Infection with *B. cinerea*; **b** treatment with 100 mmol/L H_2O_2

Resistance of *EARLI1* to *B. cinerea*

Different infection degrees were observed in wild-type Col-0, *EARLI1* overexpressing and homozygous T-DNA knockout plants. One week after inoculation with *B. cinerea*, the leaves of T-DNA knockout plants were damaged significantly and the lesion area was larger than that of wild-type leaves. In contrast, the symptom of *EARLI1* overexpressing plants was slight (Fig. 4). Hyphae of *B. cinerea* could be found on leaf surface of homozygous T-DNA knockout plants by trypan blue staining 48 h after inoculation (Fig. 5c, d), but not on leaf surface of wild-type Col-0 (Fig. 5a) and *EARLI1* overexpressing plants (Fig. 5b). All these results suggested that *EARLI1* could inhibit the growth of *B. cinerea.*

Fig. 4 Infection degree of Arabidopsis leaves by *B. cinerea*. *earli1-1* KO, leaf of homozygous T-DNA knockout line; Col-0, leaf of wild-type Col-0 plants; *EARLI1* OX, leaf of *EARLI1* overexpressing line

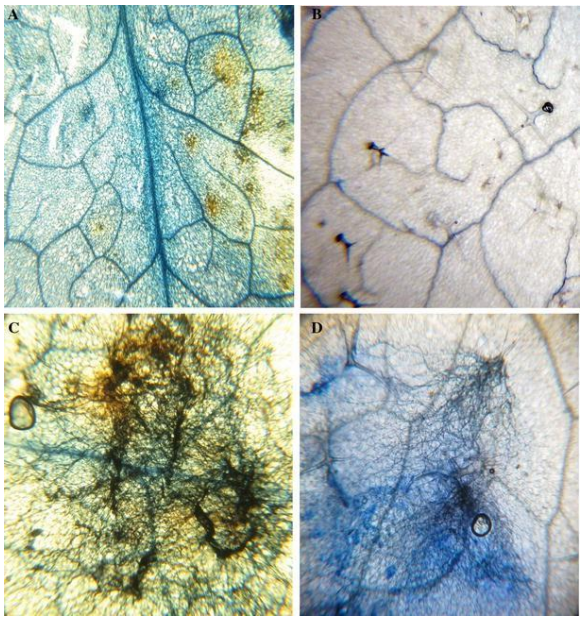

Fig. 5 Hypha staining of *B. cinerea* with trypan blue 48 h after inoculation. **a** leaf of wild-type Col-0 plant; **b** leaf of *EARLI1* overexpressing plant; **c**, **d**, leaves of homozygous T-DNA knockout plants of *EARLI1*

Inhibition of *EARLI1* to fungus gnat damage

In illumination incubator infested by fungus gnat, homozygous T-DNA knockout plants of *EARLI1* were damaged earlier than wild-type Col-0 and *EARLI1* overexpressing plants. Compared to wild-type Col-0 plants (Fig. 6a), the arrangement of the upper epidermal cells in leaves of *EARLI1* overexpressing plants was irregular and the cell wall of them was thickened obviously (Fig. 6b). The upper epidermis of *EARLI1* homozygous T-DNA knockout lines was not smooth, cells were unevenly arranged in different height (Fig. 6c). In order to determine whether the injury in leaf surface was the result of fungus infection or came from insect infestation, the damaged leaves were checked with scanning electron microscope (SEM). SEM observation showed that there were many holes in leaf surface of *EARLI1* T-DNA knockout lines, indicating the damage in leaves was derived from insect biting rather than fungus infection (Fig. 6d). Statistics analysis by Student *t* test to the data came from three repetitive experiments revealed that about 40 ± 2.6 % of the knockout mutant plants were infested by fungus gnat, while only 5 ± 0.6 % of wild-type Col-0 plants and 3 ± 0.2 % of *EARLI1* overexpressing plants were damaged after 2 weeks growth in illumination incubator. Further growth for 9 days in the same environment led to 80 ± 4.9 % of T-DNA knockout plants, 45 ± 3.6 % of wild-type Col-0 plants and 20 ± 3.1 % of overexpressing transgenic plants to be destroyed.

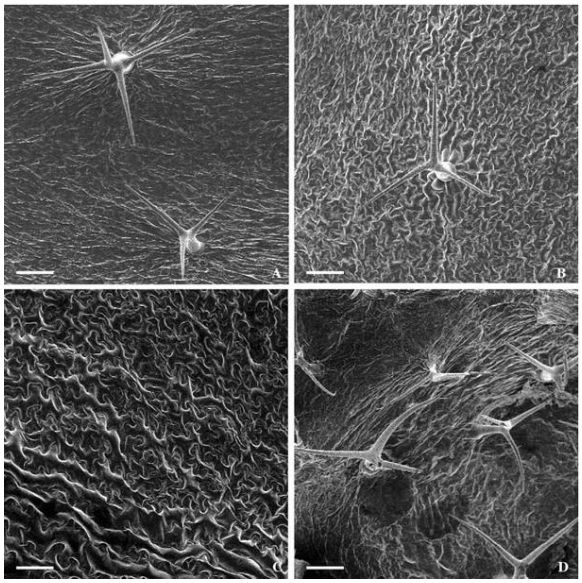

Fig. 6 Adaxial epidermis of Arabidopsis leaf under scanning electron microscope. **a** Leaf of wild-type Col-0 plant, *Bar* = 70 μm; **b** leaf of *EARLI1* overexpressing plant, *Bar* = 70 μm; **c** leaf of *EARLI1* homozygous T-DNA knockout plant, *Bar* = 200 μm; **d** leaf of *EARLI1* homozygous T-DNA knockout plant damaged by fungus gnat, *Bar* = 70 μm

Influence of EARLI1 to plasma membrane permeability of yeast cells

EARLI1 had remarkable effect on permeability of the plasma membrane of yeast cells. When SYTOX Green was added 3 h after galactose induction, a lot of yeast cells transformed by pESC-EARLI1 were permeable to SYTOX Green (Fig. 7c, d). In contrast, only a few yeast cells transformed by empty pESC-URA showed SYTOX Green fluorescence (Fig. 7a, b). It suggests that EARLI1 might repress the growth of fungus by increasing membrane permeability.

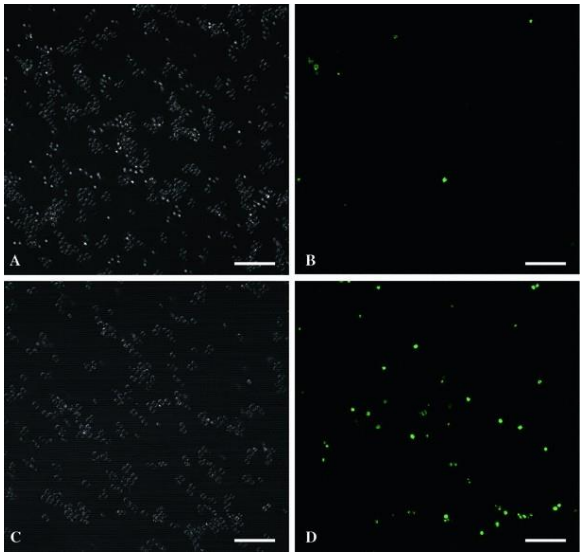

Fig. 7 Influence of in vivo expressed EARLI1 on plasma membrane permeability of *S. cerevisiae* cells. **a** Yeast cells containing pESC-URA after 3 h galactose induction under *dark field*; **b** SYTOX Green fluorescence of yeast cells containing pESC-URA after 3 h galactose induction; **c** Yeast cells containing pESC-EARLI1 after 3 h galactose induction under *dark field*; **d** SYTOX Green fluorescence of yeast cells containing pESC-EARLI1 after 3 h galactose induction. *Bar* = 50 μm. (Color figure online)

Discussion

EARLI1 encodes a secreted HyPRP which also is classified into inhibitor/seed storage/lipid transfer protein family. PRP is one kind of the five structural protein types localized in cell wall and accumulates massively when plants are subjected to physical injury (Kleis-San Francisco and Tierney 1990). PRPs may decide on the special structure of cell wall during plant growth and development, they also may play defense functions under physical damage or pathogen infection (Bradley et al. 1992). HyPRPs form a subclass of PRP and widely exist in higher plants, they contain multiple structures, including the signal peptide in N-terminus, hydrophilic PRD in the middle and hydrophobic 8CM in C-terminus. Owing to their bimodular architecture, HyPRPs have been defined as secretory cell wall proteins (José-Estanyol et al. 2004).

HyPRPs showed spatial and temporal expression patterns, they could be regulated by development stages, plant hormones and external environments. For example, strawberry *FaHyPRP* was specifically expressed in fruit (Blanco-Portales et al. 2004). The transcripts of maize *ZmHYPRP1* only accumulated in embryonic parenchyma cells and could not be found in mature plant organs except ovary before pollination (José-Estanyol et al. 1992). Alfalfa *MsPRP2* could be induced by salt (Deutch and Winicov 1995). Soybean *SbPRP* could be regulated by salicylic acid, endogenous circadian rhythm and various stresses (He et al. 2002).

In order to clarify the function of the HyPRP gene *EARLI1* in resistant response of Arabidopsis to biotic stresses, homozygous T-DNA knockout mutants were screened from progenies of the heterozygous SAIL_86_A06 line in T3 generation. In three PCR primers, two primers were designed based on the upstream and downstream sequence of *EARLI1* flanking the T-DNA insert, the third primer was designed according to the left border of T-DNA. In homozygous mutant, no product could be amplified with upstream and downstream primers of *EARLI1*, but a fragment with a certain size could be amplified with upstream primer of *EARLI1* and left border primer of T-DNA. In heterozygous mutant, two different fragments could be amplified with upstream and downstream primers of *EARLI1* or upstream primer of *EARLI1* and left border primer of T-DNA, respectively. In wild-type plants, only the combination of upstream and downstream primers of *EARLI1* could produce PCR fragment.

In the present work, *B. cinerea* Pers.ex of Garlic Sprout was used as a stimulus in expression analyses of *EARLI1*. *Botrytis* genus includes many phytopathogenic fungi with a wide host range and a necrotrophic life style (Khan et al. 2011). *B. cinerea* is thought to promote programmed cell death in plant cells surrounding the lesion by making use of the plant defense response known as hypersensitive response (HR) (Mur et al. 2008; Noda et al. 2010), which is characterized by accumulation of hydrogen peroxide. Production of reactive oxygen species (ROS) such as hydrogen peroxide is one of the earliest responses of plant tissues to the attack of pathogens and elicitors (Zhang et al. 2010b). This rapid generation and striking transient release of ROS has been defined as “oxidative burst” and has been thought to play important roles in peroxidase-catalyzed crosslinking of proteins in cell wall during pathogen infection (Wang et al. 2007). The results of RNA gel blotting showed that *EARLI1* could be activated by *B. cinerea* and hydrogen peroxide. It suggests the function of *EARLI1* is related with ROS. As a structural protein located in cell wall (Zhang and Schläppi 2007), EARLI1 is probably involved in sensing of biotic stress factors.

Production of H_2O_2 and other ROS is also associated with the growth of plants (Zhang et al. 2010a; Cui et al. 2010; Sun et al. 2010). In Arabidopsis, the transcription of hundreds of genes could be influenced by H_2O_2 based on data derived from microarray analyses (Desikan et al. 2001). In this work, the effect of H_2O_2 on *EARLI1* expression was analyzed 0 to 24 h after treatment. Transcription of *EARLI1* was enhanced along with time within 4 h and declined rapidly after 8 h, indicating EARLI1 functioned in the regulation process of plant response to external oxidative stress.

EARLI1 was also classified as a member of LTP family. Many LTPs were specifically located in epidermis and related to transfer of cutin and wax to surface of plant tissues (Carvalho and Gomes 2007; Yeats and Rose 2008).

Some LTPs were involved in biotic stress and play a role in plant defense system (Aberg et al. 2008; Kirubakaran et al. 2008). Overexpression of pepper *CALTPI* in Arabidopsis led to enhanced resistance to *B. cinerea* (Jung et al. 2005). Similarly, transgenic Arabidopsis plants overexpressing *AZI1*, *EARLI1*, *AT4G12490* also showed resistance to *B. cinerea* (Chassot et al. 2007). Our results demonstrated that overexpression of *EARLI1* could promote the resistance of Arabidopsis to *B. cinerea* and fungus gnat, and in vivo expression of *EARLI1* in yeast cells could increase the permeability of plasma membrane.

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