

Original Article

Overexpression of stress-inducible OsBURP16, the β subunit of polygalacturonase 1, decreases pectin content and cell adhesion and increases abiotic stress sensitivity in rice

Huanhuan Liu^{1,2*}, Yan Ma^{1,2*}, Na Chen^{1,2}, Siyi Guo^{1,2}, Huili Liu¹, Xiaoyu Guo^{1,2}, Kang Chong¹ & Yunyuan Xu¹

¹Key Laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China and

²Graduate University of the Chinese Academy of Sciences, Beijing 100049, China

ABSTRACT

Polygalacturonase (PG), one of the hydrolases responsible for cell wall pectin degradation, is involved in organ senescence and biotic stress in plants. PG1 is composed of a catalytic subunit, PG2, and a non-catalytic PG1 β subunit. OsBURP16 belongs to the PG1 β -like subfamily of BURP-family genes and encodes one putative PG1 β subunit precursor in rice (*Oryza sativa* L.). Transcription of OsBURP16 is induced by cold, salinity and drought stresses, as well as by abscisic acid (ABA) treatment. Analysis of plant survival rates, relative ion leakage rates, accumulation levels of H₂O₂ and water loss rates of leaves showed that overexpression of OsBURP16 enhanced sensitivity to cold, salinity and drought stresses compared with controls. Young leaves of *Ubi::OsBURP16* transgenic plants showed reduced cell adhesion and increased cuticular transpiration rate. Mechanical strength measurement of *Ubi::OsBURP16* plants showed that reduced force was required to break leaves as compared with wild type. Transgenic rice showed enhanced PG activity and reduced pectin content. All these results suggested that overexpression of OsBURP16 caused pectin degradation and affected cell wall integrity as well as transpiration rate, which decreased tolerance to abiotic stresses.

Key-words: abiotic stress; cell adhesion; pectin; polygalacturonase.

INTRODUCTION

Polygalacturonase (PG), one of the cell wall hydrolases, is responsible for wall pectin degradation during fruit ripening. PG1 is composed of a 45 kD catalytic subunit (PG2) and a 38 kD non-catalytic β subunit (PG1 β). Previous studies found tomato PG1 β protein in cell wall extracts, and the cDNA of tomato PG1 β was cloned (Zheng *et al.* 1992, 1994). The PG1 β subunit was purified from PG1 in tomato as a

heat-stable, acidic, heavily glycosylated 38 kD glycoprotein (Tucker *et al.* 1981). The PG1 β subunit functioned as a PG converter to alter the biochemical and enzymatic properties of PG2, resulting in the formation of PG1 (Tucker *et al.* 1981). PG1 β can bind to the cell wall and interact with the catalytic subunit PG2. The interaction of PG1 β with PG2 converts the thermolabile PG2 into the thermostable PG1 (Peeters *et al.* 2004).

PGs are expressed in a wide range of tissues and developmental stages in plants, and are involved in a diverse range of processes including fruit ripening, leaf and flower abscission, pod and anther dehiscence, pathogen defense and plant–host interactions (Brummell & Harpster 2001; Atkinson *et al.* 2002; Swain *et al.* 2011). For example, the PG1 complex is involved in pectin metabolism by regulating pectin solubilization or degradation during fruit ripening in tomato (Watson *et al.* 1994; Zheng *et al.* 1994). Overexpression of PG in transgenic apple leads to various novel phenotypes, including silvery-colored leaves and premature leaf shedding due to reduced cell adhesion in leaf abscission zones (Atkinson *et al.* 2002). *Arabidopsis* dehiscence zone polygalacturonase1 (ADPG1), ADPG2, and QUARTET2 (QRT2) are endo-PGs. ADPG1 and ADPG2 are essential for silique dehiscence, and QRT2 is essential for pollen grain separation (Ogawa *et al.* 2009). Few studies investigating PG or PG1 β -like proteins in rice have been reported.

The BURP domain was named for the proteins BNM2, USP, RD22 and PG1 β , which share the highly conserved sequence CH-X(10)-CH-X(23–26)-CH-X(8)-CH-W (Hattori *et al.* 1998). A previous report showed that plant BURP proteins can be divided into seven subfamilies. In rice, 17 OsBURP proteins have been found in the RD22-like, BURPV, BURPVI, BURPVII and PG1 β -like subfamilies. All the OsBURP genes but OsBURP01 and OsBURP13 are induced by at least one stress treatment such as drought, salt, cold or ABA treatment. The PG1 β -like subfamily consists of three members, OsBURP02, OsBURP14 and OsBURP16. OsBURP16 is responsive to ABA treatment and all the abiotic stress treatments including cold, salt and drought (Ding *et al.* 2009).

Correspondence: Y. Xu. Fax: +86 10 82594821; e-mail: xuyy@ibcas.ac.cn

*Equal contributors to this work.

To address the function of *OsBURP16*, we cloned *OsBURP16* from a cDNA library of cold-stressed rice. In this study, our results indicated that *Ubi::OsBURP16* transgenic plants were hypersensitive to cold, salt and drought stress compared with wild-type Zhonghua 10 (ZH10). The PG activity was higher in transgenic plants than that of ZH10, which caused a decrease in adhesion of cell walls. These results suggest that *OsBURP16* is involved in cell wall formation and abiotic stress response.

MATERIALS AND METHODS

Plant transformation, growth conditions and stress treatments

The wild-type rice cultivar Zhonghua 10 (*Oryza sativa* L. ssp. *japonica* cv. Zhonghua 10; ZH10) was used in this work. Plasmid construction and rice transformation were conducted following methods described previously (Chen *et al.* 2011). The cDNA sequence of *OsBURP16* (LOC_Os10g26940) was amplified from the cDNA library of ZH10 using gene-specific primer pairs 5'-GAA GAT CTT CGA CTT GTC TTT CCA GCA CAG C-3' and 5'-GGG GTA CCC CAC ACA CGG ATA CAT CGC ACT-3', and was cloned into the *Bam* HI and *Kpn* I sites of the pUN1301 vector and driven by the ubiquitin promoter (Ge *et al.* 2004). Transgenic plants of the T3 generation with positive β -glucuronidase (GUS) activity were used in the experiments.

To analyse the response to ABA treatment, T3-generation transgenic seedlings with positive GUS staining and wild-type seedlings were used. The seedlings were grown in the incubator under 16 h light/8 h dark at 30 °C during the day and 28 °C during the night. The light intensity was 540 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Leaves of 2-week-old seedlings were sprayed with 0.1 mM ABA solution and sampled at 0, 1, 3, 6, 12 and 24 h after treatment for RNA isolation (Ding *et al.* 2009).

For cold stress, 2-week-old seedlings were treated at 4 °C for 72 h in a low-temperature chamber under 16 h light/8 h dark, and then the seedlings were transferred to a greenhouse to recover for one week. For RNA isolation the seedlings were sampled at 0, 1, 3, 6, 12 and 24 h after treatment at 4 °C.

For salt stress, germinated seeds were placed into 96-well plates without bottom and floated in Kimura B nutrient solution (Ma & Takahashi 1990) to grow for 2 weeks in an incubator under 16 h light/8 h dark (30 °C/28 °C). The light intensity was 540 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. After two weeks, the 96-well plates with seedlings were transferred into fresh Kimura B nutrient solution containing 200 mM NaCl and treated for 4 d under the same light and temperature conditions. Four days later, the NaCl–Kimura B solution was replaced by fresh Kimura B nutrient solution, and plants were allowed to recover for one week. For RNA isolation, the seedlings were sampled at 0, 1, 3, 6, 12 and 24 h after treatment in the 200 mM NaCl–Kimura B solution.

For drought stress assays, water was withheld from 2-week-old seedlings grown in pots that contained the same weight of soil. Following drought treatment, the plants were watered

normally for 7 d. The 2-week-old seedlings were irrigated with 20% polyethylene glycol (PEG) 6000 followed by sampling at 0, 1, 3, 6, 12 and 24 h for total RNA isolation.

The recovery of the plants was photographed. Plants that had green and healthy young leaves were considered to have survived, and the survival rates were determined (Ouyang *et al.* 2010). About 50 plants of each line were used in each stress experiment, and the experiments were performed at least three times.

Southern blot and transcript measurement

The Southern blot assay of transgenic rice plants was performed according to our previous report (Chen *et al.* 2011). Genomic DNA was isolated from 2-week-old rice seedlings, and 20 μg DNA was digested with *Eco*R I or *Hind* III. The digested DNA fragments were separated on a 0.7% agarose gel through electrophoresis and then blotted onto a nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membrane was pre-hybridized in SuperHyb Solution (Tiandz, Beijing, China) at 65 °C for 4 h. PCR was used to generate [α -³²P] dCTP-labelled *GUS*, using the forward primer 5'-CAA CTG GAC AAG GCA CTA GC-3' and reverse primer 5'-AGC GTC GCA GAA CAT TAC AT-3'. After pre-hybridization, the membrane was hybridized with [α -³²P] dCTP-labelled *GUS* in SuperHyb Solution at 65 °C for 20 h and then washed with 2 \times saline–sodium citrate (SSC) plus 0.1 % sodium dodecyl sulphate (SDS) washing buffer at 65 °C for 20 min and washed twice with 1 \times SSC plus 0.1% SDS at 65 °C for 15 min. The membranes were exposed to X-ray film (Kodak Co Ltd, Xiamen, China) at –70 °C for 3–7 d, and then the signals were obtained using autoradiography.

To determine the transcriptional levels, quantitative RT-PCR (qRT-PCR) was performed. Total RNA was isolated by using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Rice cDNA was synthesized from 2 μg RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed on an Mx3000P instrument (Agilent Technologies Company, Shanghai, China) with SYBR Green Master Mix (Toyobo Co Ltd, Japan) according to the manufacturer's instructions. Gene expression levels were normalized to that of *ACTINI* (LOC_Os03g50885). Primers for *ACTINI* were 5'-TGG CAT CTC TCA GCA CAT TCC-3' (forward) and 5'-TGC ACA ATG GAT GGG CCA GA-3' (reverse). Primers for *OsBURP16* were 5'-GTA CCG TAC TGC CAA ATG GG-3' (forward) and 5'-AGC TGG TAA TCT TGC CAC CA-3' (reverse). Primers for *OsTPPI* were 5'-ACG ACT TTC TGC CAA TGA TCG-3' (forward) and 5'-GTT GCA CCT TCA ATT CCA CTG A-3' (reverse). Primers for *OsTPS1* were 5'-CAA GGT GGA GCA GGG ATT CTC-3' (forward) and 5'-TGC GAT GTT CTT CCA ATT AAA ATG T-3' (reverse). As *ACTINI* can be induced by abiotic stresses (Supporting Information Fig. S1b), we used the *OsCIPK06* gene as the internal control for the stress treatment experiments, as it is known not to be induced by ABA or abiotic stresses (Xiang *et al.* 2007). Primers for *OsCIPK06* were 5'-CGT GGT TCA AGA AGA CGT CCA-3' (forward) and 5'-AGC AGG ATC GGC AAA

GGC-3' (reverse). Three biological replications were performed, and the results were consistent.

Measurement of relative electrolyte leakage and diaminobenzidine staining for H₂O₂

Relative ion leakage was assayed following the method of Song *et al.* (2011). Four-week-old seedlings from wild type and three *Ubi::OsBURP16* transgenic lines (OE3, OE4 and OE6) were transferred into a 4 °C chamber, and another set of plants was grown in a greenhouse as control. After 60 or 108 h, 0.1 g of leaves was harvested for measurement from each of six plants. Leaf fragments were immersed in 6 mL deionized water and shaken at 200 r.p.m. at 25 °C for 2 h, and electrical conductivity was determined (C1). The samples were then boiled for 20 min, and the total conductivity was determined again (C2) after cooling to room temperature. Relative ion leakage (%) was calculated as $C1/C2 \times 100$.

DAB staining was performed following the method of Ouyang *et al.* (2010). Two-week-old plants were treated with 200 mM NaCl solution for 2 d, and the leaves were detached and immersed in 1% 3,3'-diaminobenzidine (DAB) solution in deionized water (pH 3.8). After 30 min under vacuum the samples were incubated at room temperature for 48 h in the dark. The samples were then bleached by boiling in ethanol in order to reveal brown spots, which are indicative of the reaction of DAB with H₂O₂.

Water loss, stomatal conductance and transpiration rate measurements

Leaves of wild-type and *Ubi::OsBURP16* plants grown under normal conditions for 2 weeks were detached and weighed immediately on a piece of weighing paper, and then placed on a laboratory table and weighed at designated time intervals (Chen *et al.* 2006). Three replicates were performed for each line. The extent of water loss was assessed by calculating measured mass as a percentage of the initial mass of the plants.

The fully expanded second leaves of seedlings at the tillering stage (counting from the top of the plant) were used for stomatal conductance and transpiration rate measurements using a portable gas analysis system (Li-Cor 6400, Lincoln, NE, USA). The measurements were performed under the following conditions: a constant water concentration of 10.25 ± 0.02 mmol·mol⁻¹, a constant temperature of 29.50 ± 0.02 °C and a constant CO₂ concentration of 420.20 ± 0.02 μmol·mol⁻¹. The light intensity, which was expressed by the effective radiation of photosynthesis, was 1100 ± 50 μmol·m⁻²·s⁻¹ in the environment and 1500 ± 50 μmol·m⁻²·s⁻¹ in the measuring room. At least four leaves from each sample group were measured in this experiment, and the same results were obtained.

Epidermis observation

Young leaves of 2-week-old plants were cut into 1.5 cm sections, boiled in deionized water for 10 min, and then

transferred into 95% ethanol and boiled for 1 h. The material was put into hot (96 °C) 85% lactic acid for 8 min. After the leaves became transparent, they were observed under differential interference contrast microscopy. In order to make sure cells were alive before staining with propidium iodide, fresh leaves or roots of 2-week-old plants were directly collected without treatment to remove pigments.

To count the number of stomata, leaves from 2-week-old plants were fixed in FAA fixative (89 mL ethanol, 6 mL acetic acid, 5 mL formaldehyde) overnight at room temperature and then dehydrated through an ethanol series. The samples were then replaced by isoamyl acetate, dried to a critical point in CO₂ and mounted on stubs. After the samples were sputter-coated with gold, images were obtained with a scanning electron microscope (Hitachi S-4800 FESEM, Hitachi, Tokyo, Japan). For the stomatal aperture measurement, leaves of 25-day-old plants were detached and immediately fixed by liquid nitrogen. The stomatal pictures were obtained using an environmental scanning electron microscope (Hitachi S-3000 N).

Histochemical staining for pectin and immunolocalization of methyl – ester pectin

Leaves from 2-week-old plants were excised and fixed in FAA, which was fully infiltrated into the leaf tissues under vacuum. The samples were then dehydrated with an ethanol series and embedded in paraplast (Sigma, St. Louis, MO, USA). The embedded material was cut into 8 μm sections and stained with 0.01% (w/v) ruthenium red for general localization of pectin (Atkinson *et al.* 2002; Yong *et al.* 2003). The stained samples were observed by light microscopy. Data were analysed with ImageJ software, and 10 sections of each experimental group were analysed.

Fresh roots of 3-day-old seedlings behind the root apex were sectioned to 1 – 3 mm and were directly collected into a fixative solution containing 4% paraformaldehyde in 50 mM PIPES, 5 mM MgSO₄, and 5 mM EGTA, pH 6.9 (Yang *et al.* 2008) for 1 h in 2 mL Eppendorf tubes. Then the 1–3 mm samples were cut by hand to the thickness of about 0.1–0.5 mm and were washed twice with phosphate buffered saline (PBS) and blocked with 0.2% bovine serum albumin (BSA) in PBS for 30 min at room temperature. All of these procedures were done on glass slides that had already been coated with polylysine. Homogalacturonan (HG), consisting of linear chains of 1,4-linked α-D-galacturonic acid residues, is one of the major components of pectin. Some carboxyl groups of HG are methyl-esterified. To visualize the total HG content, base de-esterification by treating with 0.1 M NaCO₃ for 30 min before adding the JIM5 antibody was needed. Monoclonal antibodies JIM5 and JIM7 (diluted 1:10 in PBS containing 0.2% BSA) were then added for 2 h. After washing three times in PBS, goat anti-rat IgG (Sigma) fluorescein isothiocyanate conjugate (diluted 1:50 in PBS containing 0.2% BSA) was added and incubated for 2 h. Before being mounted on glass slides, samples were washed three times with PBS. The samples were then mounted on glass slides and examined under a laser scanning confocal microscope (LSM

510, Zeiss, Jena, Germany) by using a digital X-Y scan system. Control samples were also examined.

Pectin chemical analysis and PG activity assay

The method of cell wall preparation was highly similar to the protocols outlined previously (Ahmed & Labavitch, 1977, Bouton *et al.* 2002). Cell walls were prepared from leaves of 2-week-old plants that were grown under normal conditions. Approximately 100 mg of leaves were boiled in 1 mL of 96% ethanol for 20 min. The homogenate was centrifuged at 1000 g for 10 min, and the supernatant was discarded. The residue was washed three times each with 70% ethanol, 96% ethanol and acetone. The samples were then exposed to vacuum overnight at 40 °C for air-drying. The prepared cell wall samples were dissolved in sulphuric acid and uronic acids and then quantified by colorimetry utilizing *meta*-hydroxydiphenyl (Ahmed & Labavitch, 1977).

The protocol for extracting PG was modified from Zheng *et al.* (1992). Leaves (2 g) of 2-week-old plants were ground with liquid nitrogen and were homogenized in 6 mL of ice-cold 0.1 M sodium citrate buffer (pH 6.0). The extracts were filtered through four layers of miracloth (Amresco, Solon, OH, USA), incubated at 4 °C for 3 h and then centrifuged at 10 000 g for 20 min at 4 °C. The supernatants were filtered through miracloth again, and proteins were subsided at 4 °C by the slow addition of solid ammonium sulphate to a final saturation of 80%, and stirred for 1 h at 4 °C. The supernatant was discarded after centrifugation at 10 000 g for 20 min at 4 °C, while the precipitate was dissolved in 2 mL of 1 M NaCl and dialysed against 1 M NaCl at 4 °C for 24 h. After dialysis, the solution was utilized for the PG activity assays. PG activity was measured according to Atkinson *et al.* (2002).

For the measurement of PG activity and pectin content after drought stress, 2-week-old ZH10 plants were treated with 20% PEG 6000 for 12 h before measurement, while other 2-week-old ZH10 plants were treated with fresh culture solution as control.

Breaking force test

To examine the mechanical strength in the leaves of the transgenic and wild-type plants, the middle sections of the third leaves (6 cm length) of 2-week-old plants were collected and immediately used for measurement. The force required to break the leaves was measured with a digital force/length tester (5848 Microtester, Instron, Norfolk County, MA, USA) according to a previous report (Ning *et al.* 2011). At least 20 leaves of each sample group were measured in this experiment, and the same results were obtained.

RESULTS

Expression patterns of *OsBURP16*

OsBURP16 is one of the three members in the PG1 β -like gene subfamily. It contains two exons and one intron and encodes a peptide with 344 amino acid residues (Ding *et al.*

2009). There is high amino acid similarity between *OsBURP16* and the known BURP domain containing protein LePG1 β in tomato (Fig. 1a). We predicted likely transmembrane helices in the structures of *OsBURP16* and the previously characterized protein LePG1 β using TMHMM Server v. 2.0 and found that the two proteins may both mainly be localized outside the cell membrane (Fig. 1b). Analysis using the Rice eFP Browser (<http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi>) showed that *OsBURP16* was mainly expressed in young leaves (Supporting Information Fig. S1a). Our quantitative RT-PCR (qRT-PCR) assays showed *OsBURP16* was expressed in various tissues, mainly in leaf lamina, leaf cushion and internodes (Fig. 1c). These results show that *OsBURP16* is highly expressed in young leaves.

The effects of ABA and abiotic stresses on the expression of *OsBURP16* were monitored by qRT-PCR to investigate the gene's role in responses to environmental factors. Under 4 °C cold stress, the transcription level of *OsBURP16* increased by about 1.5-fold compared with the untreated plants at 6 h after commencing the cold treatment, and then decreased to the level observed prior to treatment (Supporting Information Fig. S1c). For the drought stress analysis, the expression level of *OsBURP16* was up-regulated at 12 h and then decreased to the level of untreated seedlings (Supporting Information Fig. S1c). The expression levels of *OsBURP16* also clearly increased at 3 h in response to treatment with 200 mM NaCl. Treatment with 100 μ M ABA caused a decrease in the transcriptions of *OsBURP16* (Supporting Information Fig. S1c). These results suggest that *OsBURP16* is involved in responses to ABA and abiotic stresses.

Decreased pectin content and cold tolerance in *OsBURP16* transgenic plants

To investigate the function of *OsBURP16*, cDNA of *OsBURP16* was cloned into the vector pUN1301, which is driven by the maize ubiquitin (*Ubi*) promoter (Fig. 2a). This construct was introduced into the wild-type rice cultivar Zhonghua 10 (ZH10) by *Agrobacterium*-mediated transformation. Three independent *Ubi::OsBURP16* transgenic lines (OE3, OE4 and OE6) were confirmed by Southern blot analysis (Fig. 2b). qRT-PCR analysis revealed that the transcription levels of *OsBURP16* were increased 2.1-, 5.4- and 3.4-fold in the T3 progeny of OE3, OE4 and OE6, respectively (Fig. 2c). There was no obvious difference in the morphology of *Ubi::OsBURP16* transgenic rice plants compared with ZH10 at different developmental stages under normal conditions (Fig. 2d–g). As such, these three independent transgenic lines (OE3, OE4 and OE6) with relatively high expression levels of *OsBURP16* were chosen for subsequent experiments. Attempts to generate mutants or RNAi plants of *OsBURP16* have so far not been successful, but are ongoing.

As pectin is well known as an important component of the adhesive material between plant cells (Bouton *et al.* 2002), the young leaves were stained with pectin-specific ruthenium red (Atkinson *et al.* 2002). The results showed that the young

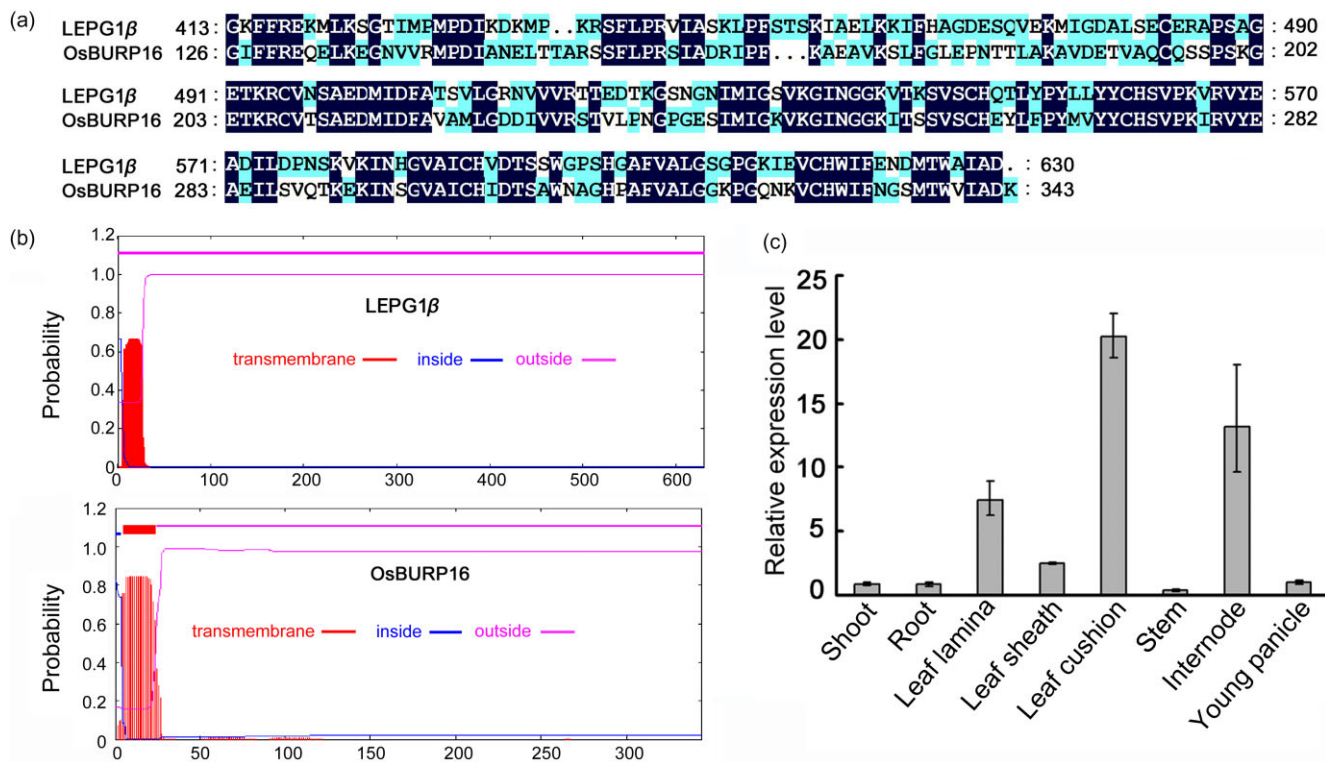


Figure 1. Characterization and expression pattern of *OsBURP16* in rice. (a) Amino acid sequence alignment of the BURP domain of *OsBURP16* with *LePG1 β* , a PG1 β subunit of tomato (*Lycopersicon esculentum* L.). (b) Prediction of transmembrane helices in *OsBURP16* and *LePG1 β* (a PG1 β subunit of tomato), performed with the software TMHMM Server v. 2.0 online (<http://www.cbs.dtu.dk/services/TMHMM>). (c) The tissue expression patterns of *OsBURP16*. The expression level in shoot was set at 1, and the *ACT1N1* gene was used as the internal control. Data represent means \pm SD of three biological replicates.

leaves of transgenic plants stained more weakly than ZH10 (Fig. 3a,e). Pectin content was also determined. As shown in Fig. 3f, the uronic acid content in ZH10 and *OsBURP16* transgenic plants was 1.1 mg·g⁻¹ and 0.9 mg·g⁻¹ fresh weight, respectively. This decrease can be partly taken as an indication of the lower galacturonic acid (GalUA) content in the *Ubi::OsBURP16* seedlings' leaves. GalUA is the sugar monomer used in the formation of the homogalacturan chains of pectin (Brummell & Harpster 2001). These results suggest that increased pectin solubilization or degradation may occur in the leaves of *Ubi::OsBURP16* seedlings as compared with the wild type.

Studies have found that the properties of the cell wall and the action of pectin methyltransferases on matrix properties may be important factors for cell adhesion in plants (Knox *et al.* 1990; Knox 1992; Willats *et al.* 2001). The degree of pectin methylation was reported to be an important factor affecting the properties of the cell wall (Yang *et al.* 2010). Additionally, pectin content and the degree of pectin methylation were reported to be related to aluminium stress in rice and maize (Eticha *et al.* 2005; Yang *et al.* 2008). PEG treatment not only reduces the total cell wall pectin content, but can also decrease the degree of methylation of pectin in root tips (Yang *et al.* 2010). We measured low-methyl-ester pectin (JIM5 epitope) and high-methyl-ester pectin (JIM7 epitope) in roots of *Ubi::OsBURP16* and wild type by

immunolocalization. The results showed that high-methyl-ester pectin content was decreased in *Ubi::OsBURP16* plants, while low-methyl-ester pectin content did not change (Fig. 3b,c). We also did the immunolocalization of low-methyl-ester pectin after base de-esterification to visualize the total HG content in root hand sections of *OsBURP16*-overexpressing transgenic seedlings (OE4) and wild-type ZH10 plants (Fig. 3b,d); the results showed that the total HG content was significantly reduced, which is consistent with the lower GalUA content in the OE4 plants (Fig. 3f), and all these results might partly be taken as an indication of lower pectin content.

PGs are essential for the degradation of pectin, catalysing random hydrolysis of α -1,4-glycosidic linkages in polygalacturonic acid (Swain *et al.* 2011). PG activity in young leaves was measured. In young leaves of the OE3 and OE4 lines, PG activity was increased by more than three- and sixfold, respectively, as compared with PG activity from ZH10 (Fig. 3g). This result indicates that overexpression of *OsBURP16* can promote the activity of PG and the degradation of pectin in the cell wall.

The cold tolerance of *Ubi::OsBURP16* plants was tested in the same manner as the analysis of *OsBURP16* expression under cold stress. *OsBURP16* transgenic lines of T3 progeny and wild-type seedlings at the trefoil stage were exposed to temperatures of 4 °C. After treatment for 8 h, leaves of line

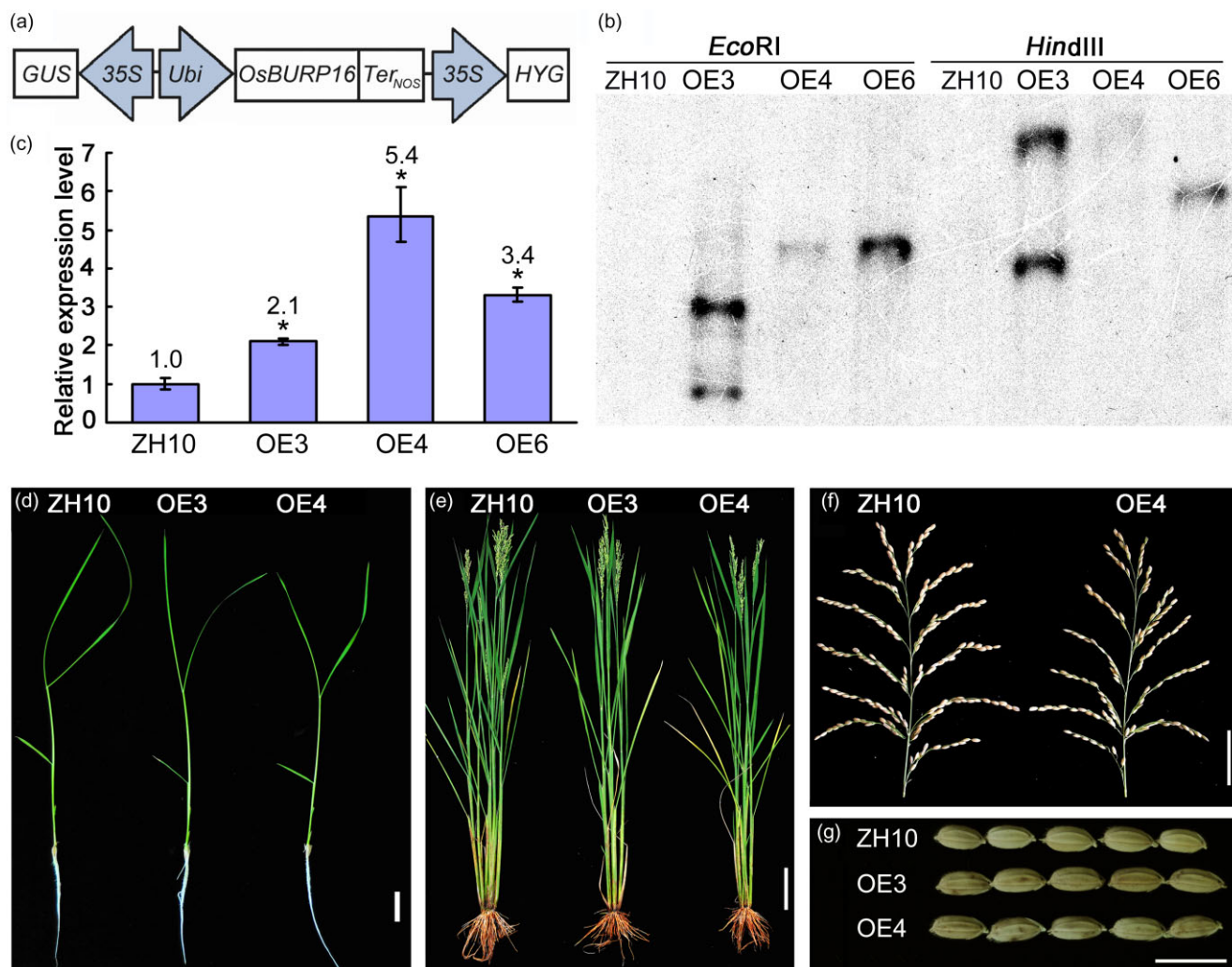


Figure 2. Generation of *Ubi::OsBURP16* transgenic rice. (a) Diagram of the *Ubi::OsBURP16* construct that was used for the generation of transgenic plants. (b) Southern blot analysis of overexpression transgenic rice lines. ZH10, wild type Zhonghua10; OE3, OE4, OE6 are the overexpression transgenic lines 3, 4 and 6. Rice genomic DNA was digested by *EcoRI* in the left four lanes and by *HindIII* in the right four lanes. (c) The expression levels of *OsBURP16* in *OsBURP16*-overexpressing lines (OE3, OE4, OE6) were determined by qRT-PCR. The expression level in ZH10 was normalized as 1. The number in each column indicates the expression levels of *OsBURP16* in each transgenic line. Data represent means \pm SD of three biological replicates. (d–g) Phenotypes of 2-week-old seedlings (d), plants at heading stage (e), spikelets (f) and seeds (g) of ZH10 and *OsBURP16* line OE3 and OE4. Scale bar = 2 cm (d), 10 cm (e), 5 cm (f) and 1 cm (g).

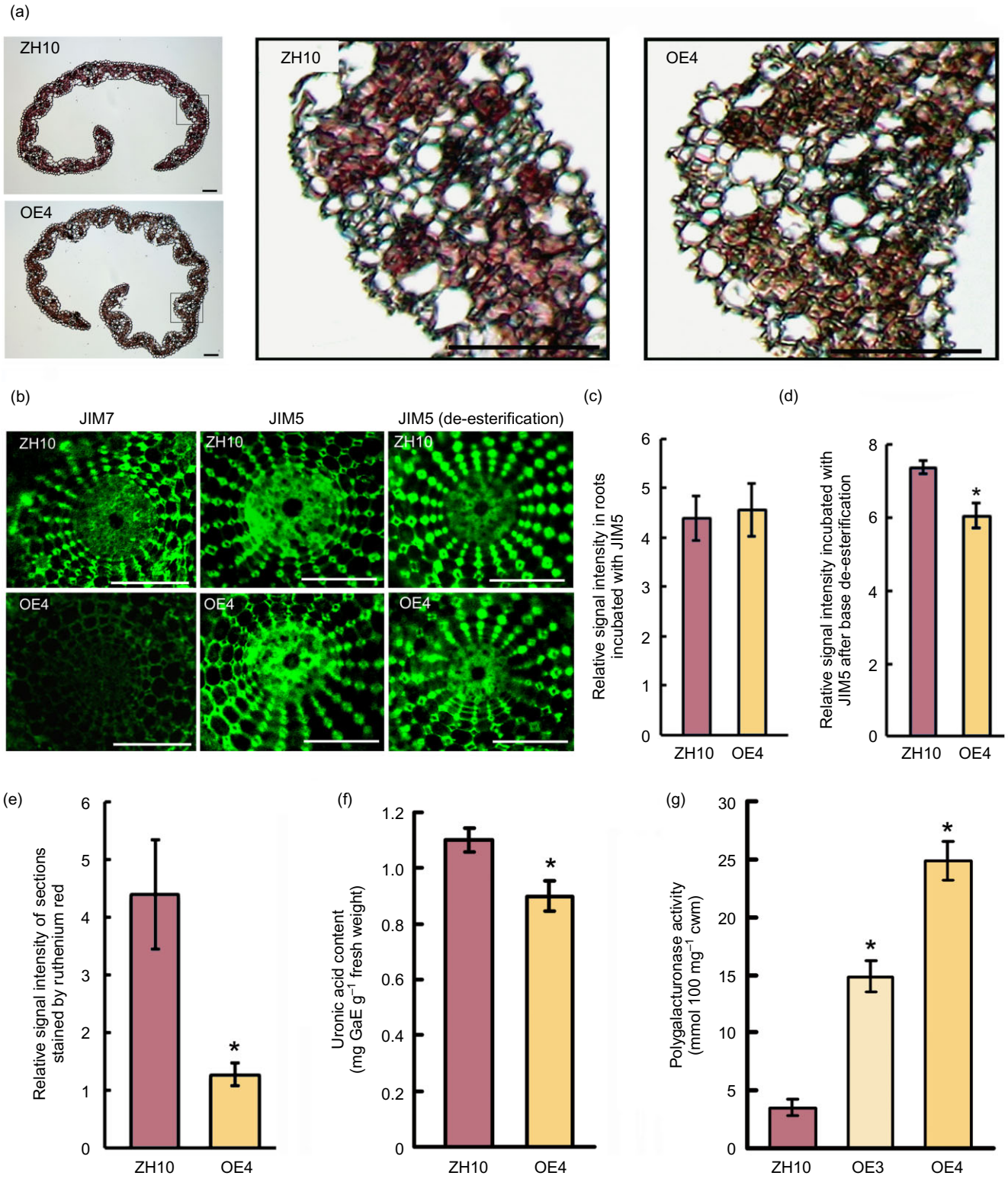
OE3 and line OE4 started rolling, while the ZH10 leaves remained normal (Fig. 4a). When the seedlings were exposed to 4 °C for 72 h and allowed to recover for 7 d in normal conditions, the survival rate of the ZH10 plants was 42%, whereas the survival rates of OE3 and OE4 were only 28% and 4% (Fig. 4b,c), respectively.

To evaluate the effects of cold stress on cell membranes, 4-week-old seedlings were treated at 4 °C for 60 or 108 h, and the relative ion leakage was measured. *Ubi::OsBURP16* lines showed higher relative electrolyte leakage than ZH10 at both 60 and 108 h. With no cold treatment, the relative ion leakage rates of OE3, OE4, OE6 and ZH10 were 18%, 25%, 19% and 21%, respectively. Following treatment at 4 °C for 60 h, the relative ion leakage rates of OE3, OE4, OE6 and ZH10 were 45%, 58%, 44% and 29%, respectively (Fig. 4d).

These results indicate that the cell membrane stability is reduced in *Ubi::OsBURP16* transgenic lines compared with the wild type.

Sensitivity of *OsBURP16* transgenic rice seedlings to salt stress

To measure the response of *Ubi::OsBURP16* transgenic rice seedlings to salt stress, 2-week-old transgenic and wild-type seedlings were treated with 200 mM NaCl for 4 d. The survival rates were calculated following 7 d of recovery in normal growth conditions. As shown in Fig. 5b, approximately 44% of wild-type plants survived, while the survival rates of *Ubi::OsBURP16* lines OE3, OE4 and OE6 were 31%, 14% and 26%, respectively (Fig. 5a,b).



Abiotic stress usually causes the generation of reactive oxygen species, such as H₂O₂, which is an important molecule in stress signaling in plants (Zhu 2001). The stress tolerance of *Ubi::OsBURP16* transgenic seedlings can be further assessed through the detection of H₂O₂. Rice seedlings were

treated with 200 mM NaCl for 2 d and were then subjected to DAB staining to detect H₂O₂ content in leaves. The untreated leaves of OE3, OE4 and ZH10 showed similar staining patterns. When treated with 200 mM NaCl for 2 d, the leaves of OE3 and OE4 exhibited more dark brown areas than those

Figure 3. Pectin and uronic acid content of cell wall fractions from *OsBURP16*-overexpressing transgenic and wild-type plants. (a) Cross sections of leaves of *OsBURP16*-overexpressing and ZH10 plants stained by ruthenium red. The black frames are enlarged in the right of the picture. Scale bar = 20 μm . (b) Immunolocalization of high- and low-methyl-ester pectin (JIM7 and JIM5, respectively) after base de-esterification in root hand sections of *OsBURP16* OE4 and ZH10 seedlings. Scale bars = 100 μm . (c) Relative signal intensity of immunolocalization of low-methyl-ester pectin (JIM5) shown in (b) with ImageJ software. Ten slides each were measured. (d) Relative signal intensity of immunolocalization of low-methyl-ester pectin (JIM5) after base de-esterification shown in (b) (ImageJ software). Ten slides each were measured; asterisk indicates significant difference at $P \leq 0.05$ compared with the wild type by Student's *t*-test. (e) Relative signal intensity of sections shown in (a) (ImageJ software). Ten slides each were measured; asterisk indicates significant difference at $P \leq 0.05$ compared with ZH10 by Student's *t*-test. (f) Uronic acid content determined by colorimetry in wild-type and *Ubi::OsBURP16* rice. The *y*-axis presents values for galacturonic acid equivalents (GaE) per 1 g fresh weight. Data represent means \pm SD of three biological replicates. The asterisk indicates significant difference at $P \leq 0.05$ compared with ZH10 by Student's *t*-test. (g) PG activity in leaves of ZH10 and *OsBURP16*-overexpressing rice (OE3 and OE4). PG activity was measured as millimoles of reducing groups per 100 mg of cell wall material ($\text{mmol} \cdot 100 \text{ mg}^{-1} \text{ cwm}$). Each column represents means \pm SD ($n = 3$) of the independent measurement. Asterisks indicate significant difference at $P \leq 0.05$ compared with ZH10 by Student's *t*-test.

of the wild type ZH10 (Fig. 5c). These results suggest that *OsBURP16* overexpression resulted in the accumulation of more reactive oxygen species in young leaves, and that the transgenic seedlings were more sensitive to salt stress than the wild type.

Decreased drought tolerance due to overexpression of *OsBURP16* with increased cuticular transpiration

A series of experiments were performed to determine the effect of overexpression of *OsBURP16* on plant tolerance to drought stress. Two-week-old plants were deprived of water for 20 d and allowed to recover at normal water supply levels for 2 weeks (Fig. 6a). Following the recovery period, the survival rate of the wild-type plants was 42%, while those of the three *Ubi::OsBURP16* lines were all less than 10% (Fig. 6b).

To observe the water loss rate *in vitro*, leaves were exposed to the air at room temperature and photographed at different times. It was found that the leaves of the overexpression lines began to wither earlier than those of the wild type. For example, leaves of *Ubi::OsBURP16* lines curled at 2 min, while the wild-type leaf curled at 6 min (Fig. 6c). Water loss is consistent with this phenotype: when the detached leaves of transgenic plants were air-dried for 45 min, the fresh weights of OE3, OE4 and OE6 leaves were less than 50% of their initial weights. Contrastingly, the fresh weight of ZH10 leaves was more than 60% of initial weight (Fig. 6d). The stomatal aperture controls CO_2 uptake and water loss to the atmosphere, thus playing an important role in abiotic stress response. The loss-of-function mutant of *OsSRO1c* showed increased stomatal aperture and sensitivity to drought and faster water loss compared with the wild-type plant, whereas *OsSRO1c* overexpression led to decreased stomatal aperture and reduced water loss, indicating the direct correlation between the stomatal aperture and drought tolerance (You *et al.* 2013). Then the stomatal density and apertures of *Ubi::OsBURP16* and ZH10 leaves were observed by scanning electron microscopy (Fig. 6e,i). No obvious differences in stoma numbers or stomatal apertures were observed between *Ubi::OsBURP16* and ZH10 (Fig. 6f,j). We further investigated the stomatal conductance and the transpiration

rate of ZH10 and OE4. The cuticle covers the leaf surface and acts as an effective barrier against water loss (Burghardt & Riederer 2003). Both cuticular transpiration and stomatal transpiration affect water loss (Karbulkova *et al.* 2008). The OE4 seedlings showed a higher transpiration rate than the wild-type seedlings (Fig. 6h), which indicates higher cuticular transpiration rates, as there was no obvious difference in the stomatal conductance and stomatal apertures (Fig. 6g,i,j). The results from the survival rate, leaf curling, water loss rate, stomatal density and transpiration rate analyses all showed that *Ubi::OsBURP16* plants have less ability to retain water compared with ZH10.

As drought stress can induce the expression of *OsBURP16*, we measured the pectin content as well as the PG activity after 12 h treatment with 20% PEG 6000. The results showed that PG activity was clearly increased while pectin content was clearly decreased (Supporting Information Fig. S2a,b). This result might partly indicate that higher levels of *OsBURP16* can promote the activity of PG and the degradation of pectin in the cell wall.

All of the above results from experiments with the *Ubi::OsBURP16* lines – cold sensitivity, higher electrolyte leakage rates, salt sensitivity, increased accumulation of reactive oxygen species, drought sensitivity, enhanced water loss and transpiration rate – indicate that *OsBURP16* overexpression transgenic seedlings are more sensitive to abiotic stress than wild-type seedlings.

Reduced cell adhesion strength in *OsBURP16* overexpression plants

As *OsBURP16* is mainly expressed in young leaves (Fig. 1c), we observed the cell–cell adhesion in the epidermis of 2-week-old transgenic rice leaves using differential interference contrast microscopy. In contrast to the wild type, the intercellular spaces of leaves in the transgenic plants were larger and appeared as transparent structures, while there was no obvious difference in their roots (Fig. 7a and Supporting Information Fig. S3). Since the spaces between the cells were larger in *Ubi::OsBURP16* transgenic plants than in the wild type, we measured the mechanical force required to break the leaves. The assay results showed that less force was required to break the leaves of *Ubi::OsBURP16* plants

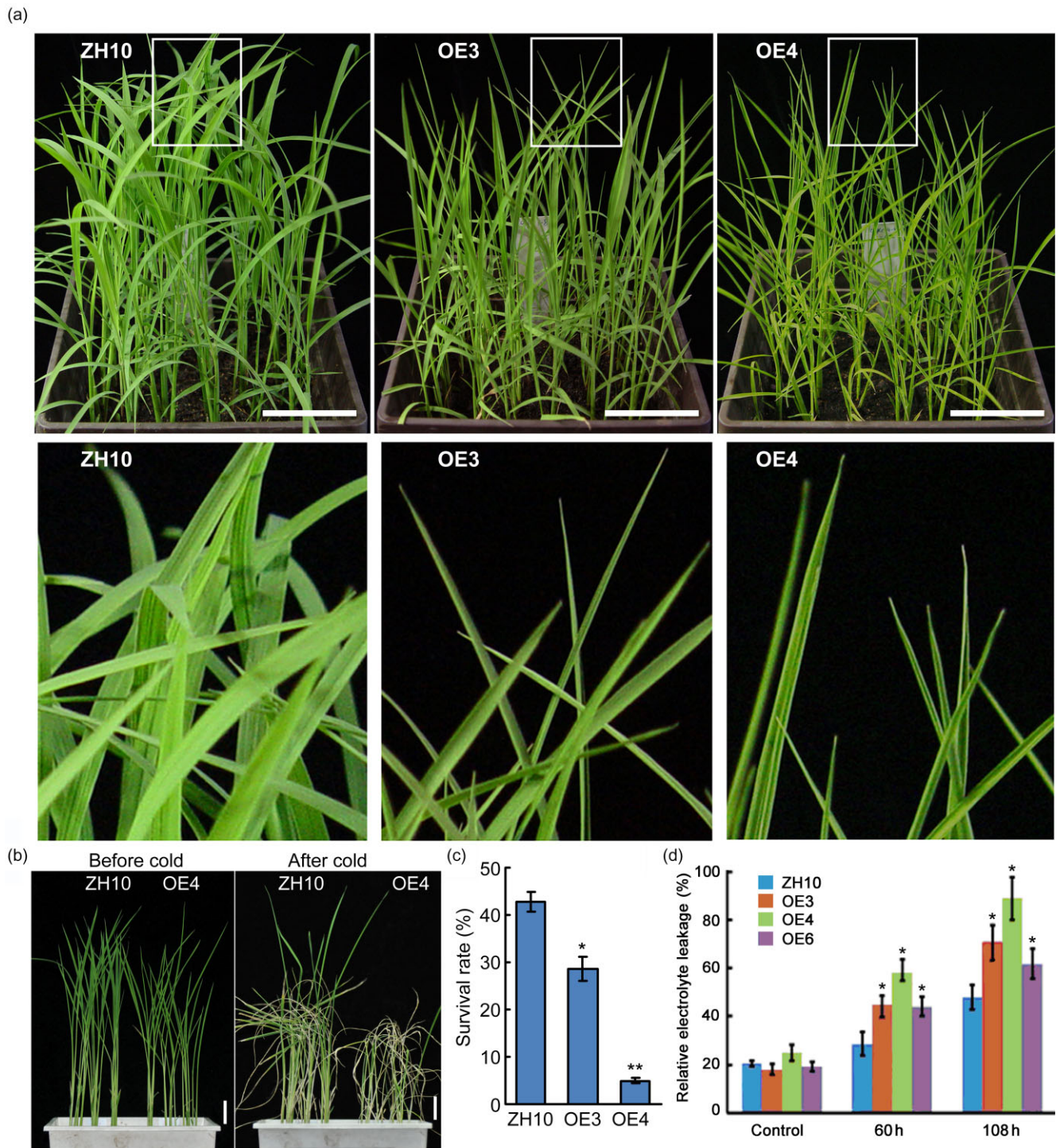


Figure 4. Performance of *Ubi::OsBURP16* transgenic rice under cold stress. (a) Seedlings of *OsBURP16*-overexpressing lines (OE) and wild-type (ZH10) at trefoil stage were treated at 4 °C for 8 h. The leaves of lines OE3 and OE4 started rolling, while the leaves of ZH10 remained normal. The boxed parts in the top panel are enlarged as a detail (lower panel). Scale bar = 5 cm. (b) Two-week-old seedlings were treated at 4 °C for 72 h, and then allowed to recover for 7 d. Scale bars = 5 cm. (c) Survival rates of the cold-treated plants after 7 d of recovery. Data represent means \pm SD ($n = 16$ for each replicate) of three independent experiments. One asterisk and two asterisks indicate significant differences at $P \leq 0.05$ and $P \leq 0.01$ compared with ZH10 by Student's *t*-test, respectively. (d) Relative ion leakage in rice leaves after 4 °C treatment for 60 h and 108 h. Data represent means \pm SD of three biological replicates. One asterisk indicates significant difference at $P \leq 0.05$ compared with corresponding ZH10 by Student's *t*-test.

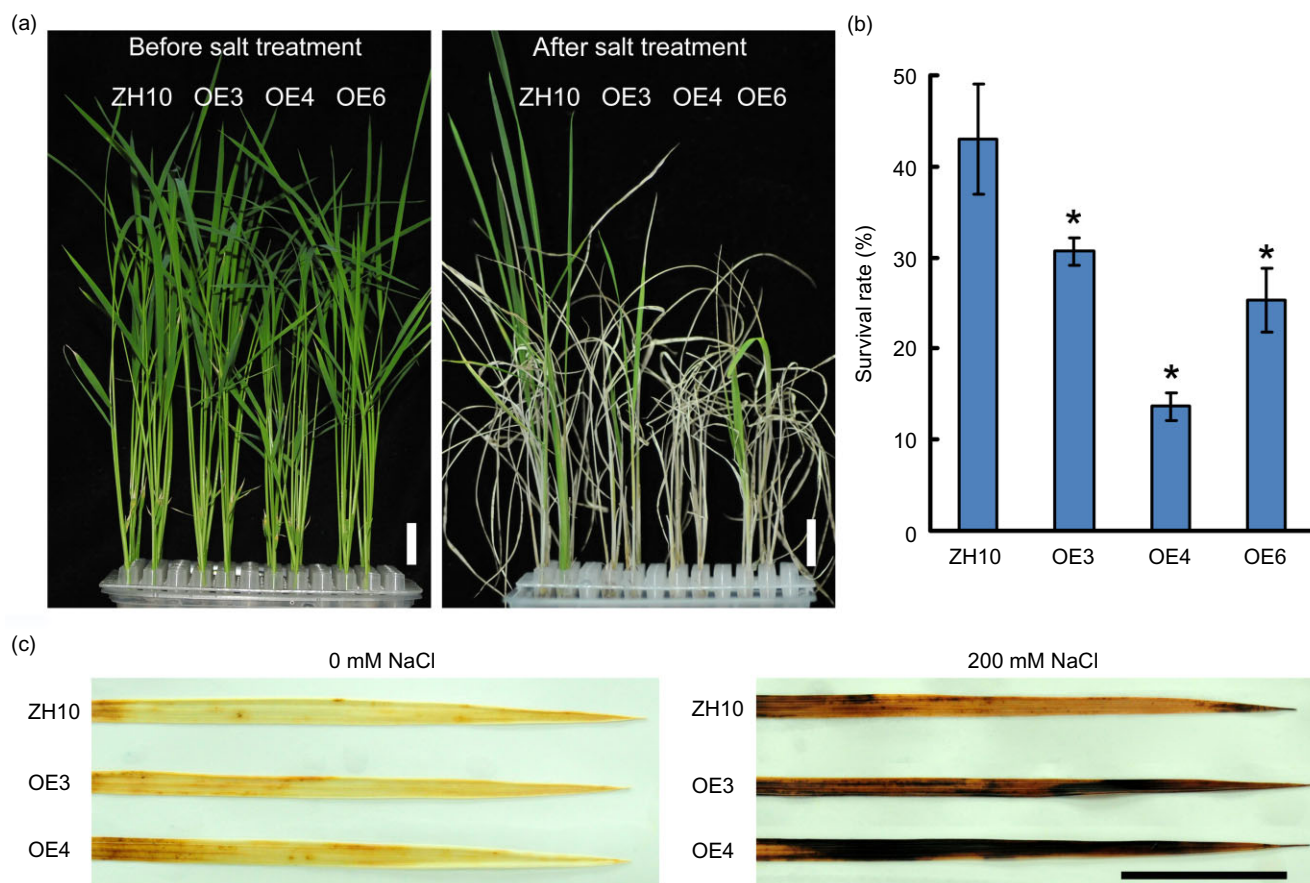


Figure 5. Performance of *Ubi::OsBURP16* transgenic rice under salt stress. (a) Plants grown for 2 weeks were treated with 200 mM NaCl for 4 d and then allowed to recover for 7 d. Scale bars = 2 cm. (b) Survival rates of the salt-treated plants after 7 d of recovery. Each column represents mean \pm SD ($n = 16$ for each replicate) of three independent experiments. Asterisks indicate significant difference at $P \leq 0.05$ compared with the wild type by Student's *t*-test. (c) 3,3'-Diaminobenzidine (DAB) staining of rice leaves from plants with 0 mM or 200 mM NaCl treatment for 2 d. The brown regions on the leaves are areas with high rates of DAB reaction with H₂O₂. Scale bar = 2 cm.

than those of the wild type. Additionally, the extension length of *Ubi::OsBURP16* leaves was shorter than that of the wild type (Fig. 7b). These results may indicate that the cell adhesion strength is reduced in *Ubi::OsBURP16* transgenic plants.

Changes in stress-related genes in *OsBURP16* overexpression plants

It is well known that accumulation of osmoregulatory substances promotes tolerance to abiotic stresses. Trehalose, an osmoregulatory substance in plants, is produced from glucose by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). Overexpression of a heterologous *TPS* gene from microorganisms (Jang *et al.* 2003) or *OsTPP1* triggers an abiotic stress pathway and confers salt and cold stress tolerance in rice (Pramanik & Imai 2005; Ge *et al.* 2008). In fact, we measured the transcript levels of *OsTPS1* and *OsTPP1*, and both of them decreased significantly in *OsBURP16* overexpression seedlings (Supporting Information Fig. S2c).

DISCUSSION

Encoding of a β subunit of PG by *OsBURP16* in rice

A previous study showed that the transcription levels of *OsBURP16* respond to ABA and abiotic stresses including drought, salt and cold (Ding *et al.* 2009), but the study did not elucidate the functions of this gene. In this study, we report that *Ubi::OsBURP16* plants were more sensitive than wild type to abiotic stresses including drought, salt and cold (Figs 4–6). Although overexpression of some stress-induced genes can enhance stress tolerance, overexpression of others can have an opposing effect. For example, the rice MAPK phosphatase gene *IBR5* is induced by PEG, ABA and H₂O₂, but its overexpression decreases drought stress tolerance (Li *et al.* 2012). *OsMAPK33* is a drought-stress-inducible gene, and its overexpression enhances sensitivity to salt stress through unfavourable ion homeostasis (Lee *et al.* 2011). *MAIF1*, a rice F-box domain gene, is induced rapidly and strongly by ABA and abiotic stresses. Overexpression of *MAIF1* reduces rice ABA sensitivity and abiotic stress

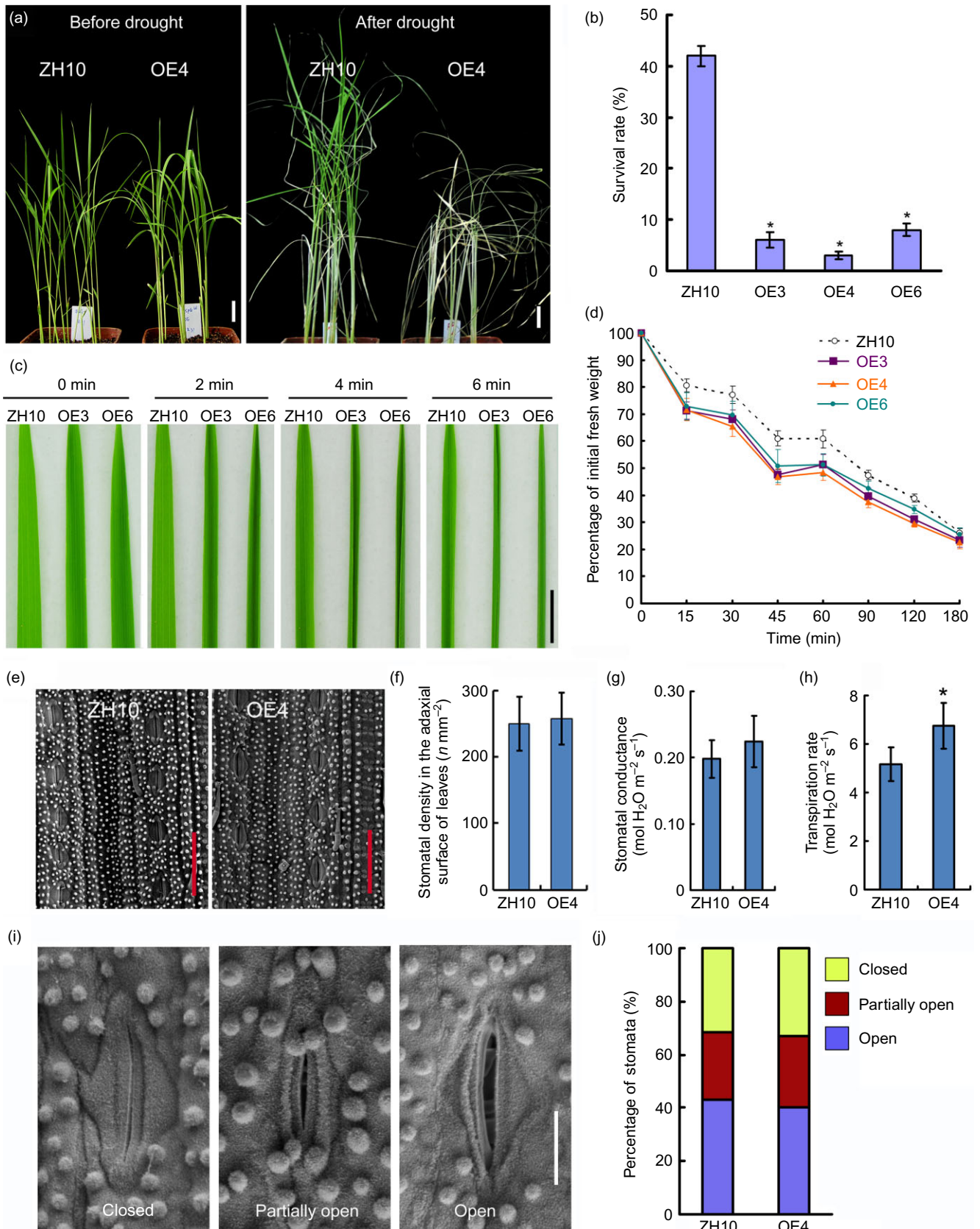


Figure 6. Performance of *Ubi::OsBURP16* transgenic rice under drought stress.

(a) ZH10 and *OsBURP16* transgenic line 4 (OE4) plants grown for 2 weeks were deprived of water for 20 d, and then allowed to recover for 2 weeks. Scale bar = 2 cm. (b) Survival rates of the drought-treated plants after 2 weeks of recovery. Each column is mean \pm SD ($n = 16$ for each replicate) of three independent experiments. Asterisks indicate significant difference at $P \leq 0.05$ compared with the wild type by Student's *t*-test. (c) Detached leaves of three-leaf-stage seedlings of ZH10, OE3 and OE6 were photographed at 0, 2, 4 and 6 minutes after detachment. Scale bar = 1 cm. (d) Dehydration of 4-week-old leaves of ZH10, OE3, OE4 and OE6. At room temperature (25 ± 2 °C), leaves were weighed at the given times from 15 min until 180 min after leaves were detached. Data represent means \pm SD of three biological replicates. (e) The stomatal densities of ZH10 and OE4 leaves were observed by scanning electron microscopy. Bars = 50 μ m. (f) Numbers of stomata of ZH10 and OE4 leaves. Data are given as means \pm SD ($n = 10$). Stomatal conductance (g) and transpiration rate (h) of ZH10 and OE4 leaves were measured. Data represent means \pm SD of three biological replicates. Asterisk indicates significant difference at $P \leq 0.05$ compared with the wild type by Student's *t*-test. (i) Environmental scanning electron microscopy images of three levels of stomatal opening. Bar = 10 μ m. (j) The percentage of stomata at each of three degrees of openness in ZH10 and OE4 plants ($n = 82$ stomata for ZH10; $n = 84$ stomata for *OsBURP16* OE4 plants).

tolerance (Yan *et al.* 2011). These genes, together with *OsBURP16*, are all induced by abiotic stresses and yet play negative roles in abiotic stress response; further investigations of the products of these genes or their downstream genes are still needed to expound their mechanisms in detail.

OsBURP16 encodes a putative precursor of PG1 β , one of the subunits that regulates PG activity (Tucker *et al.* 1981). Our results indicate that overexpression of *OsBURP16* leads to an increase in PG activity and a decrease in pectin content. The results for both immunolocalization and ruthenium red staining showed a consistent decrease in pectin content in the leaves of transgenic plants (Fig. 3). These results suggest that *OsBURP16* acts as a PG1 β subunit to regulate the activity of PG, as is the case in tomato (Zheng *et al.* 1992) and apple (Atkinson *et al.* 2002).

Effects on cell adhesion, cuticular transpiration and decreased tolerance to several stresses associated with reduced pectin content due to *OsBURP16* overexpression

The β subunit is a heat- and pressure-stable protein and is known to confer enhanced thermal resistance properties to

PG (Peeters *et al.* 2004). PG hydrolyses pectin to generate oligogalacturonides, which affect the texture and rigidity of cell walls (Atkinson *et al.* 2002; Pelloux *et al.* 2007; Ogawa *et al.* 2009). Pectin functions as an adhesive in cell wall formation, and pectin content directly influences cell wall integrity.

To evaluate the adhesion of cell walls between two adjacent cells, we performed observations under differential interference contrast microscopy and measured the mechanical force required to break the leaf. The results indicated that the leaves of *Ubi::OsBURP16* lines had larger spaces between adjacent cells and were easier to break than the wild-type leaves (Fig. 7). We can conclude that overexpression of the PG1 β -subunit gene *OsBURP16* resulted in increased PG activity (Fig. 3g) and might also result in a decrease in cell wall adhesion and integrity; however, it is possible that the decrease in force required to break the leaf is not directly related to reduced cell–cell adhesion.

The cell wall is not only the first barrier against biotic stress but is also involved in abiotic stress responses. For example, the *Arabidopsis* mutant *sos5*, which appears to have defects in the middle lamella of the cell wall and the primary cell wall, is hypersensitive to salt stress (Shi *et al.* 2003). Overexpression of the cell wall protein proline-rich protein 3

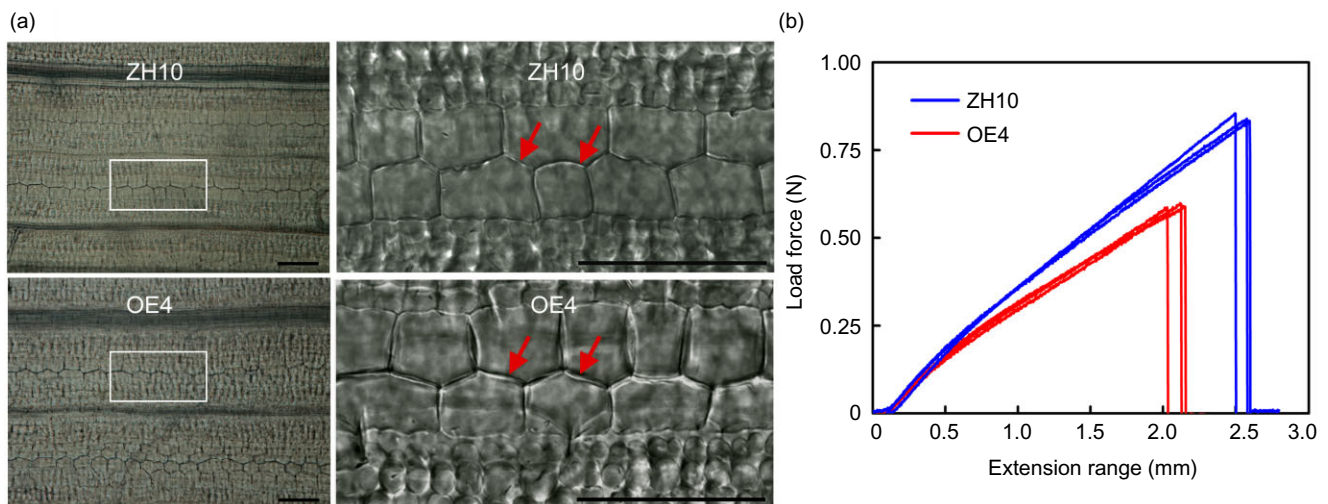


Figure 7. Phenotypic characterization of the third leaves in *Ubi::OsBURP16* transgenic rice. (a) Cellular observation of the third leaves of 2-week-old ZH10 and *OsBURP16*-overexpressing plants (OE4). The regions outlined in white are shown as details to the right. Scale bar = 50 μ m. (b) Mechanical strength measurement of the third leaves of *OsBURP16* OE4 and ZH10. Measurements of the breaking force and extension length were performed in triplicate.

(PRP3) enhances cell wall integrity and cold tolerance in rice (Gothandam *et al.* 2010). Cold acclimation induces an increased pectin content in cell walls and increased resistance to freezing (Solecka *et al.* 2008). Abiotic stresses induce expression of *PGIP*, which encodes a PG-inhibiting protein to prevent the degradation of pectin by PG (Ahsan *et al.* 2005). The *Arabidopsis AtMYB41* gene encodes an R2R3-MYB transcription factor that regulates the modification of cell wall and cuticle integrity. In *AtMYB41*-overexpression lines, more rapid water loss compared with wild type results from discontinuity in the cuticle (Cominelli *et al.* 2008). In *35S::AtMYB41* lines, *ADPG1* and *ADPG2* are up-regulated 12- and 10-fold, respectively (see supporting information in Cominelli *et al.* 2008). Both *ADPG1* and *ADPG2* encode PG in *Arabidopsis* (Ogawa *et al.* 2009). Mutation of *QUASIMODO1*, a gene that encodes a glycosyltransferase required for pectin synthesis, results in reduced pectin content, loss of cell adhesion and faster water loss than in wild type (Bouton *et al.* 2002). All of these data suggest that cell wall integrity and pectin content are associated with tolerance to abiotic stresses. These reports support our conclusions that overexpression of *OsBURP16* promotes PG activity (Fig. 3g), hydrolysing pectin (Fig. 3f) and affecting the integrity of the cell wall (Fig. 7), which may lead to more sensitivity to abiotic stresses than in wild-type plants.

Young rice seedlings are particularly sensitive to low temperature stress, which causes retardation of seedling development, yellowing, withering, reduced tillering and stunted growth (Chinnusamy *et al.* 2007). Tracing the phenomenon of leaves' becoming yellow and withering is helpful for estimating the stress tolerance of rice seedlings (Su *et al.* 2010). Leaves of *Ubi::OsBURP16* transgenic lines rolled immediately compared with wild type under cold or drought conditions, and showed more rapid water loss than that of wild type under drought stress (Fig. 4). Both cuticular transpiration and stomatal transpiration affect water loss (Karbulkova *et al.* 2008). The OE4 seedlings showed higher transpiration rate than wild-type seedlings (Fig. 6h), which indicates higher cuticular transpiration rate, as there was no obvious difference in the stomatal conductance and stomatal apertures (Fig. 6g,i,j). When the seedlings were treated with NaCl, *Ubi::OsBURP16* plants accumulated more reactive oxygen species than did wild-type plants (Fig. 5). The transgenic lines showed lower survival rates than ZH10 under all three abiotic stresses (Figs 4–6). The above studies suggest that the lower survival rate, rapid leaf curling and high water loss rate of *Ubi::OsBURP16* plants may be the result of the high cuticular transpiration rate caused by the reduced cell adhesion.

In conclusion, we found that overexpression of the precursor of PG1 β -subunit resulted in an increase of PG activity, which in turn increased the degradation of pectin in the cell wall, thereby reducing cell adhesion. The *Ubi::OsBURP16* plants, with looser, less cohesive cells, were more sensitive to abiotic stress than wild-type plants. Overexpression of *OsBURP16* then decreases pectin contents and cell adhesion and increases abiotic stress sensitivity in rice. Based on the present results, *OsBURP16* may play a role in regulating

abiotic stresses in rice, a hypothesis that could be tested in future studies using a loss of function approach.

ACKNOWLEDGMENTS

This work was supported by the Research Program from the Chinese Ministry of Agriculture (2014ZX08009-003-002), an Innovation Grant from the Chinese Academy of Sciences (CAS) (KSCX2-EW-J-1) and the National Natural Science Foundation of China (31070695). We thank Prof. Taihua Zhang (Institute of Mechanics, CAS) for help with breaking force measurements and Rongxi Jiang (Institute of Botany, CAS) for the rice transformation. We would like to thank Dr J. H. Snyder for examining the English usage in the manuscript as well as Jingquan Li (Key Lab of Plant Molecular Physiology, Institute of Botany, CAS) for help in using the confocal microscope and Yanbao Tian (Institute of Genetics and Developmental Biology, CAS) for help in using the scanning electron microscope. There are no conflicts of interest to declare.

REFERENCES

- Ahmed A.E.R. & Labavitch J.M. (1977) A simplified method for accurate determination of cell wall uronide content. *Journal of Food Biochemistry* **1**, 361–365.
- Ahsan N., Yoon H.S. & Jo J. (2005) Molecular cloning of a BcPGIP cDNA from *Brassica campestris* and its expression to several stresses. *Plant Science* **169**, 1081–1089.
- Atkinson R.G., Schroder R., Hallett I.C., Cohen D. & MacRae E.A. (2002) Overexpression of polygalacturonase in transgenic apple trees leads to a range of novel phenotypes involving changes in cell adhesion. *Plant Physiology* **129**, 122–133.
- Bouton S., Leboeuf E., Mouille G., Leydecker M.T., Talbot J., Granier F., ... Truong H.N. (2002) QUASIMODO1 encodes a putative membrane-bound glycosyltransferase required for normal pectin synthesis and cell adhesion in *Arabidopsis*. *The Plant Cell* **14**, 2577–2590.
- Brummell D.A. & Harpster M.H. (2001) Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Molecular Biology* **47**, 311–340.
- Burghardt M. & Riederer M. (2003) Ecophysiological relevance of cuticular transpiration of deciduous and evergreen plants in relation to stomatal closure and leaf water potential. *Journal of Experimental Botany* **54**, 1941–1949.
- Chen N., Xu Y.Y., Wang X., Du C., Du J.Z., Yuan M., ... Chong K. (2011) OsRAN2, essential for mitosis, enhances cold tolerance in rice by promoting export of intranuclear tubulin and maintaining cell division under cold stress. *Plant, Cell & Environment* **34**, 52–64.
- Chen Z.Z., Zhang H.R., Jablonowski D., Zhou X.F., Ren X.Z., Hong X.H., ... Gong Z.H. (2006) Mutations in ABO1/ELO2, a subunit of holo-elongator, increase abscisic acid sensitivity and drought tolerance in *Arabidopsis thaliana*. *Molecular Cell Biology* **26**, 6902–6912.
- Chinnusamy V., Zhu J. & Zhu J.K. (2007) Cold stress regulation of gene expression in plants. *Trends in Plant Science* **12**, 444–451.
- Cominelli E., Sala T., Calvi D., Gusmaroli G. & Tonelli C. (2008) Overexpression of the *Arabidopsis AtMYB41* gene alters cell expansion and leaf surface permeability. *The Plant Journal* **53**, 53–64.
- Ding X.P., Hou X., Xie K.B. & Xiong L.Z. (2009) Genome-wide identification of BURP domain-containing genes in rice reveals a gene family with diverse structures and responses to abiotic stresses. *Planta* **230**, 149–163.
- Eticha D., Stass A. & Horst W.J. (2005) Cell-wall pectin and its degree of methylation in the maize root-apex: significance for genotypic differences in aluminium resistance. *Plant, Cell & Environment* **28**, 1410–1420.
- Ge L., Chen H., Jiang J.F., Zhao Y., Xu M.L., Xu Y.Y., ... Chong K. (2004) Overexpression of *OsRAA1* causes pleiotropic phenotypes in transgenic rice plants, including altered leaf, flower, and root development and root response to gravity. *Plant Physiology* **135**, 1502–1513.

- Ge L.F., Chao D.Y., Shi M., Zhu M.Z., Gao J.P. & Lin H.X. (2008) Overexpression of the trehalose-6-phosphate phosphatase gene *OsTPPI* confers stress tolerance in rice and results in the activation of stress responsive genes. *Planta* **228**, 191–201.
- Gothandam K.M., Nalini E., Karthikeyan S. & Shin J.S. (2010) OsPRP3, a flower specific proline-rich protein of rice, determines extracellular matrix structure of floral organs and its overexpression confers cold-tolerance. *Plant Molecular Biology* **72**, 125–135.
- Hattori J., Boutillier K.A., Campagne M.M.V. & Miki B.L. (1998) A conserved BURP domain defines a novel group of plant proteins with unusual primary structures. *Molecular and General Genetics* **259**, 424–428.
- Jang I.C., Oh S.J., Seo J.S., Choi W.B., Song S.I., Kim C.H., . . . Kim J.K. (2003) Expression of a bifunctional fusion of the *Escherichia coli* genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. *Plant Physiology* **131**, 516–524.
- Karbulkova J., Schreiber L., Macek P. & Santrucek J. (2008) Differences between water permeability of stomatous and stomatous cuticular membranes: effects of air humidity in two species of contrasting drought-resistance strategy. *Journal of Experimental Botany* **59**, 3987–3995.
- Knox J.P. (1992) Cell adhesion, cell separation and plant morphogenesis. *The Plant Journal* **2**, 137–141.
- Knox J.P., Linstead P.J., King J., Cooper C. & Roberts K. (1990) Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. *Planta* **181**, 512–521.
- Lee S.K., Kim B.G., Kwon T.R., Jeong M.J., Park S.R., Lee J.W., . . . Park S.C. (2011) Overexpression of the mitogen-activated protein kinase gene *OsMAPK33* enhances sensitivity to salt stress in rice (*Oryza sativa* L.). *Journal of Biosciences* **36**, 139–151.
- Li Y.G., Feng D.R., Zhang D.L., Su J.B., Zhang Y., Li Z.Q., . . . Wang J.F. (2012) Rice MAPK phosphatase *IBR5* negatively regulates drought stress tolerance in transgenic *Nicotiana tabacum*. *Plant Science* **188**, 10–18.
- Ma J.F. & Takahashi E. (1990) Effect of silicon on the growth and phosphorus uptake of rice. *Plant and Soil* **126**, 115–119.
- Ning J., Zhang B.C., Wang N.L., Zhou Y.H. & Xiong L.Z. (2011) Increased leaf angle1, a Raf-like MAPKKK that interacts with a nuclear protein family, regulates mechanical tissue formation in the lamina joint of rice. *The Plant Cell* **23**, 4334–4347.
- Ogawa M., Kay P., Wilson S. & Swain S.M. (2009) *Arabidopsis* dehiscence zone polygalacturonase1 (ADPG1), ADPG2 and QUARTET2 are polygalacturonases required for cell separation during reproductive development in *Arabidopsis*. *The Plant Cell* **21**, 216–233.
- Ouyang S.Q., Liu Y.F., Liu P., Lei G., He S.J., Ma B., . . . Chen S.Y. (2010) Receptor-like kinase *OsSIK1* improves drought and salt stress tolerance in rice (*Oryza sativa*) plants. *The Plant Journal* **62**, 316–329.
- Peeters L., Fachin D., Smout C., van Loey A. & Hendrickx M.E. (2004) Influence of beta-subunit on thermal and high-pressure process stability of tomato polygalacturonase. *Biotechnology and Bioengineering* **86**, 543–549.
- Pelloux J., Rusterucci C. & Mellerowicz E.J. (2007) New insights into pectin methyl-esterase structure and function. *Trends in Plant Science* **12**, 267–277.
- Pramanik M.H.R. & Imai R. (2005) Functional identification of a trehalose 6-phosphate phosphatase gene that is involved in transient induction of trehalose biosynthesis during chilling stress in rice. *Plant Molecular Biology* **58**, 751–762.
- Shi H.Z., Kim Y., Guo Y., Stevenson B. & Zhu J.K. (2003) The *Arabidopsis* *SOS5* locus encodes a putative cell surface adhesion protein and is required for normal cell expansion. *The Plant Cell* **15**, 19–32.
- Solecka D., Zebrowski J. & Kacperska A. (2008) Are pectins involved in cold acclimation and de-acclimation of winter oil-seed rape plants? *Annals of Botany* **101**, 521–530.
- Song S.Y., Chen Y., Chen J., Dai X.Y. & Zhang W.H. (2011) Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. *Planta* **234**, 331–345.
- Su C.F., Wang Y.C., Hsieh T.H., Lu C.A., Tseng T.H. & Yu S.M. (2010) A novel MYBS3-dependent pathway confers cold tolerance in rice. *Plant Physiology* **153**, 145–158.
- Swain S.M. & Kay P., Ogawa M. (2011) Preventing unwanted breakups using polygalacturonases to regulate cell separation. *Plant Signaling & Behavior* **6**, 93–97.
- Tucker G.A., Robertson N.G. & Grierson D. (1981) The conversion of tomato-fruit polygalacturonase isoenzyme 2 into isoenzyme 1 in vitro. *European Journal of Biochemistry* **115**, 87–90.
- Watson C.F., Zheng L. & DellaPenna D. (1994) Reduction of tomato polygalacturonase beta subunit expression affects pectin solubilization and degradation during fruit ripening. *The Plant Cell* **6**, 1623–1634.
- Willats W.G., Orfila C., Limberg G., Buchholt H.C., van Alebeek G.J., Voragen A.G., . . . Knox J.P. (2001) Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl-esterase action, matrix properties, and cell adhesion. *Journal of Biological Chemistry* **276**, 19404–19413.
- Xiang Y., Huang Y. & Xiong L. (2007) Characterization of stress-responsive *CIPK* genes in rice for stress tolerance improvement. *Plant Physiology* **144**, 1416–1428.
- Yan Y.S., Chen X.Y., Yang K., Sun Z.X., Fu Y.P., Zhang Y.M. & Fang R.X. (2011) Overexpression of an F-box protein gene reduces abiotic stress tolerance and promotes root growth in rice. *Molecular Plant* **4**, 190–197.
- Yang J.L., Li Y.Y., Zhang Y.J., Zhang S.S., Wu Y.R., Wu P. & Zheng S.J. (2008) Cell wall polysaccharides are specifically involved in the exclusion of aluminum from the rice root apex. *Plant Physiology* **146**, 602–611.
- Yang Z.B., Eticha D., Rao I.M. & Horst W.J. (2010) Alteration of cell-wall porosity is involved in osmotic stress-induced enhancement of aluminium resistance in common bean (*Phaseolus vulgaris* L.). *Journal of Experimental Botany* **61**, 3245–3258.
- Yong W.D., Xu Y.Y., Xu W.Z., Wang X., Li N., Wu J.S., . . . Zhu Z.Q. (2003) Vernalization-induced flowering in wheat is mediated by a lectin-like gene *VER2*. *Planta* **217**, 261–270.
- You J., Zong W., Li X.K., Ning J., Hu H.H., Li X.H., . . . Xiong L.Z. (2013) The *SNAC1*-targeted gene *OsSRO1c* modulates stomatal closure and oxidative stress tolerance by regulating hydrogen peroxide in rice. *Journal of Experimental Botany* **64**, 569–583.
- Zheng L., Heupel R.C. & DellaPenna D. (1992) The beta subunit of tomato fruit polygalacturonase isoenzyme 1: isolation, characterization, and identification of unique structural features. *The Plant Cell* **4**, 1147–1156.
- Zheng L., Watson C.F. & DellaPenna D. (1994) Differential expression of the two subunits of tomato polygalacturonase isoenzyme 1 in wild-type and rin tomato fruit. *Plant Physiology* **105**, 1189–1195.
- Zhu J.K. (2001) Plant salt tolerance. *Trends in Plant Science* **6**, 66–71.

Received 11 July 2013; received in revised form 13 October 2013; accepted for publication 15 October 2013

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Expression pattern analysis of *OsBURP16* and *ACTINI*. (a) The tissue expression pattern of *OsBURP16* was predicted by the Rice eFP website (<http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi>). The color scale illustrates the microarray signal level. (b) The expression patterns of *OsACTINI* under different abiotic stresses were predicted by the Rice eFP website (<http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi>) in 7-day-old seedlings. The color scale illustrates the microarray signal level. (c) Expression profiles of *OsBURP16* under cold, drought and salt stresses and ABA treatment in 2-week-old wild-type plants. *OsCIPK06* was used as the internal control. The x-axis represents the time courses of the stress treatments. Data represent means \pm SD of three biological replicates.

Figure S2. Pectin content and PG activity in leaves of ZH10 before and after drought stress as well as relative expression levels of stress-related genes in ZH10 and *Ubi::OsBURP16* transgenic line 4 (OE4) seedlings. (a) Uronic acid content, determined by colorimetry in leaves of ZH10 before and after drought stress. The y-axis presents values for galacturonic acid equivalents (GaE) per 1 g fresh weight. Data represent means \pm SD of three biological replicates. The asterisk

indicates significant difference at $P \leq 0.05$ compared with ZH10 before drought stress by Student's *t*-test. (b) PG activity in leaves of wild type (ZH10) before and after drought stress. PG activity was measured as millimoles of reducing groups per 100 mg of cell wall material ($\text{mmol} \cdot 100 \text{ mg}^{-1} \text{ cwm}$). Each column represents means \pm SD of three biological replicates. Asterisks indicate significant difference at $P \leq 0.05$ compared with ZH10 before drought stress by Student's *t* test. (c). Relative expression levels of stress-related genes in ZH10 and *Ubi::OsBURP16* transgenic line 4 (OE4) seedlings. *OsTPP1*, trehalose-6-phosphate phosphatase gene 1 (LOC_

Os02g44230); *OsTPS*, trehalose-6-phosphate synthase gene 1 (LOC_Os08g34580). *OsCIPK06* was used as the internal control. Data represent means \pm SD of three biological replicates. Asterisks indicate significant difference at $P \leq 0.05$ compared with the wild type by Student's *t*-test.

Figure S3. Cellular observation of leaves or roots of 2-week-old ZH10 and *OsBURP16*-overexpressing plants (OE4) by staining with propidium iodide (PI). (a) Leaf sheath cells of ZH10 and *OsBURP16*-overexpressing plants (OE4). Scale bars = 50 μm . (b) Root cells of ZH10 and *OsBURP16*-overexpressing plants (OE4). Scale bars = 50 μm .