New Phytologist

Novel crosstalk between ethylene- and jasmonic acid-pathway responses to a piercing–sucking insect in rice

Feilong Ma[1,2], Xiaofang Yang[1,2], Zhenying Shi[1] and Xuexia Miao[1]

[1]Key Laboratory of Insect Developmental and Evolutionary Biology, CAS Center for Excellence in Molecular Plant Sciences, Shanghai Institute of Plant Physiology and Ecology, Innovation Academy for Seed Design, Chinese Academy of Sciences, Shanghai 200032, China; [2]University of Chinese Academy of Sciences, Beijing 100049, China

Authors for correspondence:
Zhenying Shi
Tel: +86 21 54924217
Email: zyshi@sibs.ac.cn

Xuexia Miao
Tel: +86 21 54924217
Email: xxm@sibs.ac.cn

Received: *9 December 2018*
Accepted: *5 August 2019*

New Phytologist (2020) **225:** 474–487
doi: 10.1111/nph.16111

Key words: brown planthopper, ethylene, jasmonic acid, *OsEBF1*, *OsEIL1*, *OsLOX9*, piercing–sucking insect.

Summary

- Ethylene (ET) and jasmonic acid (JA) play important roles in plant defenses against biotic stresses. Crosstalk between JA and ET has been well studied in mediating pathogen resistance, but its roles in piercing–sucking insect resistance are unclear.
- The brown planthopper (BPH; *Nilaparvata lugens*) is the most notorious piercing–sucking insect specific to rice (*Oryza sativa*) that severely affects yield. A genetic analysis revealed that *OsEBF1* and *OsEIL1*, which are in the ET signaling pathway, positively and negatively regulated BPH resistance, respectively. Molecular and biochemical analyses revealed direct interactions between OsEBF1 and OsEIL1. OsEBF1, an E3 ligase, mediated the degradation of OsEIL1 through the ubiquitination pathway, indicating the negative regulation of the ET-signaling pathway in response to BPH infestation.
- An RNA sequencing analysis revealed that a JA biosynthetic pathway-related gene, *OsLOX9*, was downregulated significantly in the *oseil1* mutant. Biochemical analyses, including yeast one-hybrid, dual luciferase, and electrophoretic mobility shift assay, confirmed the direct regulation of *OsLOX9* by OsEIL1.
- This study revealed the synergistic and negative regulation of JA and ET pathways in response to piercing–sucking insect attack. The synergistic mechanism was realized by transcriptional regulation of OsEIL1 on *OsLOX9*. *OsEIL1-OsLOX9* is a novel crosstalk site in these two phytohormone signaling pathways.

Introduction

Phytohormones, including jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), form an intricate network that underlies many resistance pathways to various biotic and abiotic stresses (Cheng *et al.*, 2013; Berens *et al.*, 2017; Guo *et al.*, 2018; Klessig *et al.*, 2018; Olate *et al.*, 2018). The brown planthopper (BPH; *Nilaparvata lugens*) is one of the most destructive insect pests of rice (*Oryza sativa*) throughout Asia. It is a piercing–sucking insect that sucks the phloem sap, which results in reduced rice growth and vigor and causes widespread death of the rice plants, known as 'hopper-burn'. Tremendous efforts have been made to clone endogenous host plant BPH-resistance genes and elucidate the underlying molecular mechanisms to provide a more practical and environmentally friendly protection than simple chemical control (Du *et al.*, 2009; Tamura *et al.*, 2014; Liu *et al.*, 2015; Wang *et al.*, 2015; Ji *et al.*, 2016; Ren *et al.*, 2016; Zhao *et al.*, 2016; Guo *et al.*, 2018). Meanwhile, phytohormone metabolic pathways involved in rice plant resistance to the BPH have long been an area of scientific interest.

A few factors in the ET-signaling pathway have been reported to act as negative regulators in BPH resistance (Du *et al.*, 2009; Lu *et al.*, 2011, 2014; Wang *et al.*, 2015; Zhang *et al.*, 2017). For example, *OsERF3* (ethylene response factor 3) negatively regulates BPH resistance, but positively regulates resistance to the striped stem borer (SSB), a chewing insect pest of rice (Lu *et al.*, 2011). RNA interference (RNAi) of the 1-aminocylopropane-1-carboxylic acid synthase gene *OsACS2* (aminocyclopropane-1-carboxylate synthase gene 2) decreases ET biosynthesis, increases resistance to the BPH, and decreases resistance to the SSB (Lu *et al.*, 2014).

Similarly, the functions of several genetic factors in the JA biosynthetic pathway against BPH have been studied. For example, silencing of *OsHI-LOX* (13-lipoxygenase gene) in the JA biosynthetic pathway increased resistance to the BPH (Zhou *et al.*, 2009), whereas a knockdown of *Osr9-LOX1* rendered the plants more susceptible to the BPH (Zhou *et al.*, 2014). The overexpression (OE) of *OsAOC* (allene oxide cyclase gene), but not *OsOPR3* (*cis*-12-oxo-phytodienoic acid reductase 3), increased BPH resistance in a JA-pathway-independent manner (Guo *et al.*, 2014). Another study indicated that the JA content was lower in resistant rice carrying *Bph29* than in a loss-of-resistance transgenic line both before and after BPH feeding, indicating that the JA pathway negatively mediated the resistance mechanism of *Bph29* (Wang *et al.*, 2015).

According to previous studies, both the ET and JA pathways play negative roles in BPH resistance. However, all these studies on the two phytohormone responses to insect infestation are in independent analyses. Few studies have focused on a synergistic mechanism between these two metabolic pathways in response to piercing–sucking insect attack.

Frequent crosstalk among different signaling pathways allows plants to orchestrate an appropriate spectrum of responses to different stresses. Evidence of the tight crosstalk between JA and ET comes from the mediation of nonspecific disease resistance (Dong, 1998). Both JA and ET concomitantly and synergistically regulate plant defenses against pathogens and necrotrophic fungi. The plant defensin1.2 (PDF1.2) protein, which protects plants against pathogens, is jointly activated by JA and ET (Penninckx *et al.*, 1996). The GCC box in the promoter of PDF1.2 is targeted by ET response factor (ERF) proteins, such as ERF1 and ORA59, which confers JA responsiveness and synergy between JA and ET (Brown *et al.*, 2003; Pre *et al.*, 2008). ETHYLENE-INSENSITIVE3 (EIN3) and its closest homologue ETHYLENE INSENSITIVE 3-like1 (EIL1) are two primary transcription factors downstream of EIN2 (Chao *et al.*, 1997; Guo & Ecker, 2003). Zhu *et al.* (2011) confirmed that JA-Zim (JAZ) domain proteins directly interact with EIN3/EIL1 and repress its transcriptional activity. JA-induced EIN3/EIL1 activation and ET-induced EIN3/EIL1 stabilization underlie the synergistic crosstalk between JA and ET in response to necrotrophic fungi in Arabidopsis (Zhu *et al.*, 2011). However, in responses to chewing insects, JA and ET act antagonistically in *Arabidopsis*, which might be mediated by MYC2 and EIN3 (Memelink, 2009; Verhage *et al.*, 2011). EIN3 interacts with and represses MYC2 to inhibit the JA-induced expression of herbivory-inducible genes and to attenuate JA-regulated plant defenses (Song *et al.*, 2014a, b; Zhang *et al.*, 2014). Thus, EIN3/EIL1 mediated the crosstalk between JA- and ET-signaling pathways in *Arabidopsis*. However, some of the conclusions in *Arabidopsis*, which is a dicotyledonous plant, cannot be verified in the monocotyledonous plant rice (De Vleesschauwer *et al.*, 2014). For example, JA or ET biosynthesis- and signaling-related genes are all critical for the positive regulation of rice resistance to *Magnaporthe oryzae*, which is another destructive disease of rice (Nasir *et al.*, 2018). Therefore, the JA–ET crosstalk plays an important role in optimizing plant responses to different kinds of stress. Nevertheless, defense-signaling pathways are often plant and insect species dependent. Thus, the crosstalk between JA and ET in mediating BPH resistance might be specific, and the detailed mechanism deserves further study.

In this study, we observed that the BPH-responsive *OsEBF1* gene in the ET-signaling pathway positively regulated BPH resistance, whereas the other ET-signaling pathway gene, *OsEIL1*, negatively regulated BPH resistance, with a rice *oseil1* mutant showing enhanced resistance to BPH. Biochemical analyses revealed the degradation of OsEIL1 by OsEBF1 through the ubiquitin-proteasome pathway, supporting the negative role of the ET-signaling pathway in BPH resistance.

RNA-sequencing (RNA-seq) data for the *oseil1* mutant indicated that many genes in the JA biosynthetic pathway were obviously downregulated; among them, the *OsLOX9* gene showed the greatest degree of downregulation. Further biochemical analyses revealed that OsEIL1 could bind to the promoter of the *OsLOX9* gene and activate its expression. Therefore, this study confirmed the negative and synergistic roles of JA and ET in mediating resistance to the BPH; *OsEIL1* and *OsLOX9* formed a novel crosstalking site between these two phytohormone-signaling pathways.

Materials and Methods

Plant and insect materials

OsEBF1OE and OsEBF1RNAi plants were generated from genetic transformation of the wild-type (WT) 'Zhonghua 11' (*Orayza sativa* L. subsp. *japonica* cv Zhonghua no. 11, ZH11), and EIL1-myc and *oseil1* mutants were generated from the WT 'Nipponbare' (*Oryza sativa* subsp. *japonica* cv Nipponbare). All the rice plants were grown in a glasshouse, with a 10 h : 14 h, light : dark photoperiod, or in the field under natural conditions in the summer in Shanghai, China.

The BPH population was originally obtained from rice fields in Shanghai, China, and maintained on the BPH-susceptible rice cv Taichung Native 1 (TN1) plants in a climate-controlled room at 26 ± 2°C, with a 12 h : 12 h, light : dark cycle and 80% relative humidity.

Measurements of BPH performance

Several methods were used to detect plants' responses to BPHs. For individual tests, individual plants at the third-leaf stage from each line were infested with 10 second- to third-instar BPH nymphs. Plant damage levels were observed daily for 7–10 d until all the plants of one line died.

For the small population analysis, *c.* 20 seeds per line were sown in 15 cm rows in a plastic box for comparison. At the third-leaf stage, each seedling was infested with 10 second- to third-instar BPH nymphs. Plant damage levels were observed daily until most of the rice seedlings of one line withered.

BPH survival rate tests and BPH weight gain measurements were performed as previously described (Du *et al.*, 2009). Then, 15 second-instar nymphs were placed on each plant and covered with a cylindrical Mylar cage. Their alive or dead states was recorded daily for 10 d ($n = 6$).

Hormone treatments and BPH performance

To evaluate the effects of ET on *OsEBF1*, the rice sheaths were treated with ethephon (0, 20, 40, 60 and 80 μM), and the samples were collected and tested at 4 h after treatment.

To investigate the effects of JA and ET on BPH performance, 10 'Nipponbare' seeds were sown in a 10 cm diameter plastic pot with a hole at the bottom. At the two- to three-leaf stage, the seedlings were sprayed with 2.4 ml of methyl jasmonate (MeJA; 0, 100 or 200 μM) or ethephon (0, 100 or 200 μM) and 100 first-instar BPH nymphs were released on the seedlings 2 h after

spraying. Each treatment was performed in triplicate. Plant damage levels were observed daily, and BPH weights were measured 7 d after infestation.

Plasmid construction and transformation of rice

The full-length cDNA of *OsEBF1* (LOC_Os06g40360) was amplified with KOD-plus DNA polymerase (Toyobo, Tokyo, Japan) using the primers EBF1OE-attB-F and EBF1OE-attB-R, and cloned into the *p*1301-35SNos vector using a Hieff-Clone™ Plus Multi One Step Cloning Kit (Yeasen, Beijing, China).

The RNAi of *OsEBF1* (EBF1RNAi) was performed by independently cloning a 237 bp complementary DNA (cDNA) fragment into the *p*1301RNAi vector in the sense and anti-sense orientations. The OsEBF1OE and OsEBF1RNAi plasmids were transformed into ZH11 using an *Agrobacterium*-mediated method with minor modifications (Hiei *et al.*, 1994).

Protein alignment

OsEBF1 and OsEIL1 sequences were downloaded from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/), and sequences of AtEBF1 and AtEIN3 were downloaded from the Arabidopsis Information Resource (https://www.arabidopsis.org/). The amino acid sequences were aligned using ClustalX v.1.83.

Phylogenetic analysis

The OsEBF1 protein sequences for 33 other species were downloaded from National Center for Biotechnology Information databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were generated using predicted full-length amino acid sequences by the maximum-likelihood method in Mega7.0 with bootstrap mode and 500 replications.

Quantitative real-time reverse transcription PCR

Total RNA was extracted from different tissues using TRIzol (Invitrogen), and then digested with DNase I. Quantitative real-time reverse transcription PCR (qRT-PCR) was performed using the SYBR Green Real-time PCR Master Mix Kit (Toyobo), and the *actin* gene served as an internal control. Each sample was run in triplicate, and the mean values of the technical replicates were recorded for each biological replicate. Data from three biological samples were collected, and the mean values with standard errors were plotted.

Subcellular localization of OsEBF1 protein in rice protoplasts

The coding sequence of *OsEBF1* was cloned into pA7-YFP to generate OsEBF1-YFP. The pA7-YFP vector was used as a control. The fusion and control plasmids were independently transformed into rice protoplasts. Fluorescence was visualized under an Olympus FV1000 fluorescence microscope (Tokyo, Japan). The experiments were repeated three times.

Yeast one-hybrid assays

The full-length cDNA of *OsEIL1* was amplified and fused into the activation domain of the vector pPC86. Fragments containing the six putative OsEIL1 binding sites of 'ATGT(C)A' or all of the deletion mutant of the six binding sites in the *OsLOX9* promoter were independently amplified and fused into the vector p178. The p178- and pPC86-derived constructs were co-transformed into the yeast strain EGY48 together with the empty p178 and pPC86 vectors. The yeasts were grown on SD selective medium (SD-His-Leu) and observed using blue coloration on a chromogenic medium. Transformants containing empty pPC86 and p178 were used as negative controls. The yeast one-hybrid assay was conducted as described by the Matchmaker One-hybrid System instructions (Clontech, Mountain View, CA, USA).

Yeast two-hybrid assays

The *OsEBF1* and *OsEIL1* sequences were independently amplified and cloned into pCR™8/GW/TOPO® and then transferred to the destination vectors pGBKT7 and pGADT7, respectively. The primers used are shown in Supporting Information Table S1. The yeast strain AH109 was independently transformed with these constructs using a lithium acetate transformation protocol (Yeast Protocols Handbook PT3024-1; Clontech).

Co-immunoprecipitation assays

The *OsEBF1* and *OsEIL1* sequences were independently amplified and cloned into pCR™8/GW/TOPO® and then transferred into the destination vectors pSPYNE-EBF1-myc and pSPYCE-EIL1-HA, respectively, using an LR reaction and then co-transformed into 'Nipponbare' protoplasts. An anti-Myc affinity gel was used for immunoprecipitation, and an anti-EIL1 antibody was used to detect OsEIL1.

Bimolecular fluorescence complementation

The OsEBF1 and OsEIL1 proteins were independently fused to both the N- and C-terminal fragments of yellow fluorescent protein (YFP) to produce pSPYNE-EBF1-myc, pSPYCE-EIL1-HA, pSPYNE-EIL1-myc, and pSPYCE-EBF1-HA. The N-terminus of OsEIL1 and the C-terminus of OsEBF1 alone were used as negative controls. The test plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and bacterial cultures of the test combinations and controls were infiltrated into *Nicotiana benthamiana* leaves. The YFP signal was evaluated using confocal microscopy 3 d after infiltration (FV10-ASW; Olympus).

Dual luciferase analysis

The plasmid p1301-35S-EIL1-Nos was transformed into *A. tumefaciens* strain GV3101 to act as an effector. The reporter

construct was prepared by cloning the *OsLOX9* promoter into the pGreenII0800-LUC vector, and subsequently co-transformed with the helper plasmid pSoup19 into *A. tumefaciens* strain GV3101 to act as the reporter. The p1301-35S-Nos plasmid was used as a negative control. The bacterial cultures from the experimental and control groups were infiltrated into opposite ends of the same tobacco leaf (*N. benthamiana*). The leaves were collected after 3 d under long-day white-light conditions and infiltrated with 150 μg ml^{-1} luciferin solution. Images were captured using a CCD camera 5 min later, and quantification was performed using the Dual-Luciferase Reporter Assay System according to the instructions (Promega, Madison, WI, USA). Five biological repeats were measured for each sample.

Electrophoretic mobility shift assay

A recombinant protein containing the N-terminus of OsEIL1 (residues 1–350) was generated according to Yang *et al.* (2015). The Cy5-labelled *OsLOX9* probe was amplified using two rounds of PCR. The DNA probes and proteins were co-incubated in the reaction buffer, purified, and incubated with the Cy5-labeled probe at 37°C for 20 min in electrophoretic mobility shift assay (EMSA) buffer (25 mM Hepes (pH 7.5), 40 mM potassium chloride, 3 mM dithiothreitol, 10% glycerol, 0.1 mM EDTA, 0.5 mg ml^{-1} bovine serum albumin, 0.5 mg ml^{-1} poly-glutamate). After incubation, the reaction mixture was electrophoresed on a 6% native polyacrylamide gel, and then labeled DNA was detected using a Starion FLA-9000 instrument (Fujifilm, Tokyo, Japan).

Measurement of ET content

Leaves from similar locations on *oseil1* mutant and WT plants at the three-leaf stage were cut into small pieces and placed into a bottle containing 500 μl water for 24 h at 28°C. Then, the ET levels in 1 ml of the headspace from each bottle were measured using a gas chromatograph equipped with a flame ionization detector (GC2014; Shimadzu, Kyoto, Japan).

Quantification of SA and JA levels in plants

The sheaths from leaves at similar locations on *oseil1* mutant and WT plants were collected and the samples homogenized according to Mao *et al.* (2017). JA and SA levels were analyzed by high-performance liquid chromatography–mass spectrometry (HPLC-MS) using labeled internal standards.

Results

Expression characteristics and the subcellular localization of *OsEBF1*

To investigate the comprehensive molecular basis of rice plant responses to a piercing–sucking insect (the BPH), we previously carried out RNA-seq on the rice resistant variety 'Rathu Heenati' (RHT) and the susceptible variety TN1 before and after BPH infestation (Wang *et al.*, 2012). The differentially expressed genes in ET biosynthesis- and signaling-related pathways that responded to BPH infestation were analyzed. The data indicated that an *EIN3-binding F-box protein 1* (*EBF1*, LOC_Os06g40360) gene was upregulated in the resistant variety RHT 8 h after BPH infestation (Fig. S1). The full-length 1884-nucleotide cDNA sequence of *OsEBF1* was obtained from rice using the rapid-amplification of cDNA ends (RACE) method, and the deduced amino acid sequence showed a 46.1% identity to that of Arabidopsis *AtEBF1* (Fig. S2a). A phylogenetic analysis of the EBF1 protein from different species revealed a high degree of conservation (Fig. S2b), indicating that they might have conserved functions.

We further analyzed the expression profile of *OsEBF1* by qRT-PCR. Among the different tissues analyzed, including leaf blade, stem, root, and leaf sheath, *OsEBF1* was mainly expressed in the leaf sheath, which is the site of BPH feeding (Fig. 1a). Then, we checked the response of *OsEBF1* to an ethephon (a potent regulator of plant growth and ripeness that has the same function as ET) treatment and found that *OsEBF1* was induced by ethephon, with the induction degree being consistent with the concentration at < 60 μM (Fig. 1b). Additionally, the *OsEBF1* gene was obviously induced at several time points after BPH infestation (Fig. 1c). Thus, we believe that the ET-signaling pathway is involved in the rice responses to the BPH.

In Arabidopsis, the AtEBF1 protein localizes to the nucleus. To better understand the function of *OsEBF1*, we analyzed the subcellular localization of an OsEBF1-YFP fusion protein in rice protoplasts and found that the fluorescence signal was clearly concentrated in the nucleus (Fig. 1d), which further indicated functional conservation between AtEBF1 and OsEBF1.

In conclusion, we deduced that *OsEBF1* was an ET- and BPH-responsive gene, and the encoded OsEBF1 protein was localized in the nucleus.

The *OsEBF1* gene positively regulates BPH resistance

To further clarify the function of the *OsEBF1* gene in rice resistance to the BPH, we constructed its OE and RNAi genetic lines. Two OsEBF1OE lines with greatly enhanced expression levels of *OsEBF1* (Fig. S3a) and two OsEBF1RNAi lines with the greatest downregulation of *OsEBF1* (Fig. S3b) were selected to investigate the response to BPH. It was revealed that the BPH resistance of the OsEBF1OE lines was enhanced significantly (Fig. 2a). However, the OsEBF1RNAi lines were more susceptible to BPHs than the WT (Figs 2b, S3c). Accordingly, the BPH weight gain on the OsEBF1OE lines was significantly lower than that on the WT, and even lower than those on the OsEBF1RNAi lines (Fig. 2c). These results indicated that the OsEBF1OE lines showed an antibiosis mechanism against BPH. We also found that the BPH survival rates are significantly greater on OsEBF1RNAi lines than those on the WT and even greater than those on the OsEBF1OE lines (Fig. 2d).

Furthermore, we verified the responses of the OsEBF1OE and OsEBF1RNAi lines to BPH infestation using a small population test (Fig. 2e,f) and recorded the mortality rates of rice seedlings (Fig. 2g,h). It was confirmed that, compared with WT plants, the OsEBF1OE lines were more resistant to the BPH, whereas the

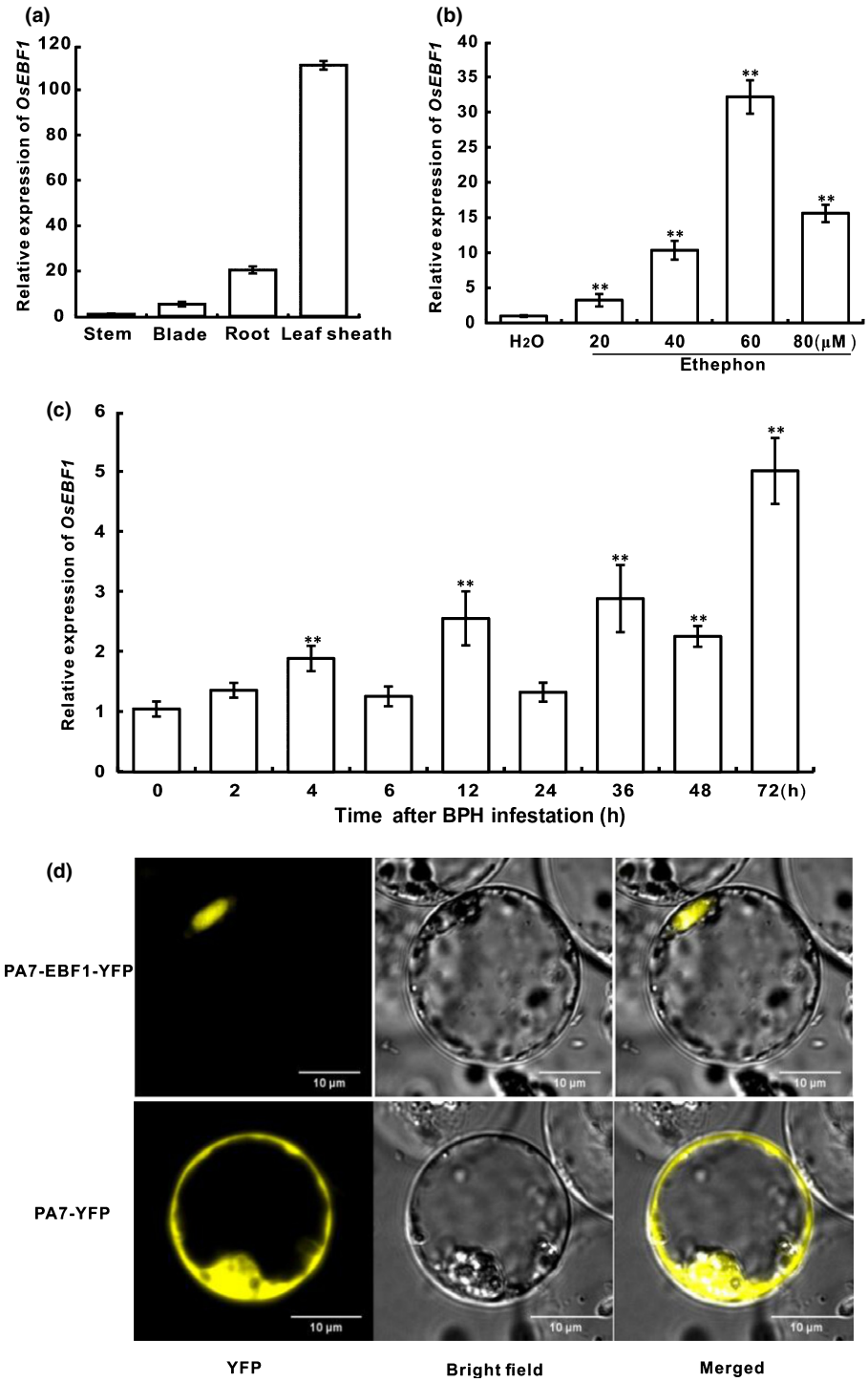

Fig. 1 Spatiotemporal expression of *OsEBF1* gene in rice and subcellular localization of OsEBF1 protein. (a) Quantitative real-time reverse transcription PCR (qRT-PCR) analysis of *OsEBF1* transcripts in different rice tissues. (b) qRT-PCR analysis of *OsEBF1* transcripts after ethephon treatment. (c) qRT-PCR analysis of *OsEBF1* transcripts after brown planthopper (BPH) attack. (d) Localization of the OsEBF1-YFP fusion protein in rice protoplasts. Error bars represent ± standard deviation ($n = 3$). In (b, c), significant difference determined by Student's *t*-test: **, $P < 0.01$.

OsEBF1RNAi lines were more susceptible to the BPHs. Thus, the *OsEBF1* gene appears to positively regulate BPH resistance through antibiosis mechanisms.

OsEBF1 mediates the ubiquitination and degradation of OsEIL1

In Arabidopsis, the *AtEBF1* gene encodes an E3 ligase and can degrade AtEIN3 through the ubiquitination pathway (Guo & Ecker, 2003; Potuschak *et al.*, 2003). To confirm the functional conservation of *OsEBF1* in rice, OsEIL1 was cloned by RACE technology. OsEIL1 is a homologous protein of Arabidopsis EIN3 (AtEIN3), with an amino acid similarity of 51.8% (Fig. S4a). First, using yeast two-hybrid analysis, we found that OsEBF1 could interact with OsEIL1 in yeast cells (Fig. 3a). Then, we used a bimolecular fluorescence complementation assay and observed positive fluorescence signals in the nucleus when OsEBF1 was fused to the N-terminus and OsEIL1 was fused to

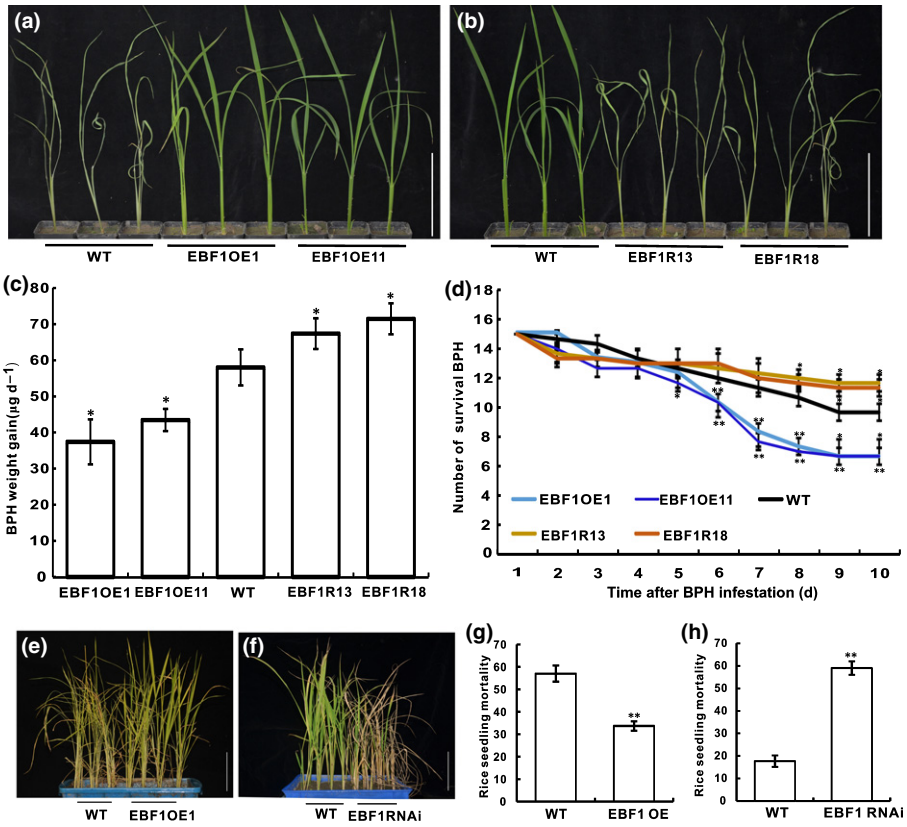

Fig. 2 *OsEBF1* positively regulates brown planthopper (BPH resistance in rice. (a) The status of the OsEBF1OE lines and the wild-type (WT) plants were individually infested with 10 second- to third-instar BPH nymphs for 7 d. (b) The status of the OsEBF1RNAi lines and the WT plants were individually infested with 10 second- to third-instar BPH nymphs for 7 d. (c) Statistical analysis of BPH weight gains after feeding on OsEBF1OE, OsEBF1RNAi, and WT plants. Error bars represent ± standard deviation ($n = 10$). (d) Statistical analysis of BPH survival rates on OsEBF1OE, OsEBF1RNAi, and WT plants after infestation for 10 d. Error bars represent ± standard deviation ($n = 10$). (e) Status of the OsEBF1OE and WT plants in small population tests for 7 d. (f) Status of OsEBF1RNAi and WT plants in small population tests for 5 d. (g) Quantitative determination of the mortality rates of OsEBF1OE and WT plants for 7 d after BPH infestation. Error bars represent ± standard deviation ($n = 3$). (h) Quantitative determination of the mortality rates of OsEBF1RNAi and WT plants for 5 d after BPH infestation. Bars: (a, b) 15 cm; (e, f) 10 cm. In (c, d, g, h), significant differences determined by Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$.

the C-terminus, or vice versa (Fig. 3b). Furthermore, a co-immunoprecipitation analysis in rice protoplasts verified the interaction between OsEBF1 and OsEIL1 *in vivo* (Fig. 3c).

Having established that OsEBF1 and OsEIL1 could interact, we tried to determine whether OsEIL1 could be ubiquitinated by OsEBF1. We first checked the protein levels of OsEIL1 in OsEBF1OE and OsEBF1RNAi plants. We found that the amount of OsEIL1 protein in the OsEBF1RNAi plants was obviously greater than those in WT and OsEBF1OE plants (Figs 3d, S4b), indicating that the expression level of *OsEBF1* affected the expression amount of OsEIL1 protein. Furthermore, OsEIL1-myc, plants were treated with 40 μM MG132 (a 26S

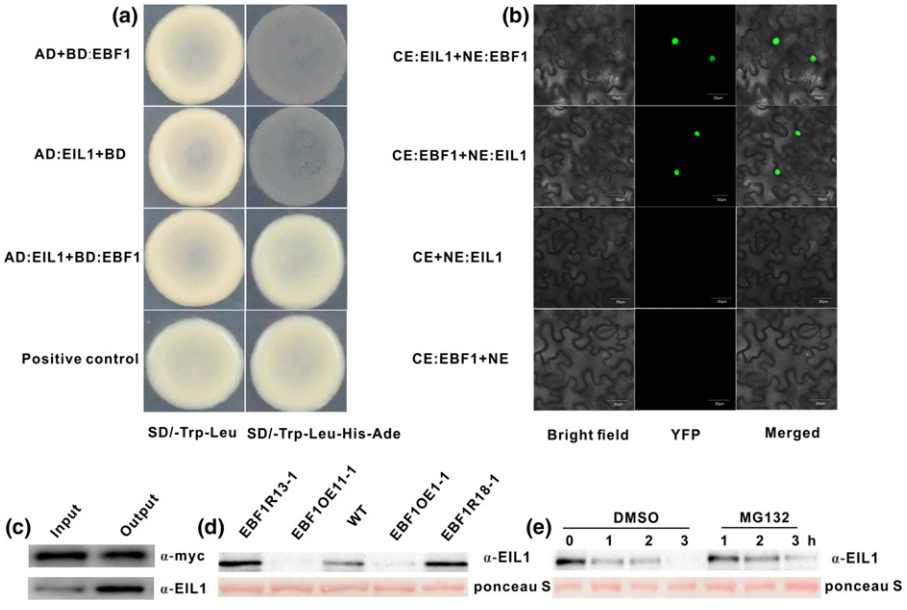

Fig. 3 Biochemical analysis of the interactions between OsEBF1 and OsEIL1, and the degradation of OsEIL1. (a) Yeast two-hybrid assay of the interaction between OsEBF1 and OsEIL1. (b) Bimolecular fluorescence complementation assays showing the interaction between OsEBF1 and OsEIL1 in *Nicotiana benthamiana*. Bars, 30 μm. (c) Co-immunoprecipitation analysis of the interaction between OsEBF1 and OsEIL1 in rice leaf protoplasts. Anti-myc was used for immunoprecipitation, and an anti-EIL1 antibody was used to detect OsEIL1. (d) Western blot to check the protein levels of OsEIL1 in the OsEBF1OE, OsEBF1RNAi, and wild-type (WT) plants. (e) Degradation rate of the OsEIL1 protein in OsEIL1-myc plant treated by 40 μM MG132 or mock of dimethyl sulfoxide at 3 h after treatment.

proteasome inhibitor), and the OsEIL1 protein was degraded much more slowly than that in the mock of dimethyl sulfoxide-treated OsEIL1-myc plants at 3 h after treatment (Fig. 3e). Thus, MG132 could inhibit the degradation of OsEIL1, which might depend on the ubiquitination pathway. Because the messenger RNA levels of *OsEIL1* were not significantly different in OsEBF1OE and OsEBF1RNAi plants (Fig. S5), we deduced that OsEBF1 does not affect the transcription of *OsEIL1* and that OsEBF1 regulates the degradation of OsEIL1 through a ubiquitination pathway.

OsEIL1 negatively regulates rice resistance to the BPH

To determine whether the ubiquitination and degradation of OsEIL1 affects the rice resistance to the BPH, the function of the *OsEIL1* gene was studied. The expression level of *OsEIL1* was obviously regulated by BPH infestation in the WT (Fig. 4a), indicating the possible involvement of *OsEIL1* in BPH resistance.

Next, one *oseil1* mutant and two OE lines (OsEIL1-myc) were detected; the expression of *OsEIL1* at both the transcriptional level (Fig. S6a,b) and the protein levels (Fig. S6c) in *oseil1* was confirmed (Yang *et al.*, 2015; Qin *et al.*, 2017). The BPH infestation results revealed that the *oseil1* mutant was more resistant to the BPHs than the WT was (Fig. 4b), whereas the two OE EIL1-myc lines were more susceptible to the BPHs (Fig. 4c). Accordingly, the BPH weight gains were significantly greater on the EIL1-myc plants than on the WT, and even greater than those on the *oseil1* mutant (Fig. 4d). Furthermore, the BPH survival rates were significantly greater on OsEIL1-myc lines than those on the WT and *oseil1* plants (Fig. 4e). All these results revealed that the *oseil1* mutant used antibiosis mechanisms in response to BPH attack.

The responses of the *oseil1* and the EIL1-myc lines to BPH infestation were further verified using a small population analysis (Fig. 4f,g), and the mortality rates of rice seedlings were determined (Fig. 4h,i). The results confirmed that the *oseil1* was more resistant to BPH than the WT was, whereas the EIL1-myc lines were more susceptible to the BPH.

The *oseil1* mutation enhanced the rice resistance to BPH, which indicated that the ET-signaling pathway negatively regulated the rice resistance to BPH.

OsEIL1 directly regulates *OsLOX9* and mediates BPH resistance

The aforementioned results indicated that the ET-signaling insensitivity would enhance rice resistance to the BPH. To further reveal the underling molecular mechanisms of OsEBF1OE and *oseil1* mutant resistance to the BPH, we carried out an RNA-seq analysis of the *oseil1* mutant. Most of the genes in the JA biosynthetic pathway was strongly downregulated in the *oseil1* mutant (Fig. 5a). Among them, *OsLOX9* (also known as *OsHI-LOX*), which has been shown to negatively regulates BPH resistance (Zhou *et al.*, 2009; Marla & Singh, 2012), was downregulated to the greatest degree. We checked the expression levels of *OsLOX9* in the *oseil1* mutant and EIL1-myc lines by qRT-PCR and found that *OsLOX9* was upregulated in the EIL1-myc lines but downregulated in the *oseil1* mutant (Fig. 5b). Thus, we deduced that *OsEIL1* regulates the expression of JA biosynthetic-related genes and that *OsLOX9* might be the gene responsible for crosstalk between the JA and ET pathways.

For further verification, we analyzed the promoter region of the *OsLOX9* gene and found six OsEIL1-binding motifs, ATGT(C)A, in the 2 kb promoter upstream of the 'ATG' start codon (Fig. 5c; Yang *et al.*, 2015). In the yeast one-hybrid assay, yeast cells with the OsEIL1 protein and the fragments containing the putative binding motifs from the *OsLOX9* promoter showed a positive blue color, whereas those with the OsEIL1 protein and deletion mutant of the OsEIL1-binding motifs did not turn blue (Fig. 5d). We also tested the binding of OsEIL1 to the promoter of *OsLOX8* and *OsAOS2* using the yeast one-hybrid system, and the results indicated that OsEIL1 cannot bind to the promoter of *OsLOX8* but can bind to the promoter of *OsAOS2* (Fig. S7). *OsAOS2* is a positive regulator of the rice blast fungus *M. oryzae* (Mei *et al.*, 2006); its role in BPH resistance will be investigated in the future. Consequently, we selected *OsLOX9* as the putative target gene for OsEIL1 in this study.

We then used the Dual-Luciferase® system and verified the activation of the *OsLOX9* promoter by the OsEIL1 protein, as indicated by an increased fluorescence signal (Fig. 5f,g). Furthermore, we tested the interaction of the recombinant OsEIL1 protein and a 40 bp length of 5′-Cy5-labeled double-stranded oligonucleotide containing the two ATGT(C)A motifs near the *OsLOX9* promoter using EMSA. The DNA–protein complex migrated more slowly than free DNA, indicating the direct interaction of OsEIL1 with the labeled DNA (Fig. 5e).

Since most of the genes in the JA biosynthetic- and signaling-related pathway were strongly downregulated in the *oseil1* mutant (Figs 5a, S8), and at least one of them, *OsLOX9*, was directly regulated by the OsEIL1 protein (Fig. 5d–g), we wondered whether the JA content was affected in the *oseil1* mutant. Therefore, we analyzed the ET, JA, and SA contents in the *oseil1* mutant using GC and HPLC–MS, and found that both the ET and JA contents were decreased (Fig. 5h,i), indicating that the *OsEIL1* deficiency in ET signaling also affected JA biosynthesis. At the same time, most of the ET-signaling marker genes were decreased (Fig. S9). Thus, the JA and ET pathways interact synergistically to regulate rice resistance to the BPH in the *oseil1* mutant, and *OsLOX9* was confirmed as a JA pathway gene that is involved in the JA–ET crosstalk. However, the SA content in the *oseil1* mutant was not altered, and the expression levels of three SA pathway-related marker genes in *oseil1* and WT showed no significant differences (Figs 5j, S10), indicating that the regulation of BPH resistance by the *OsEIL1* gene is independent of the SA pathway.

JA and ET synergistically and negatively regulate BPH resistance in rice

To further confirm the roles of JA and ET in the plant responses to BPH infestation, we treated WT rice plants with MeJA or ethephon and found that growth of the rice seedlings was not

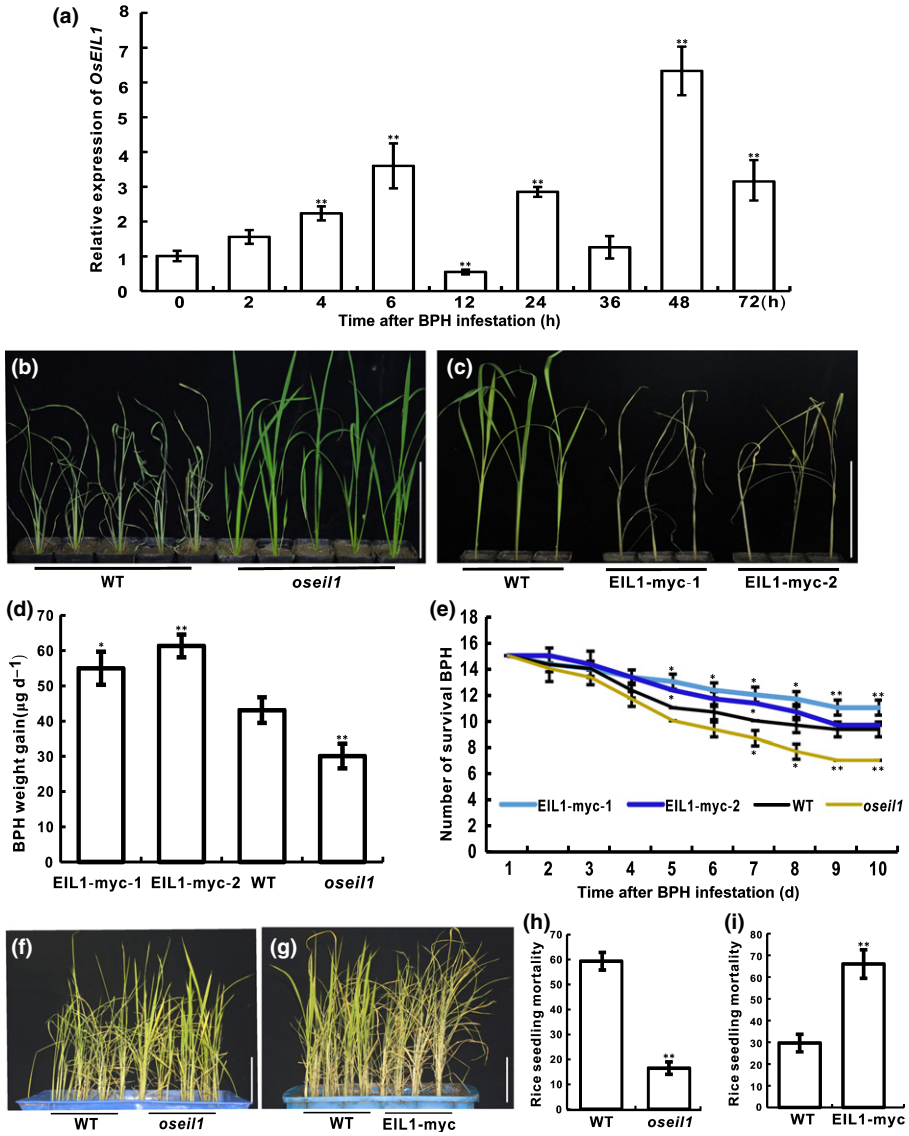

Fig. 4 *oseil1* mutant rice showed enhanced antibiosis to brown planthopper (BPH). (a) The expression levels of *OsEIL1* after BPH infestation in wild-type (WT) plants. (b) The status of *oseil1* and the WT plants that were individually infested with 10 second- to third-instar BPH nymphs for 10 d. (c) The status of EIL1-myc plants and the WT plants that were individually infested with 10 second- to third-instar BPH nymphs for 10 d. (d) Statistical analysis of BPH weight gains after feeding on OsEIL1OE, *oseil1*, and WT plants. Error bars represent ± standard deviation ($n = 3$). (e) Statistical analysis of the BPH survival rates on OsEIL1OE, *oseil1*, and WT plants at 10 d after infestation. Error bars represent ± standard deviation ($n = 10$). (f) Status of *oseil1* and WT plants in a small population test at 10 d. (g) Status of OsEIL1OE (EIL1-myc) and WT plants in a small population test at day 8. (h) Quantitative determination of the mortality rates of *oseil1* and WT plants after being infested with 10 second-instar BPH nymphs per plant for 10 d. Error bars represent ± standard deviation ($n = 3$). (i) Quantitative determination of the mortality rates of OsEIL1OE and WT plants after being infested with 10 second-instar BPH nymphs per plant for 8 d. Error bars represent ± standard deviation ($n = 3$). Bars: (b, c) 15 cm; (f, g) 10 cm. In (a, d, e, h, i), significant differences determined by Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$.

influenced significantly (Fig. S11). We then infested them with the BPHs.

Rice plants treated with 100 or 200 μM ethephon died earlier than those treated with water, indicating that they were more susceptible to the BPHs (Fig. 6a). The greater the ethephon concentration, the more susceptible the rice plants were to the BPHs (Fig. 6a). The ethephon treatment resulted in a greater rice seedling mortality rate (Fig. 6b) and greater BPH weight gain (Fig. 6c). When plants were treated with 100 or 200 μM MeJA, similar results were observed (Fig. 6d–f). These results revealed that both JA and ET have negative concentration-dependent roles in rice resistance to the BPH.

Next, we tested the superposition of the two plant hormones' responses to BPH infestation. WT plants were treated with ethephon and MeJA alone and together, at 100 and 200 μM concentrations, respectively. The plants treated with both kinds of hormones were more susceptible to the BPHs than those just treated with one kind of hormone, whether ethephon or MeJA (Fig. 6g,h), and showed increased plant mortality rates (Fig. 6i,k) and greater BPH weight gains (Fig. 6j,l). These analyses further confirmed that ethephon and MeJA synergistically and negatively regulate plants responses to BPH infestation.

Discussion

Through the genetic functional analysis of two genes, *OsEBF1* and *OsEIL1*, in the ET-signaling pathway, we demonstrated that the ET-signaling pathway plays an important role in shaping piercing–sucking herbivore-induced responses in rice plants. Specifically, OsEBF1 acts as a positive regulator and OsEIL1 acts as a negative regulator in BPH resistance. Because EBF1/2 negatively regulates the ET-signaling pathway by mediating the degradation of EIN3/EIL1 proteins (Potuschak *et al.*, 2003), and EIN3 is the major activator of ET signaling (Chao *et al.*, 1997), we deduced that the ET-signaling pathway functions negatively in BPH resistance. Several lines of evidence support this

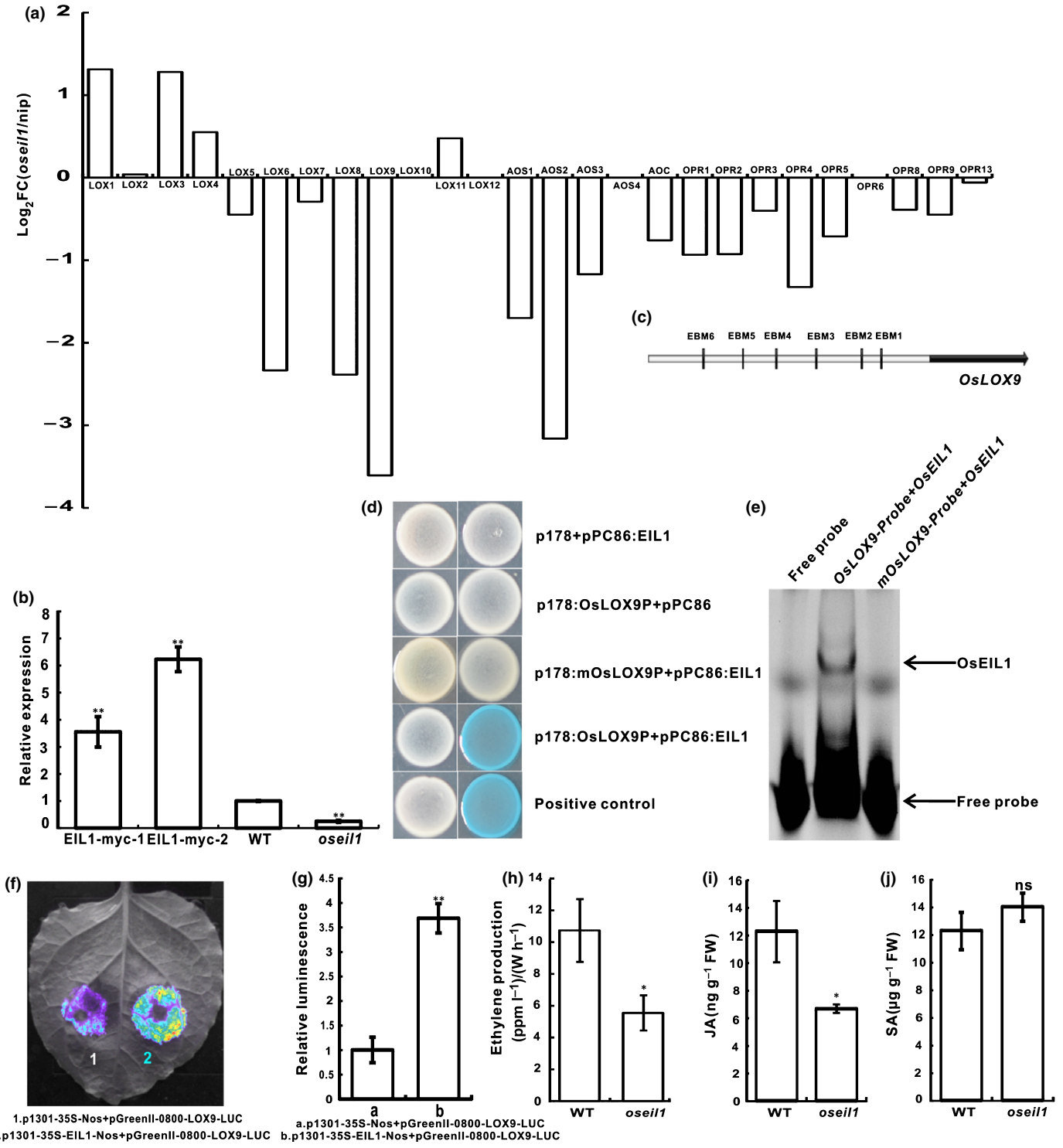

Fig. 5 Detection of direct regulation of *OsLOX9* by OsEIL1. (a) Transcriptome analysis of genes in the jasmonic acid (JA)-biosynthetic pathway in the *oseil1* mutant. (b) Quantitative real-time reverse transcription PCR analysis of the relative expression levels of *OsLOX9* in OsEIL1OE, *oseil1*, and wild-type (WT) rice plants. (c) Schematic representation of the 2 kb *OsLOX9* promoter showing the positions of putative OsEIL1-binding motifs (EBMs). (d) Yeast one-hybrid assay showing the binding of OsEIL1 to the motifs in the *OsLOX9* promoter. OsLOX9P represents the promoter region containing the EBM1–6 in (c), and mOsLOX9P represents OsLOX9P with deletions in the EBMs. (e) Electrophoretic mobility shift assay of OsEIL1 protein binding to the EBM-containing region of *OsLOX9* promoter. OsLOX9P and mOsLOX9P are as described in (d). (f) Dual-Luciferase® assay to detect the activation of the *OsLOX9* promoter by OsEIL1 in *Nicotiana benthamiana*. (g) Quantitative analysis of the comparative luminescence intensities in (f). (h–j) Ethylene, JA, and salicylic acid (SA) content in *oseil1* and WT plants. Bars, 5 cm. In (b, g–i), significant differences determined by Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$.

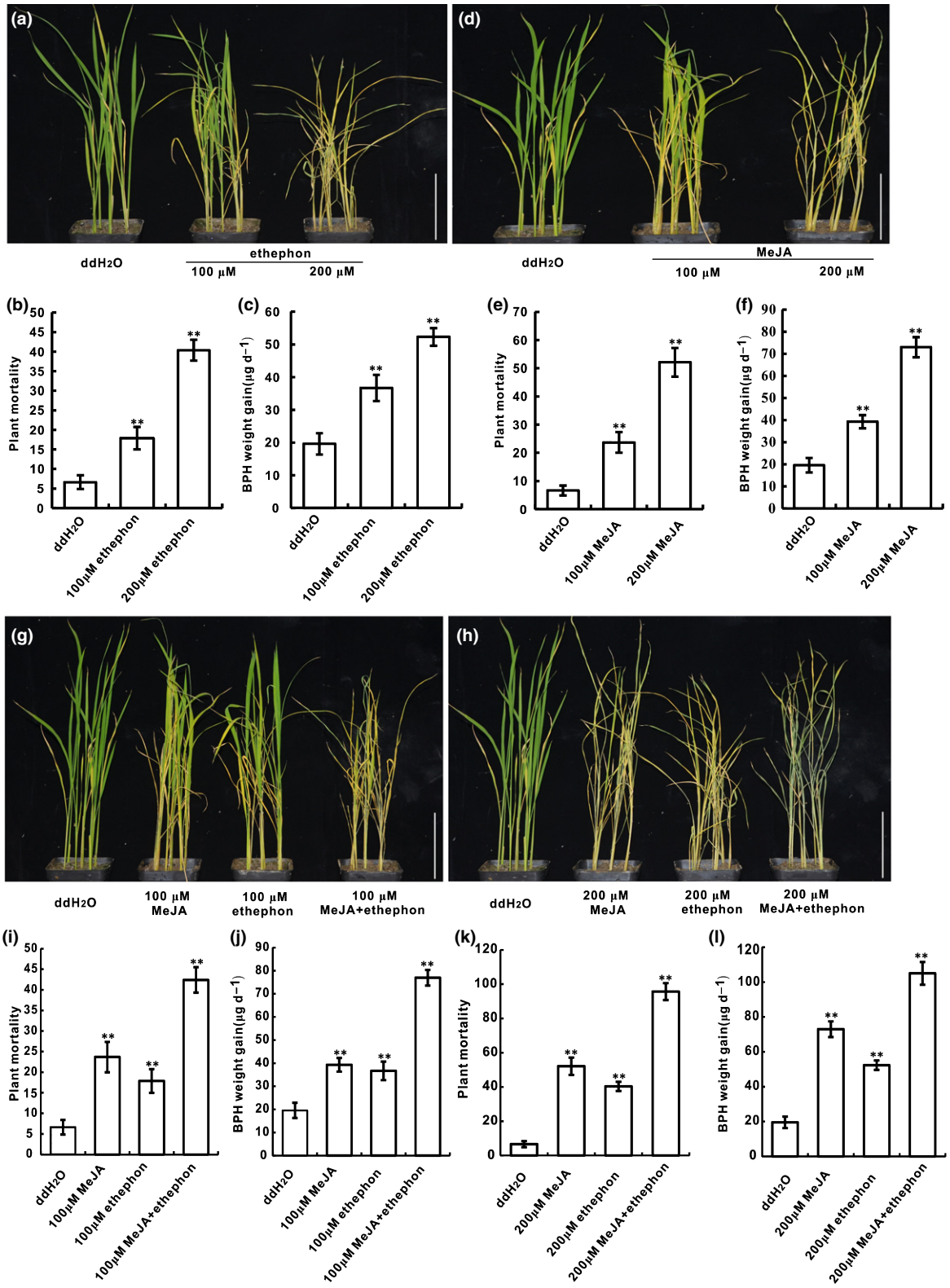

Fig. 6 Jasmonic acid and ethylene interact synergistically and negatively regulate brown planthopper (BPH) resistance in rice. (a, d) The BPH resistance phenotypes of wild-type (WT) plants treated with 2.4 ml of 0 (double-distilled water, ddH_2O), 100, or 200 μM ethephon or methyl jasmonate (MeJA); 2 h after spraying they were infested with 100 first-instar BPH nymphs for 7 d. (b, e) Statistical analyses of plant mortality rates for the different treatments in (a, d). (c, f) Statistical analyses of BPH weight gains for the different treatments in (a, d). (g, h) Comparative analyses of the BPH resistance phenotypes of WT plants treated individually with 2.4 ml of 100 or 200 μM ethephon or MeJA, or both ethephon and MeJA, and then infested with 100 first-instar BPH nymphs for 7 d. (i, k) Statistical analyses of plant mortality rates for the different treatments in (g, h). (j, l) Statistical analyses of BPH weight gains for the different treatments in (g, h). All error bars represent ± standard deviation ($n = 3$). Bars, 7 cm. Significant difference determined by Student's *t*-test: **, $P < 0.01$.

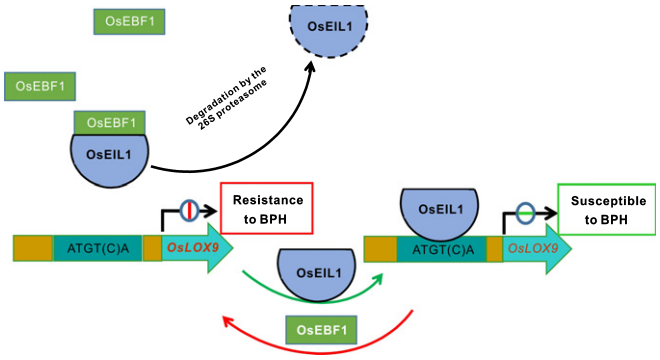

Fig. 7 Schematic representation of the synergistic and negative regulatory mechanism by which OsEBF1–OsEIL1–OsLOX9 modulates the jasmonic acid (JA) and ethylene pathways' responses to brown planthopper (BPH) attack in rice. In OsEBF1OE plants, there is an abundant level of the OsEBF1 proteins, which can interact with OsEIL1 and degrade it through the ubiquitination pathway. Consequently, there is not enough OsEIL1 to bind to the *OsLOX9* promoter and the JA synthetic pathway is blocked, which enhances plant resistance to the BPH. Conversely, if the OsEBF1 level is too low, free OsEIL1 protein will bind to the promoter region of *OsLOX9* and activate it, thereby promoting the JA biosynthetic pathway, which increases the plant's susceptibility to the BPH. The activation or repression of the *OsLOX9* gene's expression depends on the balance between the OsEIL1 and OsEBF1 proteins.

hypothesis. First, both *OsEBF1* and *OsEIL1* were induced by BPH infestation (Figs 1c, 4a). As a central factor in the ET-signaling pathway, *OsEIL1* is tightly regulated by other factors, such as EIN2 and ERF1 (Chao *et al.*, 1997; Potuschak *et al.*, 2003; Li *et al.*, 2015). Second, the overexpression lines of *OsEBF1* (Figs 2a, S3c) or knockout lines of *OsEIL1* (Fig. 4b,f) increased the resistance of rice plants to the BPHs. Finally, ethephon-treated plants were more susceptible to the BPHs (Fig. 6a–c). These data strongly suggested that ET signaling acts as a negative regulator of responses to piercing–sucking insects in rice. Moreover, this conclusion is in accordance with other studies that used different genes in the ET pathway. For example, *OsERF3* and *OsACS2* are two positive regulators of ET biosynthesis and negatively regulated resistance to the BPH (Lu *et al.*, 2011, 2014).

In this study, we also discovered that JA negatively regulates plant resistance to the BPH. Not only is there an increased susceptibility of plants to the BPH after MeJA treatments (Fig. 6d–f), but also there is a decreased expression of JA biosynthetic genes in the *oseil1* mutant (Fig. 5a). More importantly, the transcription of the *OsLOX9* gene was directly activated by OsEIL1 (Fig. 5d–f). This result is consistent with a negative function of *OsLOX9* (*OsHI-LOX* or *OsRLL*) in BPH resistance (Zhou *et al.*, 2009; Marla & Singh, 2012), but it differs from those of *AOC*, *OsHPL3*, and *OsPLDα4* (Qi *et al.*, 2011; Tong *et al.*, 2012; Guo *et al.*, 2014), which positively regulate resistance to the BPH. However, the immediate downstream product catalyzed by AOC and also the precursor of JA, 12-oxo-phytodienoic acid (OPDA), has the potential to mediate other signaling pathways independent of JA. This may occur through OPDA-specific responses (Park *et al.*, 2013; Guo *et al.*, 2014), or the inactivity of OPDA in the promotion of JAZ3–COI1 interactions (Fonseca *et al.*, 2009), even though JA and OPDA may share some initial synthesis steps (Wasternack & Hause, 2016; Wasternack & Strnad, 2016). Additionally, *OsHPL3* regulates the synthesis of green leaf volatiles (GLVs) through a branch of JA biosynthesis that is quite divergent from JA (Tong *et al.*, 2012; Ling & Weilin, 2016). The JA content in the *hpl3-1* mutant is enhanced, and this mutant is more susceptible to the BPH (Tong *et al.*, 2012). The relationship between the JA content and BPH resistance in the *hpl3-1* mutant is consistent with the negative role of JA in BPH resistance. Furthermore, both the *OsHPL3* and *OsPLDα4* genes positively mediate BPH resistance through the regulation of GLVs (Qi *et al.*, 2011; Tong *et al.*, 2012). Because OPDA might function independently of JA and because GLVs regulation by *OsHPL3* and *OsPLDα4* is a divergent branch of the JA pathway, we conclude that JA signaling function negatively regulates BPH resistance. However, further studies on the genetic factors in the JA biosynthetic and signaling pathways are still needed. Generally, plants use three resistance strategies against insects: antixenosis, to affect insects' settlement, colonization, or oviposition; antibiosis, to reduce the insect survival rate or feeding activity; and tolerance to withstand damage caused by the insects (Painter, 1951; Jing *et al.*, 2017). We discovered that OsEBF1OE lines and *oseil1* mutants showed antibiosis mechanisms in response to BPH infestation (Figs 2a, 4b).

Crosstalk between different signaling molecules allows the plant to reconcile the most appropriate signaling system and adjust the balance between growth and defense. Previous results and this study all confirmed that the JA and ET biosynthesis/signaling pathways are negative regulators of responses to the BPH. The enhanced BPH resistance by *OsHI-LOX* downregulation was attributable to greater levels of hydrogen peroxide and SA (Zhou *et al.*, 2009). The enhanced BPH resistance following *OsACS2* downregulation was attributable to two repellent volatiles: 2-heptanone and 2-heptanol (Lu *et al.*, 2014). However, the mechanisms by which ET and JA synergistically and negatively regulate rice resistance to BPH are still unclear. Using chewing insects and necrotrophic fungi, a previous study revealed the antagonism between JA and ET signaling in Arabidopsis (Song *et al.*, 2014a, b). In this study, we confirmed the synergistic and negative regulation of JA and ET signaling in responses to a piercing–sucking insect in rice, the intersection of which might come from the direct transcriptional activation of *OsLOX9* by OsEIL1 (Fig. 5). Additionally, because many other JA biosynthetic genes were downregulated in the *oseil1* mutant (Fig. 5a), there is a great possibility that OsEIL1 also directly regulates transcription of these genes. Fully elucidating the factors involved in the JA- and ET-signaling pathways and their crosstalk may help to develop strategies for breeding rice resistant to the BPH.

In summary, we concluded that the JA and ET biosynthetic or signaling pathways function synergistically and negatively regulate rice resistance to a piercing–sucking insect pest, the BPH. We proposed a model illustrating the crosstalk between the ET and JA biosynthetic pathways (Fig. 7). In OsEBF1OE plants, there is a large amount of the OsEBF1 proteins, which interacts with OsEIL1 and degrades it through the ubiquitination pathway. Consequently, not enough OsEIL1 can bind to the *OsLOX9* promoter, which leads to decreased JA biosynthesis and

increased plant resistance to the BPH. Conversely, if the amount of OsEBF1 protein is limited, the OsEIL1 cannot be degraded thoroughly enough, so that it could bind to the promoter of *OsLOX9* and activate its expression. As a result, JA biosynthesis is promoted and the plants become more susceptible to the BPH. In this model, the balance between OsEBF1 and OsEIL1 determines the activation or repression of the *OsLOX9* gene, and the JA content regulates rice resistance or susceptibility to the BPH. Crosstalk between the *OsEBF1-OsEIL1* module in the ET pathway and *OsLOX9* in the JA pathway mediates the synergistic and negative regulation of rice responses to BPH infestation.

Acknowledgements

We are very grateful to Professor Jinsong Zhang (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) for providing us the *oseil1* mutant and Professor Rongfeng Huang (Chinese Academy of Agriculture Sciences) for providing EIL1-myc seeds. We thank Professor Hongtao Liu from our institute for providing the Dual-Luciferase® system, Professor Chi-Kuang Wen for help with ET content measurement, and Dr Yining Liu for help with HPLC–MS analysis. We thank Robbie Lewis, MSc, and Lesley Benyon, PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

This work was supported by the National Basic Research Program of China (2015CB755703), the Transgenic Great Subject from the Ministry of Agriculture of China (2016ZX08009-003-001), the National Key R&D Program of China (2016YFD0100603), the National Natural Science Foundation of China (31371949; 31870232), and a grant from State Key Laboratory of Hybrid Rice (KF201805).

Author contributions

FM and XY performed the experiments; ZS and XM designed the research; FM, ZS and XM interpreted the result and wrote the paper.

ORCID

Zhenying Shi https://orcid.org/0000-0002-8441-2162
Xuexia Miao https://orcid.org/0000-0003-3244-0126

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Fold changes of selected genes in the ethylene-biosynthetic and -signaling pathways from RNA-seq data.

Fig. S2 Amino acid sequence analysis and phylogenetic tree of EBF1 proteins.

Fig. S3 Verification of the gene expression levels in OsEBF1OE and OsEBF1RNAi transgenic lines and their performance after BPH infestation.

Fig. S4 Protein alignment and the degradation of the OsEIL1 protein in different plants.

Fig. S5 qRT-PCR analysis of *OsEIL1* transcripts in OsEBF1OE, OsEBF1RNAi and WT plants.

Fig. S6 Evaluation of OsEIL1OE and *oseil1* plants.

Fig. S7 Yeast one-hybrid assay of OsEIL1 with the binding motifs from the promoter of *OsLOX8* and *OsAOS2*.

Fig. S8 Expression analysis of the JA pathway-related genes in the *oseil1*and the WT.

Fig. S9 Expression analysis of the ET-signaling marker genes in the *oseil1* and the WT plants.

Fig. S10 Expression analysis of the SA-pathway marker genes in the *oseil1* mutant and the WT.

Fig. S11 The rice seedlings phenotype 2 h after Ethephon and MeJA treatments.

Table S1 Primers used in this study.

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