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RESEARCH PAPER

Modulation of ethylene responses by *OsRTH1* overexpression reveals the biological significance of ethylene in rice seedling growth and development

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Abstract

Overexpression of *Arabidopsis Reversion-To-ethylene Sensitivity1* (*RTE1*) results in whole-plant ethylene insensitivity dependent on the ethylene receptor gene *Ethylene Response1* (*ETR1*). However, overexpression of the tomato *RTE1* homologue *Green Ripe* (*GR*) delays fruit ripening but does not confer whole-plant ethylene insensitivity. It was decided to investigate whether aspects of ethylene-induced growth and development of the monocotyledonous model plant rice could be modulated by rice *RTE1* homologues (*OsRTH* genes). Results from a cross-species complementation test in *Arabidopsis* showed that *OsRTH1* overexpression complemented the *rte1-2* loss-of-function mutation and conferred whole-plant ethylene insensitivity in an *ETR1*-dependent manner. In contrast, *OsRTH2* and *OsRTH3* overexpression did not complement *rte1-2* or confer ethylene insensitivity. In rice, *OsRTH1* overexpression substantially prevented ethylene-induced alterations in growth and development, including leaf senescence, seedling leaf elongation and development, coleoptile elongation or curvature, and adventitious root development. Results of subcellular localizations of OsRTHs, each fused with the green fluorescent protein, in onion epidermal cells suggested that the three OsRTHs were predominantly localized to the Golgi. *OsRTH1* may be an *RTE1* orthologue of rice and modulate rice ethylene responses. The possible roles of auxins and gibberellins in the ethylene-induced alterations in growth were evaluated and the biological significance of ethylene in the early stage of rice seedling growth is discussed.

Key words: *Arabidopsis*, coleoptile curvature, ethylene, OsRTH1, rice, RTE1.

Introduction

Ethylene is a gaseous plant hormone, and *Arabidopsis* has been used as a eudicotyledonous model plant for study of ethylene signal transduction. Major components of the signalling pathway have been identified (Guzman and Ecker, 1990; Chao *et al.*, 1997; Hua and Meyerowitz, 1998; Johnson and Ecker, 1998; Alonso *et al.*, 1999; Wang *et al.*, 2002, 2003; Guo and Ecker, 2003; Resnick *et al.*, 2008). Ethylene signalling components were identified and functionally demonstrated in tomato, which indicated conserved signalling machinery across dicot species (Wilkinson *et al.*, 1995; Tieman and Klee, 1999; Tieman *et al.*, 2000, 2001; Adams-Phillips *et al.*, 2004; Zhong *et al.*, 2008).

At the top of the ethylene signal transduction hierarchy is a small family of ethylene receptors. In the absence of ethylene, ethylene receptors constitutively suppress responses that are ethylene inducible. Ethylene binding prevents the suppression of ethylene receptors, and responses can proceed (Klee, 2004). *Arabidopsis* has five ethylene receptors: ethylene response1 (ETR1), ETR2, ethylene response sensor1 (ERS1), ERS2, and ethylene

insensitivity4 (EIN4) (Bleecker and Kende, 2000; Hall *et al.*, 2007). A suppressor screen of the dominant ethylene-insensitive *etr1-2* mutation revealed *Reversion-to-Ethylene Sensitivity 1* (*RTE1*) as a positive regulator of the ethylene receptor gene *ETR1*. *Arabidopsis* RTE1 is predominantly localized to the Golgi and can physically associate with ETR1 at the endoplasmic reticulum (ER) (Resnick *et al.*, 2006; Zhou *et al.*, 2007; Dong *et al.*, 2008, 2010). RTE1 overexpression can promote ETR1 receptor signalling and results in whole-plant ethylene insensitivity throughout development (Zhou *et al.*, 2007). *Green Ripe* (*GR*) is an *RTE1* homologue of tomato, and elevated *GR* expression delays fruit ripening but does not confer whole-plant ethylene insensitivity (Barry and Giovannoni, 2006). The ethylene signalling machinery may be highly conserved in higher plants but may be differentially regulated in different plant species.

Current knowledge of the role of ethylene in plants comes mainly from studies of dicotyledonous plant species. Rice is a monocot and a major crop in Asia; two major subspecies (*japonica* and *indica*), which probably diverged >0.44 million years ago, are widely cultivated (Ma and Bennetzen, 2004). The roles of ethylene in rice were primarily revealed in submergence responses of *indica* cultivars. Upon flooding, submerged deep-water rice plants produce ethylene, which induces biosynthesis of gibberellins (GAs) and degradation of abscisic acid (ABA), thus favouring internodal elongation so that submerged shoots can grow above the water surface to obtain oxygen. Accompanying internodal elongation is ethylene-induced cell death, which facilitates the emergence of adventitious roots from the node of stem sections (Metraux and Kende, 1983; Raskin and Kende, 1984; Satler and Kende, 1985; Saika *et al.*, 2007; Fukao and Bailey-Serres, 2008*a*). Two quantitative trait loci (QTLs) responsible for the internodal elongation of deep-water rice have been cloned: these are *SNORKEL* genes (*SK1* and *SK2*) encoding proteins of the ethylene response factor (ERF) family (Hattori *et al.*, 2009). Of note, *SK1* and *SK2* are ethylene inducible but their functions in internodal elongation depend on GAs. Another type of survival strategy for rice is avoiding energy consumption. *Submergence 1A* (*Sub1A*), encoding an ERF protein, is present in a few *indica* cultivars and confers tolerance to complete submergence by restricting GA responses. Although *Sub1A* expression is ethylene inducible, it can function independently of ethylene actions (Fukao *et al.*, 2006; Xu *et al.*, 2006; Fukao and Bailey-Serres, 2008*b*).

A recent study showed the rice ethylene receptor homologue *OsETR2* with a role in promoting flowering of the *japonica* cultivar *Zhonghua 11* (*ZH11*) (Wuriyanghan *et al.*, 2009). Two aspects of ethylene-induced growth alteration, seedling height and primary root elongation, were modulated to different degrees in *japonica* cultivars overexpressing *OsETR2*, *OsEIN2* (*Ethylene-Insensitive 2*), and *OsEIN3* by chemical treatments that replaced ethylene or eliminated ethylene production (Jun *et al.*, 2004; Mao *et al.*, 2006; Wuriyanghan *et al.*, 2009). Other aspects of ethylene response that alter rice seedling growth and development have yet to be studied. Of note, primary root growth and plant height can be modulated by auxins and GAs. Given that ethylene modulates *Arabidopsis* primary root growth and apical hook formation by modulating the function of DELLA protein (a GA response repressor) and auxin biosynthesis and polar transport, whether ethylene-induced alterations in rice growth are affected by the corresponding plant hormones needs to be investigated (Achard *et al.*, 2003; Stepanova *et al.*, 2005).

It was decided to investigate whether any *OsRTH* genes are functionally conserved in ethylene signalling in rice and whether *OsRTH* overexpression may address the functional significance of ethylene-dependent alterations in growth. The ethylene antagonist 1-methylcyclopropene (1-MCP) was used to evaluate ethylene responses modulated by *OsRTH1* overexpression in rice. *OsRTH1* overexpression conferred ethylene insensitivity to an extent similar to or even greater than that with 1-MCP treatment. The possible synergistic effects of ethylene with other plant hormones, auxins and GAs, on the ethylene-induced alterations in growth were evaluated. *OsRTH1* may be functionally conserved in ethylene signalling, and the biological significance of ethylene in rice seedling growth is discussed.

Materials and methods

Plant materials and gas treatments

Rice (*Oryza sativa* L. ssp. *japonica* cv. *Zhonghua 11*, designated *ZH11*) was used throughout this study. Ethylene concentrations were measured by gas chromatography (GC) using a flame ionization detector (FID). 1-MCP (Rohm & Haas China, Beijing) was released in water as per the manufacturer's instructions, and the concentration was determined by GC/FID. Unless specified, 5 μl l^{-1} 1-MCP and 100 μl l^{-1} ethylene were applied to rice. Growth conditions and ethylene treatment for Arabidopsis were as described (Xie *et al.*, 2006). For analysis of the triple-response phenotype in *Arabidopsis* seedlings, 20 μl l^{-1} ethylene was applied.

Laser scanning confocol microscopy (LSCM)

For subcellular localization study of fluorescence protein fusions, corresponding transgenes were delivered into onion epidermal cells by particle bombardment as described (Zhou *et al.*, 2007). LSCM involved the Olympus FluoView FV1000 and FV10-ASW1.7 Viewer for data acquisition at the Core Facility Center of the Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences.

Clones and transgenes

OsRTH1, *OsRTH2*, and *OsRTH3* were cloned by PCR. For green fluorescent protein (GFP) fusion, corresponding clones were released by *Bam*HI and ligated with *GFP* on *pRTL2*. The primers were OsRTH1-F (5′-CCGAATTCATGGCACCAAACAAAATT TCC TC-3′) and OsRTH1-R (5′-CCGGATCC TCAGCACACTA-GATCCTTCATG-3′); OsRTH2-F (5′-ATGGATCCATGGA GGTTGAAGCTGCTTG-3′) and OsRTH2-R (5′-ATGGATCCT-CAGCAGACCAAGCCCTTGA-3′); and OsRTH3-F (5′-ATG-GATCCATGGAAACCGACAGAAGCCA-3′); and OsRTH3-R (5′-ATGGATCCCTACAACTCTACAAGGCTCT-3′).

Real-time quantitative reverse transcription-PCR (qRT-PCR)

qRT-PCR involved use of the StepOne real-time PCR system (Applied Biosystems). Total RNA was isolated by use of TaKaRa RNAiso Plus. cDNA was synthesized from mRNA by use of the PrimeScript RT reagent Kit and TaKaRa SYBR Premix Ex Taq. *Ubiquitin* and *actin* were used as the internal calibrator for *Arabidopsis* and rice, respectively. Melting curve analysis for each primer set suggested no non-specific priming (data not shown). Each measurement was repeated three times with three independent biological samples (n=3×3). The ubiquitin primers for qRT-PCR were as described by Zhang and Wen (2010) or the following: Sub 1C-F (5′-CTGCTCCGACGACCTGAT-3′) and Sub 1C-R (5′-TTAGGCGAGTCGCATGTCAA-3′); ADH2-F (5′-CCCATC CCTGGATTCAGGT-3′) and ADH2-R (5′-CACGAGGTAGGT GCTGATTGA-3′); SC129-F (5′-TGACGGTGTACGGTCCGA-T-3′) and SC129-R (5′-TCGGCGTACTGGTCACAGAT-3′); OsActin-F(5′-GAAGATCACTGCCTTGCTCC-3′) and OsActin-R (5′-CGATAACAGCTCCTCTTGGC-3′); and OsRTH1-F (5′-ACTCATTTGTGGCAAACTGCTT-3′) and OsRTH1-R (5′-ATCCTTCATGCAGTATACAGCA-3′).

Leaf senescence test and rice seedling growth

The rice leaf senescence test was as described (Kao and Yang, 1983) and involved 100 μl l^{-1}ethylene. Chlorophyll content was measured 4 d after the treatment (Zhang and Wen, 2010). Rice seedlings were grown in an environmentally controlled phytotron (28±1 °C, 12 h/12 h day and night, 50–70% humidity, and average illumination at 482 μmol m^{-2} s^{-1}).

Results

Sequence and gene structure of evolutionarily representative plant RTHs

RTHs from other evolutionarily representative plant species could be retrieved from phytozome (http://www.phytozome.net/); their sequences are not shown in this study. The RTH sequence and gene structure of evolutionarily representative plant species, including *Physcomitrella patens*, Selaginella moellendorffii, *Arabidopsis*, and rice were compared (Banks *et al.*, 2011).

In addition to the putative, C-terminal transmembrane domains, these RTHs have two conserved regions (CR1 and CR2) and two non-conserved regions (NCR1 and NCR2). NCR1 may not have a role in RTE1 function because the NCR1-lacking NΔ49rte1 isoform is still functional in ethylene signalling (Zhou *et al.*, 2007) (Fig. 1A). The structures of *AtRTH*, *OsRTH1* (Os01g0711600), and *OsRTH3* (Os03G0799500) are similar to that of *RTE1*.

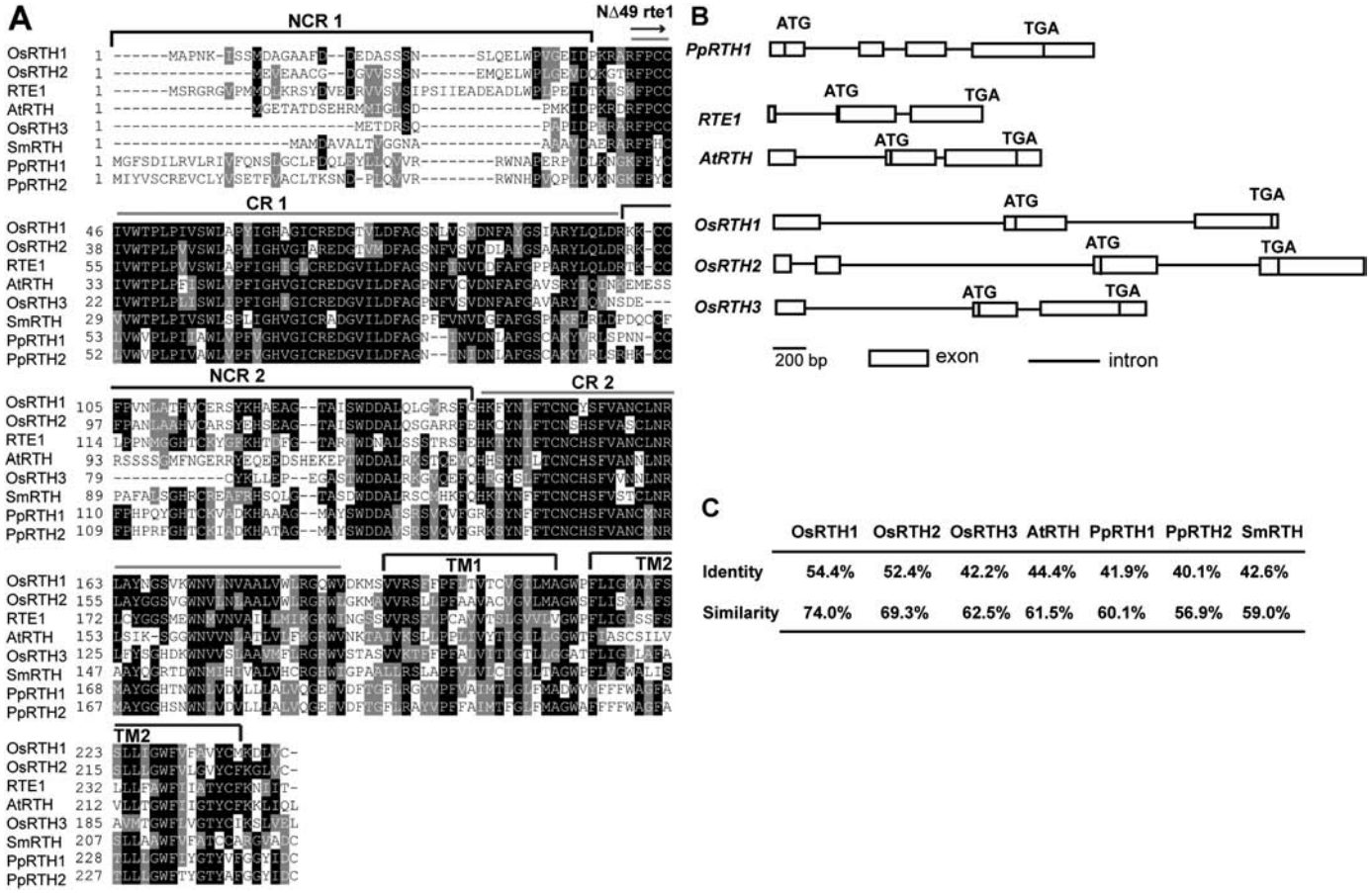

Fig. 1. RTH sequence and gene structure. (A) Sequence alignment of evolutionarily representative plant RTHs. NCR, non-conserved region; CR, conserved region; TM, transmembrane domain; NΔ49rte1, the rte1 isoform lacking the N-terminus; the arrow indicates the start of NΔ49 rte1. (B) RTH gene structure. Rectangle indicate the exons and lines indicate the introns. (C) Sequence identity and similarity of plant RTHs compared with RTE1. Os, rice (*Oryza sativa*); At, *Arabidopsis*; Sm, *Selaginella*; Pp, *Physcomitrella*.

They have three exons and two introns. The putative start codon is located at the beginning of exon 2 and the stop codon is located at the end of exon 3. *PpRTH1* and *OsRTH2* (Os05g0539800) are structurally distinct from *RTE1* and have three introns and four exons (Fig. 1B). Because of the lack of genomic sequence information, the gene structures for the moss *PpRTH2* and lycophyte *SmRTH* could not be compared. On pairwise sequence alignment of RTHs and RTE1 (http://www.ebi.ac.uk/Tools/psa/emboss_needle/), RTE1 had the highest sequence identity with and similarity to OsRTH1, and the lowest with AtRTH, OsRTH3, and RTHs of lower plants (Fig. 1C).

OsRTH1 and RTE1 had the highest sequence identity and similarity in gene structure. Although OsRTH2 and RTE1 showed high sequence homology, their gene structures differed. *OsRTH3* and *AtRTH* were similar to *RTE1* in gene structure but had relatively poor protein sequence identity and similarity. These results agree with a previous phylogenetic analysis showing OsRTH3 and AtRTH in the same clade, and RTE1, OsRTH1, and OsRTH2 in the same clade (Barry and Giovannoni, 2006). Thus, *OsRTH1* probably has a role in ethylene signalling in rice.

Cross-species complementation test of OsRTHs *in* Arabidopsis

RTE1 overexpression leads to ethylene insensitivity in *Arabidopsis*, which depends on *ETR1* (Resnick *et al.*, 2006; Zhou *et al.*, 2007). Cross-species complementation testing was used to determine whether any *OsRTH* genes are functionally conserved in ethylene signalling in *Arabidopsis*.

Ethylene treatment promotes the apical hook curvature and inhibits the hypocotyl and primary root growth of *Arabidopsis* etiolated seedlings, namely the seedling triple-response phenotype. With ethylene treatment, the seedling triple-response phenotype was prevented in wild-type (Col-0) seedlings expressing *35S:OsRTH1*. Overexpression of the transgene was confirmed by RT-PCR (Fig. 2A). *ETR1* is the only wild-type ethylene receptor gene in the receptor quadruple mutant *ers1-2 etr2 ein4 ers2*, designated (*ETR1*)4-LOF (LOF indicating loss of function), which shows the seedling triple-response phenotype with ethylene treatment (Liu *et al.*, 2010; Liu and Wen, 2012). *35S:OsRTH1* expression substantially rescued the hypocotyl growth inhibition of (*ETR1*)4LOF (Fig. 2B). As expected, *OsRTH1* overexpression did not rescue the growth inhibition of ethylene-treated *ETR1*-defective *etr1-7*, and both *etr1-7* and the transformation lines showed a seedling triple-response phenotype (Fig. 2C). Ethylene insensitivity conferred by *etr1-2* depends on *RTE1* (Resnick *et al.*, 2006). With ethylene, *etr1-2 rte1-2* seedlings showed a short seedling hypocotyl, and *35S:OsRTH1* expression rescued the growth inhibition (Fig. 2D).

Unlike *OsRTH1*, neither *OsRTH2* nor *OsRTH3* overexpression prevented the ethylene-induced growth inhibition in wild-type (Col-0) seedlings. Overexpression of these transgenes was confirmed by RT-PCR (Fig. 2E). Neither transgene could complement the *rte1-2* loss-of-function mutation, and the *etr1-2 rte1-2* transformation mutant, which expressed *35S:OsRTH2* or *35S:OsRTH3*, showed the ethylene-induced seedling triple-response phenotype (Fig. 2F).

The effect of *OsRTH1* overexpression on other aspects of the ethylene response was examined. Wild-type (Col-0) but not ethylene-insensitive *etr1-2* rosettes showed the leaf senescence phenotype with ethylene treatment (20 μl l^{-1}) (Fig. 2G, H). The expression of *35S:OsRTH1* prevented the ethylene-induced leaf senescence phenotype in the wild type, (*ETR1*)4LOF, and *etr1-2 rte1-2*, but not in *etr1-7* (Fig. 2I–L).

The degrees of leaf senescence were quantified by measuring the chlorophyll *a* content. The chlorophyll *a* content was greatly decreased with ethylene treatment in wild-type (Col-0) leaves and slightly decreased in *etr1-2* leaves. With the *35S:OsRTH1* transgene, the chlorophyll *a* content was slightly reduced in wild-type, *etr1-2 rte1-2*, and (*ETR1*)4LOF leaves, and greatly reduced in *etr1-7* leaves (Fig. 2M). *Senescence Associated Gene12* (*SAG12*) expression is specifically associated with leaf senescence progression (Noh and Amasino, 1999; Grbić, 2003). Progression of leaf senescence was quantified by measuring *SAG12* expression. With ethylene treatment for 48 h, the *SAG12* level in wild-type leaves was substantially increased, up to 190-fold. *SAG12* expression in *etr1-2* leaves was extremely low and not induced. Wild-type plants expressing *35S:OsRTH1* showed relatively minor *SAG12* induction, <4-fold. The *SAG12* level was highly induced, up to 130-fold, in *etr1-7* expressing *35S:OsRTH1*. *etr1-2 rte1-2* and (*ETR1*)4LOF expressing *35S:OsRTH1* did not show *SAG12* induction (Fig. 2N).

Thus, OsRTH1 may be functionally conserved in regulating ETR1 receptor signalling when heterogeneously expressed in *Arabidopsis*. The other two OsRTH genes examined were unable to affect ethylene responses at the transcriptional level.

GFP-fused OsRTHs are associated with the Golgi

GFP-tagged RTE1 is predominantly localized to the Golgi apparatus when ectopically expressed in onion epidermal cells and in *Arabidopsis* (Zhou *et al.*, 2007). Therefore, ectopically expressed RTE1 and its orthologues can correctly localize to the Golgi apparatus in cells of dicots (*Arabidopsis*) and monocots (onion). The subcellular localization of OsRTHs in onion epidermal cells was evaluated.

GFP was individually fused to OsRTH1, OsRTH2, aOsRTH3, and AtRTH. The resulting clones were co-expressed with a fluorescence protein-fused organelle marker in onion epidermal cells. GFP–OsRTHs co-localized with the Golgi-mCherry marker G-rk (Nelson *et al.*, 2007) (Fig. 3A–C). *AtRTH* is the only *RTE1* homologue in *Arabidopsis* and is believed not to be functional in ethylene signalling (Rivarola *et al.*, 2009). The subcellular localization of AtRTH was evaluated to address whether the RTE1 homologue may localize in compartments distinct from the Golgi apparatus. GFP–AtRTH co-localized with the ER-mCherry marker ER-rb (Nelson

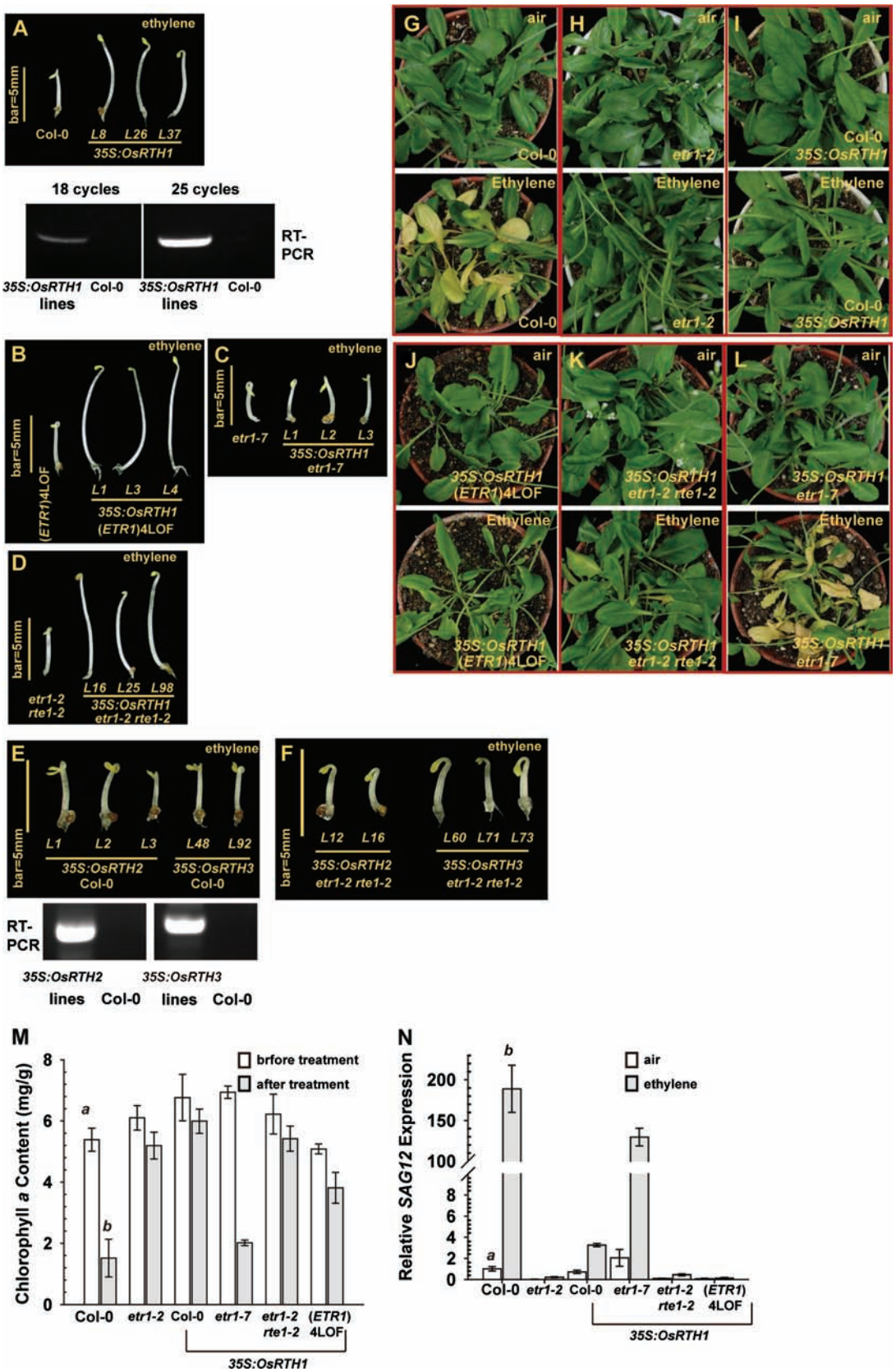

Fig. 2. Functional analyses of plant RTHs in *Arabidopsis*. The seedling triple-response phenotype of ethylene-grown wild type (Col-0) and *35S:OsRTH1* transformation lines (A), (*ETR1*)4LOF and a mutant expressing *35S:OsRTH1* (B), *etr1-7* and a mutant expressing *35S:OsRTH1* (C), and *etr1-2 rte1-2* and a mutant expressing *35S:OsRTH1* (D). (E) The seedling triple-response phenotype of the wild type (Col-0) expressing *35S:OsRTH2* and *35S:OsRTH3*. (F) Seedling triple-response phenotype of *etr1-2 rte1-2* expressing *35S:OsRTH2* and *OsRTH3*. Leaf senescence phenotype of the wild type (Col-0) (G) and ethylene-insensitive *etr1-2* (H); phenotype of wild type (Col-0),

et al., 2007) and in the nucleus (Fig. 3D). The subcellular localization of GFP–OsRTHs in rice cells was examined, but the fluorescence of GFP–OsRTHs was found to be extremely poor in the resulting transformation rice lines and it was not possile to determine their localizations (data not shown).

The data suggest that AtRTH1 localizes to the ER and within the nucleus. The possibility that AtRTH may localize to other organelles if expressed in *Arabidopsis* cells cannot be excluded. Nevertheless, this possibility does not affect AtRTH being less likely to be associated with the

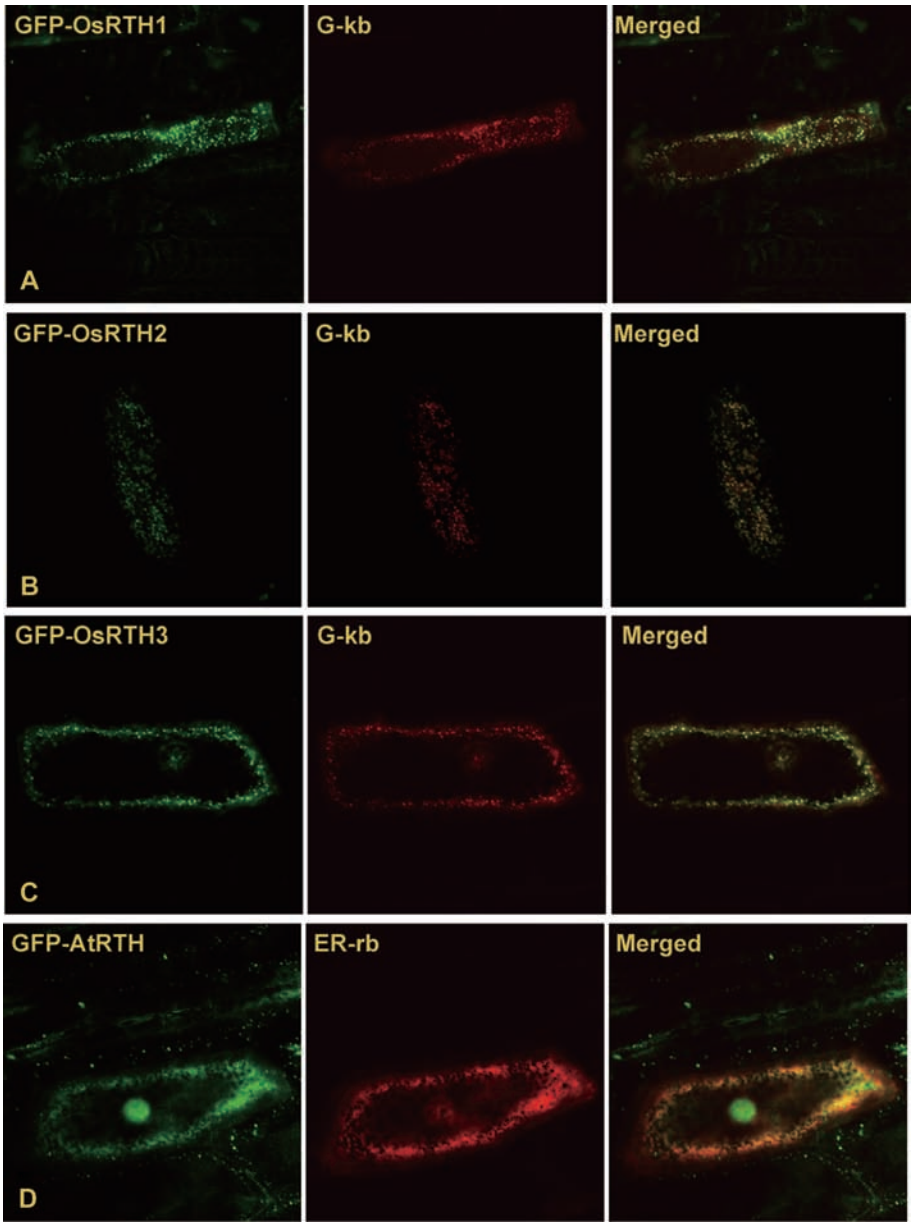

Fig. 3. Subcellular localizations of GFP–RTHs in onion epidermal cells. Subcellular localizations of GFP–OsRTH1 (A), GFP–RTH2 (B), GFP–RTH3 (C), and GFP–AtRTH (E) determined by laser scanning confocol miscroscopy in onion epidermal cells co-expressing G-kb, the Golgi marker, and ER-rb, the ER marker.

(*ETR1*)4LOF (J), *etr1-2 rte1-2* (K), and *etr1-7* (L) expressing *35S:OsRTH1*. Air and ethylene indicate the phenotype of the same plants before and after the treatment, respectively. Chlorophyll *a* measurement (M) and *SAG12* expression (N) of the wild type (Col-0), *etr1-2*, and *35S:OsRTH1* transformants in the corresponding mutation background as indicated. Error bars indicate the standard error (SE) for the means of five measurements. RT-PCR, analysis of the mRNA level of corresponding transgenes at the translational level. *a* (air) and *b* (ethylene) indicate a statistically significant difference (α=0.01) between the wild type and mutant or transformation lines.

Golgi apparatus. In contrast, OsRTHs may all localize predominantly in the Golgi apparatus.

OsRTH1 *overexpression prevents ethylene-induced rice leaf senescence*

Complementation testing revealed that *OsRTH1* is an *RTE1* orthologue. Elevated *RTE1* expression results in a hypermorph that causes ethylene insensitivity. Next experiments were carried out to examine whether elevated *OsRTH1* expression may confer ethylene insensitivity in rice. *OsRTH1* overexpression and treatment with the ethylene antagonist 1-MCP were compared in terms of degree of ethylene response.

Ethylene can promote rice leaf senescence (Kao and Yang, 1983). Here it was found that detached leaf fragments of wild-type rice (*ZH11*) showed the leaf senescence phenotype, and the chlorophyll *a* content decreased to 13% after 4 d (96 h) in air. 1-MCP treatment attenuated the leaf senescence phenotype, and the chlorophyll *a* content was ~28%. *ZH11* expressing *35S:OsRTH1* did not show the leaf senescence phenotype, and the chlorophyll *a* content was relatively high (39–59%), regardless of 1-MCP treatment (Fig. 4A, B). With 96 h ethylene treatment, leaf fragments of *ZH11* were completely yellow, and the chlorophyll *a* content was only 3.6%. *35S:OsRTH1* transformation lines did not show the leaf senescence phenotype, and the chlorophyll *a* content was still high after ethylene treatment, with the chlorophyll *a* content lower in the transformation line *L2* than in the other lines (19.4% versus ~40%) (Fig. 4A, B).

The ethylene evolution in *ZH11* and *35S:OsRTH1* lines was examined to evaluate whether the degree of leaf senescence in transformation lines was affected by ethylene production. The ethylene evolution of *ZH11*, *L7*, *L10*, and *L17* was similar to but slightly lower than that of *L2* and *L6* (Fig. 4C). To confirm that prevention of the ethylene-induced senescence phenotype resulted from *OsRTH1* overexpression, the mRNA level of *OsRTH1* in each transformation rice line was compared with that in *ZH11*. *OsRTH1* was overexpressed in each transformation line, and *OsRTH1* expression was higher in lines *L7* and *L10* than in *L2*, *L6*, and *L17* (Fig. 4D). Thus, the *OsRTH1* level in transformation lines was sufficient to suppress the ethylene-induced leaf senescence to a great extent.

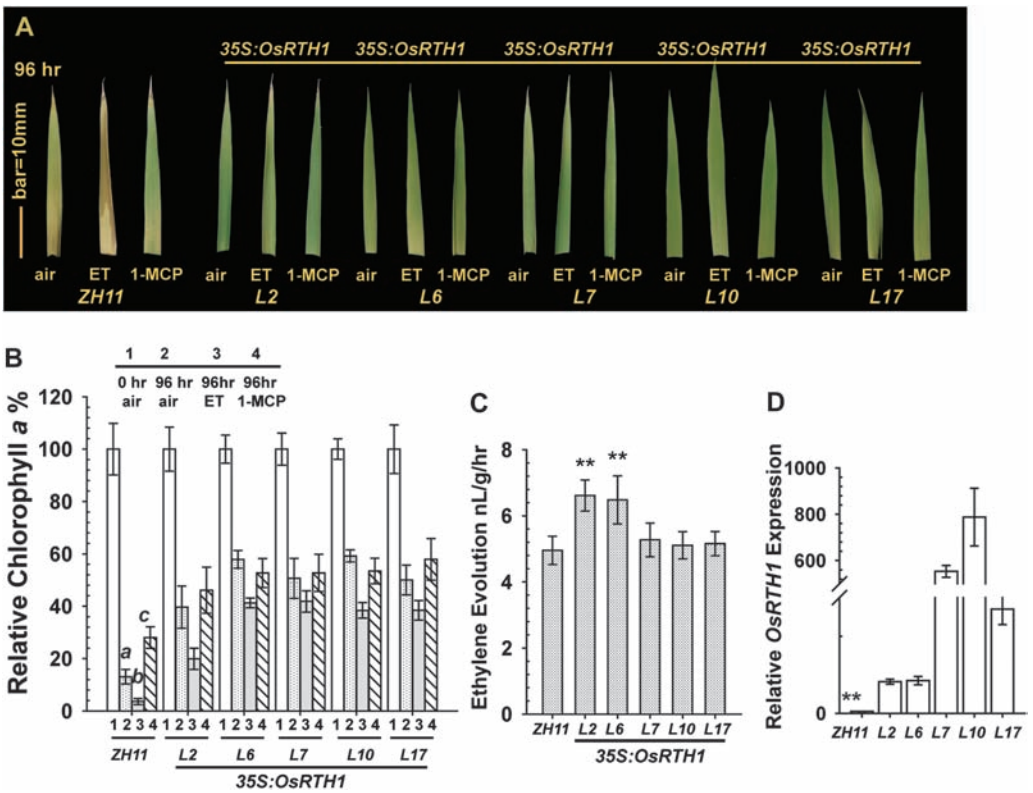

Fig. 4. Rice leaf senescence test. (A) Senescence phenotypes of rice leaf in air, ethylene (ET), and 1-MCP. (B) Chlorophyll *a* content (%) relative to that before treatment (0 h). Data are the mean ±SD of five biological repeats. (C) Ethylene evolution of *ZH11* and transformation rice lines. (D) Relative *OsRTH1* expression in *ZH11* and transformation rice lines (*L*). Data are the mean ±SD of each measurement. Ethylene, 100 μl l^{-1}; 1-MCP, 5 μl l^{-1}. *a*, *b*, and *c*, statistically significant difference (Fisher's LSD, α=0.01) between the wild type (*ZH11*) and transformation rice lines for air (*a*), ethylene (ET, *b*), and 1-MCP (*c*) treatments. **Significant difference (Fisher's LSD, α=0.01) among *ZH11* and transformation rice lines.

The inhibition of rice leaf senescence progression was stronger with *OsRTH1* overexpression than with 1-MCP treatment. The ethylene evolution of air-grown *35S:OsRTH1* lines was not reduced and the delay in leaf senescence was not due to alterations in endogenous ethylene production.

OsRTH1 *overexpression attenuates the expression of ethylene-inducible genes*

Because elevated *OsRTH1* overexpression efficiently prevented ethylene-induced leaf senescence, whether *OsRTH1* overexpression could repress the expression of various ethylene-inducible genes was next examined.

At the submergence locus, *Sub1C* is ethylene inducible in *japonica* and *indica* cultivars (Fukao *et al.*, 2006). A 4 h ethylene treatment was sufficient to elevate *Sub1C* expression to nearly 8-fold in the wild-type rice (*ZH11*) (Fig. 5A, B). In contrast, *Sub1C* expression was highly attenuated in *35S:OsRTH1* lines with ethylene treatment (Fig. 5B). *ADH2* encodes alcohol dehydrogenase2, and its expression can be induced by ethylene (Fukao *et al.*, 2006). With ethylene treatment, *ADH2* expression was ~3-fold higher in *ZH11* than in *35S:OsRTH1* lines (Fig. 5C). *SC129* (AK104680), possibly encoding a glutathione *S*-transferase, is ethylene inducible (Jun *et al.*, 2004). It was found *SC129* expression in *ZH11* was induced 3.5-fold by ethylene, with its induction weak in *35S:OsRTH1* lines (Fig. 5D).

OsRTH1 *overexpression prevents ethylene-induced alterations in coleoptile growth and development*

Coleoptiles protect rice seedlings and are closed in darkness; ethylene promotes the coleoptile elongation of etiolated rice seedlings (Ku *et al.*, 1970; Satler and Kende, 1985). Experiments were carried out to examine whether *OsRTH1* overexpression can prevent ethylene-induced coleoptile elongation.

Etiolated rice seedlings with a coleoptile length of 2 mm were subjected to air, 1-MCP, or ethylene treatment in the dark for 5 d, and coleoptile length was measured. In air, the coleoptile length was 5–6 mm longer in *ZH11* than in the transformation lines. As expected, 1-MCP had a similar effect to the *35S:OsRTH1* transgene in preventing coleoptile elongation, and coleoptile length was similar in 1-MCP-treated *ZH11* and the transformation lines (Fig. 6A). Ethylene treatment substantially promoted coleoptile elongation, and the length was much longer, by 12–15 mm, in *ZH11* than in the transformation lines (Fig. 6B). Therefore, *OsRTH1* overexpression effectively prevented ethylene-induced coleoptile elongation in dark-grown rice seedlings.

In addition, experiments were conducted to determine whether ethylene had any effect on the growth and development of light-grown seedling coleoptiles. Etiolated rice seedlings with a coleoptile length of 2–3 mm were transferred to growth under light for 4 d with various ethylene concentrations. Ethylene-treated *ZH11* seedlings showed exaggerated coleoptile curvature, with minimal curvature of air-grown *ZH11*. An amount of 1 μl l^{-1} ethylene was sufficient to produce the curvature phenotype. In contrast, seedlings of the *35S:OsRTH1* transformation line *L6* showed a relatively straight coleoptile, regardless of ethylene treatment (Fig. 6C). Consistent with the phenotype of the *35S:OsRTH1* transformation line *L6*, the other *OsRTH1* overexpression lines produced a short and relatively straight coleoptile when grown under light, regardless of ethylene treatment (Supplementary Fig. S1 available at *JXB* online). Therefore, ethylene promoted the coleoptile curvature of light-grown seedlings.

The abaxial side of the coleoptile of ethylene-grown *ZH11* was greener than that of air-grown *ZH11*. Ethylene treatment had little effect on the greening of the abaxial side of transformation coleoptiles (Fig. 6D). Therefore, the chlorophyll content was measured in *ZH11* and *35S:OsRTH1* transformation line *L6* coleoptiles. Ethylene treatment increased the chlorophyll content in *ZH11* but not in *35S:OsRTH1* line *L6* coleoptiles. The chlorophyll *a*/*b* content in *ZH11* coleoptiles was identical with 10 μl l^{-1} and 100 μl l^{-1} ethylene treatment (Student's *t*-test; $P > 0.01$). The chlorophyll content was identical in coleoptiles of air- and ethylene-grown *35S:OsRTH1 L6* and air-grown *ZH11* (F test; $P=0.3515$ and $F=1.1947 < Fcrit=4.459$) (Fig. 6E). Thus, ethylene could produce coleoptile curvature and greening in light-grown seedlings.

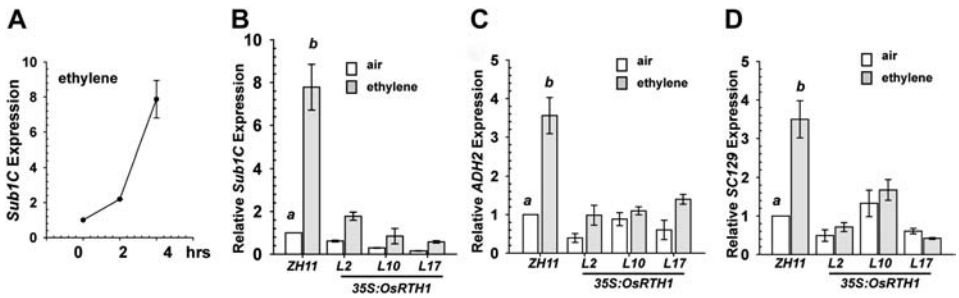

Fig. 5. Gene expression analyses. (A) Kinetics of *Sub1C* induction by ethylene treatment. Expression of *Sub1C* (B), *ADH2* (C), and *SC129* (D) of *ZH11* and *35S:OsRTH1* transformation lines in air (white bars) and ethylene (grey bars). Data are the mean ±SE of three independent measurements with three repeats ($n=3\times3$). *a* (air) and *b* (ethylene): significant difference (Fisher's LSD, $\alpha=0.01$) between *ZH11* and transformation rice lines.

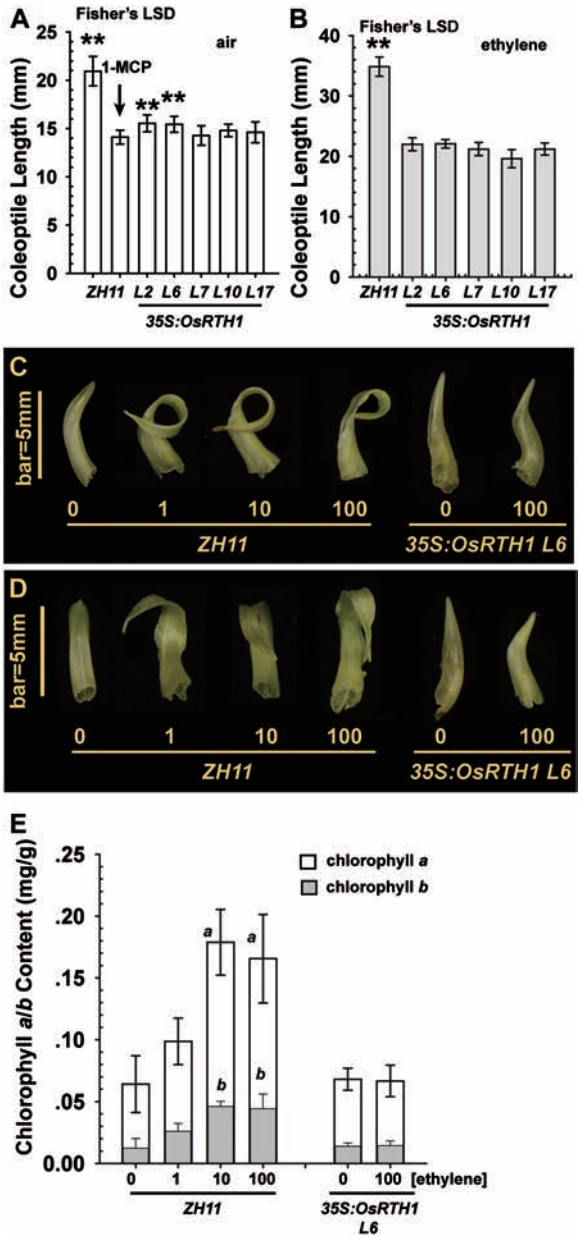

Fig. 6. Seedling coleoptile growth. Measurement of the coleoptile length of etiolated rice seedlings grown in air (A) and ethylene (B). The coleoptile phenotype of light-grown *ZH11* and *35S:OsRTH1* (line *L6*) seedlings in air and ethylene, viewed from the side (C) and back (D). (E) Chlorophyll content of coleoptiles of light-grown *ZH11* and *35S:OsRTH1* line *L6*. Numbers in (C), (D), and on the *x*-axis (E) indicate the ethylene concentration (μl l^{-1}). Data are the mean ±SD for (A) and (B), $n \geqslant 15$, and mean ± SE for (E) of 3–5 measurements. **Significant difference (Fisher's LSD, α=0.01) between 1-MCP-treated *ZH11* and air-grown *ZH11* and transformation rice lines (A), or ethylene-treated *ZH11* and transformation rice lines (B). *a* (chlorophyll *a*) and *b* (chlorophyll *b*) indicate identical chlorophyll contents between *ZH11* treated with 10 μl l^{-1} and 100 μl l^{-1} ethylene (Student's *t*-test, $P > 0.05$).

The degree of greening was associated with the chlorophyll content. Elevated *OsRTH1* expression antagonized ethylene effects on coleoptile growth and development.

OsRTH1 *overexpression inhibits ethylene-induced leaf elongation and development*

1-Aminocyclopropane-1-carboxylic acid (ACC) is the immediate ethylene biosynthesis precursor. ACC-treated rice seedlings are taller than untreated seedlings (Jun *et al.*, 2004; Wuriyanghan *et al.*, 2009). Here the effect of ethylene on rice seedling growth was evaluated. Germinating rice seedlings with a coleoptile of 2–3 mm were cultured hydroponically for 7 d in an environmentally controlled phytotron. *ZH11* was taller than *35S:OsRTH1* transformation lines regardless of ethylene treatment (Fig. 7A). Plant height can be determined by the internode and leaf length. Experimnents were conducted to determine whether the internodes elongated with ethylene treatment. By peeling off outer leaves, we showed that the last leaf was attached to the grain and found no sign of internodal elongation regardless of ethylene or 1-MCP treatment (Fig. 7B).

The next aim was to examine whether leaf elongation resulted in taller seedlings. The rice leaf is composed of a leaf sheath and leaf blade, except that the first leaf does not have a blade. The first and second leaves of *ZH11* and transformation lines were similar in length; however, the sheath of the third leaf was longer, by ~1 cm (or 10%), in *ZH11* than in the *35S:OsRTH1* transformation lines (Fig. 7C). 1-MCP treatment substantially inhibited growth of the third leaf of *ZH11*, and the length of each leaf was similar to that of air-grown *35S:OsRTH1* lines. As expected, 1-MCP did not affect the leaf length of *35S:OsRTH1* lines (Fig. 7C, D). Air-grown *ZH11* had three leaves at day 7. Of note, with ethylene treatment, *ZH11* seedlings had four leaves, whereas *35S:OsRTH1* seedlings had three. The third leaf sheath of ethylene-treated *ZH11* was ~2 cm (or 25%) longer than that of *35S:OsRTH1* lines (Fig. 7E, 7F). Therefore, ethylene promoted the elongation of the third sheath and the development of the fourth leaf at the stage examined. The third sheath elongation was responsible for the tall ethylene-treated *ZH11*.

1-MCP treatment and *OsRTH1* overexpression had the same inhibitory effect on leaf growth and elongation promoted by ethylene. Ethylene may promote the leaf development so that ethylene-grown *ZH11* seedlings had one more leaf at the stage examined.

Ethylene and GAs have different effects on seedling leaf elongation

Ethylene can induce endogenous GA biosynthesis in *indica* deep-water cultivars (Metraux and Kende, 1983; Raskin and Kende, 1984; Saika *et al.*, 2007; Jackson, 2008). Whether ethylene-induced leaf elongation of *ZH11*, a *japonica* cultivar, could be due to elevated GAs was next examined.

When supplemented in hydroponic culture, GA_3 had little effect on leaf growth at concentrations <0.1 μmol l^{-1} (Fig. 8A). GA_3 of 1.0 μmol^{-1} and 10 μmol l^{-1} substantially promoted the elongation of the second sheath and third blade (Fig. 8A). With GA_3 at 1.0 μmol l^{-1}, *35S:OsRTH1* transformation lines and 1-MCP-treated *ZH11* showed

a leaf growth pattern similar to that of GA_3-treated *ZH11*, except that the third sheath was shorter, by ~2–3 cm, for *OsRTH1* overexpression lines and 1-MCP-treated *ZH11* than *ZH11* in the presence of GA_3 treatment (Fig. 8B, C).

Thus, high GA_3 treatment differentially promoted rice leaf elongation. The effects of GA_3 and ethylene on leaf growth patterns were distinct. GAs increased the effects of 1-MCP treatment or *OsRTH1* overexpression on inhibition

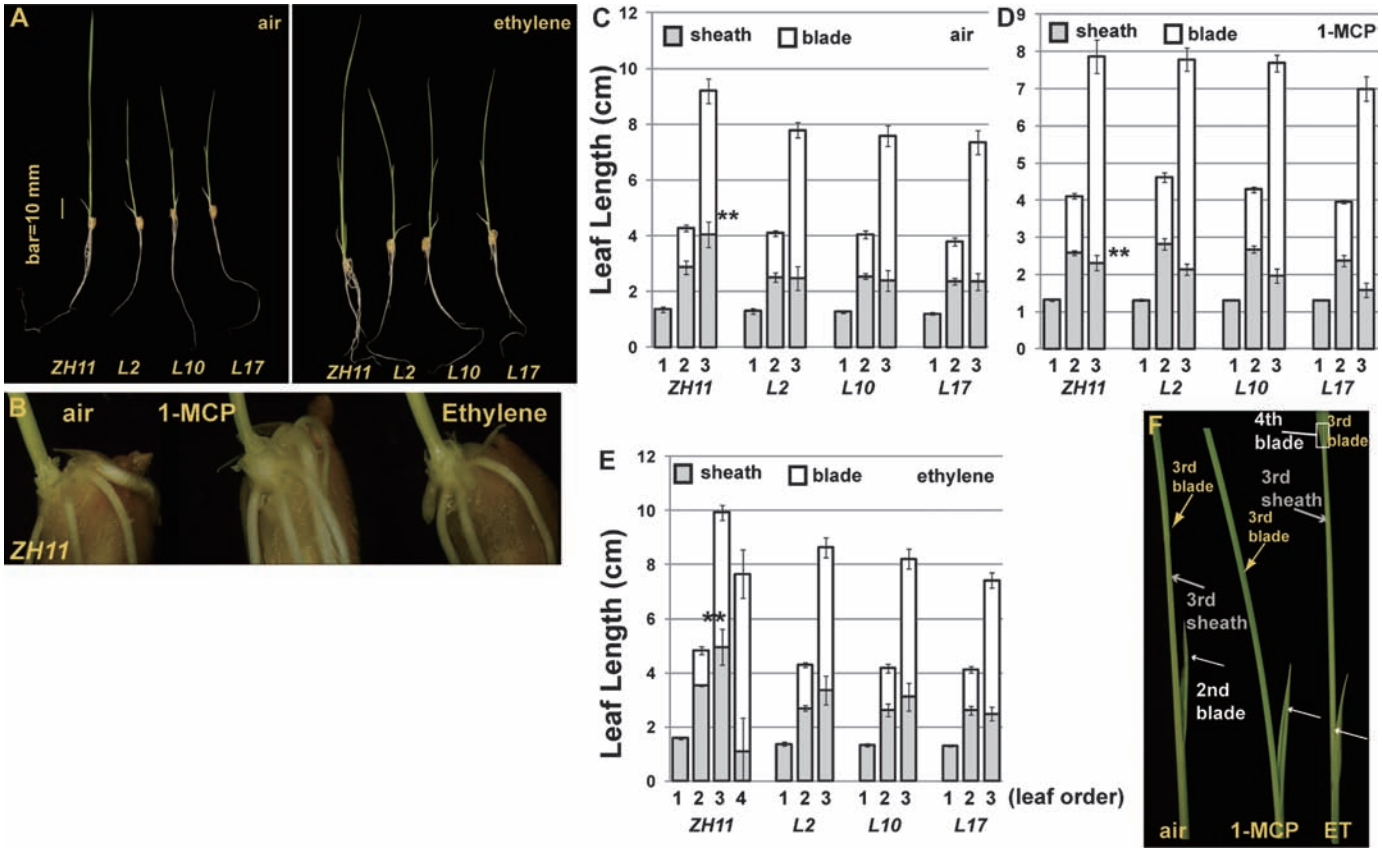

Fig. 7. Ethylene promotes the leaf growth of *ZH11*. (A) Seedling phenotype of *ZH11* and *35S:OsRTH1* transformation lines in air and ethylene. (B) The internode is not visible in rice seedlings with the outer leaves removed. Length of individual leaves of rice seedlings grown in air (C), 1-MCP (D), and ethylene (E); the *x*-axis indicates the leaf order. (F) Shoot phenotype of *ZH11* rice seedlings grown in air, 1-MCP, and ethylene (ET). The second blade is indicated by a white arrow; the third sheath by a grey arrow; the third blade by a yellow arrow; and the fourth leaf by a white box. **Significant difference (Fisher's LSD, α=0.01) comparing the third-leaf sheath length between *ZH11* and transformation rice lines.

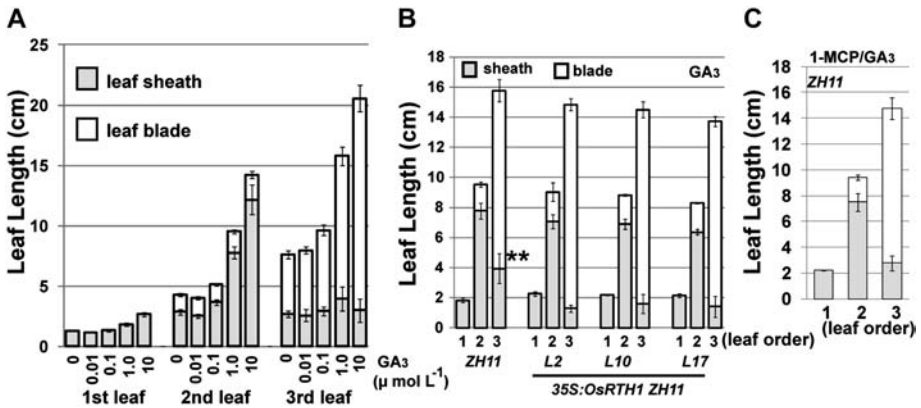

Fig. 8. Effect of gibberellins on rice seedling growth. (A) Leaf length of *ZH11* seedlings grown with GA_3. (B) Leaf length of *ZH11* and *35S:OsRTH1* transformation lines with 1 μM GA_3. The *x*-axis indicates leaf order. (C) Leaf length of *ZH11* seedlings with GA_3 and 1-MCP. The *x*-axis indicates leaf order. *ZH11*, wild type; *L2*, *L10*, and *L17* are three independent transformation lines. At least 15 seedlings were scored for each measurement ($n \geqslant 15$). Data are the mean ±SD for each measurement. Ethylene: 100 μl l^{-1}. **Significant difference (Fisher's LSD, α=0.01) comparing the third-leaf sheath length between *ZH11* and transformation lines in (B).

of elongation of the third sheath. Thus, ethylene-induced leaf growth may not be primarily due to the effects of GAs.

OsRTH1 *overexpression prevents ethylene-induced adventitious root growth*

The rice root system mainly consists of a primary root and numerous adventitious roots. The adventitious root, also called the crown root, plays a major role in nutrition and water uptake (Inukai *et al.*, 2005; Osmont *et al.*, 2007; Rebouillat *et al.*, 2009). The internodal adventitious root initiation, induced by ethylene, is coupled with the internodal elongation of *indica* deep-water cultivars (Mergemann and Sauter, 2000). The effects of ethylene and *OsRTH1* overexpression on the adventitious root growth of *ZH11* (a *japonica* cultivar) were thus examined.

Rice seedlings with a coleoptile of 2–3 mm were hydroponically grown for 7 d. Without exogenous ethylene, *ZH11* showed approximately two more adventitious roots than did the transformation lines. Ethylene treatment increased the number of adventitious roots of *ZH11* but not *35S:OsRTH1* lines by about two. 1-MCP treatment reduced the number of adventitious roots of *ZH11* by two and had little effect on *35S:OsRTH1* lines. 1-MCP-treated *ZH11* and *35S:OsRTH1* lines were similar in adventitious root number (Fig. 9A).

Auxins are important to rice adventitious root growth (Yamamoto *et al.*, 2007). The roles of auxins in ethylene-induced adventitious root growth were evaluated. Auxin [1-naphthaleneacetic acid (NAA)]-treated *ZH11* had about one more adventitious root than untreated *ZH11*. The auxin transporter inhibitor 3-nitropropionic acid (NPA) severely inhibited adventitious root growth, and treated rice seedlings had only one adventitious root. *ZH11* seedlings with ethylene treatment alone and those with ethylene and auxin treatment had the same number of adventitious roots. Auxin (NAA) alone or ethylene and auxin had minor effects on adventitious root growth of *35S:OSRTH1* lines. With NAA treatment, there were 3–4 fewer adventitious roots in *35S:OsRTH1* lines than in *ZH11*. The prevention of endogenous ethylene perception of *ZH11* by 1-MCP reduced the adventitious root number, regardless of auxin treatment. The adventitious root number of 1-MCP/NAA-treated *ZH11* was similar to that of NAA-treated *35S:OsRTH1* lines (Fig. 9A).

Ethylene and 1-MCP are gases, and their efficacy on root growth may be altered to an unknown extent because of the physical barrier of the aqueous solution. The effects of ethylene on the adventitious root growth of rice seedlings grown on wet tissues to eliminate the aqueous barrier were evaluated. Air-grown *ZH11* seedlings had 2–3 more adventitious roots than did the *35S:OsRTH1* transformation lines. Ethylene treatment increased the number of adventitious roots by two for *ZH11* but not for *35S:OsRTH1* lines. 1-MCP treatment reduced the number of adventitious roots by 2.5 for *ZH11* but not for *35S:OsRTH1* lines. *ZH11* and the *35S:OsRTH1* lines were similar in adventitious root number with 1-MCP treatment. Auxin (NAA) treatment

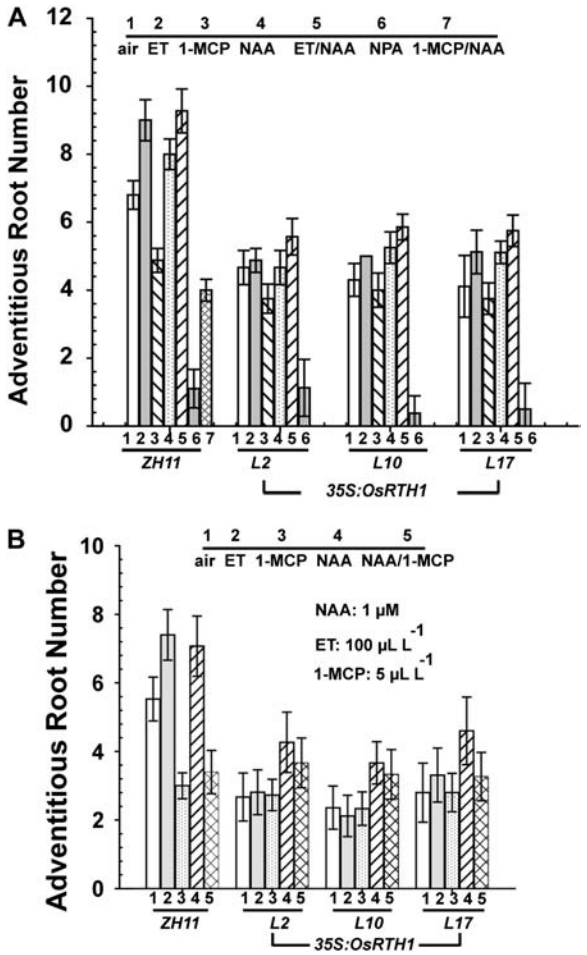

Fig. 9. Adventitious root growth of *ZH11* and *35S:OsRTH1* lines. Adventitious root number of hydroponically grown (A) and wet tissue-grown (B) *ZH11* and *35S:OsRTH1* lines. *ZH11*, wild type; *L2*, *L10*, and *L17* are three independent transformation lines. At least 15 seedlings were scored for each measurement ($n \geqslant 15$). NAA, 0.1 μM; NPA, 1 μM; ethylene, 100 μl l^{-1}; 1-MCP, 5 μl l^{-1}.

increased the number of adventitious roots by 1.5 for *ZH11* and by 1–2 for the *35S:OsRTH1* lines. Preventing endogenous ethylene perception by 1-MCP reduced the adventitious root number of *ZH11* and NAA-treated *ZH11* by 2 and 2.5, respectively. The adventitious root number of *ZH11* was identical with 1-MCP and 1-MCP/NAA treatments (Student's *t*- test and P=0.104). *ZH11* and the *35S:OsRTH1* lines were identical in adventitious root number in the presence of NAA and 1-MCP (F test; F=0.949 < *Fcrit* 2.769, and P=0.423) (Fig. 9B).

Therefore, auxins were essential for basal-level adventitious root growth, and the effects of an excessive amount of exogenous auxins on root growth were marginal. In *indica* deep-water rice, ethylene-induced adventitious root growth is coupled with internodal elongation. Here it was shown that adventitious root growth induced by ethylene was independent of internodal elongation in the *japonica* cultivar *ZH11*. 1-MCP treatment and *35S:OsRTH1* had the same effects on inhibition of ethylene-induced adventitious root growth. Auxin-induced adventitious root growth

was substantially attenuated by 1-MCP treatment or *35S:OsRTH1* expression, which implies the important roles of ethylene in auxin-induced adventitious root growth.

Discussion

In this study, *OsRTH1* overexpression suppressed various aspects of ethylene response in *Arabidopsis* and rice, and complemented *Arabidopsis rte1-2* mutation. Thus, the ethylene signalling machinery is highly conserved across higher plant species and favours *OsRTH1* as an orthologue of *Arabidopsis RTE1*, although O*sRTH1* functions were not inferred from phenotypes of loss-of-function mutants. Of note, *rte1* loss-of-function mutants do not exhibit a discernible phenotype (Zhou *et al.*, 2007; Resnick *et al.*, 2008); rice mutants defective in *OsRTH1* may not display a discernible phenotype, and the mutants may not be the best for inferring *OsRTH1* functions.

Three OsRTH genes are identified in the rice genome, and *OsRTH2* and *OsRTH3* were unable to confer ethylene insensitivity when overexpressed. The rice genome has undergone gene loss after whole-genome duplication to form a stabilized diploid (Ma and Bennetzen, 2004, 2006; Salse *et al.*, 2008). Truly redundant genes are evolutionarily unstable, and subfunctionalization or neofunctionalization stabilizes duplicated loci in the genome (Nowak *et al.*, 1997). The localization of GFP–AtRTH to the ER and nucleus may indicate functional divergence of AtRTH, which does not have a role in ethylene signalling (Rivarola *et al.*, 2009). In contrast, the present study suggested that the three OsRTHs, like RTE1, predominantly associated with the Golgi apparatus. The absence of non-Golgi OsRTHs may indicate loss of the non-Golgi OsRTHs during the rice genome diploidization, with Golgi-associated OsRTHs retained. Among these OsRTHs, only OsRTH1 acquired functions in ethylene signalling. Although it was not possible to demonstrate whether OsRTH2 and OsRTH3 are expressed at the translational level, analyses of gene structure and sequence identity and a previous phylogenetic analysis do not favour the two OsRTHs being functionally conserved in ethylene signaling (Barry and Giovannoni, 2006). Although within the same clade as RTE1 and OsRTH1, OsRTH2 may have not acquired functions in ethylene signalling. Alternatively, OsRTH2 could have lost its functions in ethylene signalling after mutation accumulation during evolution. AtRTH and OsRTH3 are in the same clade (Barry and Giovannoni, 2006), in agreement with results showing that *OsRTH3* overexpression failed to confer ethylene insensitivity and complemented *rte1-2* mutation.

Previous studies of ethylene effects on rice growth and development involved ACC or ethephon, chemicals that replace ethylene gas; two aspects of ethylene-induced growth alterations were differentially alleviated in *japonica* rice expressing *OsETR2*, *OsEIN2*, or *OsEIN3* (Jun *et al.*, 2004; Mao *et al.*, 2006; Wuriyanghan *et al.*, 2009). The growth alterations by ACC or ethephon included an increase in seedling height and a minor elongation in the primary root. Other aspects of growth and development that are modulated by ethylene must be investigated. Auxins and GAs may also affect root growth in *Arabidopsis* (Achard *et al.*, 2003; Stepanova *et al.*, 2005); whether the ethylene-induced alterations in rice seedling growth result from synergistic actions of these hormones is unknown.

Treatment with ethylene, rather than a chemical replacement, was used to evaluate the effects on aspects of rice growth and development, and 1-MCP treatment was used to evaluate the effects of *OsRTH1* overexpression on the prevention of ethylene-induced alterations in growth. Ethylene promoted various aspects of rice growth and development, including coleoptile elongation of etiolated rice seedlings, coleoptile curvature and greening of light-grown rice seedlings, and the growth and development of rice seedling leaves and adventitious roots. Although GAs may promote leaf elongation, the leaf growth patterns of ethylene- and GA-treated rice seedlings were distinct, which indicates differential regulation of rice leaf growth by the two hormones. Auxins play major roles in adventitious root development; however, the effects of an excess amount of auxins were marginal. In contrast, the increase in adventitious root number caused by ethylene indicated a collaboration of auxins and ethylene in adventitious root development. Unlike in deep-water rice, in the *japonica* cultivar *ZH11*, adventitious growth promoted by ethylene was not associated with internodal elongation.

The ethylene-dependent alterations in growth of rice seedlings may reveal the biological significance of ethylene in rice seedling growth and development. Etiolated rice seedlings show coleoptile elongation induced by ethylene (Ku *et al.*, 1970; Satler and Kende, 1985). Conceivably, when seedlings are germinated in the dark environment of soil, leaves are enclosed within a coleoptile. With ethylene production, the coleoptiles elongate and penetrate through the soil while protecting the shoots. Once the seedlings emerge from the soil and perceive light, ethylene promotes the coleoptile curvature, which facilitates exposure of seedling leaves to light. Meanwhile, ethylene-induced coleoptile greening may provide energy, from photosynthesis, for seedling growth. Once seedlings grow under light, ethylene promotes the growth and development of shoots and adventitious roots. The leaf elongation and development, promoted by ethylene, may facilitate early-stage rice seedling growth, and the increase in adventitious root number may help rice seedlings stand in the soft, muddy field and facilitate nutrition uptake. This study suggests that ethylene plays important roles in coordinating various environmental (darkness and light) and internal cues during early-stage rice seedling growth and development.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Coleoptile phenotype of light-grown *35S:OsRTH1* transformation rice lines grown in air and ethylene.

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