

RESEARCH PAPER

Ethylene is involved in the regulation of iron homeostasis by regulating the expression of iron-acquisition-related genes in *Oryza sativa*

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Received 3 August 2010; Revised 4 September 2010; Accepted 6 September 2010

Abstract

Plants employ two distinct strategies to obtain iron (Fe) from the soil. In Strategy I but not Strategy II plants, Fe limitation invokes ethylene production which regulates Fe deficiency responses. *Oryza sativa* (rice) is the only graminaceous plant described that possesses a Strategy I-like system for iron uptake as well as the classic Strategy II system. Ethylene production of rice roots was significantly increased when grown under Fe-depleted conditions. Moreover, 1-aminocyclopropane-1-carboxylic acid (ACC) treatment, a precursor of ethylene, conferred tolerance to Fe deficiency in rice by increasing internal Fe availability. Gene expression analysis of rice iron-regulated bHLH transcription factor *OsIRO2*, nicotianamine synthases 1 and 2 (*NAS1* and *NAS2*), yellow-stripe like transporter 15 (*YSL15*) and iron-regulated transporter (*IRT1*) indicated that ethylene caused an increase in transcript abundance of both Fe (II) and Fe (III)-phytosiderophore uptake systems. RNA interference of *OsIRO2* in transgenic rice showed that ethylene acted via this transcription factor to induce the expression of *OsNAS1*, *OsNAS2*, *OsYSL15*, and *OsIRT1*. By contrast, in *Hordeum vulgare* L. (barley), no ethylene production or ethylene-mediated effects of Fe response could be detected. In conclusion, Fe-limiting conditions increased ethylene production and signalling in rice, which is novel in Strategy II plant species.

Key words: Ethylene, gene expression, iron homeostasis, rice, signalling pathway.

Introduction

Iron (Fe) is predominantly present as oxidized Fe (III) compounds in soils, which are poorly soluble under high pH conditions and not readily available to plants (Kim and Guerinot, 2007). Under Fe-deficiency conditions, plants induce morphological and physiological changes to maximize iron uptake and utilization. Higher plants have evolved two strategies for Fe acquisition. The reduction strategy, Strategy I, is employed by all plant species except

the grasses. The Strategy I approach involves pumping protons by H⁺-ATPases to acidify the rhizosphere and increase Fe solubility in the soil. A ferric chelate reductase (FRO) reduces Fe (III) to Fe (II), and Fe (II) transporters (*IRT*s) transport Fe into cells (Kim and Guerinot, 2007; Walker and Connolly, 2008; Giehl *et al.*, 2009). Grass plants use the chelation strategy (Strategy II) (Kim and Guerinot, 2007; Walker and Connolly, 2008). In this

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; ACO, ACC oxidase; AdoMet, S-adenosyl-L-methionine; DMA, 2'-deoxymugineic acid; EREBP, ethylene response element binding protein; FW, fresh weight; MA, mugineic acid; NA, nicotianamine; RNAi, RNA interfering; SPAD, soil-plant analyser development; STS, silver thiosulphate; qRT-PCR, quantitative real-time PCR.

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approach, grass plants synthesize and release mugineic acid family phytosiderophores (MAs) from their roots to chelate Fe (III). The Fe (III)–MA complexes are then taken up through Fe (III)–MA transporters (Curie *et al.*, 2001; Murata *et al.*, 2006; Lee *et al.*, 2009; Inoue *et al.*, 2009).

Rice is one of the most important grasses, responsible for approximately 21% of the calorific supply of the world's population, which reaches almost 80% in some South-East Asian countries. As in other graminaceous plants, rice employs a Strategy II system for Fe uptake through the Fe (III)–MAs system. MAs are synthesized from methionine via nicotianamine (NA). The expression of genes that encode key enzymes of this pathway are induced by Fe deficiency in rice and barley with a significant increase in MAs synthesis (Inoue *et al.*, 2003; Kobayashi *et al.*, 2005; Bashir *et al.*, 2006; Nagasaka *et al.*, 2009). The levels of transcripts that encode Fe (III)–MAs transporters, *ZmYS1*, *OsYSL15*, and *HvYS1* in maize, rice, and barley, respectively, are also increased under Fe-deficient conditions (Curie *et al.*, 2001; Lee *et al.*, 2009; Ueno *et al.*, 2009). Mutation in the rice nicotianamine aminotransferase gene (*naat1*) diminished the production of 2'-deoxymugineic acid (DMA) and Fe (III) uptake (Cheng *et al.*, 2007). However, *naat1* rice plants grow normally when Fe (II)-EDTA was supplied, indicating that *naat1* rice plants can use Fe (II) as an iron source. It has been shown that rice can directly absorb Fe (II) using a Strategy I-like system (Ishimaru *et al.*, 2006, 2007; Cheng *et al.*, 2007). Two Fe-deficiency induced genes from rice, *OsIRT1* and *OsIRT2*, have been identified as Fe (II) transporters (Ishimaru *et al.*, 2006).

It has been shown that ethylene production is induced in roots by Fe deficiency in Strategy I plants (Romera *et al.*, 1999; Romera and Alcantara, 2004). Treatment of *Arabidopsis*, tomato, and cucumber plants with 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, induced the expression of ferric reductase (*AtFRO2*, *LeFRO1*, *CsFRO1*), iron transporter (*AtIRT1*, *LeIRT1*, *CsIRT1*), H⁺-ATPases (*CsHAI*), and transcription factors (*LeFER*, *AtFIT*), that regulate the iron deficiency response. Conversely, the addition of ethylene inhibitors, such as silver thiosulphate (STS), Co²⁺, aminoethoxyvinylglycine (AVG), and aminooxylacetic acid (AOA), markedly repressed the expression of these genes (Romera and Alcantara, 1994; Lucena *et al.*, 2006; Waters *et al.*, 2007). Thus, Fe limitation enhanced the ethylene production that, in turn, increases Fe acquisition mediated via *LeFER* (or *AtFIT*) transcriptional regulation (Lucena *et al.*, 2006; Giehl *et al.*, 2009).

In contrast to Strategy I plants, research indicates that ethylene is not involved in the regulation of Fe-deficiency stress responses by Strategy II plants (Welch *et al.*, 1997; Romera *et al.*, 1999). As rice has both Strategy I and II systems for Fe uptake, ethylene may be involved in regulating the Fe-deficiency responses. The expression of several rice *ACC* synthases (ACS) and *ACC* oxidases (ACO) were up-regulated under Fe-deficient conditions as determined from studies using microarray analysis (Zheng *et al.*, 2009), suggesting a role for ethylene in signalling under Fe-limited conditions. In this study, it has been

demonstrated that ethylene is involved in the Fe-deficiency response in rice, but not in barley.

Materials and methods

Plant materials, growth conditions, and treatments

Rice (*Oryza sativa* L. cv. Nipponbare) seeds were germinated in tap water for 2 d and transferred into culture solution prepared as previously described by Yoshida *et al.* (1976). The initial nutrient solution contained 1.425 mM NH₄NO₃, 0.323 mM NaH₂PO₄, 0.513 mM K₂SO₄, 0.998 mM CaCl₂, 1.643 mM MgSO₄, 0.009 mM MnCl₂, 0.075 μM (NH₄)₆Mo₇O₂₄, 0.019 mM H₃BO₃, 0.155 μM CuSO₄, 0.070 mM citric acid, and 0.152 μM ZnSO₄ with 0.125 mM EDTA-Fe(II) or no Fe. Seedlings were grown in a growth chamber at 30/22 °C day/night temperatures with a 12/12 h light/dark regime (450 mmol photons m⁻² s⁻¹) until they were 3 weeks old. Seedlings were then transplanted to 1.0 l glass vessels with different treatments. Nutrient solution was refreshed daily and the pH of the solution was adjusted to 5.5. 1 μM of ACC, 50 μM STS, or 50 μM CoCl₂ were added as indicated.

Barley (*Hordeum vulgare* L.) genotype CV Zhemai 1 was used in this study. Seeds were surface-sterilized in 2% (v/v) H₂O₂ for 10 min, rinsed several times with deionized water, and then germinated in hermetically-sealed plastic containers with sterilized moist quartz sand at 20 °C with a 12/12 h light/dark regime (450 mmol photons m⁻² s⁻¹). Germinated seeds were transferred into a hydroponic solution culture for 5 d. The composition of the basic nutrient solution was 0.365 mM (NH₄)₂SO₄, 0.547 mM MgSO₄, 0.091 mM K₂SO₄, 0.183 mM KNO₃, 0.365 mM Ca(NO₃)₂, 0.182 mM KH₂PO₄, 0.02 mM Fe-citrate, 4.5 μM MnCl₂, 0.38 μM ZnSO₄, 0.16 μM CuSO₄, 0.047 mM HBO₃, and 0.056 μM H₂MoO₄. The pH of the solution was adjusted to 6.5 every other day. Five-day-old seedlings were transferred into solution cultures with or without Fe for 1 week prior to sampling. All physiological experiments were carried out in triplicate.

Measurement of chlorophyll content

Soil–plant analyser development (SPAD) values were determined on the fully expanded youngest leaves of 3-week-old seedlings with a portable chlorophyll meter (SPAD-502, Minolta Sensing, Japan).

Measurement of ethylene production

Ethylene production in the roots of rice seedling was analysed using intact roots of treated seedlings by placing detached roots into 15 ml glass vials containing 1 ml water and rapidly sealing with a gas-proof septum. The sealed vials were incubated in a dark growth chamber for 4 h at 30 °C. One millilitre of gas was withdrawn from the airspace of each vial using a gas-tight syringe (Focus GC, Thermo, USA) and injected into a gas chromatograph (Focus GC, Thermo, USA) equipped with a capillary column (CP-carboPLOT P7, Varian, CA, USA) and flame-ionization detector for ethylene determination. Ethylene production was calculated on the basis of fresh weight (FW) of root samples.

Measurement of the total and soluble Fe

To determine total Fe roots of seedling plants were washed three times with deionized water and dried at 80 °C. Dried samples were ground to fine powders and digested with 5 ml of 11 M HNO₃ for 5 h at 150 °C. Fe concentrations were measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7500ce, CA, USA).

Extraction of water-soluble Fe was performed as previously described (Zheng *et al.*, 2009). Briefly, approximately 0.5–1 g of shoot samples were ground in liquid nitrogen and extracted in 5 vols of deionized water at room temperature. After centrifugation,

the supernatant was recovered. Fe concentration was measured as described above.

Construction of *OsIRO2* RNA interfering (RNAi) rice

The *OsIRO2* RNA interfering construct was made using the Gateway™ cloning system according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). In brief, a fragment of *OsIRO2* was amplified from cDNA (the reverse transcript from total RNA as described below) with primers IRO2-F (5'-CACCTCGTGCAACATCAGCTCACT-3') and IRO2-R (5'-AGCAGAGGCGGATGATCTCCT-3'). The amplified fragment was cloned into pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA) and verified by DNA sequencing. The cDNA insert was introduced into the final vector pH7GWIWG2 (Karimi *et al.*, 2002) in both sense and antisense orientations to construct the binary vector of the *OsIRO2* RNA interfering construct. The above construct was used to generate *OsIRO2* RNAi transgenic plants via *Agrobacterium*-mediated transformation as previously described (Chen *et al.*, 2003; Wang *et al.*, 2008).

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from plant samples using the TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's recommendations. First-strand cDNAs were synthesized from total RNA using SuperScript II reverse transcriptase (Invitrogen, CA, USA). Quantitative RT-PCR (qRT-PCR) was performed using SYBR Premix Ex Taq™ (Perfect Real Time) Kit (TaKaRa Biomedicals, Tokyo, Japan) on a LightCycler 480 (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Triplicate quantitative assays were performed at each cDNA sample. The housekeeping gene *OsACTIN* was used as an internal control. The relative level of expression was calculated using the formula $2^{-\Delta\Delta C_t}$. All the primers and annealing temperatures used for these PCR reactions are given in Supplementary Table S1 at *JXB* online.

Statistical analysis of data

Data were compared with one-way analysis of variance and differences between groups were compared with LSD *t* test ($P < 0.05$).

Results

Iron deficiency induced ethylene production in rice roots

To investigate the effect of Fe supply on ethylene production in rice, 3-week-old rice seedlings were transferred in nutrient solution without Fe for 5 d. A Minolta SPAD-502 meter was used to measure the relative chlorophyll content of leaves. Five days of Fe deprivation (–Fe) resulted in a significant decline of chlorophyll concentration and induced chlorotic symptoms in newly developed leaves (Fig. 1A, B). The Fe concentration in shoots decreased significantly (Fig. 1C). Re-supply of Fe for 2 d restored the Fe concentration to normal levels (Fig. 1C), indicating that the Fe concentrations in leaves could readily be restored following Fe re-supply. The level of ethylene production was inversely correlated with the Fe concentration in plants (Fig. 1D). While removal of Fe from the medium significantly increased the ethylene production from 2.43 to 3.89 nl g⁻¹ FW h⁻¹ (Fig. 1D), re-supply of Fe for 2 d resulted in ethylene production returning to normal levels (Fig. 1D).

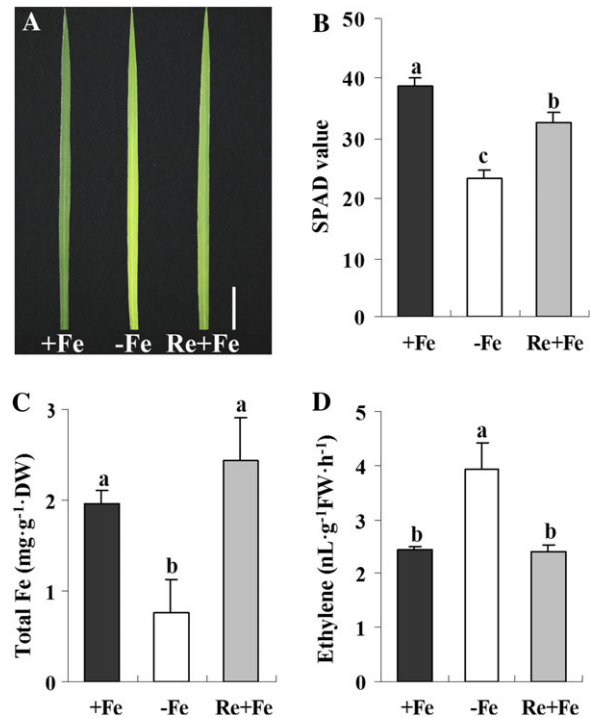


Fig. 1. Effects of Fe deficiency and resupply on rice seedlings. Three-week-old seedlings were transferred to Fe-depleted (–Fe) nutrient solution for 5 d. The –Fe treatment caused leaf chlorosis (A) (Bar=3 cm), decreased SPAD value (B), decreased total Fe content (C), and increased root ethylene production (D). Two days after Fe was resupplied (Re+Fe) to the seedlings, all the above changes were reversed (A–D). Values are the means ± standard error of three replicates. Points with different letters are significantly different at $P < 0.05$ (LSD test, $P < 0.05$).

Iron deficiency induced the expression of specific *OsACS* and *OsACO* genes

There are six putative *ACS* genes and seven putative *ACO* genes in the rice genome (Rzewuski and Sauter, 2008). *AtACS10* and *AtACS12*, which display aminotransferase activity instead of ACS activity (Yamagami *et al.*, 2003). According to phylogenetic analysis of the *Arabidopsis* and rice *ACS* gene family, *OsACS6* is the closest orthologue of *AtACS10* and *AtACS12* (Iwai *et al.*, 2006). The gene was excluded from the analysis due to putative function loss of ACS activity. *OsACO6* was also excluded because it is a pseudogene (Rzewuski and Sauter, 2008). Our previous microarray data indicated that the transcription of *ACS* and *ACO* genes are induced by Fe deficiency (see Supplementary Table S2 at *JXB* online; Zheng *et al.*, 2009). To determine which of these genes are responsible for the induction of ethylene production by Fe deficiency, the expression of *ACS* and *ACO* genes were analysed by qRT-PCR. Five days of Fe deprivation resulted in an increase of expression for *OsACS1*, *OsACS2*, *OsACS3*, *OsACO1*, *OsACO2*, *OsACO3*, and *OsACO7* (Fig. 2).

To test if the induction of *ACS* and *ACO* gene expression could be reversed by the re-supply of Fe, seedlings grown in –Fe nutrient solution for 5 d were transferred back to

medium with 0.125 mM EDTA-Fe(II) for 2 d. qRT-PCR analysis indicated that the expression of *OsACS1*, *OsACS2*, *OsACS3*, *OsACO1*, *OsACO2*, and *OsACO3* were restored to the normal (Fig. 2). Strikingly, expression of *OsACO7* remained high after Fe re-supply. The marked changes in *OsACS2*, *OsACS3*, *OsACO1*, and *OsACO2* expression mirrored changes in ethylene levels, suggesting that these *ACS* and *ACO* genes are responsible for the increased of ethylene synthesis in roots under Fe depletion.

Ethylene contributes to iron-deficiency tolerance in rice

To evaluate the role of ethylene on iron homeostasis in rice plants, 3-week-old seedlings were transferred to either a normal or an Fe-deficient nutrient solution supplemented with 1 μ M ACC. While 10 d of Fe deprivation resulted in

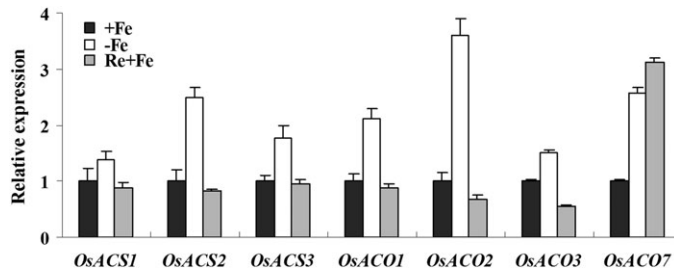


Fig. 2. Effect of Fe deficiency and resupply on expression of ethylene biosynthesis enzymes. Three-week-old seedlings were transferred to Fe-depleted (-Fe) nutrient solution for 5 d and were resupplied with Fe for 2 d. After treatment, total root RNA was extracted and used to synthesize cDNA. qRT-PCR was performed using gene-specific primers. Values are the means \pm standard error of three replicates.

a visible chlorotic leaf phenotype, application of ACC alleviated the symptoms (Fig. 3A–C). Under Fe-sufficient condition, the SPAD values of seedlings with or without ACC supply were similarly high (Fig. 3B). The SPAD value in the seedlings of -Fe+ACC treatment was significantly higher than that of -Fe (Fig. 3B). Analysis of the total and soluble Fe concentrations of seedlings in +Fe, +Fe+ACC, -Fe, and -Fe+ACC showed that the addition of ACC did not change the total Fe concentrations (Fig. 3D), but significantly increased soluble Fe concentration in the +Fe- and -Fe-treated seedlings (Fig. 3E).

Regulation of Fe deficiency response genes by ethylene in rice

To determine whether ethylene participates in -Fe responsive signalling, the expression of genes typically induced in response to Fe deficiency, *OsIRO2*, *OsNAS1*, *OsNAS2*, *OsYSL15*, *OsIRT1*, and *IDEF1* and ethylene responsive element binding protein-encoding genes *OsEBP1* (Loc_Os03g64260) and *OsEBP89*, were analysed by quantitative RT-PCR in roots grown in normal or Fe-deficient medium supplemented with 1 μ M ACC. Under Fe-sufficient conditions, the expression of both Fe deficiency and ethylene-responsive genes was strongly induced by ACC treatment (Fig. 4). Although Fe deficiency induced the expression of *OsIRO2*, *OsNAS1*, *OsNAS2*, *OsYSL15*, *OsIRT1*, *OsEBP1*, and *IDEF1*, addition of ACC further increased these inductions (Fig. 4). Fe deficiency has no effect on the expression of ethylene responsive gene *OsEBP89*.

To confirm the involvement of ethylene signalling in the induction of these genes, inhibitors of ethylene signalling, STS and CoCl_2 , were added to the nutrient solution.

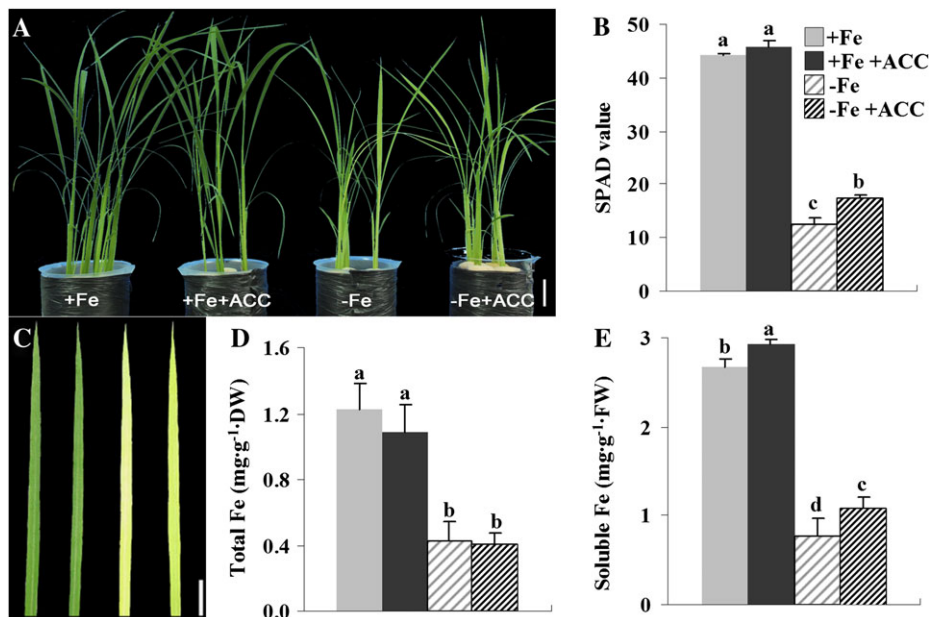


Fig. 3. Physiological responses of rice seedlings to ACC treatment under both Fe-sufficient and -deficient conditions. Three-week-old seedlings were transferred to +Fe and -Fe nutrient solution with or without 1 μ M ACC for 10 d. (A–E) Growth performance, SPAD values, leaf chlorosis phenotypes, and total and soluble Fe concentrations, respectively. Bar=5 cm (A) and 3 cm (C). Values are the means \pm standard error of three replicates. Points with different letters are significantly different at $P < 0.05$ (LSD test, $P < 0.05$).

Addition of both inhibitors repressed the induction of Fe deficiency response genes by -Fe (Fig. 5). Application of the ethylene inhibitors has no significant effect on the total or soluble Fe concentration in rice seedling (data not shown).

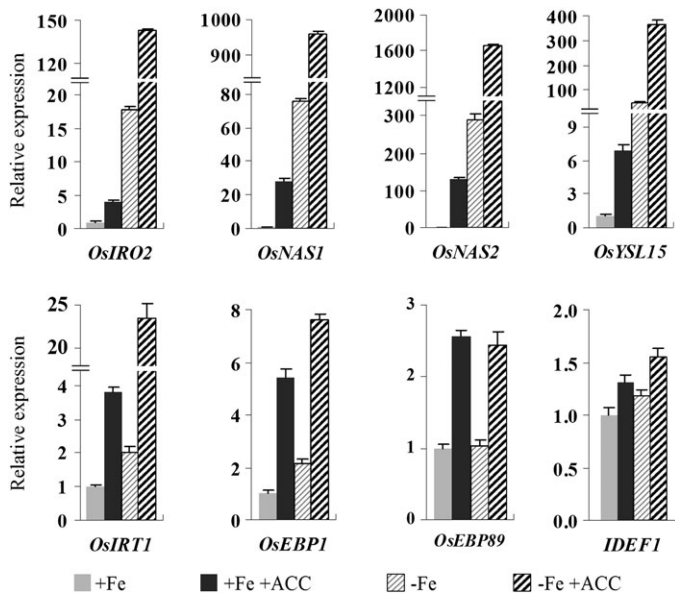


Fig. 4. Regulation of *OsIRO2*, *OsNAS1*, *OsNAS2*, *OsYSL15*, *OsIRT1*, *OsEBP1*, *OsEBP89*, and *OsIDEF1* expression by Fe deficiency and precursors of ethylene synthesis in rice seedlings. Three-week-old seedlings were transferred to +Fe and -Fe nutrient solution incubated with or without 1 μ M ACC for 5 d. After treatment, total root RNA was extracted and used to synthesize cDNA. qRT-PCR was performed using gene-specific primers. Values are the means \pm standard error of three replicates.

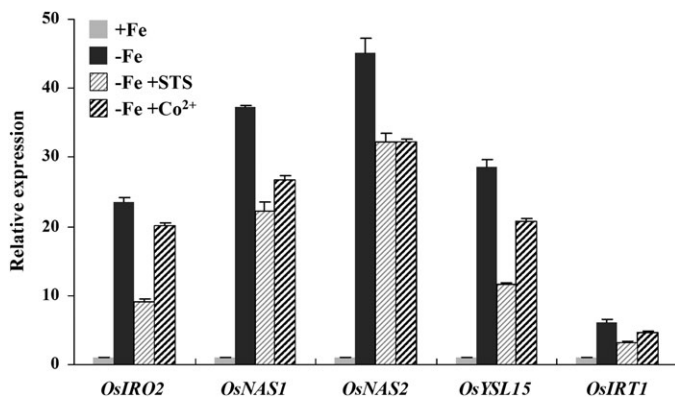


Fig. 5. Effect of ethylene inhibitors on the expression of Fe-deficiency responsive genes. Three-week-old seedlings were transferred to +Fe and -Fe nutrient solution for 5 d. Some of the -Fe plants were treated with 50 μ M STS or 50 μ M CoCl_2 . After treatment, total root RNA was extracted and used to synthesize cDNA. qRT-PCR was performed using gene-specific primers of *OsIRO2*, *OsNAS1*, *OsNAS2*, *OsYSL15*, and *OsIRT1*. Values are the means \pm standard error of three replicates.

OsIRO2 is required for the ethylene-mediated Fe regulation

OsIRO2 has been reported to be a novel regulator in Strategy II Fe response signalling (Ogo *et al.*, 2006, 2007). To determine whether *OsIRO2* was involved in the ethylene-mediated Fe response, transgenic lines with reduced *OsIRO2* transcript levels were produced through RNA interference. Out of the ten independent lines generated, two lines (Ri1 and Ri2) with significantly reduced expression of *OsIRO2* were selected for the study (Fig. 6). Under Fe-deficient condition, the expression of *OsYSL15*, *OsNAS1*, and *OsNAS2* but not *OsIRT1* were moderately suppressed in RNAi plants compared with wild-type plants (Fig. 6), which is consistent with a previous report (Ogo *et al.*, 2007). Application of ACC greatly induced the expression of these genes in the wild-type seedlings (Fig. 6), but the extent of the induction was much lower in the Ri1 and Ri2 seedlings. This indicates that *OsIRO2* is involved in the ethylene-mediated regulation of these Fe deficiency response genes (Fig. 6).

Ethylene is not involved in the Fe deficiency response in barley

To determine whether ethylene production is induced by Fe deficiency in other grass species, 5-d-old barley seedlings were transferred into hydroponic solution cultures with or without Fe. However, ethylene production in barley was not significantly changed by Fe deficiency (Fig. 7A). Application of ACC had no significant effect on the expression of Fe-deficiency response genes in either Fe-sufficient or -deficient conditions (Fig. 7B).

Discussion

It has been previously documented that Fe limitation can induce ethylene production and that ethylene signalling can increase Fe acquisition in Strategy I plants but not in Strategy II plants (Romera *et al.*, 1999; Romera and

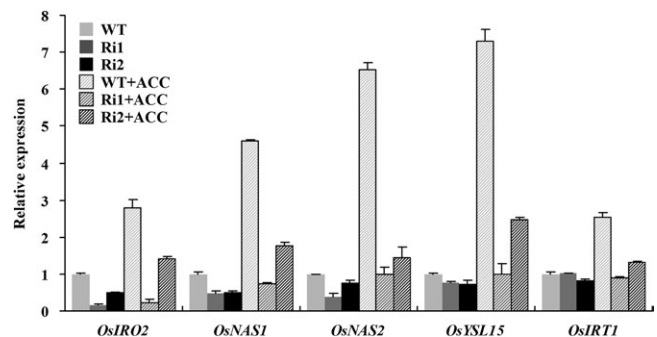


Fig. 6. Expression analysis of *OsIRO2*, *OsNAS1*, *OsNAS2*, *OsYSL15*, and *OsIRT1* in *OsIRO2* RNAi plants. Three-week-old seedlings were transferred to -Fe nutrient solution with or without 1 μ M ACC for 5 d. After treatment, total root RNA was extracted and used to synthesize cDNA. qRT-PCR was performed using gene-specific primers. Values are the means \pm standard error of three replicates.

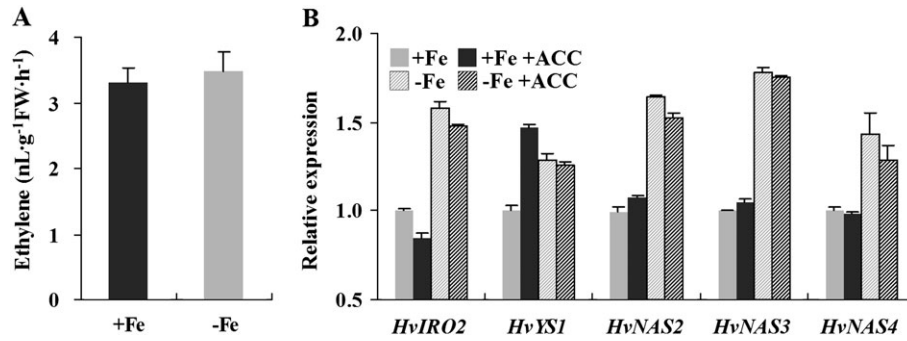


Fig. 7. Effect of Fe deficiency on ethylene production (A) and the expression of -Fe responsive genes (B) in barley. Five-day-old seedlings were transferred to +Fe and -Fe nutrient solution with 1 μ M ACC for 1 week. After treatment, roots were sampled for ethylene measurements and RNA extraction. qRT-PCR was performed using gene-specific primers. Values are the means \pm standard error of three replicates.

Alcantara, 2004). A similar involvement of ethylene is reported here in the Fe-deficiency response in rice. Fe deficiency induced ethylene production, while a re-supply of Fe reduced ethylene production to normal levels (Fig. 1). The pattern of ethylene production was correlated with the expression pattern of *OsACS1*, *OsACS2*, *OsACS3*, *OsACO1*, *OsACO2*, and *OsACO3*, indicating that these *ACS* and *ACO* genes may be responsible for the increase in ethylene synthesis in roots under Fe depletion (Fig. 2). Moreover, ACC treatment induced Fe starvation tolerance in rice seedlings and expression of Fe-deficiency response genes (Figs 3, 4). By contrast, a similar phenomenon was not observed in barley under similar experimental conditions (Fig. 7).

The growth of rice in submerged conditions has resulted in the adoption of a Strategy I-like system to take up Fe (II) directly through Fe (II) transporters (Ishimaru et al., 2006; Cheng et al., 2007). This is in addition to the typical Strategy II iron uptake system through the secretion of mugineic acid family phytosiderophores (MAs) from roots present in grasses (Inoue et al., 2003, 2009; Lee et al., 2009). The genes encoding enzymes that participate in the MAs biosynthetic pathway are highly induced during Fe deficiency (Higuchi et al., 1999; Takahashi et al., 1999; Nakanishi et al., 2000; Kobayashi et al., 2005; Zheng et al., 2009). As both MAs and ethylene are synthesized from the amino acid methionine via the intermediate *S*-adenosyl-methionine (AdoMet), there are potential interactions between MAs secretion and ethylene synthesis. Compared with barley, rice secretes much less MAs from its roots and is more sensitive to iron deficiency (Mori et al., 1991; Ishimaru et al., 2006). This may in part be due to the use of AdoMet in ethylene synthesis. However, both Fe deficiency and ACC treatment strongly induced the expression of *OsNAS1* and *OsNAS2*, which promote MA biosynthesis (Fig. 4). Measurements of the concentration of NA and secreted MA in ACC-treated plants would understand the interactions between ethylene and MA synthesis in rice plants.

In Strategy I plants, ethylene regulates an array of Fe deficiency responses mediated via *LeFER* (or *AtFIT1*) transcriptional regulation (Lucena et al., 2006). In this study,

it was demonstrated that application of ACC greatly induced the expression of Fe response genes, whereas addition of inhibitors of ethylene signalling or synthesis prevented induction of these genes under Fe-deficiency condition (Figs 4, 5). ACC also significantly induces expression of these Fe deficiency response genes even under conditions with sufficient or normal supply of Fe (Fig. 4). These results demonstrate that the ethylene signalling pathway is involved in the regulation of both Fe (III)-MAs and Fe (II) uptake systems in rice. Although STS or CoCl_2 repressed the induction of genes induced under Fe-deficient conditions, the expression of these genes under -Fe+STS or -Fe+Co condition was still higher than that in Fe-sufficient condition (Fig. 5), suggesting the existence of other signal pathway(s) independent of ethylene signalling in Fe deficiency responses, as proposed in Strategy I plants (Lucena et al., 2006; Giehl et al., 2009). *OsEBP1* and *OsEBP89* are AP2/EREBP (Ethylene Response Element Binding Protein) transcription factors involving ethylene signalling (Yang et al., 2002; Mao et al., 2006). ACC treatment induced expression of *OsEBP1* and *OsEBP89* under Fe-sufficient and -deficient conditions indicating the activation of ethylene signalling (Fig. 4). Consistent with previous studies (Ogo et al., 2007; Zheng et al., 2009), expression of *OsEBP1* was also induced by Fe deficiency. The extent of induction of *OsEBP89* expression by the addition of ethylene is much lower than that of *OsEBP1*. Thus, *OsEBP89* is less sensitive to ethylene than *OsEBP1*, which may have accounted for the fact that the expression of *OsEBP89* was not significantly induced by Fe deficiency (Fig. 4). It is also possible that Fe deficiency triggers tissue-specific ethylene signalling, which could not induce the expression of *OsEBP89*. The similar expression pattern of *OsEBP1* and other Fe-deficiency response genes indicated that *OsEBP1*, along with other potential ethylene responsive transcription factors, may act as the convergence point that links ethylene and Fe signalling.

In RNAi lines of *OsIRO2* plants, the transcript abundance of *OsNAS1*, *OsNAS2*, and *OsYSL15* were decreased, but not for *OsIRT1* (Fig. 6). This was expected because *OsIRO2* is the key regulator of the Fe (III)-MAs uptake system and directly regulates the expression of *OsNAS1*, *OsNAS2*, and *OsYSL15* under Fe deficiency (Ogo et al.,

2007). Analysis of RNAi lines of *OsIRO2* indicated that ethylene induction of *OsNAS1*, *OsNAS2*, and *OsYSL15* was under the control of *OsIRO2* (Fig. 6). It is interesting that *OsIRO2* also mediates ethylene induced expression of *OsIRT1*. It has been reported that *IDEF1* is the central transcription factor upstream of *OsIRO2* and *OsIRT1* (Kobayashi *et al.*, 2007, 2009). Overexpression of *IDEF1* enhanced Fe-deficiency resistance without changing the total Fe content in rice (Kobayashi *et al.*, 2007), similar to what was observed in this study. Although it has been reported that *IDEF1* is constitutively expressed (Kobayashi *et al.*, 2010), it was found that expression of *IDEF1* is slightly induced by ACC treatment, especially under Fe-deficient conditions (Fig. 4). It needs to be investigated whether the induction of *IDEF1* expression by the application of ACC contributes to the increased expression of Fe deficiency responsive genes and tolerance to Fe deficiency. It is also possible that ethylene may directly regulate *OsIRO2* and downstream genes by other transcription factors, such as *EILs* or *EREBPs*. We have searched the ethylene responsive elements, GCC-box (Broglie *et al.*, 1989), OsEIL-binding sequence (Hiraga *et al.*, 2009), and PERE (Itzhaki *et al.*, 1994) in the promoters (–1500 bp from the start code) of these genes. The results showed that all the promoters of these genes contained at least one ethylene response *cis*-acting elements (see Supplementary Table S3 at *JXB* online). Identification of factors that mediate ethylene-regulated Fe starvation signalling would provide a unifying model to explain the physiological response observed in the study.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table 1. Gene-specific primers used in this work.

Supplementary Table 2. Expression data of *ACS* and *ACO* genes extracted from microarray analysis (Zheng *et al.*, 2009).

Supplementary Table 3. Ethylene response *cis*-acting elements in promoters of iron acquisition related genes in rice.

Acknowledgements

We gratefully acknowledge Dr Feibo Wu who provided us with the barley seeds and the hydroponic culture protocol for barley used in this study. This work was supported by the National Natural Science Foundation (30770191 and 30871585), the Ministry of Agriculture (2009ZX08010-013B and 2009ZX08009-1238), and Outstanding Young Scientist Funds in Zhejiang Province (R3090229).

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