Brassinosteroids are involved in response of cucumber (Cucumis sativus) to iron deficiency

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INTRODUCTION

Iron (Fe) is one of the essential nutrients for plant growth and development (Curie and Briat 2003). Although Fe is the fourth most abundant element in the earth’s crust, its availability to plants is often limited due to its low solubility, especially in calcareous, high pH soils (Romera and Alcántara, 2004). Plants have developed various strategies to cope with Fe deficiency in those soils. These strategies are classified as strategy I in non-graminaceous monocots and dicots, and strategy II in graminaceous monocots (Römheld and Marschner, 1981). In strategy I plants, several morphological and physiological processes are involved in the mobilization and uptake of Fe in response to Fe deficiency. These include subapical swelling with abundant root hairs, induction of transfer cells, and increases in ferric reductase (FRO) activity, acidyfication of the rhizosphere, increases in the number of Fe2+ transporters (IRT), and release of flavins and phenolics (Römheld and Marschner, 1986; Curie and Briat 2003; Hell and Stephan 2003; Romera and Alcántara, 2004). These responses are regulated by Fe status in the plant, but the underlying mechanisms have not been fully understood.

Several Fe deficiency-responsive genes encoding FRO (AtFRO2), IRT (AtIRT1) and H+-ATPase (AtHA7) have been identified in Arabidopsis (Eide et al., 1996; Robinson et al., 1999; Colangelo and Guerinot, 2004). Homologues of these genes have also been reported in tomato, pea and cucumber (Eckhardt et al., 2001; Waters et al., 2002; Li et al., 2004; Santi et al., 2005; Waters et al., 2007). Several genes involved in Fe acquisition have been reported to be up-regulated in response to Fe deficiency, but the signalling cascades associated with these events remain to be unravelled. Lucena et al. (2006) showed that the response of Fe-deficiency is not only dependent on root Fe content, but signals from aerial parts may also be involved. In this context, systemic signals have been suggested to play a role regarding Fe deficiency-induced responses (Romera et al., 1992; Vert et al., 2003).

Phytohormones and messenger molecules such as auxin, ethylene, nitric oxide (NO) and mono oxide have been reported to be involved in the regulation of Fe deficiency-induced physiological processes (Römheld and Marscher, 1986; Ivanov et al., 2012). For instance, auxin synthesis is enhanced under Fe deficiency, and exogenous application of auxin analogues promotes the induction of the root FRO in bean and
cucumber (Li and Li, 2004) and Trifolium pratense (Zheng et al., 2003). Furthermore, recent studies revealed that NO is also involved in the regulation of Fe deficiency-induced responses by improving internal Fe availability (Graziano et al., 2002; Graziano and Lamattina, 2007; Jin et al., 2009). More recently, Chen et al. (2010) demonstrated that there is crosstalk between auxin and NO in mediation of Fe deficiency-dependent processes such that NO is a downstream signal of auxin under Fe deficiency, leading to the induction of FRO via FIT-mediated transcriptional regulation in Strategy I plants.

As a class of plant polyhydroxysteroids, brassinosteroids (BRs) exist ubiquitously in plants (Noguchi et al., 1999; Divi and Krishna, 2009). BRs play important roles in a wide range of developmental processes in plants, including seed germination, root growth, floral initiation and flowering (Sasse, 2003; Divi and Krishna, 2009). There is emerging evidence demonstrating that BRs also participate in the response of plants toiotic and abiotic stresses (Bajguz and Hayat, 2009; Divi and Krishna, 2009). For instance, exogenous application of BR ameliorates the inhibitory effect of salt stress on seed germination and seedling growth of Arabidopsis and Brassica napus (Kagale et al., 2007).

Ethylene, which plays a diverse role in regulation of numerous physiological processes in plants (Pierik et al., 2006), has also been shown to be involved in sensing and responding to mineral stress such as deficiencies of phosphorus and Fe (Romera and Alcántara, 2004; Li et al., 2009) and aluminum toxicity (Sun et al., 2010). Similar to ethylene, BRs also play regulatory roles in many physiological processes (Sasse, 2003). However, little information is available on the involvement of BR in response of plants to nutrient deficiency in the literature. In the present study, we investigated the role of BR in the response of cucumber seedlings to Fe deficiency, and explored the possible interaction between BR and ethylene in response to Fe deficiency.

**MATERIALS AND METHODS**

**Plant materials**

Cucumber (Cucumis sativus L. ‘Zhongnong 8’) seeds were sterilized in 5% (v/v) sodium hypochlorite solution for 10 min and allowed to germinate on filter paper. The germinated seeds were pre-cultured hydroponically in aerated quarter-strength Hoagland’s solution (pH 5.8) with sufficient supply of Fe (50 μM Fe-EDTA) in a controlled environment with a light/dark regime of 14/10 h, temperature of 20 °C/26 °C and a light intensity of 230 μmol m⁻² s⁻¹. After 1 week pre-culture, the seedlings were subjected to further experiments.

**Determination of FRO activity**

Cucumber plants were pre-cultured in nutrient solution with 50 μM Fe-EDTA for 1 week and exposed to varying concentrations of Fe (0, 1, 5 and 50 μM Fe-EDTA) for 4 d. For the effect of 24-epibrassinolide (EBR) on FRO activity, cucumber seedlings were cultured in the Fe-sufficient solution (50 μM Fe-EDTA; Fe50) for 1 week. Thereafter they were transferred to Fe-deficient (1 μM Fe-EDTA; Fe1) and Fe50 media for another 2 d and treated with varying concentrations of EBR (0, 0.01, 0.1, 0.5 μM EBR) for 24 h. After treatments, root FRO activity was determined as described below.

FRO activity was assayed following the protocols described by Waters et al. (2007). Briefly, intact plants were pretreated for 30 min in a nutrient solution without micronutrients (pH 5.5), and then transferred to 50 mL Fe(III) reduction assay solution for 1 h. The assay solution consisted of nutrient solution without micronutrients, 100 μM Fe³⁺ EDTA and 300 μM ferrozine (pH 5.0). The conditions for the measurement of Fe(III) reduction were identical to the growth conditions described above. The FRO activity was determined spectrophotometrically by measuring the absorbance (562 nm) of the Fe²⁺ ferrozine complex formed. After the reduction assay, roots were excised and weighed. Reduction rates were determined using an extinction coefficient of 29 800 M⁻¹ cm⁻¹. Since 0.5 μM EBR and 1 μM FeEDTA were found to be significant in terms of its effect on FRO activity under Fe-deficient conditions, this concentration was used in the following experiments. To determine the effect of BR biosynthesis inhibitor brassinazole (BRz) on FRO activity, cucumber seedlings were pre-cultured in nutrient solution containing 50 μM FeEDTA for 1 week, and then transferred to either Fe1 or Fe50 medium for another 2 d treated with or without 5 μM BRz for 24 h. After treatments, root FRO activity was determined as described above.

**Measurement of pH and ethylene emission**

Cucumber seedlings pre-cultured in nutrient solution containing 50 μM Fe-EDTA for 1 week were transferred to Fe1 and Fe50 medium for 2 d, and then treated with 0.5 μM EBR for 24 h. The pH of the nutrient solution was determined on the fourth day of treatment. The change in pH value in the medium was determined with a pH meter. The pattern of pH change around roots was visualized by placing seedlings in a medium containing quarter-strength Hoagland’s solution without micronutrients and the pH indicator bromocresol purple (0.006 %), solidified with 0.7 % agar. The pH of the medium was adjusted to 6.0 with NaOH. Cucumber seedlings pre-cultured in nutrient solution containing 50 μM Fe-EDTA for 1 week were transferred to Fe1 and Fe50 media for 2 d. The seedlings were then exposed to Fe1 and Fe50 media supplemented with 0.5 μM EBR for 24 h, and thereafter they were transferred to medium supplemented with bromocresol purple for 2 h.

To measure ethylene production, 7-d-old cucumber seedlings grown in Fe-sufficient medium were incubated in Fe50 and Fe1 solutions for 2 d, then treated with 0.5 μM EBR in the Fe50 and Fe1 media for 24 h. After treatment, ethylene emission of cucumber roots was determined. One milliliter of the headspace was taken from the vials, and then injected into a gas chromatograph equipped with an alumina column (GDX502) and a flame ionization detector (GC-7AG; Shimadzu Japan) to measure the ethylene concentration as described previously (Sun et al., 2007).

**Measurement of CHL and Fe content**

Seven-day-old cucumber seedlings pre-cultured in nutrient solution containing 50 μM were transferred to Fe1 and Fe50 media in the absence and presence of 0.5 μM EBR for 1 week. Afterwards, CHL in leaves and Fe content in shoot...
and root were measured. Newly formed leaves were weighed and then ground with aqueous acetone (80% v/v) and centrifuged at 10 000 g for 5 min. Absorbance (A) readings of the supernatant were recorded at 645 and 663 nm. Total chlorophyll (CHL) content (mg L⁻¹) was calculated as 8·02ₐ₆₆₅ + 20·21ₐ₆₄₅, calculated values were expressed as mg CHL g⁻¹ fresh weight. Root and shoot samples were separated and digested with concentrated nitric acid and hydrogen peroxide, and the total Fe content was determined by inductively coupled plasma mass spectrometry.

Expression patterns of genes encoding FRO and IRT

Quantitative real-time PCR (qRT-PCR) was used to investigate the effect of EBR on the expression patterns of genes encoding CsFRO1 (accession number AY590765) and CsIRT1 (accession number AY590764) in cucumber roots. Total RNAs were extracted from roots with Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Promega; Promega, Madison, WI, USA). Total RNAs were reverse-transcribed into First-strand cDNA in a 20-µL volume with M-MLV reverse transcriptase (Promega). The samples that were diluted to 100 µL with water and 5 µL of each sample (approx. 8 ng RNA equivalent) were PCR amplified using SYBR GreenER™ qPCR SuperMix Universal (Invitrogen) in a 25-µL reaction, containing 5 µL diluted cDNA, 12·5 µL SYBR GreenER™ qPCR SuperMix Universal, 0·5 µL Rox Reference Dye, 1 × 10⁻⁶ M forward primer, 1 × 10⁻⁶ M reverse primer and 5 µL water. The Mx3000P™ Real-Time PCR System (Agilent Technology, Santa Clara, CA, USA) was used to run quantitative RT-PCR with the following three primer pair combinations: CsFRO1 5′-TCAAACTAGCGGGAAGAC-3′ and 5′-GG TAGAAAACGGGAGCAT-3′; CsIRT1 5′-GGCATCTTTACTAAAACC-3′ and 5′-CAC CAATGACTCGCAAT-3′.

In addition, a housekeeping gene, CsActin1, was employed as a control: 5′-TTGAAATCCAAGGCGGAATG-3′ and 5′-TGCGACCACTGGCATAAAG-3′ primers were designed across exon–exon junctions of cDNA to avoid potential problems due to contaminating genomic DNA. Amplification efficiency for each primer pair was calculated using serial cDNA dilutions after correcting the cycle threshold values according to the amplification efficiency. The expression values of the two genes were normalized to the Fe50 treatment.

Statistical analysis

Analysis of variance was conducted between the different treatments. Significant differences between treatments were evaluated by LSD multiple range tests (P ≤ 0·05) using the SAS statistical software.

RESULTS

Contrasting effect of EBR on FRO activity of Fe-deficient and Fe-sufficient roots

As shown in Fig. 1A, FRO activity in cucumber roots was negatively correlated with Fe concentrations in the medium. For instance, FRO activity was reduced from 0·44 to 0·06 µM Fe³⁺ g⁻¹ root f. wt h⁻¹ when Fe concentrations in the medium increased from 0 to 50 µM Fe²⁺. To determine whether BR is involved in the Fe deficiency-induced changes in FRO activity, the effect of external supply of EBR at varying concentrations (0–0·5 µM) on FRO activity in cucumber roots grown under Fe-sufficient (50 µM Fe-EDTA, defined as F50) and Fe-deficient (1 µM Fe-EDTA, defined as Fe1) was investigated.
In general, EBR had opposite effects on FRO activity of Fe-deficient and Fe-sufficient seedlings. For instance, EBR at concentrations >0.1 μM significantly enhanced FRO activity of seedlings grown in Fe50 medium (Fig. 1B). In contrast, EBR significantly reduced FRO activity of Fe-deficient seedlings, and the inhibitory effect was positively dependent on the EBR concentrations (Fig. 1B). A similar effect of EBR on FRO activity of Fe-sufficient and Fe-deficient roots was also visualized by changes in colour around roots due to the formation of Fe(II)-ferrozine complex, as shown by the more intense colour of Fe-deficient roots of cucumber seedlings (Fig. 1C).

The involvement of BRs in Fe deficiency-induced changes in FRO activity was further evaluated by investigating the effect of BRz, an inhibitor of BR biosynthesis, on FRO activity of Fe-sufficient and Fe-deficient roots. There was no change in FRO activity of Fe-sufficient roots when treated with BRz. In contrast, FRO activity of Fe-deficient roots was significantly enhanced by the same concentration of BRz (Fig. 2).

No effect of EBR on Fe deficiency-induced acidification of rhizosphere

To test whether BR is involved in the Fe deficiency-induced acidification of rhizosphere, the effect of EBR on medium pH was investigated. A marked reduction in medium pH from 6.0 to 4.8 was observed when cucumber seedlings were cultured in Fe1 medium (Fig. 3A). The Fe deficiency-induced acidification was not affected by addition of EBR (Fig. 3A). Moreover, the pH value in Fe50 medium was not affected by EBR (Fig. 3A). The effect of BR on acidification of rhizosphere in Fe-sufficient and Fe-deficient roots was further visualized by monitoring the colour change of the pH indicator bromocresol purple, which turns yellow once the pH falls below 5.2 (Fig. 3B). As shown in Fig. 3B, the Fe deficiency-induced change in colour was independent of EBR. These results suggest that BR may not be involved in the processes associated with Fe deficiency-dependent acidification of the rhizosphere.

Suppression of Fe deficiency-induced ethylene production by EBR

Previous studies showed that Fe deficiency triggers ethylene production in strategy I plants, and that ethylene plays a regulatory role in response to Fe deficiency (Romera and Alcántara, 2004). To test whether BR may play a role in the Fe deficiency-induced ethylene production, the effect of EBR on ethylene emission from Fe-sufficient and Fe-deficient roots was examined. As shown in Fig. 4, ethylene production from cucumber roots was significantly enhanced by exposing cucumber seedlings to Fe1 medium. Similar to Fe-deficient treatment, EBR can also evoke ethylene production from Fe-sufficient roots (Fig. 4), while the Fe deficiency-induced ethylene production was suppressed by EBR (Fig. 4).

Fe deficiency-induced chlorosis enhancement by EBR

To test further whether BR affects Fe deficiency-induced changes in physiological processes, the effect of EBR on CHL content of cucumber seedlings grown in Fe1 and Fe50 medium was also studied. No apparent chlorosis was observed in young leaves of cucumber seedlings grown in Fe1 medium for 1 week. However, chlorosis became evident when EBR was added to Fe1 medium. In contrast, there was no apparent effect of EBR on leaf chlorosis in cucumber seedlings grown in Fe50 medium. Accordingly, we found that treatment with EBR led to a significant decrease in CHL content of Fe-deficient seedlings, while the same concentration of EBR
had no effect on CHL content in Fe-sufficient cucumber seedlings (Fig. 5B).

**Opposite effects of EBR on Fe contents in shoots and roots**

No significant changes in shoot Fe contents were observed after exposure of cucumber seedlings to Fe1 medium for 1 week (Fig. 6A). In contrast, exposure of cucumber seedlings to the Fe1 medium led to a marked decrease in root Fe contents (Fig. 6B). EBR had contrasting effects on Fe contents in roots and shoots regardless of Fe supply. For instance, treatment with EBR led to decreases in Fe contents in shoots by 30% and 50% in Fe-sufficient and Fe-deficient seedlings, respectively (Fig. 6A), while there were approx. 50% increases in root Fe contents of both Fe-sufficient and Fe-deficient seedlings.
Fe-deficient cucumber seedlings were significantly reduced by EBR (Fig. 7A). The increased CsFRO1 transcript in Fe-sufficient seedlings, while it markedly reduced CsIRT1 transcript in Fe-deficient seedlings (Fig. 7B).

**DISCUSSION**

There is increasing evidence supporting the involvement of BR in physiological processes such as plant growth and development, as well as responses of plants to numerous abiotic and biotic stresses (Sasse, 2003). However, there has been limited information on the role of BR in response of plants to nutrient stress. In the present study, the physiological function of BR in the response of cucumber seedlings to Fe deficiency was evaluated. Our results demonstrate that an exogenous supply of BR (EBR) stimulated the activity of FRO in the Fe-sufficient seedlings, while it suppressed the Fe deficiency-induced FRO activity (Fig. 1). Moreover, our results reveal that EBR downregulated the expression of CsFRO1 in the Fe-deficient cucumber roots. The involvement of ethylene in the response of plants to Fe deficiency in strategy I plants has been demonstrated (Romera and Alcántara, 2004). A functional link between BR and ethylene has been reported in the literature. For instance, BR stimulates ethylene emission in arabidopsis by stabilizing ACS protein (Hansen et al., 2009). Our previous results also showed that exogenous application of BR alleviated the inhibitory effect of salt stress on cucumber-seed germination by stimulating ethylene production (Wang et al., 2011). In the present study, we found that EBR had a contrasting effect on FRO activity of the Fe-sufficient and Fe-deficient seedlings, suggesting that crosstalk between BR and ethylene may exist in the modulation of FRO activity. In addition to the increase in FRO activity, acidification of the rhizosphere is another commonly observed response of strategy I plants to Fe deficiency (Romera and Alcántara, 2004). The observation that Fe deficiency induced a decrease in pH of the growth medium is in agreement with previous reports. Unlike FRO activity, application of EBR had no impact on the Fe deficiency-induced decrease in pH, implying that BR is unlikely to be involved in the acidification of the rhizosphere by Fe deficiency.

Chlorophyll is a major component for chloroplasts and has been used as an indicator of Fe status in plants (Graziano et al., 2002). In the present study, we did not find a significant reduction in CHL content in response to Fe deficiency (Fig. 5). This may be due to a relatively short duration (7 d) used in the present study for exposure of cucumber seedlings to the Fe-deficient medium, as appearance of symptoms associated with Fe deficiency often occurs after longer term of exposure of plants to an Fe-deficient medium (Romera and Alcántara, 2004). Moreover, our findings that the CHL contents in leaves of Fe-sufficient plants were not affected by EBR (Fig. 5) are consistent with those reported in the literature (Yu et al., 2004; Xia et al., 2009). In addition, we found that EBR significantly enhanced leaf yellowing and reduced CHL contents in the Fe-deficient seedlings (Fig. 5), suggesting that BR may negatively regulate CHL contents in the Fe-deficient plants.

Exposure of cucumber seedlings to Fe-deficient medium for 7 d did not affect Fe contents in shoots, while the same treatment led to a significant decrease in Fe contents in roots when treated with EBR (Fig. 6B). In addition to Fe contents, we also determined the total amount of Fe absorbed in plants on the basis of per weight roots and the ratio of Fe contents in shoots to those in roots in the absence and presence of EBR under Fe-sufficient and Fe-deficient conditions. This showed that treatment with EBR reduced the total amount of Fe absorbed in plants and also the ratio of Fe in the shoot to root (Fig. 6C) under both Fe-sufficient and Fe-deficient conditions, suggesting that EBR mainly affects Fe translocation from roots to shoots.

**Regulation of expression of CsFRO1 and CsIRT1 in an Fe-dependent manner**

To assess further the role of EBR in Fe deficiency-dependent changes in physiological processes, we studied the effect of EBR on FRO and Fe transport at the transcriptional level. There were marked increases in transcripts of CsFRO1 and CsIRT1 when cucumber seedlings were exposed to Fe1 solution (Fig. 7A, B). The CsFRO1 transcripts in both Fe-sufficient and Fe-deficient cucumber seedlings were significantly reduced by treatment with EBR (Fig. 7A). Unlike CsFRO1, treatment with EBR had a contrasting effect on transcript of CsIRT1 in Fe-sufficient and Fe-deficient seedlings. For instance, EBR increased CsIRT1 transcript in Fe-sufficient seedlings, while it markedly reduced CsIRT1 transcript in Fe-deficient seedlings.

![Fig. 7](https://example.com/fig7.png)

**Fig. 7.** Effect of EBR on expression of *FRO1* (A) and *IRT1* (B) of cucumber roots. Seven-day-old cucumber seedlings grown in Fe1 and Fe50 for 3 d were treated with 0 μM or 0·5 μM EBR for 24 h. Relative mRNA level was normalized based on the mRNA in roots treated in 50-μM FeEDTA solution. Data are means ± s.e. of three replicates. Means with different letters are significantly different (*P* ≤ 0·05) with regard to treatments within the same Fe supply.
(Fig. 6). The reduced Fe contents in roots have been suggested to act as a cue to initiate a response of plants to Fe deficiency (Bienfait et al., 1987). Fe contents in leaves are usually positively correlated with CHL contents. In the present study, we found that EBR reduced the Fe contents in shoots by 30%, but it had no impact on leaf CHL contents in Fe-sufficient seedlings. It has been shown that a certain proportion of Fe is unavailable to plants because it remains insoluble in the apoplasm of mesophyll cells (Graziano et al., 2002), and that the reduction of Fe(III) in the apoplasm is a prerequisite for Fe transport across the plasma membrane (Brüggemann et al., 1993). EBR and Fe deficiency had similar effect on FRO activity such that application of EBR increased FRO activity in roots of Fe-sufficient cucumber seedlings (Fig. 1B). It is conceivable that EBR presumably may also regulate FRO activity in leaf mesophyll cells, thus leading to enhanced availability of Fe to sustain normal CHL contents. The reduction in Fe contents in shoots under Fe-deficient conditions is likely to account for the positive correlation between shoot Fe concentration and CHL content. In contrast to Fe contents in shoots, exogenous application of EBR significantly increased Fe contents in roots (Fig. 6B). We showed further that EBR markedly suppressed the ratio of Fe contents in roots to those in shoots under both Fe-sufficient and Fe-deficient conditions (Fig. 6C), indicating that EBR may probably enhance Fe uptake and/or suppress Fe translocation from roots to shoots.

In summary, our results demonstrate that BRs are likely to play a negative role in regulating Fe-deficiency-induced FRO, expressions of CsFRO1 and CsIRT1, as well as Fe translocation from roots to shoots. Our results also highlight that crosstalk between BRs and ethylene may be involved in regulating the response of plants to Fe deficiency, which warrants further investigation.

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LITERATURE CITED


