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The effects of ethylene on the HCl-extractability of trace elements during soybean seed germination

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ABSTRACT

Background: Ethylene is capable of promoting seed germination in some plant species. Mobilization of metals such as Fe, Cu, Mn, and Zn in mature seeds takes place when seeds are germinating. However, whether ethylene is involved in the regulation of soybean seed germination and metal element mobilization during early seed germination stage remains unknown. In the present study, seeds were treated with ethylene synthesis inhibitor aminoethoxyvinylglycine (AVG) and ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and double distilled H_2O (ddH_20) treatment was used as control. Ethylene emission, ACC synthase (ACS) expression, ACS enzyme activity and Ca, Zn, Mn, Cu and Fe content in hypocotyls were qualified to analyze the relationship between ethylene and mobilization of these elements.
Results: The results showed that ACS expression, ACS enzyme activity and ethylene emission peaked at 1 and 7 d after sowing. AVG inhibited ethylene production, promoted the hypocotyls length, ACS expression and its activity, concentrations of total and HCl-extractable Zn, and HCl-extractable Fe in hypocotyls, while ACC caused opposite effects. AVG and ACC treatment had no significantly effects on total and HCl-extractable Ca, Cu and HCl-extractable Mn. Total Mn concentration was promoted by AVG at 1, 3, and 5 d significantly, while ACC treatment tended to have no significantly effects on Mn concentration.
Conclusion: These findings suggested that ethylene is at least partly involved in the regulation of soybean seed germination. Remobilization of Zn and Fe may be negatively regulated by ethylene.

1. Introduction

Seed germination involves regulation of a series of metabolic processes by plant hormones [1]. In some plant species, including soybean (*Glycine max* (L.) Merr.), it has been shown that detectable ethylene production begins with the onset of germination, *i.e.*, with radicle emergence [2]. Moreover, many reports indicate that ethylene promotes seed germination [3,4]. The triple response, as described by Neljubov [5], indicates the plant sensitivity to ethylene. Understanding the mechanisms underlying the control of seed germination and its regulation by ethylene is not only of academic interest, but is also important for improving crop production and yield [6].

Minerals accumulated during seed development constitute less than 3% of the seed dry mass, yet they form an important pool of essential nutrients [7]. These mineral reserves are mobilized during germination and are a source of cofactors for enzymes, which are required for rapid growth [8]. The cotyledon of a soybean seed is very important for seed germination, seedling establishment, as well as growth and survival because it serves as the main nutrient resource for young seedlings [1]. Large seed reserves of mineral nutrients may be of importance in order to support plant adaptation in micronutrient-deficient soils. Mobilization of metals such as Fe, Cu, Mn, and Zn in mature seeds takes place during germination and early seedling development. However, there is limited knowledge regarding the transport mechanisms of these immobile metal elements during the process of seed germination.

A large number of solutions have been used for metal extraction in order to assess the bioavailability of trace elements [8]. Single extractants may broadly be divided into three main classes: (1) weak replacement ion salts: $MgCl_2$, $CaCl_2$ and NH_4NO_3, (2) dilute solutions of either weak acids: acetic acid, or strong acids: HCl and HNO_3; and (3) chelating agents: pentetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) [9]. The chelating agents DTPA and EDTA reduce the activity of the free metal ions in solution by forming complexes with the free metal ions. The second type, acid extractants are able to release into solution and can be considered as bioavailability [10,11]. Input of total and extractable metals in the growing hypocotyls is vital for enhancing seed germination and seedling growth. The transport and bioavailability of immobile metal element are dependent on a number of factors in addition to plant

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Table 1
The primers used for RT-PCR and qRT-PCR amplification of GMACS gene.

Target gene	Primer	Primer sequence (5′–3′)	Length (bp)	GC %	Amplification length (bp)
Actin	Actin-F	5′-ACCTCGACATACTGGTGTTATGGTT-3′	25	44.00	
	Actin-R	5′-ATACCTCTTTTGGATTGGGCTTC-3′	23	43.40	81
ACS	ACC-F	5′-CACCTCAAATCCCGGTCAA-3′	19	54.55	
	ACC-R	5′-AGCAACTGGAGCACACGAAG-3′	20	40.00	105

growth regulators [12,13]. Little information is available regarding the ethylene regulation of immobile metal element transport during soybean seed imbibition and germination by far. The current study was carried out to examine ethylene metabolism, and its influence on the mobilization and bioavailability of immobile metal element during soybean seed germination, as well as to determine the dynamics of Fe, Mn, Zn, Ca, and Cu mobilization in hypocotyls.

2. Materials and methods

2.1. Materials and treatments

Soybean seeds of Tiefeng-31 were imbibed in 100 mg/L of aminoethoxyvinylglycine (AVG), 75 μmoL/L of 1-aminocyclopropane-1-carboxylic acid (ACC) and double-distilled H_2O (ddH_2O), respectively for 4 h and sowed in vermiculite. After thorough watering, the seeds were incubated in an illumination incubator at 25°C and 65% relative humidity for 7 d. Each treatment was performed in triplicates (100 seeds). At 1, 3, 5 and 7 d after sowing, hypocotyls of three treatments were sampled for further analysis.

2.2. Ethylene qualification

Fresh hypocotyls (0.5 g fresh weight) were sealed in an 8 mL vial and incubated for 4 h in darkness at 30°C. Then, the accumulated ethylene was quantified by gas chromatography with a glass column (2 mm × 2 m) of Porapak N (Waters, Milford, MA, USA) at 80°C [14]. Peak areas were determined with a Chromatopak C-R6A system (Shimadzu, Kyoto, Japan).

2.3. Quantification of ACC synthase (ACS) activity

ACS was extracted and assayed using high performance liquid chromatography (HPLC) as previously described [15].

2.4. Quantification of ACS expression by RT-PCR and qRT-PCR

Total RNA was extracted from 100 mg hypocotyls using TRIZOL Reagent method (Invitrogen, Carlsbad, CA). The first strand cDNA synthesis and RT-PCR were performed using One Step RNA PCR kit (Takara Biochemicals, Kyoto, Japan) using oligo-dT and random oligonucleotide primers, followed by amplification of the resulting DNA using polymerase chain reaction. The reaction was performed in an Eppendorf master cycler, which began with an initial denaturation step at 95°C for 3 min, followed by 35 cycles of 15 s at 94°C, 30 s at 45°C, 1.5 min at 72°C, and a final 7 min extension at 72°C. qRT-PCR analysis was performed following the method described by Cheng [14]. Primers used for RT-PCR and real-time amplification of GMACS cDNA were designed according to the sequences of soybean ACC (GMACS) gene. The primer sets are listed in Table 1.

2.5. Quantification of total and HCl-extracted Ca, Zn, Mn, Cu and Fe

Ca, Zn, Mn, Cu and Fe in hypocotyls were quantified using dry ashing and atomic absorption spectrometry method described by Altundag [16]. Ca, Zn, Mn, Cu and Fe were extracted with 0.03 M HCl solution described by Maki [17]. Then, their concentration was determined with atomic absorption spectrometry [16].

2.6. Data collection and statistical analysis

All experiments were conducted in triplicates. Statistical analysis was carried out with ANOVA process of SAS version 8.01 (SAS Institute, Inc., Cary, NC, USA). Means were compared using least significant difference (LSD) *t*-test at the 5% level of significance.

3. Results

3.1. Effects of ACC and AVG treatment on hypocotyls growth

Dry weights and lengths of hypocotyls in germination seeds were measured during the various stages of seed germination (Table 2). In the control, the hypocotyls length increased from 0.22 ± 0.02 cm at 1 d to 4.50 ± 0.24 cm at 7 d, and their dry weight increased from 0.0076 ± 0.0005 g at 1 d to 0.0208 ± 0.0015 g at 7 d. The results obtained from AVG and ACC treatments indicated that: a) AVG treatment strongly promoted hypocotyl length; b) the growth of hypocotyl length appeared to be more sensitive to AVG treatment after 5 to 7 d of incubation (the hypocotyl elongation stage); c) the effects of ACC on hypocotyls length were opposite to that caused by AVG, and d) in contrast to hypocotyl lengths, hypocotyl dry weight appeared to be insensitive to AVG or ACC treatments.

Table 2
Changes of hypocotyls growth, ACS expression by qRT-PCR, ACS activity, ethylene production during seed germination stage.

Treatment	Time (d)	Hypocotyls length (cm)	Hypocotyls dry weight (g)	ACS expression	ACS activity (nmoL/g·h)	Ethylene (ppm/h·g)
AVG	1	0.22 ± 0.01 g	0.0075 ± 0.0002 d	0.180 ± 0.042 g	206.88 ± 10.20 fg	4.21 ± 0.23 e
	3	1.06 ± 0.03 f	0.0097 ± 0.0003 cd	0.120 ± 0.004 g	144.41 ± 5.11 hg	1.90 ± 0.15 f
	5	4.57 ± 0.176 b	0.0142 ± 0.0019 b	0.112 ± 0.022 g	35.59 ± 2.15 i	0.85 ± 0.07 f
	7	8.49 ± 0.15 a	0.0211 ± 0.0013 a	0.890 ± 0.039 e	254.60 ± 2.67 ef	11.85 ± 0.28 d
Control	1	0.22 ± 0.02 g	0.0076 ± 0.0005 cd	1.003 ± 0.006 d	355.20 ± 27.43 e	12.41 ± 0.57 d
	3	1.02 ± 0.02 f	0.0095 ± 0.0004 cd	0.333 ± 0.007 f	53.46 ± 1.11 hi	2.74 ± 0.28 ef
	5	2.57 ± 0.19 d	0.0141 ± 0.0033 b	0.147 ± 0.009 g	34.72 ± 1.80 i	1.86 ± 0.13 f
	7	4.50 ± 0.24 b	0.0208 ± 0.0015 a	1.523 ± 0.058 c	785.23 ± 7.40 d	20.75 ± 0.91 b
	1	0.22 ± 0.02 g	0.0081 ± 0.0003 cd	1.776 ± 0.106 b	1234.68 ± 195.38 b	21.59 ± 0.66 b
ACC	3	1.01 ± 0.02 f	0.0099 ± 0.0012 c	0.943 ± 0.022 de	871.27 ± 22.06 cd	11.60 ± 0.75 d
	5	1.75 ± 0.25 e	0.0149 ± 0.0007 b	1.458 ± 0.028 c	946.31 ± 29.13 c	16.95 ± 2.30 c
	7	2.850 ± 0.19 c	0.0211 ± 0.001 a	2.807 ± 0.055 a	1436.97 ± 71.45 a	39.12 ± 3.08 a

Data represent the mean ± SD. Different letters within the same column indicated significant difference at 5% level by LSD.

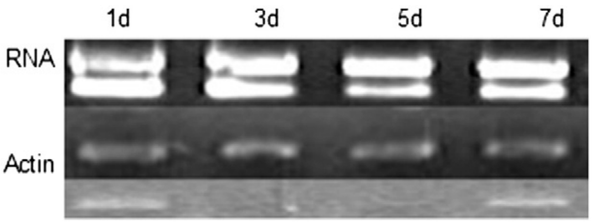

Fig. 1. ACS expression changes during seed germination stage by RT-PCR analysis.

3.2. Effects of ACC and AVG treatments on ethylene emission

To analyze the effects of ACC and AVG treatments on ethylene emission, GMACS expression, ACS activity and ethylene emission were determined. Maximum of GMACS mRNA, ACS activity and ethylene production were observed from 1 d to 7 d (Fig. 1, Table 2). High value of ethylene emission exhibited an early onset at 24 h, followed by a 5-fold and 10-fold decline respectively at 3 d and 5 d, and subsequently reached the maximum level at 7 d. In agreement with ethylene changes, GMACS expression peaked at 1 d and 7 d (Fig. 1, Table 2). Similarly, ACS activity showed maximum activity at 7 d in the control. GMACS expression, ACS activity, as well as ethylene production were detectable in the hypocotyls from 1 d to 7 d in AVG treatment, and the levels of these peaked at 7 d. The levels of ACS activity and ethylene were respectively 3-fold and 2-fold lower than those obtained in the control (Table 2). On the other hand, in comparison to the control, ACC treatment led to a 2-fold increase in ACS activity and ethylene production after 7 d (Table 2). GMACS expression was in accordance with the initial stimulation of ACS activity and ethylene production.

3.3. Total and HCl-extractable Ca, Zn, Mn, Cu and Fe in hypocotyls

In the AVG, ACC and control treatments, Ca, Zn, Mn, Cu and Fe concentrations differed from each other in different degrees (Fig. 2). In general, total Zn concentration increased with time, and it was significantly promoted and deduced by AVG and ACC treatments respectively at 5 d and 7 d compared with the control (Fig. 2). At the same sampling day, AVG and ACC treatments had no significant effects on total Ca and Cu (Fig. 2). AVG treatment significantly

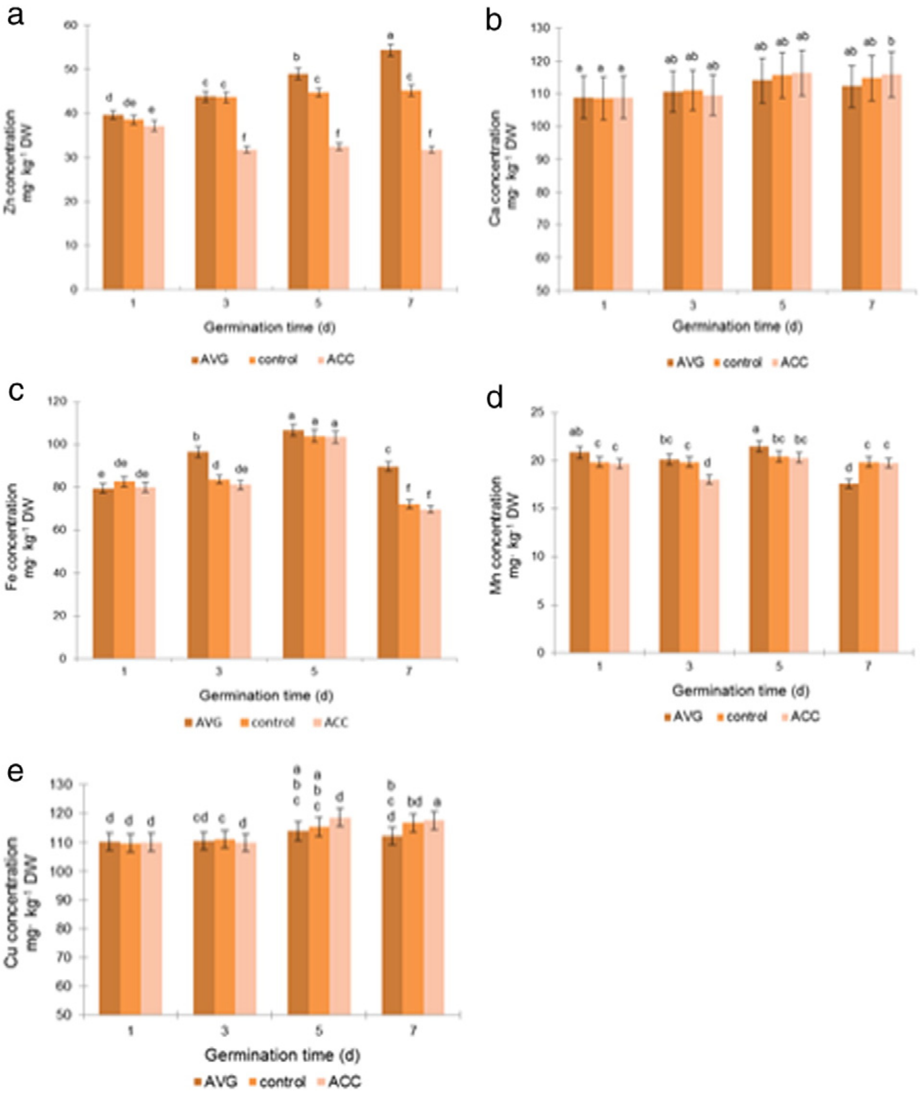

Fig. 2. The effect of AVG and ACC treatments on total Zn, Ca, Fe, Mn and Cu concentration in the hypocotyls during soybean seed germination. a: Total Zn concentration in the hypocotyls after AVG and ACC treatments; b: Total Ca concentration in the hypocotyls after AVG and ACC treatments; c: Total Fe concentration in the hypocotyls after AVG and ACC treatments; d: Total Mn concentration in the hypocotyls after AVG and ACC treatments, and e: Total Cu concentration in the hypocotyls after AVG and ACC treatments. Data represent the mean ± SD. Different letters within the same figure indicated significant difference at 5% level by LSD.

promoted total Fe concentrations at 7 d compared with the control, while ACC treatment had no significant effects on total Fe concentration (Fig. 2). Compared with the control, AVG treatment promoted Mn concentration at 1, 3, and 5 d significantly, while ACC treatment tended to have no significant effects on Mn concentration (Fig. 2). The AVG treatment promoted HCl-extractable Zn and Fe concentration in hypocotyls, while ACC treatment showed opposite effects (Fig. 3). HCl-extractable Zn and Fe in AVG treatment were significantly higher than the control at 7 d (Fig. 3). In contrast, those in ACC were significantly lower than the control at 7 d (Fig. 3). Compared with the control at the same sampling day, AVG and ACC treatment had no significant effects on HCl-extractable Ca, Mn and Cu (Fig. 3). Taken together, total Zn, HCl-extractable Zn and Fe concentration were promoted and deduced by AVG and ACC treatment respectively in hypocotyls of soybean seeds.

4. Discussion

4.1. Ethylene is at least partly involved in the regulation of soybean seed germination

Numerous studies demonstrate that the ability to germinate correlates with ethylene production, suggesting that ethylene is involved in the regulation of seed germination and dormancy [4,18]. However, Gianinetti *et al.* [19] concluded that endogenous ethylene is not required for dormancy breakage in many species, and germination does not strictly depend on ethylene produced by the seed itself. Increased ethylene production during germination is associated with an increase in ACO activity, as well as a progressive accumulation of ACS and ACO transcripts [6,20]. Our study showed that an early endogenous ethylene emission peak was observed at 1 d after seed sowing, followed by radicle protrusion through the seed coat, and the maximum of ethylene emission was observed at 7 d. After ethylene biosynthesis was promoted by ethylene precursor ACC, hypocotyl dry weight increased significantly (Table 2). However, in AVG and control treatment, there was no significant difference in hypocotyl dry weight at the same day after sowing. In the control, levels of GMACS expression and ACS activity were in accordance with ethylene production at 1 and 7 d. Taken together, these results indicated that ethylene was at least partly involved in the regulation of seed germination and hypocotyl elongation, and soybean seed germination was associated with an increase in transcripts and activity of ACS.

4.2. Ethylene and Zn remobilization during soybean seed germination

Zinc (Zn) is essential for many plant functions as it works as a metal cofactor in transcription factors and other enzymes of DNA metabolism. Zn is required for ethylene response because it is a component of ethylene receptor. Month-old Zn-deficient tomato plants hardly respond to ethylene even overnight, and show no ethylene-induced epinasty. Thus, Zn deficiency might lead to

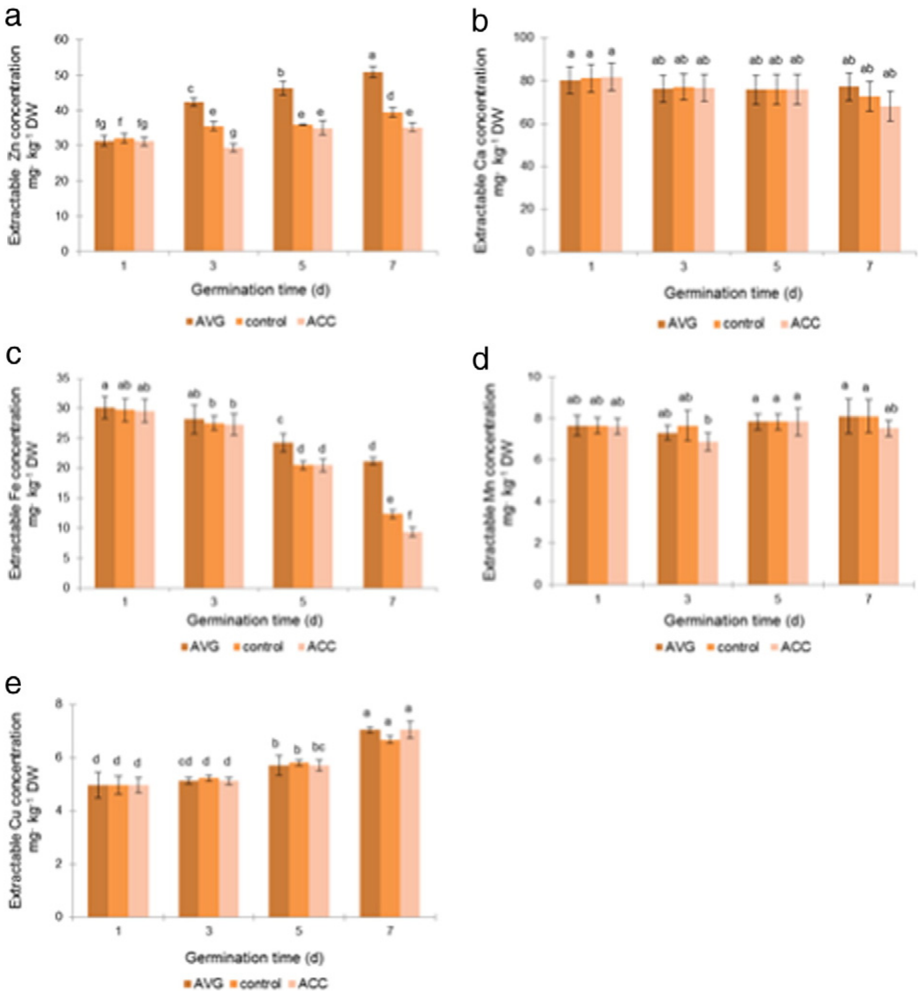

Fig. 3. The effect of AVG and ACC treatments on HCl-extractable Zn, Ca, Fe, Mn and Cu in the hypocotyls during soybean seed germination stage. a: HCl-extractable Zn level in hypocotyls after AVG and ACC treatments; b: HCl-extractable Ca level in hypocotyls after AVG and ACC treatments; c: HCl-extractable Fe level in hypocotyls after AVG and ACC treatments; d: HCl-extractable Mn level in hypocotyls after AVG and ACC treatments, and e: HCl-extractable Zn level in hypocotyls after AVG and ACC treatments. Data represent the mean ± SD. Different letters within the same figure indicated significant difference at 5% level by LSD.

reduced ethylene-mediated response [21]. It has also been suggested that there is a substantial remobilization of Zn from seed pools into the developing roots (radicle) and coleoptile during wheat seed germination [22]. However, the effect of ethylene on Zn uptake or remobilization remains unknown. In the present study, the total and HCl-extractable Zn concentration in hypocotyls increased with seed germination time in the control, and the results confirmed that there existed a remobilization of Zn from seed pools into hypocotyls. Total Zn and HCl-extractable Zn concentration were promoted and deduced by AVG and ACC treatment respectively (Fig. 2, Fig. 3). As a component of ethylene receptor, promoted total and HCl-extractable Zn concentration in AVG treatment may be beneficial for the sensibility maintenance of ethylene receptor. Similarly, reduced HCl-extractable Zn concentration may weaken ethylene receptor sensibility in ACC treatment. In brief, Zn remobilization may be negatively regulated by ethylene, and Zn remobilization likely in turn kept the sensibility of ethylene receptor to ethylene within a reasonable limit in order to adapt to restricted nutrition circumstance.

4.3. Ethylene and Fe remobilization during soybean seed germination

Iron (Fe) plays an important role in the respiration and photosynthetic processes of plants. It is present in several enzymes of redox system and is also implied in many enzymatic systems [21]. It has been shown that ACC addition to *Arabidopsis*, tomato, and cucumber plants enhanced the expression of genes that respond to low Fe supply and mediate Fe uptake and assimilation [23]. On the contrary, ethylene production increases under Fe deficiency in the roots of several dicots and non-grass monocots [24]. In general, HCl-extractable Fe showed obvious declining trend (Fig. 3) due to the rapid increase of hypocotyl dry weight. Total and HCl-extractable Fe concentration was promoted and deduced by AVG and ACC treatment in varying degrees respectively (Fig. 2, Fig. 3). The results likely indicated that Fe remobilization was negatively regulated by ethylene, which was not consistent with the previous reports [25]. Mobilization of vacuolar Fe stores is crucial to support *Arabidopsis* early development until efficient systems for Fe acquisition from the soil take over [26]. In the present study, Fe reserves in cotyledon of seeds may be enough to meet the early development need of seedling. Thus, ethylene may promote Fe remobilization or uptake only when Fe deficiency was obvious. When Fe stores in seeds were abundant during seeds germination, ethylene may have a negative effect on Fe remobilization, and its detail mechanism needs further research.

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Conflict of interest

The authors declare that they have no conflict of interest.

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