

# Phytotoxicity and Iron Homeostasis Disturbance of Bismuth on *IRT1* Promoter Knockout *Arabidopsis thaliana*

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**Abstract:** Bismuth (Bi) is used in semiconductors and water pipes as a substitute for lead. The Bi concentration in the soil has not been investigated, and there is no environmental quality standard for Bi in Japan. We previously reported Bi accumulation and phytotoxicity in *Arabidopsis thaliana* (*A. thaliana*) and *Solanum lycopersicum*, which were presented that Bi disturb iron (Fe) homeostasis in both plants. However, the mechanism of Bi phytotoxicity remains unclear. We examined the toxic effect of Bi for the expression of Fe-related genes in *A. thaliana*. The microarray analysis revealed an increase of the Fe regulation cascade including the subgroup Ib transcription factor. We tried to clear the effect to Fe homeostasis using the *Iron-Regulated Transporter 1* (*IRT1*) promoter knockout line. Bi inhibits the root growth and lateral root development, while Bi induces Fe contents. In wild type, Fe was located in the stem cell niche (SCN) without Bi condition. Fe location in *IRT1* promoter knockout line was disturbed by 2  $\mu$ M Bi treatment. Moreover, the cell death in root was found in 2  $\mu$ M Bi treated-roots. These results suggest that Bi disturbed the Fe homeostasis, Fe overaccumulation enhanced cell death in *A. thaliana*, and Bi damaged SCN in the root tip.

**Keywords:** Bismuth (Bi), Iron (Fe), Iron-Regulated Transporter 1 (*IRT1*), Stem Cell Niche (SCN), Quiescent Center (QC)

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## 1. Introduction

Bismuth (Bi) is considered as one of the minor metals. In Japan, Bi is used as an ingredient in some pharmaceuticals and is included in the Japanese Pharmacopoeia 17<sup>th</sup> edition (JP17) [1]. Bi subnitrate is an antiulcer drug with gastrointestinal mucosa convergence due to the protection of the alimentary canal mucosa [2]. Bi is also a used medicine in Sri Lanka, where Bi subsalicylate is used as a gastrointestinal medicine [3]. Aside from its pharmaceutical uses, Bi is also used in semiconductors and water pipes as a lead (Pb) substitute in Japan.

The toxicity of excess Bi in animals has been previously reported, and Bi has lower toxicity compared to other metals such as Pb [4, 5]. However, there is no environmental quality standard for Bi in Japan. To date, there are some reports about Bi concentration in the soil worldwide. In Japan, Bi was detected from the soil and the river around the Bi smelting areas [5]. Furthermore, Bi drained from metalliferous mining and smelting areas was also detected

from a rice paddy [4]. In England, Bi drained from metalliferous mining and smelting areas was detected from the soil and the pasture herbage [6]. In Brazil, Bi was detected in fertilizers [7]. However, little is known about its phytotoxicity in plants [8].

Recently, we reported Bi accumulation in *Arabidopsis thaliana* (*A. thaliana*) [9] and *Solanum lycopersicum* (*S. lycopersicum*) [10]. In wild type *A. thaliana*, the root elongation and shoot growth were significantly inhibited by Bi in a dose-dependent manner. The Bi concentration in the root was approximately 70 nmol g<sup>-1</sup> fresh weight (FW), which was seven-fold higher compared to the shoot in 2  $\mu$ M Bi-treated plants. Furthermore, Bi increased the iron (Fe) content in the root. Microarray analysis revealed candidate genes such as iron-regulated transporter 1 coding gene (*IRT1*). *IRT1* is essential for ferrous Fe uptake, which mainly depends on *IRT1* in the root of *A. thaliana* [11]. In *S. lycopersicum*, Bi also inhibited the growth and accumulated in a dose-dependent manner [10]. However, Bi decreased the Fe content in the root. These results suggest that Bi might

disturb Fe homeostasis in plants. However, the mechanism of Bi phytotoxicity remained unclear.

Fe is an essential nutrient for plants. Fe functions as a cofactor of many enzymes and plays an important role in the processes such as respiration or photosynthesis. However, the excess Fe is toxic for plants, because it generates the hydroxyl radical by the Fenton reaction [12]. Thus, plants strictly regulate their Fe content in order to avoid both deficiency and excess in Fe [13, 14]. Fe regulatory cascade, including the expression of *IRT1*, has been reported in *A. thaliana* [15]. The bHLH transcription factor plays a key role in the Fe regulatory cascade. The subgroup IVc bHLH transcription factors bHLH34, bHLH104, ILR3 (bHLH105), and bHLH115 are involved in activating the subgroup Ib genes [16–18]. The E3 ligase BTS degrades the subgroup IVc bHLH transcription factors [15, 19, 20]. The FER-like iron deficiency-induced transcription factor (FIT) is induced by Fe deficiency and forms a heterodimer with the subgroup Ib bHLH transcription factors (bHLH38, bHLH39, bHLH100, and bHLH101) to activate the transcription of *IRT1* and Ferric reduction oxidase 2 (FRO<sub>2</sub>) during Fe deficiency [21, 22]. However, the feedback mechanism of this cascade when there is excess in Fe remains unclear.

*A. thaliana* has a stem cell niche (SCN) in the root tip, and it includes four Quiescent center (QC) cells [23]. The root tissue is known to be maintained by SCN. The stem cell divides in the meristem zone (MZ) and produces cells to constitute a root. There is a transition zone (TZ) in the base of MZ, TZ stops cell division, and the root cell starts elongation and differentiation. Finally, the cell completes the differentiation program [24]. Fe has been found to be located to the SCN of the root in *A. thaliana* [25]. As described above, we reported the growth inhibition of the root and the effect of the Fe-related gene expression in the presence of Bi. Thus, we hypothesized that Bi influences the SCN of the root.

In this study, we examined Bi toxicity effect for the expression of Fe-related genes in *A. thaliana*. Also, we tried to clear the effect to the root growth and Fe homeostasis using the *IRT1* promoter knockout line. In addition, we investigated the Bi damage to the SCN and the induction of cell death in the root.

## 2. Materials and Methods

### 2.1. Plant Material and Growth Conditions

The seeds of *Arabidopsis thaliana* cv. Col-0 was obtained from Inplanta Innovations, Inc. (Kanagawa, Japan), and the *IRT1* promoter knockout line (SALK\_024525) was obtained from the *Arabidopsis* Biological Resource Center (ABRC). The seeds were surface sterilized in 70% ethanol for 2 min and in 10% commercial bleach with detergent (Kitchen Haite, Kao, Tokyo, Japan), which includes sodium hypochlorite (NaOCl), alkyl ether sulphate sodium salt, and NaOH for 7 min. The seeds were then rinsed with sterilized water five times. The seeds were planted in a Murashige and Skoog (MS) medium containing B5 vitamins with 0.8% agar and 1% sucrose [26]. The MS agar plates were supplemented with various

concentrations of Bi(NO<sub>3</sub>)<sub>3</sub>. The plate without Bi was used as a control. All cultures were maintained at 23°C under a 16 h light and 8 h dark cycle. Plants were grown in an MLR-350 growth chamber (Sanyo, Osaka, Japan) for 2 weeks.

### 2.2. RNA Isolation and Microarray Assays

After the incubation under the treatments described above, the total RNA from two biological replicates for the control and 2 μM Bi-treatment plants were isolated using a RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA concentrations and integrity were assessed spectrophotometrically.

The microarray analysis was performed with *Arabidopsis* 4x44K Ver. 4.0 (Agilent Technologies). The RNA was amplified using Affymetrix labeling according to standard Affymetrix protocols (Low Input Quick Amp Labeling Kit, Agilent Technologies). Hybridization, labeling, scanning, and data extraction were also performed according to standard Affymetrix protocols. Iron-related genes were selected, and the *Arabidopsis* Genome Initiative (AGI) list and calculated ratios between the control and Bi-treated plants were presented in Table 1.

**Table 1.** Effect of Bi on expression of Fe-related genes in wild-type.

Genes	Systematic Name	Fold Change
Subgroup IVc bHLH genes		
<i>bHLH34</i>	AT3G23210	1.03
<i>bHLH104</i>	AT4G14410	1.03
<i>bHLH115</i>	AT1G51070	0.99
<i>ILR3</i>	AT5G54680	0.96
Subgroup Ib bHLH genes		
<i>bHLH38</i>	AT3G56970	21.8
<i>bHLH39</i>	AT3G56980	8.06
<i>bHLH100</i>	AT2G41240	180
<i>bHLH101</i>	AT5G04150	19.1
Fe homeostasis regulation		
<i>FIT</i>	AT2G28160	1.09
<i>BTS</i>	AT3G18290	3.85

### 2.3. Inhibition of Growth and Root Formation of *IRT1* Promoter Knockout Line

The five-seeds of the *IRT1* promoter knockout line were grown in MS agar plates. After 2 weeks incubation, the germination rates were calculated, and the plants were photographed. The root length was then determined, and plants were harvested. The plants were separated to the shoot and root and their fresh weights (FWs) were measured. The development of the lateral root was evaluated over one cell from the epidermis of the root, and the number of lateral roots were counted. The length of the long side of the cell in the root of the elongation differentiation zone (EDZ) was measured. A total of 20 cells from each cell of the EDZ were measured and its mean was estimated n=1. All experiments were repeated at least three times.

### 2.4. Phytotoxicity of Bi to *IRT1* Promoter Knockout Line

Bi phytotoxicity was estimated using Lugol and Evans blue staining. Roots treated or untreated with Bi<sup>3+</sup> were fixed

and dehydrated using acetic acid and ethanol solution for 1 h. Subsequently, the roots were replaced by sequentially soaking them in an ethanol series (70%/PBS and 30%/PBS) each for 5 min. After the treatment, the solution was replaced by PBS without ethanol and the roots were soaked for 5 min. The whole plants were then stained with Lugol solution (Sigma-Aldrich Japan, Tokyo, Japan) for 10 min or with 0.5 g L<sup>-1</sup> Evans blue solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 10 min. The roots were washed three times with PBS and immersed in a tissue-clearing reagent, TOMEI (Tokyo chemical Industry Co., Ltd., Tokyo, Japan), for photograph [27]. All figures represented the staining detected in whole plants of three independent experiments.

### 2.5. Determination of Bi and Fe Contents in *IRT1* Promoter Knockout Line

After 14 days, plants were harvested and were separated into shoots and roots. The Bi and Fe concentrations in various tissues of control and the Bi treatment were determined by atomic absorption spectrophotometry (ContraAA700, Analytic Jena, Germany) after digesting the samples with concentrated nitric acid [28, 29].

### 2.6. Effect of Bi to the Expression of *IRT1* in Root

After incubation under the treatment described above, total RNA from roots was isolated using a RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. First-strand complementary DNA was synthesized from 1 µg of total RNA using the oligo dT (18) primer and random primers (ReverTra Ace, TOYOBO, Japan).

PCR reactions were carried out using *Act8* (At1G49240) as the reference gene. *IRT1* (AT4G19690) gene sequences from *Arabidopsis thaliana* have been published in the NCBI database (<http://www.ncbi.nlm.nih.gov/pubmed>). Sequences were chosen using the Primer3 software to design primers [30]. qRT-PCR was performed using the Thunderbird SYBR qPCR Mix (Toyobo, Japan) and CFX96 (Bio-Rad, Tokyo, Japan). The primer sequences used for *IRT1* were as follows:

5'- AAGCTTTGATCACGGTTGG -3' (forward) and

5'- TTAGGTCCCATGAACTCCG -3' (reverse). For *Act8*,

the sequences were as follows:

5'- GATCACAGCTCTTGCCCCG -3' (forward) and

5'- ACAGTCCAATTTTACCTGCTGGA -3' (reverse).

### 2.7. Effect of Bi to Fe Distribution in the *IRT1* Promoter Knockout Line

The five-seeds of the *IRT1* promoter knockout line were grown in MS agar plates. After 2 weeks of incubation, the lateral roots number from primary to tertiary were determined. Roots were fixed and dehydrated as described above. The solution was replaced by Perls, and the roots were vacuumed for 30 min and left standing in a room condition for 1 h. The roots were washed three times with MilliQ water and were determined in EDZ using a microscope (IX83,

Olympus, Tokyo, Japan). All figures are represented the staining detected in the roots of three independent experiments.

### 2.8. Statistical Analysis

All experiments were performed at least three times. Data are presented as means of the three replicates. Differences between each treatment were determined by Student's *t*-tests. A *P*-value <0.05 was considered significant.

## 3. Results

### 3.1. Bi Response Genes in a Wild Type *Arabidopsis*

To identify the iron-related genes expression levels between those with or without Bi treatment, a microarray analysis was performed. The expression levels of iron-related genes, such as *BTS*, IVc *bHLH*, Ib *bHLH*, and *FIT*, were shown in Table 1. In Bi-treated plants, *BTS*, which was one of the important regulators of the *IRT1* expression cascade, increased the coding gene (Accession no. NC\_003075.7) in the NCBI database for about 3.8-fold compared with the control. In the subgroup IVc *bHLH*, including *bHLH34* (Accession no. NC\_003074.8), *bHLH104* (Accession no. NC\_003075.7), *bHLH105* (*ILR3*, Accession no. NC\_003076.8), and *bHLH115* (Accession no. NC\_003070.9), which were degraded by the E3 ligase *BTS*-dependent degradation [19, 20], the expression levels remained the same (Table 1). It has been reported that when the expression of subgroup IVc *bHLH* was suppressed, the expression of subgroup Ib, such as *bHLH38* (Accession no. NC\_003074.8), *bHLH39* (Accession no. NC\_003074.8), *bHLH100* (Accession no. NC\_003071.7), and *bHLH101* (Accession no. NC\_003076.8) was increased [31]. It was shown that the expression levels of subgroup Ib increased (Table 1).

### 3.2. Effect of Bi on Growth of *IRT1* Promoter Knockout Line

*Arabidopsis* seeds of the *IRT1* promoter knockout line (*IRT1*) were sowed in a MS agar medium with various Bi concentrations in a growth chamber. After 2 weeks of incubation, the germinate plantlets were counted and the phenotypes were observed (Figure 1). The germination ratio was decreased to approximately 50% in the presence of a 3.0 µM Bi and was 0% when seeds were exposed to a 6.0 µM Bi. The 1 µM Bi-treated plantlets were inhibited the root growth. Among the 2 µM Bi-treated plantlets, the structure of the shoot and root were formed and the tissues were friable to harvest. In the root, there was no significance between the root lengths of control and 0.1 µM Bi-treated plants. However, the root lengths of 2 µM Bi-treated plantlets were about 50% more than the control. The 2 µM Bi-treated root lengths were decreased by a Bi dose-dependent manner. The plantlets were then separated into shoots and roots, and the FWs were measured. The FWs of the shoots and roots were noticeably lower for 4 or 5 µM Bi-treated plants. The 5 µM Bi-treated plant roots failed to extend.

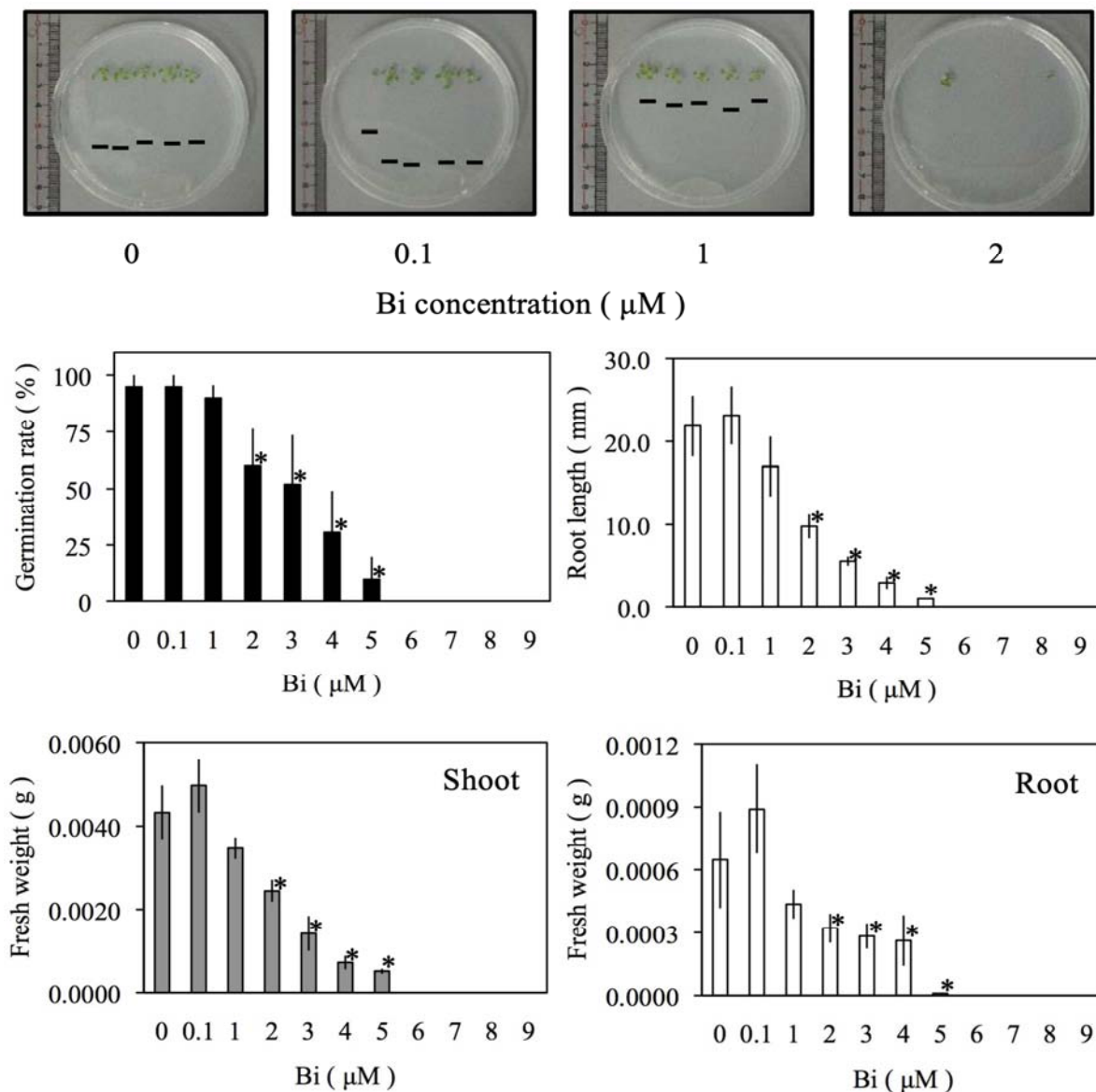


Figure 1. Effect of Bi to the phenotype and the growth of *IRT1* promoter knockout *Arabidopsis*.

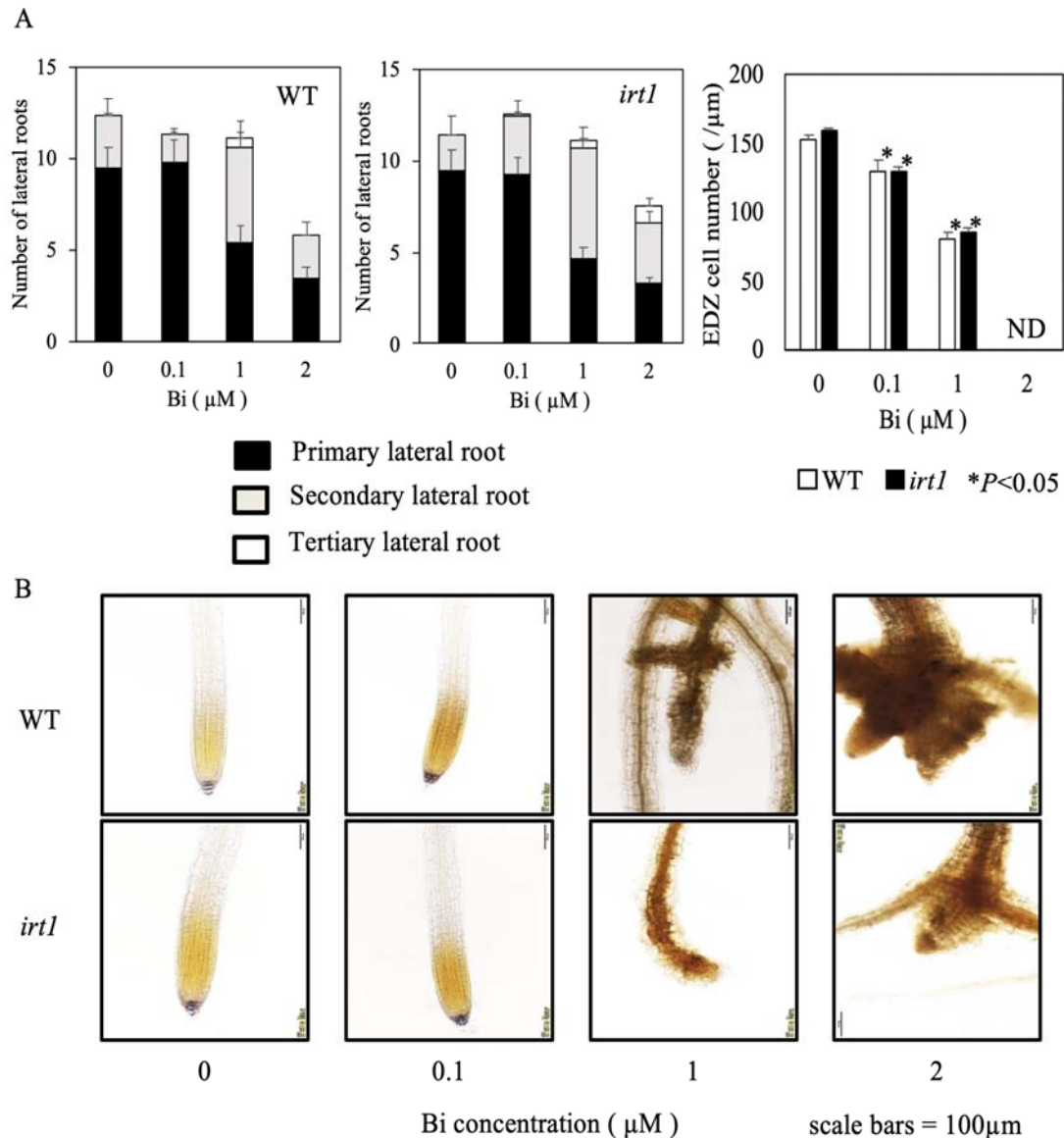
Two-week-old plantlets were photographed. The effect of Bi on the germination of seeds was evaluated by counting the germinated seedlings. The effect of Bi on the seedling was evaluated by measuring the root length and the fresh weight of plant tissues. Two-week-old plantlets were separated to shoot and root. The fresh weight of each tissue was measured immediately. Data are means  $\pm$  SE ( $n=3-10$ ). Asterisks indicate a significant difference from Bi-treated plants and control plants ( $p<0.05$ ).

### 3.3. Effect of Bi to the Root Formation of *IRT1* Promoter Knockout Line

In the lateral root development, there were no differences in the number of primary lateral roots between the wild type (WT) and *IRT1* mutant under 1  $\mu\text{M}$  Bi (Figure 2A). However, the number of the WT secondary lateral root was reduced in a 0.1  $\mu\text{M}$  Bi, while that of the *IRT1* mutant was increased. Moreover, the tertiary lateral root of the WT was not shown in a 2  $\mu\text{M}$  Bi. In root cell elongation of the primary root, although both roots of WT and *IRT1* mutant were significantly decreased by Bi treatment, there was no difference between the roots of WT and *IRT1* mutant. Also,

we could not distinguish the primary root from the lateral root.

Next, to estimate the effect of Bi to the root cap, the amyloplast in the columella cells were stained and the root tip was observed. There was no difference between the root formation of WT and *IRT1* mutant in all tested conditions (Figure 2B). However, both lines showed the morphological change to Bi concentrations especially at 1 and 2  $\mu\text{M}$  Bi. Both lines of the root tips in 2  $\mu\text{M}$  Bi appeared disordered with multiple roots. And more, although the amyloplast were observed in the columella cells in 0 and 0.1  $\mu\text{M}$  Bi, the amyloplast were not shown in Bi of 1 and 2  $\mu\text{M}$ . Moreover, the primary roots of both lines were not extended in a 2  $\mu\text{M}$  Bi.



**Figure 2.** Effect of Bi to the root formation of *IRT1* promoter knockout *Arabidopsis*.

Two-week-old plantlets were separated to shoot and root. The effect of Bi on the development of lateral roots was evaluated by counting the primary, secondary and tertiary lateral roots (A). And also, the cell length of the elongation differentiation zone (EDZ) was measured. The effect of Bi on the primary root formation was observed by Lugol staining (B). Data are means  $\pm$  SE ( $n=3-10$ ). Asterisks indicate a significant difference from Bi-treated plants and control plants ( $p<0.05$ ).

### 3.4. Bi and Fe Accumulation of *IRT1* Promoter Knockout Line

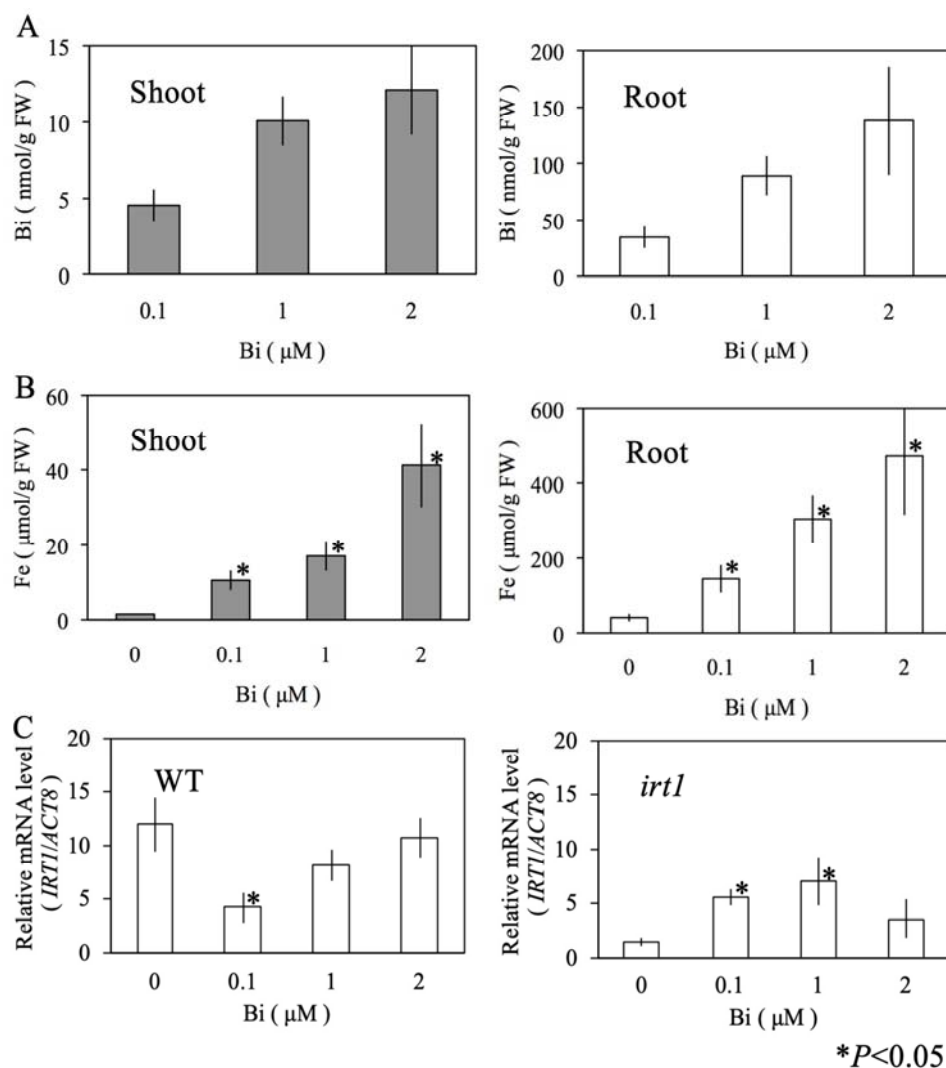
To estimate the accumulation of Bi in the root tissue, the Bi contents in each organ were measured. Bi-untreated plants cannot detect Bi in *IRT1* mutant. Bi was detected in a dose-dependent manner in the shoot and root (Figure 3A). In a 2  $\mu$ M Bi treated plant, the Bi concentration of roots was about 130 nmol g<sup>-1</sup> FW, which was 10-fold higher than that of the shoot.

Next, Fe accumulation in each organ was measured. The Fe concentrations in the shoot and root were also detected in a dose-dependent manner (Figure 3B). In a 2  $\mu$ M Bi-treated *IRT1* mutant, the Fe concentration of the roots was about 450  $\mu$ mol g<sup>-1</sup>

FW. The Fe concentration in a 2 Bi-treated shoot and root was 10-fold higher than that of the untreated shoot and root.

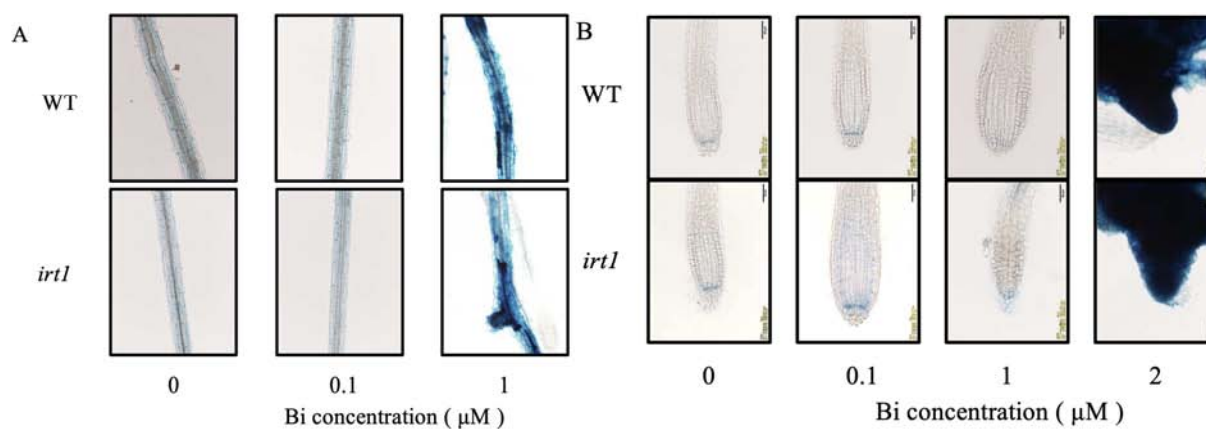
### 3.5. Effect of Bi on *IRT1* Expression Level in Root

*IRT1* mRNA contents were determined in both lines to confirm the *IRT1* expression level in the root (Figure 3C). In Bi-treated WT, the expression level of only the 0.1  $\mu$ M Bi-treated plant was significantly decreased compared with the untreated plant. In Bi-untreated *IRT1* mutant, the expression was not detected in the root compared with WT. However, Bi increased the expression levels of 0.1 and 1  $\mu$ M Bi-treated *IRT1* mutant. Also, the expression level of a 2  $\mu$ M Bi-treated *IRT1* mutant was approximately 40% more than that of WT.



**Figure 3.** Accumulation of Bi and *IRT1* expression on *IRT1* promoter knockout in *Arabidopsis*.

The concentrations of Bi (A) and Fe (B) in each organ was determined by an atomic adsorption of spectrometer. Tissue Bi concentrations were expressed as mmol g<sup>-1</sup> FW and tissue Fe concentrations were expressed as μmol g<sup>-1</sup> FW. Expression level of *IRT1* was determined using qRT-PCR (C). Transcript accumulation was quantified as a value relative to *Act8* transcript accumulation. Data are means ± SE (*n*=3-10). Asterisks indicate a significant difference from the shoot and root (*p*<0.05).



**Figure 4.** Localization of Fe in Bi treated *IRT1* promoter knockout *Arabidopsis*.

Two-week-old plantlets were separated to shoot and root. Fe in root was stained by Perls and observed under microscope. The root elongation differentiation zone (EDZ) (A) and the root tip (B) were observed. The experience was performed at least three times.

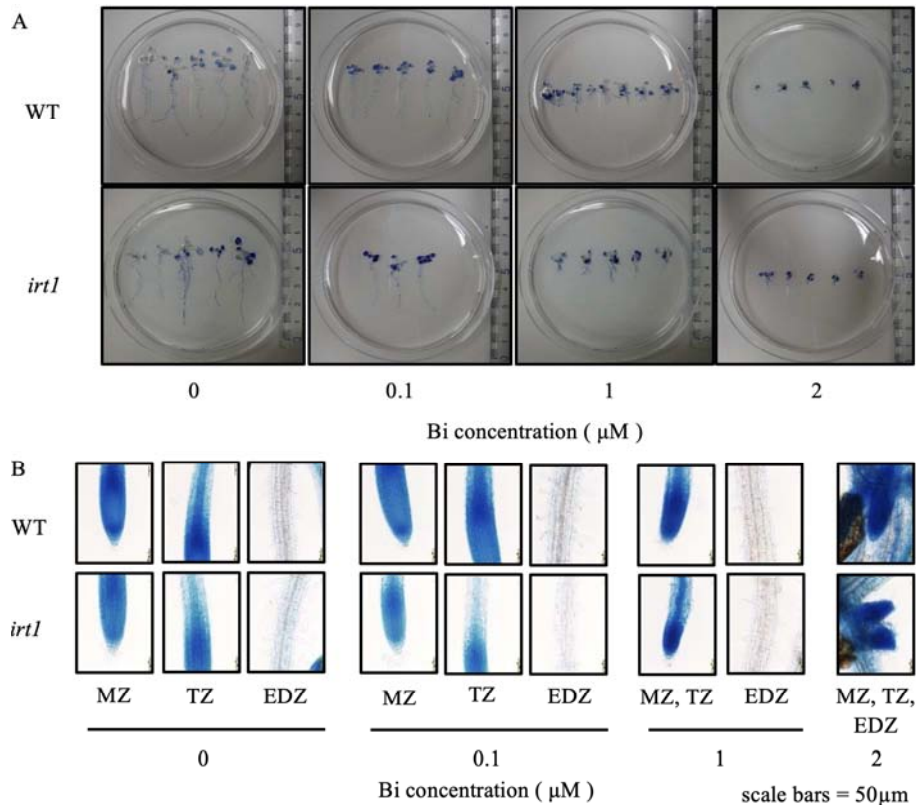


### 3.6. Effect of Bi on Fe Localization in the Root

The mature zone of roots was not harvested in both lines. There was no difference between WT and *IRT1* mutant in all tested conditions (Figure 4A). Bi-untreated and 0.1  $\mu\text{M}$  Bi-treated roots were slightly stained in the mature zone. However, 2  $\mu\text{M}$  Bi-treated roots were markedly stained in both lines.

In the root tip, Fe localizes Quiescent center (QC) cells in Bi-untreated and 0.1  $\mu\text{M}$  treated roots (Figure 4B). However, Fe was not observed in 1  $\mu\text{M}$  Bi-treated roots of

WT. The 2  $\mu\text{M}$  Bi-treated roots were stained not only QC cells but also whole roots. On the other hand, Fe localizes QC cells in Bi-untreated roots of *IRT1* mutant. In 0.1  $\mu\text{M}$  treated roots of *IRT1* mutant, Fe was stained QC cells and meristem extracellular. Fe did not localize QC cells and was observed slightly over the root tip in 1  $\mu\text{M}$  Bi-treated roots. Similarly, 0.1  $\mu\text{M}$  Bi-treated roots of *IRT1* mutant, Fe was stained extracellular. However, in 2  $\mu\text{M}$  Bi-treated roots of *IRT1* mutant, Fe was stained the whole roots, same as WT.



**Figure 5.** Induction of the cell death by Bi in *IRT1* promoter knockout *Arabidopsis*.

The effect of Bi on the cell death was evaluated by staining of Evans blue. The whole plant was photographed (A). Moreover, the plantlets were separated to the shoot and root. The root elongation differentiation zone (EDZ) and the root tip were observed (B). The experience was performed at least three times.

### 3.7. Damage of Bi to *Arabidopsis Thaliana*

The cell death was observed by Evans blue staining (Figure 5). In whole plants, the same staining was observed in WT and *IRT1* mutant. The roots were not strongly stained in 0 and 0.1  $\mu\text{M}$  Bi-treated plants (Figure 5A). However, the root, especially the root tip, was strongly stained in the 2  $\mu\text{M}$  Bi-treated plant. In the shoot, the strong staining was not observed in untreated plants. Among the 0.1  $\mu\text{M}$  Bi-treated shoots, strong staining was observed in WT and *IRT1* mutant.

In the root tip, meristem zone (MZ), transition zone (TZ), and EDZ were observed using a microscope. Although MZ and TZ were stained, EDZ was not stained in the Bi-untreated and 0.1 and 1  $\mu\text{M}$  treated WT (Figure 5B). In 2  $\mu\text{M}$

Bi-treated roots, we could not recognize the root morphology development. However, the 2  $\mu\text{M}$  Bi-treated root was extensively stained. The same tendency was observed in the *IRT1* mutant root. The 2  $\mu\text{M}$  Bi-treated root of *IRT1* mutant was also extensively stained.

## 4. Discussion

There are only a few reports on the Bi concentration in the soil. Moreover, the phytotoxicity remains unclear. Recently, we reported the effect of Bi on the growth and gene expression of *Arabidopsis thaliana* [9]. The vegetative plant *Solanum lycopersicum* was also inhibited the growth by Bi [10]. Bi has high toxicity to plants and disturb their Fe contents. However, the mechanism of growth inhibition and the collapse of Fe

homeostasis have not been cleared in detail. In this study, we assess the involvement of Fe-related genes and its toxicity to the root with the *IRT1* promoter knockout line.

At first, the expression of Fe-related genes was analyzed by a microarray analysis. The Fe-related gene expression levels of 2  $\mu$ M Bi-treated WT *Arabidopsis thaliana* were shown in Table 1. Various subgroup Ib promoters (*bHLH38/39/100/101*) were upregulated. This result correlates with our previous report that Bi enhances *IRT1* expression [9]. Recently, the E3 ligase BTS was reported in the degradation of subgroup IVc bHLH transcription factors by the proteasomal feedback system [19, 20]. Taken together, the expression of *BTS* was upregulated and *BTS* degraded subgroup IVc in proteasomal degradation feedback system. Thus, subgroup IVc promoters (*bHLH34/104/115/ILR3*) were not upregulated. These results suggest that Bi disturbs Fe homeostasis, causing a change in the expression levels of Fe regulation genes transcription factors. On the other hand, FIT, which bound subgroup Ib to an express *IRT1* [31], was not upregulated. And also, the bHLH100 and bHLH101 could control Fe homeostasis independently of FIT [32]. These suggest that Bi might inhibit unknown transcription factor or expression regulation mechanisms in Fe-regulated genes.

Next, to analyze the involvement of *IRT1*, we used the *IRT1* promoter knockout line. The effect of Bi to the whole plant was observed (Figure 1). The growth of *IRT1* mutant was inhibited in a Bi dose-dependent manner. The phenotype of 0.1  $\mu$ M Bi-treated plantlets showed no significant difference compared with untreated plantlets [9]. The root length and growth were suppressed under a Bi 2  $\mu$ M condition. Interestingly, the roots of Bi-treated plants were elongated from the agar medium to the air. These results suggest that the defect of *IRT1* cannot alleviate the toxicity of Bi.

To estimate the effect of Bi to root phenotype, the development of the lateral root and the cell elongation of the primary root were determined (Figure 2). The number of secondary lateral roots in a 0.1  $\mu$ M Bi-treated *IRT1* mutant was increased compared to the WT. Moreover, the tertiary lateral roots in 2  $\mu$ M Bi-treated plants only showed in *IRT1* mutant. The total lateral root number of *IRT1* mutant was more than that of WT. These results suggest that the defect of *IRT1* slightly rescued the development of the lateral root under Bi presence condition. However, there was no significance in the cell number of EDZ between WT and *IRT1* mutant, and was decreased in a Bi dose-dependent manner. These results suggest that the root cell elongation was inhibited by Bi and *IRT1* was not involved in this inhibition. It was known that the plant cell is surrounded by the cell wall, and cell elongation is caused under high pressure when the cell wall is relaxed [33]. This relaxation of the cell wall has also been reported as being caused by the plant hormone auxin [34]. It is assumed that the inhibition of root cell elongation causes a disorder in plant hormones by Bi.

We then estimated the effect to columella cells using by staining the amyloplast. There was no difference between WT and *IRT1* mutant, and each line was damaged under 1

and 2  $\mu$ M Bi condition (Figure 2). Four layers of columella cells were observed in 0 and 0.1  $\mu$ M Bi conditions. However, the amyloplast of 1  $\mu$ M Bi-treated plants were observed. Also, 2  $\mu$ M Bi-treated plants did not form a layer of columella cells. We thought that Bi may inhibit the differentiation activity of columella stem cells and/or may collapse the columella cells caused by cell death.

Next, we examined the accumulation of Bi in shoots and roots of *IRT1* mutant. However, we were not able to get the tissue from a 3  $\mu$ M Bi-treated plant. As shown in Figure 3A, the Bi concentration of the root was 10-fold higher than that of the shoot, suggesting that Bi was not transported to the shoot. Remarkably, the Fe contents in each tissue were increased in proportion to Bi contents (Figure 3B). It suggests that Bi enhances Fe concentration in *IRT1* mutant. In *IRT1* mutant, the T-DNA is inserted in *IRT1* promoter region, which is -577 bp upstream of *IRT1* start codon [35]. Nishida et al. revealed that the *IRT1* mutant was not induced the *IRT1* expression by Fe starvation. We confirmed the expression level of *IRT1* in *IRT1* mutant under Bi presence conditions. In the absence of Bi, the expression level of *IRT1* mutant was less than that of WT (Figure 3C). However, the *IRT1* expression was induced in 0.1 and 1  $\mu$ M Bi-treated *IRT1* mutants. We assume that the formation might be changed as caused by the binding of Bi to the subgroup Ib transcriptional factor, and this factor and RNA polymeraseII were weakly bound to the *IRT1* promoter region near the *IRT1* coding gene.

An excess Fe is toxic due to reactive hydroxyl radicals generated by the Fenton reaction [12]. The Fe uptake mechanisms in the root have been revealed, and the uptake is highly regulated and induced only under Fe deficiency [15]. Thus, we tried to stain Fe in the root tissue to elucidate the contribution of Fe for Bi toxicity. Although Fe was located in Quiescent center (QC) cells in untreated WT and *IRT1* mutant, Fe was distributed all over the root in 2  $\mu$ M Bi-treated WT and *IRT1* mutant (Figure 4B). Fe overaccumulation was revealed in our result (Figure 3B), exclusive with *IRT1* expression levels in 2  $\mu$ M Bi-treated roots of WT and *IRT1* mutant (Figure 3C). These results suggested that Fe influxes into the tissue without cell viability. We assume that root cells were injured by Bi and Fe overaccumulation, and Bi toxicity was enhanced by the reactive hydroxyl radicals. Thus, resulting in the death of the root cells in 2  $\mu$ M Bi-treated roots.

It has been reported that metal ions, such as aluminum or cadmium, induce cell death [36, 37]. To confirm the cell death, we stained the dead cells by Evans blue (Figure 5). In WT and *IRT1* mutant shoots, the cell death was caused by 0.1  $\mu$ M Bi treatment. On the other hand, in WT and *IRT1* mutant root tips, the cell death was slightly increased in 1 and 2  $\mu$ M Bi treatment. Considering Fe locations in roots (Figure 4), these results suggest that the induction of cell death was caused directly by Bi, and was not caused by Fe overaccumulation in a 1  $\mu$ M Bi treatment. However, 2  $\mu$ M Bi treated roots showed Fe overaccumulation and cell death (Figure 5). Thus, we assumed that the Fe overaccumulation might enhance cell death by the Fenton reaction. Interestingly, the cell death was not induced in



the EDZ of WT and *IRT1* mutant. It suggests that the target of Bi toxicity is the root tip and the stem cells, including QC, which might be collapsed by Bi toxicity.

## 5. Conclusion

The 2  $\mu$ M Bi has toxicity to *A. thaliana*, especially in its root tip. This phytotoxicity is caused by the disturbance of the Fe regulation cascade, such as the subgroup Ib transcription factor. As a result, Bi inhibits the root growth and lateral root development. Furthermore, Bi induces Fe content without induction of *IRT1* expression. Fe might flow in root tissue in Bi including the environment. However, this flow may not concern *IRT1*. The cell death in the root was found in 2  $\mu$ M Bi-treated roots including SCN. These results suggest that Bi disturbed the Fe homeostasis, and Fe overaccumulation is one of the reasons of causing the cell death in *A. thaliana*. To the best of our knowledge, this is the first elucidation of Bi enhances Fe homeostasis disorder in phytotoxicity.

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