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Original Paper

Sweet potato expressing the rice Zn transporter *OsZIP4* exhibits high Zn content in the tuber

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Abstract Zinc (Zn) is an essential element for humans as well as for plants. Sweet potato is a major staple crop in the world, and is an attractive target crop for genetic engineering because its suitability for clonal propagation and its self-incompatibility. *OsZIP4* is a rice Zn transporter that increases Zn concentrations, especially in roots, when it is artificially overexpressed in rice. In our study we induced an embryogenic callus from the apex of sweet potato (*Ipomoea batatas* L. Lam. cv. Kokei No. 14), and obtained *OsZIP4* overexpressed lines driven by the CaMV35S promoter through *Agrobacterium* infection. Three transgenic lines were confirmed by RT-PCR for their *OsZIP4* expression in roots and leaves. Zn concentrations in roots and leaves of *OsZIP4* transgenic plants grown on LS medium were approximately 2.3 times and 1.3 times higher, respectively than those of wild type plants. In addition, the iron content of tubers was two times higher than wild type (WT) lines, and that of leaves was up to 1.2 times higher. Root tubers of transgenic lines were obtained under soil culture. The Zn concentration of transgenic sweet potato tubers was 2.2 times higher than WT lines, but there were no differences in shoot length or fresh weight between the transgenic and WT lines. These results indicate that introducing the *OsZIP4* gene in to sweet potato is a potential method to improve the Zn nutrition of livestock and humans.

Key words: Bio-fortification, *OsZIP4*, transgenic sweet potato, zinc transporter.

Zinc (Zn) is an essential nutrient for most organisms and has important roles in numerous physiological processes. Zn is included in many enzymes that participate in a wide variety of metabolic processes, such as synthesis and/or degradation of carbohydrates, lipids and proteins (Vallee and Galdes 1984; Wu et al. 1992; Wu and Wu 1987). Several molecules associated with DNA and RNA synthesis are also Zn metalloenzymes, such as polymerase, transcriptase and transcription factor (Wu and Wu 1987).

Zn deficiency is a global nutritional problem especially in developing countries (Sayre et al. 2011). Deficiency arrests normal growth processes and causes abnormal DNA and RNA synthesis in various tissues and organisms (Falchuk et al. 1975; Ho 2004; Scrutton et al. 1971; Swenerton et al. 1969). It is estimated that Zn deficiency causes problems like growth retardation, skin diseases, hypogonadism, immune-dysfunction and cognitive impairment in 25% of the world's population (Cakmak 2008; Cakmak 2009; Gomez et al. 2009). One bio-fortification strategy to generate health benefits for large numbers of people is to increase the nutrient content of the edible parts of crops (Nestel et al. 2006; Uzoma et al. 2012; Zhu et al. 2007).

Sweet potato, *Ipomoea batatas* (L.) is the sixth most important crop in the world (Vietmeyer 1986). Most sweet potato is cultivated in developing countries to provide edible storage roots and foliage for humans and animals (Sihachakr and Ducreux 1987). The conventional breeding of many sweet potato varieties is complicated due to its hexaploid genome, and the incompatibility and climate restrictions for florescence (Noh et al. 2010; Sihachakr and Ducreux 1987). Genetic engineering offers great potential for the quick development of new improved varieties of sweet potato. For example, the use of biotechnology could enable the bio-fortification of such staple foods, which is required as a strategy to avoid nutrition shortages in developing countries (Uzoma et al. 2012).

Rice (*Oryza sativa* L.) has a transporter, OsZIP4, which is located in the plasma membrane. It controls Zn translocation in the plants, and it has been reported that it is responsible for the accumulation of Zn in rice plants, mainly in the roots (Ishimaru et al. 2007). Therefore, it is possible that this *OsZIP4* gene (Zn and Iron (Fe) transporter-like Protein) could be used to promote Zn accumulation in other staple food crops, such as sweet potato.

Abbreviations: RT-PCR, Reverse Transcription Polymerase Chain Reaction; IRT, Iron Regulated Transporter; ZIP, Zinc regulated transporters, Iron regulated transporter like Protein.

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In this study, we show that overexpression of the *OsZIP4* gene in sweet potato enhanced Zn content in tubers as well as in roots and leaves. Furthermore, these lines also improved Fe content in sweet potato roots, tubers and leaves.

Materials and methods

Plant material

Sweet potato (*Ipomoea batatas* L. cv. Kokei No. 14), which is a popular variety for experiments in Japan, was kindly provided by The National Agriculture and Food Research Organization (NARO), Kyushu Okinawa Agricultural Research Center. Shoots of the sweet potato were sprouted from root tubers in the soil for 2 weeks at room temperature (Day: 16 h of light over 200 μmol photons $m^{-2} s^{-1}$; Dark: 8 h). The shoots were then sterilized with 2% sodium hypochlorite solution (WAKO, Japan) and transferred to LS solid medium containing 0.32% gelrite (WAKO, Japan) and cultured (Linsmaier and Skoog 2006).

Sections of stem, about 3 cm in length and with at least 2 nodes, were cut from middle part of the plant stems and sub-cultured in vessels on fresh LS medium.

Sweet potato transformation

The pIG121Hm plasmid with *OsZIP4* was used as previously reported by Ishimaru et al. (2007). The plasmid pIG121Hm was used for vector control (VC).

Embryogenic calli from apexes of sweet potato (Figure 1A) were prepared according to the method of Otani et al. (1998). The embryogenic calli were cultured for more than 6 weeks, until they were approximately 0.5 cm to 1.0 cm in size (Figure 1B). Then the calli were genetically transformed using *Agrobacterium* (*Agrobacterium tumefaciens* strain EHA105) infection. Regenerated lines (T0) were obtained and selected after growing on LS medium containing 25 $mg l^{-1}$ hygromycin (Figure 1C). The transformed sweet potatoes were grown for 1 month and then transplanted in to 15 cm diameter pots containing 700 g soil (Nippi YOSAIBAIDO SP200, JA, Japan) to induce tuber roots (Figure 1D). One hundred milliliters of nutrient solution containing 0.4 mM K_2SO_4 0.1 mM KCl, 0.1 mM KH_2PO_4, 2 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 10 μM H_3BO_3, 50 μM $MnSO_4$, 0.1 μM $CuSO_4$, 3 μM $ZnSO_4$, 0.05 μM Na_2MoO_4, and 10 μM $FeSO_4$ was added to each pot every three days. Sweet potato tubers were obtained after culturing in the pots for more than 3 months.

Expression analysis of introduced genes in transgenic plants

Genomic DNA was extracted from the regenerated plants using ISOPLANT II (NIPPON GENE, Japan), and analyzed by polymerase chain reaction (PCR). Total RNA was extracted from leaves and roots of transgenic lines using RNeasy mini kit (Qiagen, USA). The extracted RNA was reverse transcribed

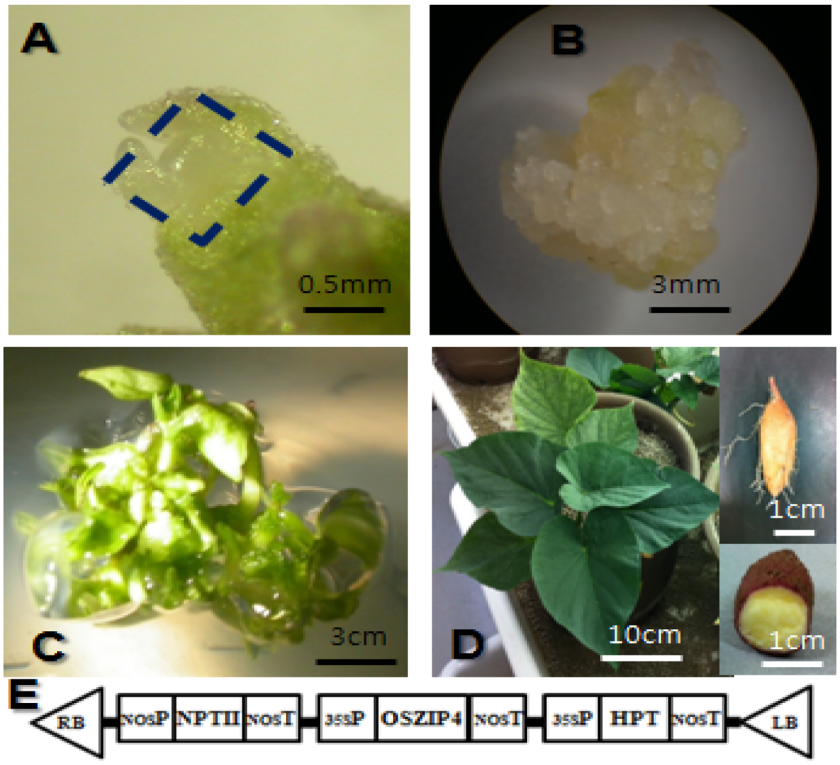

Figure 1. Transformation of sweet potato. A) Apex of stem, B) embryogenic callus 6 weeks after apex cultivation, C) regeneration, D) regenerated plant and tuber, E) structure of pIG121Hm with *OsZIP4*; nosP: *nos* promoter, nosT: *nos* terminator, 35sP: CMS 35s promoter, NPTII: neomycin phosphotransferase gene HPT: hygromycin phosphotransferase gene, LB: left border, RB: right border.

by PrimeScript RT reagent kit (Perfect Real time, Takara, Japan). Total DNA and cDNA were used as templates for PCR amplification with ExTaq™ and a pair of gene specific primers of the *OsZIP4* gene, Forward 5′-ATG AGG CAG AGC ACG CG and Reverse 5′-TCA TGC CCA TAT GGC AAG CA, to determine *OsZIP4* expression. The primers used for internal control in RT-PCR were *beta-actin* forward 5′-GTT GTA GCA CCA CCT GAA AGG A and reverse 5′-CTG CCT TGG CAA TCC ACA TCT GTT. The amplification was performed using a Thermal Cycler (LifePro, BIOER, USA) with an initial denature at 94°C for 2 min, followed by a cycling stage with 30 cycles of 98°C for 15 s, and then either 55°C with *OsZIP4* or 50°C with *beta-actin* for 30 s followed by 72°C for 30 s or 72°C for 60 s. There was no genomic contamination in any of the RNA samples, and the sizes of the amplified fragments were confirmed by electrophoresis and sequencing (data not shown).

Total cDNA was mixed with 2x QuantiTect SYBR Green PCR Master Mix (Qiagen) based on the manual procedure. The mixed liquids were prepared on capillaries (20u LightCycler Capillaries, Roche).

Quantitative real time PCR was performed with specific *OsZIP4* primers for 152 bp, Forward 5′-AAC AAT CCG GCA CAG GGT TGT CTC and Reverse 5′-TGC CTT CAA AGA ACT GGT GGA AGC and *actin* primer for 99 bp Forward 5′-AGG TTG TAG CAC CAC CTG AAA GGA and Reverse 5′-CTG CCT TGG CAA TCC ACA TCT GTT. It comprised an initial denature at 95°C for 15 min, followed by cycling stage with over 35 cycles of 94°C for 15 s, and then either 55°C with *OsZIP4* or 50°C with *actin* for 30 s, followed by 72°C for 30 s. Then a melt curve was made at the end of each amplification cycle and the single product was confirmed using a LightCycler (Roche).

Dilution series of 2*x*, 1*x*, 1/5*x* and 1/10*x* cDNA were made for each product and these were compared against calibration curves for each gene. The standard curves with correlation coefficients higher than 0.99 were used for quantification.

Determination of metal contents

Metal contents were measured in the leaves, stems, roots and tubers of the transgenic plants. The organs were dried for more than 2 days at 70°C in the oven. The dried materials (20–100 mg) were wet ashed with 3 ml of 13 M HNO_3 for 30 min at 220°C using a microwave system (MARS Xpress, CEM, USA). The metal concentrations were measured using inductively coupled plasma atomic emission spectrometry (SPS1200VR; Seiko, Tokyo, Japan) at wavelengths of 213.856 nm (Zn), 238.204 nm (Fe), 293.930 nm (Mn), and 324.754 nm (Cu).

Statistical analysis

A standard Student's *t*-test was performed to determine differences between wild type (WT)/VC lines and transgenic lines.

Results

Selection of transgenic sweet potato lines

The *OsZIP4* gene, driven by the 35S promoter (35S-*OsZIP4*) in the plasmid pIG121Hm, was introduced into embryogenic calli induced from sweet potato apices (Figure 1E). Transgenic plants were regenerated from the embryogenic calli over more than 6 months (Figure 1D). 35s-*OsZIP4* transgenic sweet potato lines were obtained and confirmed by genomic PCR (data not shown). Three transgenic lines No. 4, 5 and 17 had root Zn contents of 497, 680 and 599 $\mu g\,g^{-1}$, respectively, that were significantly higher than the average Zn root content of 299 $\mu g\,g^{-1}$ in WT lines (p-value <0.05; Figure 3). The transgenic lines of *OsZIP4* mRNA (1219 bp) were expressed in both the leaves and roots (Figure 2). Moreover, OsZIP4 (152 bp) expression was measured against *beta-actin* expression using quantitative real time PCR with *OsZIP4* (152 bp) against *actin*. The three lines were selected for further analysis of metal concentrations in the roots, shoot, roots and root tubers.

Metal concentrations of transgenic sweet potato

We compared the three transgenic lines with VC and WT to confirm whether the transgenic lines had increased growth and metal concentrations. The root Zn contents of No. 4, 5 and 17 transgenic lines were approximately 1.6, 2.3 and 2.0 times higher than that in WT, and the respective Zn leaf contents were also 1.2, 1.3 and 1.6

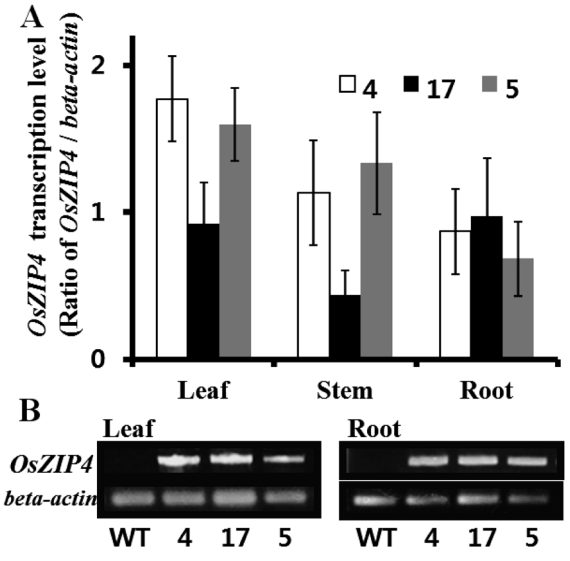

Figure 2. *OsZIP4* expression in transgenic sweet potato lines. A) Relative quantitative analysis of real time PCR. *OsZIP4* expression is shared with the constitutive gene *beta-actin* in transgenic lines. No OsZIP4 expression was detected in WT, so the *OsZIP4*/*beta-actin* ratio could not be calculated for WT (error bars show SE, n=three independent root samples). B) Reverse transcription PCR of *OsZIP4* in roots and leaves. *beta-actin measurements* were internal standards. None of the bands included DNA because there is no band without reverse transcriptase.

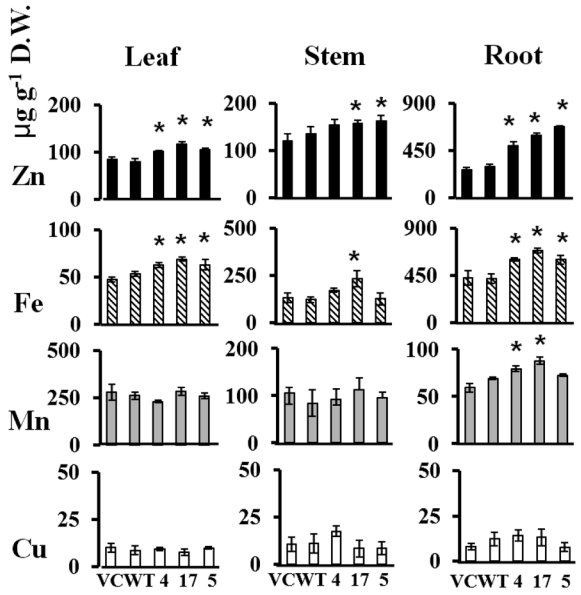

Figure 3. Metal concentrations in transgenic lines, vector control (VC) and wild type (WT) on LS medium. The concentrations of Fe, Zn, Mn and Cu are expressed as micrograms per gram of dry weight (n=3) in roots and leaves. Error bars show SE and the symbol * indicates statistical differences (p<0.05)

Table 1. Root tuber metal concentrations and fresh weights of wild type and transgenic lines. The values are averages±standard error (n=3); Values with the symbol* indicate significant differences comparing with WT (p<0.05).

	WT	4		5	
	$\mu g g^{-1}$	$\mu g g^{-1}$	Ratio	$\mu g g^{-1}$	Ratio
Zn	9.77±2.3	*20.9±8.7	2.1	*21.8±4.3	2.2
Fe	355±81	*548±43	1.5	*620±98	1.7
Mn	56±24	66.9±20	1.2	67±23.8	1.2
Cu	0.64±0.17	0.72±0.06	1.1	0.99±1.2	1.5
Weight (g)	1.84±0.71	1.66±0.53		2.04±0.96	

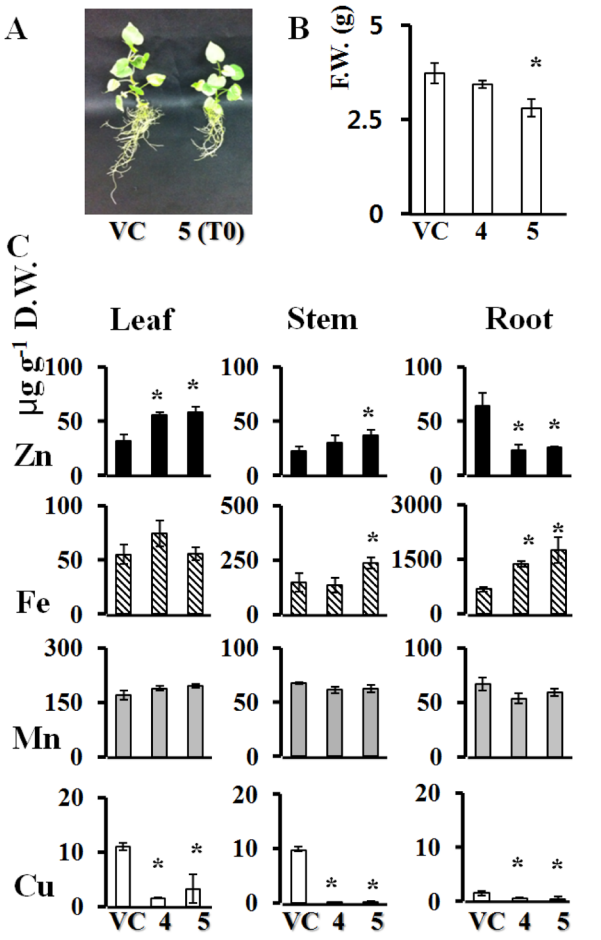

Figure 4. Metal concentrations and growth of transgenic lines and VC under Zn deficiency Plants were cultured solid LS medium and then transferred to LS medium without $ZnSO_4$ for 30 days, after which metal concentrations were measured. A) Phenotype of VC and transgenic line No. 5 (T0). B) Fresh weight of entire plant of VC and transgenic lines C) Metal concentrations in transformants and VC. The concentrations of Fe, Zn, Mn and Cu are expressed as micrograms per gram of dry weight (n=3). Error bars show SE and the symbol * indicates statistical differences (p<0.05)

times higher (Figure 3). Furthermore, the respective transgenic plants accumulated approximately 1.4, 1.4 and 1.6 more Fe in their roots than the WT plants, and 1.2, 1,2 and 1,3 times more Fe in their leaves (Figure 3). However, the concentration of Cu in the leaves, shoots and roots and Mn concentration of leaves and shoots of the *OsZIP4* transformants were not significantly different from those in WT (Figure 3). Furthermore, there were no differences in shoot length between the transgenic and WT lines (data not shown). No root length measurements were made because the plants were an irregular branching type. Additionally, total plant fresh weights of the transgenic and WT lines were similar (data not shown).

The Zn content in sweet potato tubers (about to 3 cm length and 1 cm diameter or over in size) harvested from the soil pots was more than 2 times higher in the 35s-OsZIP4 transgenic lines than in WT (Table 1). The Fe concentration in root tubers was also at least 1.5 fold higher in the transgenic lines than in the WT lines, but there was much less difference in Mn or Cu concentrations between the transgenic lines and WT lines.

Zn and Fe concentrations under Zn deficient conditions

The growth of the transgenic plants was significantly decreased on LS medium containing no Zn (Figure 4A). The transgenic lines were compared with the VC plants to confirm that the increased growth and metal concentrations were due to the *OsZIP4* transporter functioning in the transgenic lines, and that it was not a gene insertion effect. The respective fresh weights of transgenic lines 4 and 5 were 0.94 and 0.75 times those of VC plants (Figure 4B). The Zn content of the transgenic plant roots was also lower than in VC, but

it was higher in the leaves (Figure 4C). In contrast, the Fe concentration in roots of transgenic line No. 5 was almost 2.6 times higher (1,763 $\mu g\,g^{-1}$) than that of VC (678 $\mu g\,g^{-1}$) (Figure 4C). There was no significant difference in Mn concentrations between the transgenic and VC plants in either roots or leaves.

Discussion

Zn is an important nutrient for many of physiological functions in plants. The *OsZIP4* gene is known to be related to Zn translocation and accumulation in rice plants (Ishimaru et al. 2007). In this report, we were successfully able to obtain 35s-*OsZIP4* transgenic sweet potato lines. Three of those transgenic lines, No. 4, 5 and 17, had higher Zn concentrations in roots and leaves, and two lines, No. 4 and No. 5, also showed higher Zn contents in tubers.

OsZIP4 is a Zn transporter responsible for the translocation of Zn into the cell (Ishimaru et al. 2005). However, over-expression of *OsZIP4* in rice caused a 10-fold increase in Zn accumulation in roots and resulted in decreased shoot length (Ishimaru et al. 2007), suggesting that extremely high Zn concentrations may inhibit rice growth. However, in our study the *OsZIP4* over-expressed sweet potato lines did not show any decrease in either shoot length or fresh weight, even though the Zn concentration increased 2.3 times in roots and 1.3 times in leaves of these plants grown on LS medium. So, our data suggest that it may be possible to increase the Zn content of sweet potato artificially, without inhibiting the growth of the transgenic sweet potato plants.

Zn deficiency in soil is a critical constraint on the yields of many crops (Cakmak et al. 1996), including sweet potato (Courtney and Bonte 2006). *OsZIP4* overexpression lines of sweet potato were thought to improve Zn concentration and growth of plants growing under Zn deficiency. However, the growth of the 35S-*OsZIP4* transgenic lines was impeded compared with VC (Figure 4A). Moreover, the Zn concentration in roots of the transgenic lines was lower than those of VC. Therefore, in Zn deficient conditions, plants from *OsZIP4* expression lines may not accumulate more Zn from the surrounding soil in their roots, but instead translocate Zn from the roots to the shoots. This could explain why the Zn content in the roots of the *35s-OsZIP4* expressed line plants was lower than in the WT lines, even though the Zn concentration in the leaves was higher. Irregular metal distribution in the studied plants may be one reason for the inhibition of the plants' growth. The root Fe concentration of the No. 5 transgenic plants grown in LS medium without Zn was much higher (1,763 $\mu g\,g^{-1}$) than that of VC (Figure 4C). This value for the transgenic plant in Zn deficient medium was 4.2 times higher than the equivalent value for WT grown in normal LS medium (419 $\mu g\,g^{-1}$) (Figure 3). In addition, the growth of the *OsZIP4* transgenic sweet potato lines under Zn deficient conditions was also decreased (Figure 4). Therefore, these data suggest that *OsZIP4* may act indirectly to accumulate Fe in transgenic sweet potato roots, and this excess metal concentration might influence plant growth (Hall 2002; Ishimaru et al. 2007; Nations et al. 2008), and have inhibited the sweet potato growth.

The Zn concentrations in tubers of the 35S-*OsZIP4* transgenic lines were also higher than in the WT lines, showing a similar trend to the root Zn contents (Table 1, Figure 3). In the transgenic sweet potatoes, *OsZIP4* was highly expressed throughout the whole plant, including the tubers, suggesting that the *OsZIP4* gene may have translocated the Zn into the plant roots and tuber cells. The fact that the Fe concentrations in the transgenic root tubers were also higher than in the WT lines (Table 1), suggests that the transgenic sweet potato lines can accumulate both Zn and Fe in the tuber directly from the root.

The sweet potato tuber is used for human consumption, but leaves are not used as food. However, the leaves are also edible and can provide a good supply of nutrients to organisms. For example, sweet potato leaves have been used as a suitable nutrient source for the growth and development of rabbits (Abonyi et al. 2012). Sweet potato foliage production is almost 5 ton/ha, and therefore it is also a potential source of feed for livestock (Dominguez 1992). In addition, sweet potato leaves can be harvested all through the year, even from plants grown on un-used land (Adewolu 2008; Tial et al. 2009). Our results showed that Fe and Zn concentration in leaves from *OsZIP4* overexpressing lines of sweet potato with were higher than those of the WT and VC lines (Figure 3), and this suggests that leaves of *OsZIP4* sweet potato could be a good source of Zn and Fe nutrients.

Previous report mentioned that sweet potato has high fiber, low saturated fat and an abundance of antioxidants (Park et al. 2014). Moreover, sweet potato is also considered to be tolerant to environmental stress. Therefore, sweet potato could be used to improve nutrient supply as part of the bio-fortification strategy. To achieve such a strategy it would be necessary to improve the quality of sweet potato, and this could be more easily achieved using genetic engineering technology than using conventional breeding. In our study, the 35s-*OsZIP4* transgenic sweet potato showed improved Zn and Fe contents in tubers and leaves. Therefore, our preliminary study suggests that genetically modified sweet potato with the *OsZIP4* gene is a possible strategy to improve nutritional quality in tuber plants.

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