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Expression of Zinc Transporter Genes in Oat *(Avena sativa* L.) as Influenced by Zinc-Solubilizing Bacteria

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A B S T R A C T

Mineral deficiency mostly zinc is a well-documented problem in food and forage crops affecting the crop production and subsequently the human and animal health. Oat (*Avena sativa* L.) is winter crop in many parts of the world and is used as multipurpose crop for grain and forage. Oat is sensitive to Zn, which is major dry fodder and thereby causes Zn deficiency in animals. Application of Zinc solubilizing bacteria (ZSB) could be a sustainable agronomic approach to increase the soil availability of Zn for nutritionally rich oat. In the present study, the role of zinc solubilizing bacteria on regulation of Zn-regulated transporters (OsZIP1, OsYSL2 and OsYSL6) was assessed by quantative real-time reverse transcription PCR and semi-quantative PCR, in control (without ZSB inoculation) and in treated (with ZSB inoculation) of leaf, stem and root. The expression of OsZIP1 in treated leaf and stem was more observed as compared to control whereas there is no change in root expression. The expression of OsYSL2 was more in leaf and root as compared to control. The expression of OsYSL6 was higher in treated stem and root as compared to control. These result suggested that OsZIP1, OsYSL2 and OsYSL6 in oat is an oat zinc transporters could be responsible for the Zinc transportation.

Introduction

Mineral deficiency is a well-documented problem in food and forage crops, reducing the probabilities of nutritional security. Mineral deficiency/imbalances in livestock feeds and forages have been observed in tropical and sub-tropical regions (McDowell, 1996), which often limits the performance of the animals (Corah, 1996). Several trace or inorganic elements are essential for normal growth, production and reproduction of animals that includes zinc, cobalt, copper, iodine, iron, manganese, molybdenum, selenium and perhaps chromium and fluorine, among them zinc has immense importance

(McDowell, 1996). Zn is essential for normal growth, production, reproduction, health and immunity of animals and its plays role in many physio-biochemical processes. Deficiency of Zn reduces feed intake, growth, listlessness, excessive salivation, testicular growth, cracked hooves, fertility and skin lesions or slowed wound healing in animals (Graham *et al.,* 2001). Zn requirement of animal is 20-75 mg/kg, whereas the tolerable level of dietary zinc is suggested to be 300 to 1000 mg/kg diet but the availability of Zn in oat is 24-30 mg/kg (Miller et al. 1994; Ashraf Y, 1996).

Oat (*Avena sativa* L.) is an economically important crop and ranks sixth in world cereal production after wheat, rice, maize, barley and sorghum (FAO, 2012). It is an important winter forage crop in many parts of the world and is also grown as multipurpose crop for grain, pasture and forage (Ruwali et al. 2013). Differing from other cereal grains such as wheat and barley, it is rich in the antioxidants α-tocotrienol, α- tocopherol, and avenanthramides, as well as total dietary fibre including the soluble fibre β-glucan. Oat is the major source of dry fodder-low in Zn concentrations, particularly when grown on Zn-deficient soil of India (Ruwali *et al.,* 2013). Singh *et al.,* 2005 reported that average Zn content 30-32mg/kg in Oat at different cutting stages. Ashraf, 1996 reported that average Zn content in different genotype of Oat is 24-30 mg/kg. In recent years, with the advent of exaggerated dairy industry, the oat have fascinated the attention of breeders for its improvement due to its nutritious quality fodder for livestock and its grains as animal feed with high net energy gains (Stevens *et al.,* 2004).

Several zinc solubilizing bacteria (ZSB) were characterized from tropical and temperate soils to provide available Zn (Hafeez *et al.,* 2013). For example *Bacillus* and *Pseudomonas* from rice, wheat and soybean, *Gluconacetobacter* from sugarcane capable of solubilizing Zn compounds into oxide, carbonate and phosphate were reported (Saravanan *et al.,* 2011). Inoculation of these bacteria enhanced the Zn uptake in several crops such as rice (Vaid *et al.,* 2014), maize (Goteti *et al.,* 2013), wheat (Rana *et al.,* 2012) and soybean (Sharma *et al.,* 2012) were earlier reported. However nothing is known in oat to explore molecular mechanism of the microbe-soil-plant interactions.

Several genes are responsible for transportation and accumulation of minerals in plants. The available Zn from soil is taken to root membrane transport mechanisms in rice (Bashir et al 2010) and Zn and Fe regulated transporters like protein (ZIP) family (Guerinot 2000). Several reports for Zn uptake from soil, translocation from root to shoot as well as for storage in grains and their molecular mechanism, genes involved OsIRT1, OsIRT2, OsZIP1, OsZIP3, OsZIP4, OsZIP5, OsZIP7, and OsZIP8 were studied (Ramesh *et al.,* 2003; Ishimaru *et al.,* 2005; Yang *et al.,* 2009; Lee *et al.,* 2010). Ishimaru *et al.,* 2005 reported that OsITR1 and OsITR2 are responsible for transport of iron from soil to root, whereas OsZIP1, OsZIP3, OsZIP4, OsZIP5, and OsZIP8 are rice plasma membrane Zn transporters and are induced by Zn deficiency (Ramesh *et al.,* 2003; Ishimaru *et al.,* 2005; Yang *et al.,* 2009; Lee *et al.,* 2010; Suzuki *et al.,* 2012). Chen et al. (2008) reported the differential expression pattern of ZIP genes (OsZIP1, OsZIP3, and OsZIP4) in rice. These ZIP genes varied their expression levels at different growth stages of rice from germination to grain filling (Ishimaru *et al.,* 2011). Krithika and Balachandar, (2016) studied the expression of Zinc transporter genes as inoculation by Zn solubilizing PGPR in rice. They studied the expression patterns of *OsZIP1*, *OsZIP4*, and *OsZIP5* in root and shoot of rice. The study of molecular mechanism and understanding the interaction between oat plant and Zn solubilizing bacteria

in terms of Zn transporter genes expression would help to alleviate the Zn deficiency as well as to improve the Zn fortification. In the present work, we have reported the role of zinc solubilizing bacteria on regulation of Zn-regulated transporters (OsZIP1, OsYSL2 and OsYSL6) was assessed by quantative real-time reverse transcription PCR and semi-quantative PCR, in control (without ZSB inoculation) and in treated (with ZSB inoculation) of leaf, stem and root.

Materials and Methods

Plant material

Oat (*Avena sativa* L.) variety JHO-822 from Indian Grassland Fodder and Research Institute, Jhansi, U.P. was used the experiment.

Seed inoculation

The seeds of oat (*Avena sativa* L.) variety JHO-822 were inoculated with Zinc solubilizing bacteria (ZSB) @ 200gm/10kg seed before sowing. Zinc solubilizing bacteria (ZSB) was purchased from Division of Microbiology, Indian Agricultural Research Institute, New Delhi. The required quantity of the cultures, (*i.e.* @ 200 gm culture per 10 kg seed) was mixed to 10% sugar solution to form slurry. The slurry was sprinkled on seeds and mixed with hand to make a uniform coating over the seeds. The required quantity of charcoal was added to absorb the moisture and then seeds were sown immediately (Rao and Tilak, 1977).

Genomic DNA isolation and PCR amplification

Genomic DNA was extracted from oat and rice by using CTAB method (Doyle and Doyle, 1987) with some modifications. PCR amplification were carried out in oat and rice with primer designed using the free software of Primer 3. The primer details are provided in Table 1.

RNA extraction

Total RNA from leaf, stem and root of oat was extracted separately by following the procedure of RNA Express reagent (Himedia Inc.) according to the manufacturer's instructions. Total RNA concentration was measured with a Nanodrop UV/Visible Spectrophotometer (DeNovix DS11 Spectrophotometer). The residual genomic DNA in the RNA preparation was digested with RNase-free Dnase I (Chromous Biotech Pvt. Ltd. India).

Real-Time RT-PCR analysis

Single-stranded cDNA was prepared from Total RNA by Reverse Transcription using Oligo-dT Primer. To 20µg total RNA in 30µl total volume, 2µl of the Oligo-dT Primer (1µg/µl) was added and incubated at 65 °C for 10 min. After a quick chill on ice, 10µl 5X Buffer, 5µl 0.1M DTT and 2µl 10mM dNTPs were added and incubated at 42°C for 10 min. Finally 1µl M-MLV RT (Chromous Biotech Pvt. Ltd. India) was added and kept at 42°C for1 hr. Reaction was terminated by incubation at 65 °C for 10 min. The single-stranded cDNA prepared was used in Real-Time PCR. Real Time PCR was carried out on a Realplex master cycler (Eppendrof Inc.) using the SYBR Green I dye-based detection system. The total Real Time-PCR volume of 20 µl contained 10µl 2xQ-PCR master mix, 150 ng each of forward and reverse primers and 4 µl of the cDNA samples as recommended by Q-PCR kit for SYBER green real time PCR (Chromous Biotech Pvt. Ltd. India). PCR was initiated with a pre-incubation at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 15 second, annealing at 55°C for 15 second and extension at 68°C for 20 second. To determine the specificity of the reaction,

melting curve analysis was done. Data analysis of real-time PCR was carried out using mathematical model of Pfaffl (Pfaffl 2001).

Semi Quantative PCR

Semi Quantative PCR was carried out using the cDNA synthesis kit (Chromous Biotech Pvt. Ltd. India), according to the manufactures instructions.

Results and Discussion

Genomic DNA isolation and PCR amplification

Total genomic DNA was extracted from rice and oat. PCR amplification was carried out in rice as control and Oat, to check the efficiency of primers in rice and oat (Fig. 1).The amplification pattern of genes OsYSL2, Oszip1, OsYSL6 were same observed as expected size in rice and oat.

Real Time PCR analysis

RNA from control and treated oat plants were subjected for real time PCR analysis. The expression level of OsZIP1 gene was increased by 0.4 fold change in treated leaf as compared to control leaf, whereas in stem and root it was increased by 0.5 and 0.1 fold change respectively. For OsYSL2 not any significant fold changes had observed in stem whereas the expression was same in leaf and root. The fold change was much higher in root as compared to leaf and stem for Os YSL6 gene in treated as compare to control (Table 2)

Semi quantitative PCR analysis

The expression level of OsZIP1 gene in treated leaf was much higher as compared to control leaf, whereas in treated stem it was slightly increased but in root it was same as control (Fig. 2). The expression level of OsYSL2 gene in treated leaf and treated root was higher than control leaf and control root. But in treated stem there was not any change in the expression as compared to control stem (Fig. 3). The Expression level of OsYSL6 gene in treated leaf was higher as compared to control leaf, where as in treated stem it was much higher than the control stem. But in treated root it was slightly much higher as compared to control root (Fig. 4).

Table.1 Primer used for Real-Time PCR and semiquantative PCR

SN	Target gene	Primer name	Sequences
1.	OsZIP1	OsZIP1-F OsZIP1-R	5-CGGTTCTACACCACGGACTT-3 5-TCGAGGAACTCGACGAAGAT-3
2.	OsYSL2	OsYSL2-F OsYSL2-R	5-AGAACACCGTTGTCCAGACC-3 5-TAAGGAGCCCAACGAAGCTA-3
3.	OsYSL6	OsYSL6-F OsYSL6-R	5-GTTTGTCTGGGAGAGGGTGA-3 5-CTGGTAAGGGATGGCTTGAA-3
4.	Tubulin	Tubulin-F Tubulin-R	5-TCTTCCACCCTGAGCAACTC-3 5-GAGTTGCTCAGGGTGGAAGA-3

Table.2 Expression level of target genes in different plant part of Oat

SN	Target gene	Plant part	Fold Change (Treated/Control)
1.	OsZIP 1	Leaf	0.4
		Stem	0.5
		Root	0.1
2.	OsYSL 2	Leaf	0.1
		Stem	0.0
		Root	0.1
3.	OsYSL 6	Leaf	0.2
		Stem	0.4
		Root	1.4

Fig.1 Amplification pattern of OsYSL2, OsZIP1 and OsYSL6 in rice and oat. M: 1Kb ladder; R1: amplified product of OsYSL2 from rice; O1: amplified product of OsYSL2 from oat; R2: amplified product of OsZIP1 from rice; O2: amplified product of OsZIP1 from oat; R3: amplified product of OsYSL6 from rice; O1: amplified product of OsYSL6 from oat

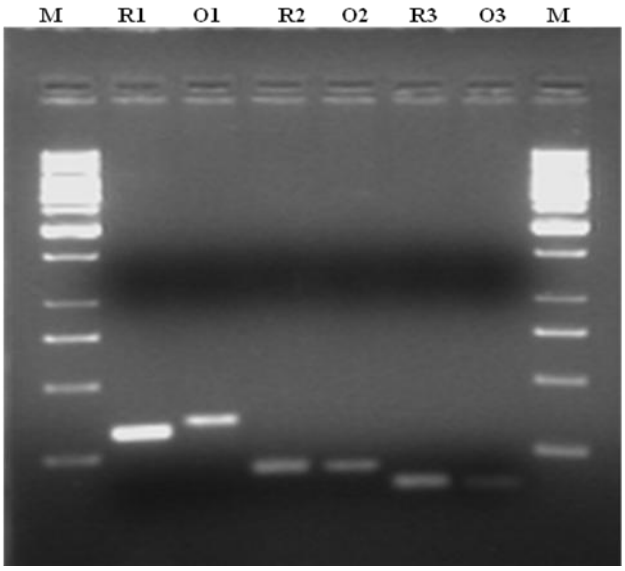


Fig.2 Semi-Quantative PCR showing expression pattern of OsZIP1 and tubulin in different plant part of oat in control and treated. CL: Control Leaf; CS: Control Stem; CR: Control Root; TL: Treated Leaf; TS: Treated Stem; TR: Treated Root

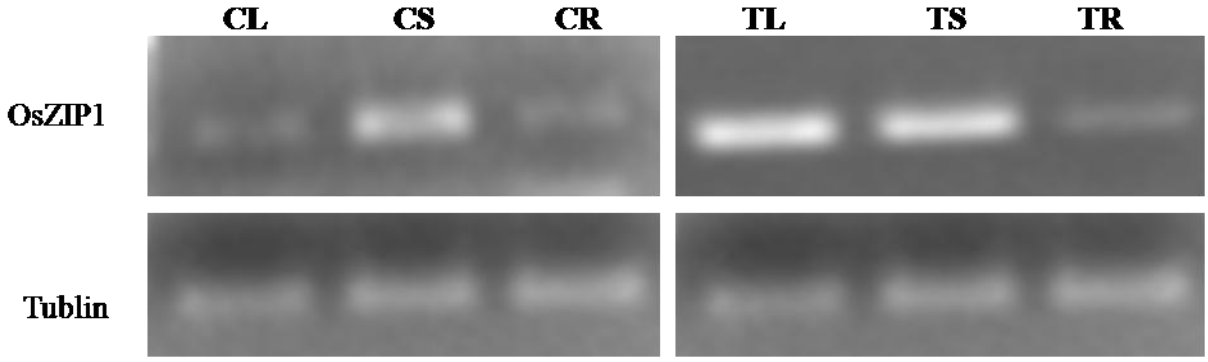


Fig.3 Semi-Quantative PCR showing expression pattern of OsYSL2 and tubulin in different plant part of oat in control and treated. CL: Control Leaf; CS: Control Stem; CR: Control Root; TL: Treated Leaf; TS: Treated Stem; TR: Treated Root

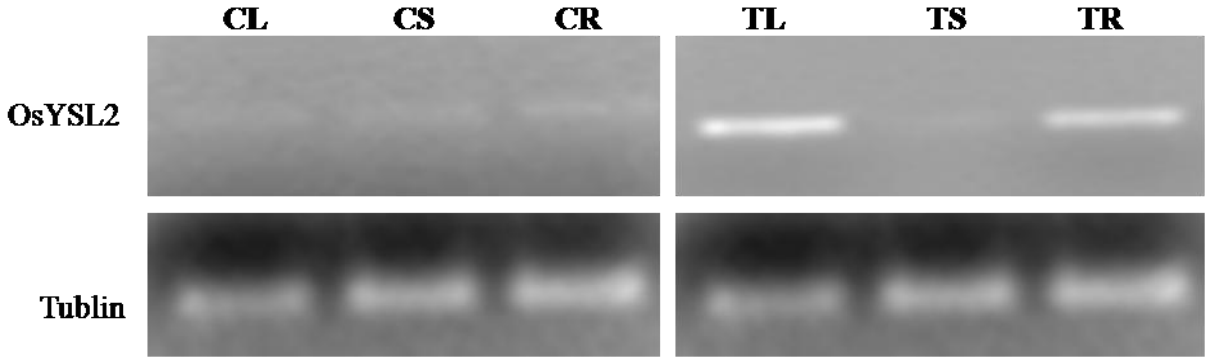
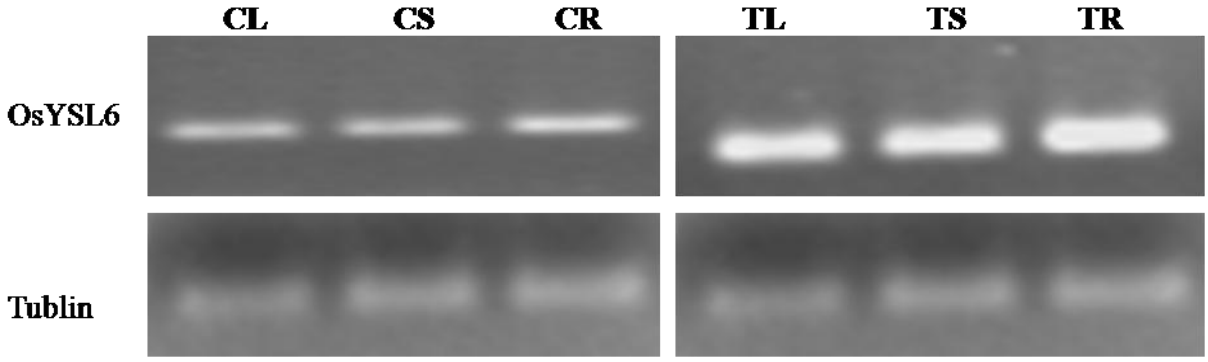


Fig.4 Semi-Quantative PCR showing expression pattern of OsYSL6 and tubulin in different plant part of oat in control and treated. CL: Control Leaf; CS: Control Stem; CR: Control Root; TL: Treated Leaf; TS: Treated Stem; TR: Treated Root



Plants evolved various mechanisms for transportation, translocation and assimilation of mineral nutrient from soil to plant. The present study framed in keeping in the view of all mechanism involved in mineral transportation, translocation and assimilation in plants. In the present study by exploiting the expression analysis of genes involved in the transportation of zinc has been studied. The molecular mechanism for the transportation of zinc is a complicated process; however the exact expression analysis study gives inside the mechanism of transportation of zinc in plant. In the present investigation transporters genes which may be responsible for the transportation of zinc were studies by their expression studies.

Expression analysis of OsZIP1 gene

The expression level of OsZIP1 gene in treated leaf was much higher as compared to control leaf, where as treated stem it was slightly increased but in root it was same as control. The ZIP family transporters are well characterized in plants (Ramesh *et al.,* 2003). Guerinot (2000), Maser *et al.,* (2001) suggested their involvement in uptake system for zinc. Ramesh *et al.,* (2003); Ishimaru *et al.,* (2005); Chen *et al.,* (2008) demonstrated that most of ZIP family genes are induced by zinc deficiency and their expression pattern varied between root and shoot system. In our study we have observed that in control (normal soil), the expression of ZIP1 gene was less as compared to zinc treated soil (zinc efficient soil). The expression pattern varied between leaf, shoot and root system. The same result were observed by Ramesh *et al.,* (2003); Ishimaru *et al.,* (2005), where they showed that expression of ZIP1 gene was higher level in root than shoot under zinc deficient condition. Chen *et al.,* (2008) also observed that OsZIP1 was up regulated in zinc deficient root, but no visible transcript in shoot of both zinc efficient and zinc inefficient rice genotype. Ramegowda *et al.,* (2013) found that by over expressing OsZIP1 finger millet showed higher expression of this gene in leaves under zinc sufficient condition. In the present work we found that higher expression of ZIP1 in shoot than in root. This is in accordance with the earlier finding that zinc abundance reduces the OsZIP1 expression (Ramesh *et al.,* 2003; Ishimaru *et al.,* 2005).

Expression analysis of OsYSL2 gene

YSL2 transporter gene belong the family of YSL, they are oligopeptide transporter family (Yen *et al.,* 2001). The expression level of OsYSL2 gene in treated leaf and treated root was higher than control leaf and control root. But in treated stem there was no change in the expression as compared to control stem. Curie *et al.,* (2001) showed analysis of some YSL transporter family member and their involved in transport of zinc. Koike *et al.,* (2004) showed high expression of OsYSL2 in the leaves but in root of iron deficient plant. They also demonstrate that OsYSL2 expression was induced in the leaves of iron deficient plant. Schaaf *et al.,* (2004) reported ZmYS1 analogs of YSL2 also transported zinc and copper with iron. Curie *et al.,* (2001) reported that iron deficiency induced opposing the expression OsYSL2 in leaves. In our study a similar pattern was observed in control, where expression was lower however; in Zinc treated soil the expression of YSL2 was much higher in leaves.

Expression analysis of OsYSL6 gene

OsYSL6 is another member of YSL family (Curie *et al.,* 2009). The Expression level of OsYSL6 gene in treated leaf was higher as compared to control leaf, whereas in treated stem it was much higher than the control stem. But in treated root it was much higher as compared to control root. OsYSL6 was

constitutively expressed in both leaves and root of either iron sufficient and deficient plant (Koike *et al.*, 2004). In our study the same result were obtained in leaves, stem and root, in both the condition either Zn sufficient or Zn deficient, But the expression level was more in Zn sufficient soil.

It is concluded that the present investigation involved the study of expression of zinc transporters genes (OsZIP1, OsYSL2 and OsYSL6) through quantitative real time PCR and qualitative semi-quantitative PCR in Oat. The study involved the selection of appropriate zinc transporter genes from NCBI and their expression analysis. Three transporter genes specially OsZIP1, OsYSL2and OsYSL6 studied in control plant (without ZSB inculcation) and treated (with ZSB inculcation) in plant part such as leaf, stem and root. We proved that the inoculation of ZSB under controlled condition can able to regulate some of the Zn-regulated transporters family genes and thereby controlled the Zn uptake in oat. The expressions of OsZIP1, OsYSL2 and OsYSL6 in oat were slightly higher in treated as compared to control. These results are evident that the ZSB inoculation could regulate the Zn uptake and translocation in oat plant.

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