Advances in Bioresearch
Adv. Biores., Vol 12 (2) March 2021: 23-29
©2021 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.12.2.2329

Advances in Bioresearch

ORIGINAL ARTICLE

Expression of ZmZIP1 a gene involved in Zinc Transport after nutrient application in Oat *(Avena Sativa* L.)

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ABSTRACT
Mineral deficiency mostly Zinc (Zn) is one of the well-documented problems in food and forage crops affecting agricultural production and subsequently human and animal health. Although many studies have been performed on the biofortification of staple crops with Zn, few studies have focused on forage crops. Oat (Avena sativa L.) is a winter crop in many parts of the world and is used as a 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 micronutrients could be a sustainable agronomic approach to increase the soil availability of Zn for nutritionally rich oats. In this study, the role of micronutrient application on regulation of Zn-regulated transporter gene (ZmZIP1) was assessed by quantitative real-time reverse transcription PCR and semi-quantitative PCR, in control (without micronutrient application) and in treatment condition (with micronutrient application) in different tissues of whole life cycle of oat. ZmZIP1 gene undoubtedly upregulated in all tissues with ≤ 1 or ≥ 1 fold change especially up to 80 DAE, whereas at plant maturity particularly at 100 DAE ≥ 1 fold change were observed in all tissues. These results suggest that ZmZIP1, in oat is an oat zinc transporter could be responsible for the Zinc transportation and will inform genetic engineering strategies aimed at increasing the efficiency of crop Zn biofortification.
Keywords: *Zinc, Micronutrient, Oat (Avena sativa L.), ZIP transporters, DAE (Days after Emergence) of plant*

Received 24.10.2020 Revised 22.11.2020 Accepted 27.02.2021

How to cite this article:
R Ranjan, V. S. Chauhan, M Chaudhary, K. K. Singh, Shahid Ahmed, and K K Dwivedi. Expression of ZmZIP1 a gene involved in Zinc Transport after nutrient application in Oat *(Avena Sativa* L.). Adv. Biores. Vol 12 [2] March 2021. 23-29

INTRODUCTION

Mineral deficiency or mineral imbalances 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 subtropical regions [18], which often limit the performance of the animals [4]. 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 [18]. Zn is essential for normal growth, production, reproduction, health and immunity of animals and it plays a 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 [7]. 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 [19, 1].

Oat (*Avena sativa* L.) is an economically important crop and ranks sixth in world cereal production [6]. It is also used as forage crop and grown as a multipurpose crop for grain, pasture and forage in many parts of the world [23]. Oat is the major source of dry fodder-low in Zn concentrations, particularly when grown on Zn-deficient soil of India [23, 25]. [24] reported that average Zn content 30-32mg/kg in Oat at different cutting stages. [1] reported that the average Zn content in different genotypes of Oat is 24-30 mg/kg.

Several genes are responsible for transportation and accumulation of minerals in plants. The available nutrient especially Zn and Fe from soil is taken to root membrane transport mechanisms in rice [2]. Various ZIP family proteins which Zn and Fe regulated transporters like protein are responsible to regulate the mechanism of transportation [8]. Genes involved for Zn uptake from soil, translocation from root to shoot as well as for storage in grains such as OsIRT1, OsIRT2, OsZIP1, OsZIP3, OsZIP4, OsZIP5, OsZIP7, and OsZIP8 were studied [22, 10, 27, 13, 14] . OsZIP1, OsZIP3, OsZIP4, OsZIP5, and OsZIP8 are rice plasma membrane Zn transporters and are induced by Zn deficiency [22, 10, 27, 13, 26], whereas OsITR1 and OsITR2 are responsible for transport of iron from soil to root [10]. In rice the expression of Zinc transporter genes as inoculation by Zn solubilizing PGPR were studied. They obsrved the expression patterns of *OsZIP1*, *OsZIP4,* and *OsZIP5* in the root and shoots of rice [12]. [3] 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 [9]. Phylogenetic tree of ZmZIPs with Rice ZIPs (OsZIPs), Arabidopsis ZIPs (AtZIPs), *Hordeum vulgare* ZIPs (HvZIPs), *Medicago truncatula* ZIPs (MtZIPs), Wild Emmer Wheat ZIP (TdZIP1), and *Glycine max* ZIP (GmZIP1) were constructed and revealed that the predicted amino acid sequences of ZmZIPs were closely related to ZIPs from rice, Arabidopsis, Wheat, Lucern and Barley, indicating that those ZIPs may share a common evolutionary ancestor [15]. The study of molecular mechanism and understanding the interaction between oat plant and nutrient application 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 micronutrient application on regulation of Zn-regulated transporter gene (ZmZIP1) was assessed by quantitative real-time reverse transcription PCR and semi-quantitative PCR, in control (without micronutrient application) and in treated (with micronutrient application) in different tissues of whole life cycle of oat.

MATERIAL AND METHODS

Plant Material

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

Nutrient application

Seeds of released variety JHO-822 were planted at a density of twenty plants per square in 30cm deep pots, and later thinned to a maximum density of ten plants per pot. In treatment condition, plants were watered as needed (usually twice a week) by following the sub-irrigation of a nutrient solution of the following composition: 0.23M $CaCl_2$, 0.95M NH_4NO_3, 0.319M $NaH_2PO_4.2H_2O$, 0.574M KCl, 0.327M $MgCl_2$.6H2O, 0.207M $NaSO_4$, 0.207M FeNaEDTA, 0.0489M H_3BO_3, 0.022M $MnCl_2.4H_2O$, 0.016M $ZnCl_2$, 12.4mM $CuCl_2$ $2H_2O$, 1.1mM $[NH_4]_6Mo_7O24.H_2O$, 0.84mM $NiCl_2.6H_2O$ and 0.84mM $CoCl_2.6H_2O$ [11], whereas in case of control condition, plants were watered as needed.

Sample collection

Tissue samples were collected at different growth stages from the whole life cycle of the oat plant, i.e. 20, 40, 60, 80 and 100 days after emergence (DAE) of the plant. The leaf and root samples were collected at 20 DAE, whereas at 40 and 60 DAE leaf, stem and root were collected. At 80 DAE i.e. maturity of the plant leaf, stem, root and florets and at 100 DAE leaf, stem, root and grain were collected from both the sets of experiments.

Genomic DNA isolation and PCR amplification

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

RNA extraction

Total RNA from all the tissues at different growth stages of the life cycle were 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 (Eppendorf 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 SYBR 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 the mathematical model of Pfaffl [20].

Semi-Quantitative PCR

Semi-Quantitative PCR was carried out using the cDNA synthesis kit (Chromous Biotech Pvt. Ltd. India), according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Genomic DNA isolation and PCR amplification

Total genomic DNA was extracted from maize and oat. PCR amplification was carried out in maize as control and oat, to check the efficiency of primers in maize and oat (Fig. 1).The amplification pattern of gene ZmZIP1 was the same observed as expected size in maize and oat.

Expression analysis by Real Time PCR and Semi quantitative PCR

Plants evolved various mechanisms for transportation, translocation and assimilation of mineral nutrients from soil to plant. The present study is framed in keeping in view of all mechanisms 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 plants. In the present investigation transporters genes which may be responsible for the transportation of zinc were studied by their expression studies. Despite several genes encoding Zn transporters having been identified and characterized in plants, the mechanisms of Zn uptake and transport in oat are still unknown. In the present study, the role of micronutrient application on regulation of Zn-regulated transporter gene (ZmZIP1) was assessed by quantitative real-time reverse transcription PCR and semi-quantitative PCR, in control (without micronutrient application) and in treatment (with micronutrient application) in different tissues of whole life cycle of Oat.

RNA from control and treated oat plants at different growth stages were subjected for real time PCR analysis. The expression level of the ZmZIP1 gene was increased by 0.5 fold change in treated leaf as compared to control leaf, whereas in root it was increased by 0.9 fold change at 20 DAE. At 40DAE the fold changes were 0.6, 0.8 and 1.1 in leaf, stem and root respectively in treatment compared to control condition. The fold change at 60DAE were 1.3, 0.9 and 1.7 in leaf, stem and root respectively in treated as compared to control. Whereas at 80DAE the fold changes were 0.7, 0.6, 0.7 and 0.2 in leaf, stem, root and florets respectively as compared to treated versus control. At grain stage i.e. at 100DAE, the expression in terms of fold change was 1.1, 1.6, 1.8 and 1.7 in leaf, stem, root and seed respectively (Table 2). The expression pattern by semi-quantitative PCR of the ZmZIP1 gene in the leaf and root of control and treated were almost the same at 20DAE, there were not any significant changes observed (Fig. 2). At 40DAE the expression was slightly changed in different tissues of the plant in treated versus control condition. In leaf and root expression were same observed, whereas in stem it was drastically decreased in treated as compared to control (Fig. 3). The expression in leaf and root were decreased but in stem it was the same in treated and controlled at 60DAE (Fig. 4). The expression level was low in stem and florets, whereas in leaf and root it was high in both treated and controlled at 80DAE (Fig. 5). At 100DAE the pattern of expression in leaf and root were high and same in treated and control, whereas in stem it was low and same in both conditions. But the expression was more observed in treated seed as compared to control seed (Fig. 6).

Expression of ZmZIP1 gene in various tissues at different times of days after emergence of the oat was different. At 20DAE, no significant changes were observed whereas at 40DAE the expression was conversely differ with 60DAE. At 80DAE and 100 DAE expression was comparatively similar however it was slightly high at 100DAE. Although several workers studied the expression of ZIP in various crops but no one observed the expression in the whole life cycle of the plant. The ZIP family transporters are well characterized in plants [22]. [8] & [17] suggested their involvement in the uptake system for zinc. [22], [10] & [3] demonstrated that most 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 was varied in different tissues at different growth stages of the whole life cycle of the

plant. The same results were observed by [22] & [10], where they showed that expression of ZIP1 gene was higher level in root than shoot under zinc deficient condition. [3] also observed that OsZIP1 was upregulated in zinc deficient root, but no visible transcript in both zinc efficient and zinc inefficient rice genotypes. [21] found that by over expressing OsZIP1 finger millet showed higher expression of this gene in leaves under zinc sufficient condition. Few maize transporter genes ZmZIP3, ZmZIP4, ZmZIP5 and ZmZIP7 were upregulated in maize root in Zn deficient condition. [16], this is according to our result. 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 [22, 10]. In conclusion, this is the first study to characterize the expression of gene related to Zn transporter following the nutrient application in different tissues of the whole life cycle of oat and provide the new molecular insights to the response of the Zn transport related processes. The present investigation involved the role of micronutrient application on regulation of Zn-regulated transporter gene (ZmZIP1) was assessed by quantitative real-time reverse transcription PCR and semi-quantitative PCR, in control (without micronutrient application) and in treated (with micronutrient application) in different tissues of whole life cycle of oat. We proved that the nutrient application under controlled condition is able to regulate some of the Zn-regulated transporters family genes and thereby controlled the Zn uptake in oat. The expression of the ZmZIP1 gene in oats was slightly higher in treated as compared to control. These molecular results are evident that the nutrient application could regulate the Zn uptake and translocation in oat plants.

Table 1: Primer used for Real-Time PCR and semi-quantitative PCR

SN	Target gene	Primer name	Sequences
1.	ZmZIP1	ZmZIP1-F ZmZIP1-R	5-CTCCCTGATTGAGGGTTTCA-3 5-AGAAATGCCTAGAGCGACCA-3
2.	Tubulin	Tubulin-F Tubulin-R	5-TCTTCCACCCTGAGCAACTC-3 5-GAGTTGCTCAGGGTGGAAGA-3

Table 2: Expression level of ZmZIP1genes in different tissues of Oat at different days after emergence (DAE)

Day after Emergence (DAE) of plant	Plant part	Fold Change (Treatment/Control)
20th	Leaf	0.5
	Root	0.9
40th	Leaf	0.6
	Stem	0.8
	Root	1.1
60th	Leaf	1.3
	Stem	0.9
	Root	1.7
80th	Leaf	0.7
	Stem	0.6
	Root	0.7
	Florets	0.2
100th	Leaf	1.1
	Stem	1.6
	Root	1.8
	Seed	1.7

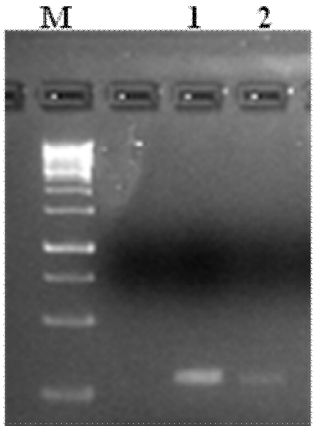

Fig. 1: Amplification pattern of ZmZIP1 in maize and oat. M: 1KB ladder; 1: amplification product of ZmZIP1of maize; 2: amplification product of ZmZIP1of Oat

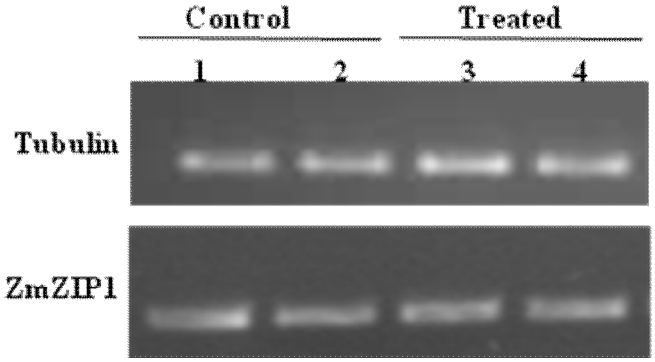

Fig. 2: Semi-quantitative PCR showing expression pattern of ZmZIP1 and tubulin in different tissues of Oat in control and treated at 20 DAE. 1: Control leaf; 2: Control root; 3: Treated leaf; 4: Treated root

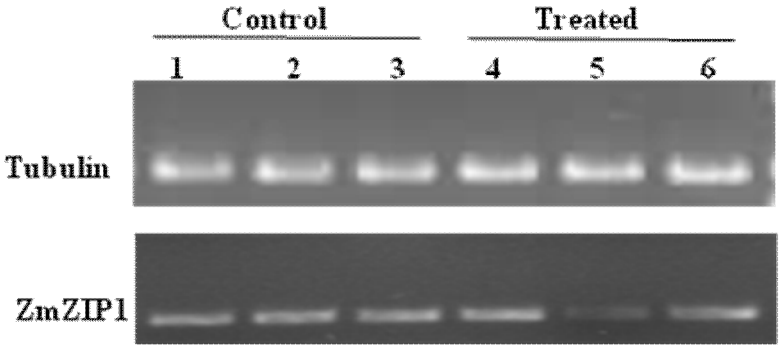

Fig. 3: Semi-quantitative PCR showing expression pattern of ZmZIP1 and tubulin in different tissues of Oat in control and treated at 40 DAE. 1: Control leaf; 2: Control stem; 3: Control root; 4: Treated leaf; 5: Treated Stem; 6: Treated root

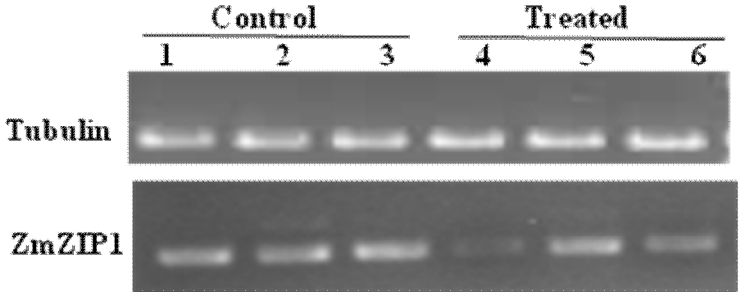

Fig. 4: Semi-quantitative PCR showing expression pattern of ZmZIP1 and tubulin in different tissues of Oat in control and treated at 60 DAE. 1: Control leaf; 2: Control stem; 3: Control root; 4: Treated leaf; 5: Treated Stem; 6: Treated root

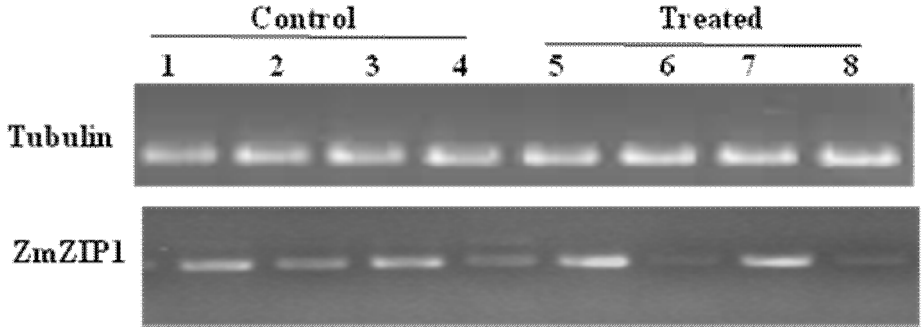

Fig. 5: Semi-quantitative PCR showing expression pattern of ZmZIP1 and tubulin in different tissues of Oat in control and treated at 80 DAE. 1: Control leaf; 2: Control stem; 3: Control root; 4: Control floret; 5: Treated leaf; 6: Treated Stem; 7: Treated root; 8: Treated floret

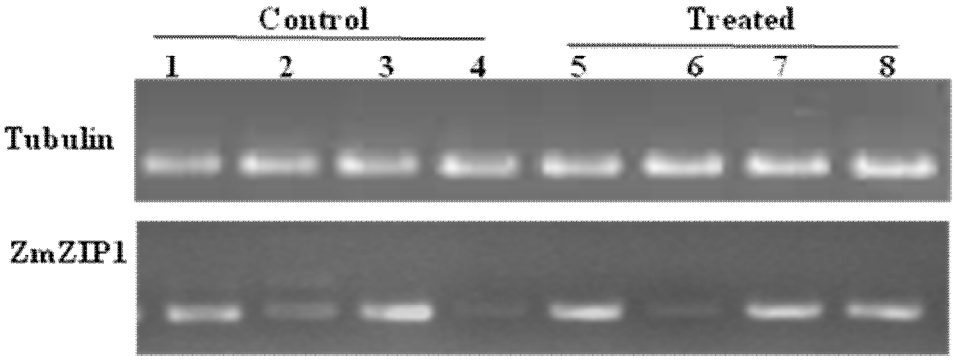

Fig. 6: Semi-quantitative PCR showing expression pattern of ZmZIP1 and tubulin in different tissues of Oat in control and treated at 100 DAE. 1: Control leaf; 2: Control stem; 3: Control root; 4: Control seed; 5: Treated leaf; 6: Treated Stem; 7: Treated root; 8: Treated seed

ACKNOWLEDGMENTS

Authors gratefully acknowledge Director, Indian Grassland and Fodder Research Institute (IGFRI), Jhansi and Indian Council of Agricultural Research (ICAR), New Delhi, India for carrying out the present investigation

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