Identification, expression analysis, and molecular modeling of *Iron-deficiency-specific clone 3 (Ids3)*-like gene in hexaploid wheat

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Abstract

Graminaceous plants secrete hydroxylated phytosiderophores encoded by the genes iron-deficiency-specific clone 2 (*Ids2*) and iron-deficiency-specific clone 3 (*Ids3*). An effort was made to identify a putative ortholog of *Hodeum vulgare Ids3* gene in hexaploid wheat. The protein structure of TaIDS3 was modeled using homology modeling and structural behavior of modeled structure was analyzed at 20 ns. The simulation trajectory using molecular dynamics simulation suggested the model to be stable with no large fluctuations in residues and local domain level RMSF values (<2.4 Å). In addition, the ProFunc results also predict the functional similarity between the proteins of HvIDS3 and its wheat ortholog (TaIDS3). The *TaIds3* gene was assigned to the telomeric region of chromosome arm 7AS which supports the results obtained through bioinformatics analysis. The relative expression analysis of *TaIds3* indicated that the detectable expression of *TaIds3* is induced after 5th day of Fe starvation and increases gradually up to 15th day, and thereafter, it decreases until 35th day of Fe-starvation. This reflects that Fe deficiency directly regulates the induction of *TaIds3* in the roots of hexaploid wheat. The identification of *HvIds3*-like gene in wheat has opened up new opportunities to enhance the nutrient quality in wheat through biofortification program.

Keywords Phytosiderophores · *Ids3* · Hexaploid wheat · Chromosomal assignment · Fe deficient · Homology modeling · Molecular dynamics simulations

Introduction

Iron (Fe) deficiency is a major abiotic stress, especially in calcareous soil (~30% of the world's cultivated soils) with extremely low solubility of Fe (owing to high pH levels) leading to its poor availability uptake by plants. This in turn causes severe yield losses. Furthermore, the poor uptake of other minerals by plants also reduces the overall mineral content in grains, which is essential to meet the required

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human dietary needs (Cakmak 2008; Farre et al. 2017; Waters et al. 2018).

The manifestation of Fe deficiency in calcareous soils has been recognized as Fe chlorosis and lime-induced chlorosis. Under adverse conditions of Fe deficiency, graminaceous plants secrete Fe chelators called mugineic acid family phytosiderophores (MAs) from their roots (Takagi 1976). MAs are highly effective in solubilisation and mobilization of Fe in calcareous soil (Treeby et al. 1989) and are involved in the uptake of Fe through roots (von Wiren et al. 1995) through Fe^{3+} -MA transporters in the form of Fe^{3+} -MAs complex. The secretion of MAs increase under deficiency of Fe and is correlated with plant's tolerance to Fe deficiency (Ceballos-Laita et al. 2018). The quantity and different forms of phytosiderophores secreted by roots play an important role in providing tolerance to plants under Fe-deficient condition. The hydroxylated forms of phytosiderophores like 3-hydroxymugineic acid (HMA) and 3-epihydroxymugineic acid (epi-HMA) have been found more efficient in chelation and subsequent uptake of trace minerals (von Wiren et al. 2000). In *Hordeum vulgare*, the genes responsible for hydroxylation of phytosiderophores have been recognized as *iron-deficiency-specific clone 2* (*Ids2*) and *iron-deficiency-specific clone 3* (*Ids3*) (Nakanishi et al. 2000). The *Ids3* gene encodes an enzyme dioxygenase which hydroxylates the C-2' position of 2'-deoxymugineic acid (DMA) and 3-epihydroxy-2'-deoxymugineic acid (epi-HDMA) and converts DMA to MA or epi-HDMA to epi-HMA. The *Ids2*, on the other hand, hydroxylates C-3' position of MA and DMA and converts MA to epi-HMA or DMA to epi-HDMA. MA, epi-HDMA, and epi-HMA are more stable under mildly acidic conditions and might be more favourable for inter-mineral translocation (Kobayashi and Nishizawa 2012). The gene *Ids2* has 3411 bp long coding sequence which encodes a protein of 338 amino acids (<http://www.uniprot.org/uniprot/Q40061>), while *Ids3* has a 4904 bp long coding sequence which encodes a protein of 339 amino acids (<http://www.uniprot.org/uniprot/Q40062>). The expression of *Ids3* is always much stronger than that of *Ids2* in Fe-deficient condition indicating predominant conversion of DMA to MA (Nakanishi et al. 2000).

The biosynthetic pathway for mugineic acid production is well documented in graminaceous crops (Ma and Nomoto 1993). All MAs share the pathway from methionine to DMA and the DMA is then converted to other MAs like HMA, epi-HMA, and epi-HDMA. The pathway up to DMA synthesis is conserved in all graminaceous plants, but different types of phytosiderophores are synthesized during hydroxylation steps (Takahashi 2003). It is reported that wheat cultivar Chinese Spring (CS) used in the present study produces only one phytosiderophore, namely, DMA (non-hydroxylated form), whereas barley cv. Betzes and rye each synthesizes four types of MAs, i.e., DMA, MA, epi-HDMA, and epi-HMA (Nakanishi et al. 2000; Mori et al. 1990; Neelam

et al. 2011). It has been reported that wheat and its wild progenitors do not have the ability to secrete MA and HMA from the beginning of their evolution, because they lack they lack the *IDS3* enzyme need for their synthesis (Singh et al. 2000). Despite the critical interplay of *Ids3* in nutrition, no efforts were made in the past to study *Ids3* gene in wheat genome. However, the recent availability of wheat reference genome sequence it has become possible to identify ortholog of *HvIds3* in wheat.

In view of the above, the present investigation was aimed at the identification and chromosomal assignment of the wheat ortholog of *Ids3* gene and to analyze its expression pattern under Fe-deficient conditions in hexaploid wheat. We also modeled the 3D structure of TaIDS3 followed by molecular dynamics (MD) simulations. This study provides an insight about the *TaIds3* gene in wheat, both at the gene level and the protein structural level.

Materials and methods

Plant growth conditions

Seeds of wheat cv. CS were surface sterilized and soaked on filter paper with distilled water and incubated in dark at 25 °C until germination. Three-day-old seedlings were transplanted and cultured hydroponically in 2.0 L plastic boxes with continuous air flow under Fe-sufficient (+Fe) and Fe-deficient (−Fe) conditions. The 1× concentration of the nutrient solution for hydroponic culture consisted of 2.0 mM $\text{Ca}(\text{NO}_3)_2$, 0.7 mM K_2SO_4 , 0.1 mM KCl, 0.1 mM KH_2PO_4 , 0.50 mM MgSO_4 , 0.15 mM Fe^{3+} -EDTA, 10 μM H_3BO_3 , 0.5 μM MnSO_4 , 0.5 μM ZnSO_4 , 0.2 μM CuSO_4 , and 0.01 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$. The nutrient solution was replaced once per week and its pH adjusted to 5.5–5.6 daily with 1 N HCl. For expression study under Fe starvation, wheat cv. CS was grown at 23–25 °C under a 16/8 h light (~160 $\mu\text{mol}/\text{m}^2 \text{ s}$)/dark regi.

Identification and characterization an ortholog of *HvIds3* gene in wheat using in-silico approach

In-silico analysis was performed to identify the putative ortholog of *HvIds3* gene in hexaploid wheat. Full-length genomic sequence of *HvIds3* gene (Accession Number AB024058.1) was retrieved from NCBI (National Center of the Biotechnology Information) (<http://www.ncbi.nlm.nih.gov/>) in FASTA format. Similarity searches were carried out using genomic sequence of *HvIds3* as a query against the wheat chromosome survey sequences of IWGSC-URGI available at <http://urgi.versailles.inra.fr/using> the BLAST search engine (<http://www.ncbi.nlm.nih.gov/BLAST>). The results of BLAST analysis were used to identify 'putative'

wheat ortholog using the criteria followed by Kumar et al. (2016a, b). The exon/intron structure prediction in the selected wheat contigs was carried out using FGENESH+ (<http://linux1.softberry.com/berry.phtml>) program. Conserved domain search (CD Search) program was used to predict functional domain(s) in the sequences of *HvIds3* and its wheat ortholog *TaIds3* (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>).

Multiple sequence alignment (MSA) between *HvIds3* and three copies of *TaIds3* (*TaIds3*-7AS, *TaIds3*-7BS, and *TaIds3*-7DS) was performed using CLUSTALW2 (www.ebi.ac.uk/Tools/msa/clustalw2) available on EMBL-EBI (www.ebi.ac.uk).

Homology modeling, verification, and function prediction

The 3D structure of TaIDS3 protein was predicted using homology modeling-based Swiss-Model server (<http://swissmodel.expasy.org/>) using the following criteria as described by Kumar et al. (2017) and Nagaraju et al. (2018). To evaluate the modeled structure of TaIDS3, Ramachandran plot was calculated by analyzing phi (Φ) and psi (Ψ) torsion angles and covalent bond quality using PROCHECK program of PSVS server (http://psvs-1_5-dev.nesg.org/). Flexible structure Alignment T by Chaining AFPs (Aligned Fragment Pairs) with Twists (FATCAT) structure alignment tool (<http://fatcat.burnham.org/>) was used for the alignment of 3D structure model of proteins. ProFunc (<http://www.ebi.ac.uk/thornton-srv/databases/ProFunc/>) was used to predict the biochemical function of TaIDS3 based on the predicted 3D model. Sub cellular localization of HvIDS3 and TaIDS3 proteins was predicted using consensus algorithms of Target P 1.1 (<http://www.cbs.dtu.dk/services/TargetP/>) and PLANT-mPLoc (<http://www.csbio.sjtu.edu.cn/cgi-bin/PlantmPLoc.cgi>), respectively.

Cytogenetic stocks

To facilitate the chromosomal assignment of wheat ortholog of *Ids3* gene to specific, chromosome arm and deletion bin, following cytogenetic stocks in the background of wheat cv. CS were used: (1) 22 nullisomic–tetrasomic (NT) lines (Sears 1954, 1966); (2) two ditelosomic (Dt) lines, i.e., 7AS and 7AL (Sears and Sears 1978); and (3) 8 homozygous deletion (Del) lines of chromosome 7AS (Endo and Gill 1996). Seeds of NT, Dt, and Del lines were procured from the Wheat Genetic and Genomic Resources Center at the Kansas State University, Manhattan, USA and multiplied in the greenhouse facility at the G. B. Pant University of Agriculture and Technology, Pantnagar, India.

DNA isolation and primer designing

The genomic DNA from fresh leaves of cytogenetic stocks and wheat cv. CS was extracted using CTAB (Cetyltrimethyl ammonium bromide) method with some modifications (Maroof et al. 1994). To confirm the chromosomal location of *TaIds3*, a pair of 7AS chromosome specific primer, i.e., *TaIds3* 7AS F (TTGGTTACGACCTGCAAGAAA) and *TaIds3* 7AS R (ACCATTACCCAAGGTGCTCG) was designed using primer blast software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

Polymerase chain reaction (PCR)

PCR amplification was carried out in a volume of 20 μ L in Applied Biosystems (Veriti 96 well) thermal cyclor. The reaction mixture contained 80–100 ng of template DNA, 1 $\times$ PCR buffer, 1.5 mM MgCl₂, 0.25 mM dNTP, 500 nM of each PCR primer (forward and reverse) and 1.0 U *Taq* DNA polymerase (New England Biolabs). PCR cycling conditions were as follows: 95 °C for 2.30 min followed by 35 cycles of 95 °C for 50 s, 56 °C for 40 s and 68 °C for 1.0 min with a final extension at 68 °C for 5 min. The PCR products were resolved using 10% polyacrylamide gel electrophoresis (PAGE) and visualized following silver staining (Tegelstrom 1992).

RNA extraction and real-time reverse-transcription PCR (RT-PCR) analysis

100 mg root samples were harvested at 5, 10, 15, 25, and 35 days after the plants were exposed to +Fe and –Fe conditions and used immediately for RNA isolation. Root tissues were frozen in 2 mL micro-centrifuge tubes and disrupted under frozen conditions using two stainless steel beads (5 mm diameter) in the TissueLyser (Qiagen, USA). Total RNA was extracted from the disrupted tissue using the GeneJET Plant RNA purification kit (Fermentas Life Sciences) according to the manufacturer's protocol in duplicates. The quantity and quality of the total RNA were evaluated using a nanophotometer (Nanodrop 1000, Thermo-scientific) and visualized following 1% agarose gel electrophoresis. Approximately 1 μ g of total RNA was reverse transcribed to cDNA in 20 μ L reaction using oligo-dT and M-MLV reverse transcriptase (Fermentas Life Sciences). Real-time PCR primers were designed to amplify a 100–200 bp fragment in untranslated regions. The primer pair, i.e., *Ids3*cDNA F (CGTCGGCGACACCAAGA) and *Ids3*cDNA R (CACCTCGCGGAGCTTTTG) was designed from the conserved region of mRNA of all predicted

TaIds3 orthologs using Primer Express® Software version 3.0 (Applied Biosystems).

Real-time RT-PCR was performed with a StepOnePlus Real-Time PCR System using SYBR Select Master Mix (Applied Biosystems, USA). Reactions were performed in a total volume of 10 µL with 0.2 µM of each primer, 1×SYBR premix, 50 ng cDNA and ddH₂O. Reactions were cycled under the following conditions: initial denaturation at 95 °C for 2 min, followed by 40 cycles composed of 15 s denaturation at 95 °C and 1.0 min of annealing/extension at 60 °C. To verify specific amplification, melting curve analysis was performed at 65–95 °C with the fluorescence continuously being monitored. Data were analyzed via $2^{-\Delta\Delta C_T}$ method with the v2.3 Software (Applied Biosystems, USA) (Livak and Schmittgen 2001). A primer pair, i.e., ADP rf F (GGGTTC TACGAGGGTCTTGA) and ADP rf R (TCCAGCACGTTT GTTCTTG) was designed to check the expression level of housekeeping gene *TaADP* ribosylation factor which was used as an internal control (Paolacci et al. 2009). For all real-time PCR analysis, two biological replicates and three technical replicates of each biological replicate were used.

Molecular dynamics simulations

Molecular dynamics (MD) simulations have become one of the most important techniques in biophysics which is helpful to understand the behavior of biological macromolecules on ns-to-ms time scales (Gajula et al. 2015, 2016). To check the stability of modeled structure of *TaIDS3*, molecular dynamics (MD) simulation carried out by Desmond v3.6 (Shivakumar et al. 2010; Guo et al. 2010; Jee et al. 2017) implemented in Schrodinger-maestro 2017 (Schrodinger Release 2017-2). Protein model preparation was performed by assigning bond orders, hydrogen atoms added in protein model with neutral pH 7.0 applied by PROPKA method (Li et al. 2005). Total 5307 TIP3P (Jorgensen et al. 1983) water solvent atoms were added in truncated octahedron simulation box under periodic boundary conditions and shape and size was set at 10.0 Å buffered distance. To neutralize the simulation system electrically, added Na⁺/Cl[−] ions to stabilize box charge and placed randomly in the solvated system. After preparing simulation box (Box size = 19,882) was proceeded to energy minimization by applying 800 steps of steepest descent algorithm followed 2000 steps of conjugate gradient algorithm with 120.0 kcal/mol/Å convergence threshold energy under NPT ensemble (Kumar et al. 2017). The 300 K temperature was applied using Nose–Hoover chain method (Nosé 1984; Hoover 1985) and one atmospheric pressure applied by Martyna–Tobias–Klein (MTK) Barostat method (Martyna et al. 1994). For both ensemble classes (NPT and NVT), OPLS2005 force field was used (Jorgensen et al. 1996; Kaminski et al. 2001). Finally,

simulation system was set in relaxation state and run for 20 ns. Trajectory recorded in each 4.8 ps.

The 20 ns MD simulation trajectory was analyzed by simulation event and interaction diagram program module available in Desmond v3.6. Root-mean-square deviation (RMSD) value and root-mean square fluctuation (RMSF) were selected for protein stability analysis and for determination of fluctuation/thermal motion in protein residues during simulation, respectively (Jee et al. 2017). We analyzed the protein contribution of secondary structure elements (SSE) during simulation in structure stabilization.

Results

In-silico mapping and functional annotation

Putative wheat ortholog of *HvIds3* gene was identified using different bioinformatics-based algorithms. The results suggested the presence of 2OG-FeII Oxy domain (Pfam Id-CL0029) belonging to dioxygenase protein family in wheat ortholog of *HvIds3* (Fig. S1). Furthermore, *HvIds3* sequence was aligned against wheat survey sequences available on IWGSC database. BLASTN results showed 74% sequence similarity with IWGSC_chr7AS_ab_k71 contig of chromosome 7AS, 88% sequence similarity with IWGSC_chr7BS_ab_k71 contig of chromosome 7BS and 78% with IWGSC_chr7DS_ab_k71 contig of chromosome 7DS in wheat genome. The predicted structure of 7AS and 7BS contained four exons and the structure belonging to 7DS contained three exons. *TaIds3-7AS* and *TaIds3-7BS* coded for 339 and 309 amino acid long proteins, respectively, while *TaIds3-7DS*-coded 242 amino acids length protein which was comparable to the protein of the known gene *HvIds3* of barley. Further MSA results predicted by CLUSTALW2 revealed that *TaIds3-7AS* shared high similarity with *HvIds3* as compared to *TaIds3-7BS* and *TaIds3-7DS* (Fig. S2). To confirm the chromosomal location of *TaIds3* in hexaploid wheat genome, contig IWGSC_chr7AS_ab_k71 was used as query in BLAST against *T. aestivum* DNA database in Ensembl plants database, which gave a hit on telomeric region of 7AS.

Out of three predicted wheat orthologs, only *TaIds3* on 7AS had complete gene structure (Fig. 1a). Transcription start site (TSS) for *TaIds3* on 7BS and 7DS could not be predicted. CD search for these predicted genes showed that *TaIds3-7AS* with 2OG-FeII Oxy domain belonging to DIOX protein family is similar to the gene *HvIds3*, while the complete domain was not present in *TaIds3-7BS* and *TaIds3-7DS* (Fig. 1b). The machine-learning program PLANT-mPLoc suggests cytoplasmic localization of *HvIds3*, *TaIds3-7AS*, *TaIds3-7BS*, and *TaIds3-7DS* encoded proteins.

Fig. 1 **a** *TaIds3* gene structure; **b** representation of functional domains and their respective super families; **c** interactive ribbon view and surface representation of TaIDS3 protein: right: view from back of molecule via vertical rotation by 180°. Figure was prepared using UCSF-Chimera

Protein structure modeling, evaluation, and phylogeny analysis

Swiss-model software (homology-based algorithm) was used to generate 3D model of wheat proteins encoded by *Ids3* gene based on 1w9yA template from the Protein Data Bank (PDB) at Root-Mean Square Deviation (RMSD) 2.0. UCSF-CHIMERA visualized the different chemical shapes of modeled TaIDS3 (Fig. 1c). The 3D structure of TaIDS3 protein was submitted to Protein Model Database (PMDb) (<https://bioinformatics.cineca.it/PMDB/>), and assigned the id PM0078983, which can be freely accessed for further structural-based annotations. Dihedral statistics of Ramachandran plot for TaIDS3 protein structure revealed a total of 82.9, 15.2, and 1.9% residues in most favoured, additionally allowed, and generously allowed, respectively (Fig. 2). The Ramachandran plot generated by PROCHEK and Richardson's lab also showed the excellent geometry of the protein model. The overall G-scores -0.57 suggested that the predicted model was acceptable. Verify3D server gave the score 0.35 indicated that the model generated was of good quality (Table 1).

The structural alignment of proteins encoded by *HvIds3* and *TaIds3-7AS* using FATCAT superposition showed 57.25% similarity at protein structural level (Fig. S3). Pro-Func server was used to determine the biochemical function of *Ids3* in wheat showing catalytic activity (59.07) and oxidoreductase activity (49.52). The phylogenetic tree generated the individual branches for *TaIds3*, *HvIds3*, *HvIds2*, and *OsIds2* (Fig. 3). The clustering pattern showed that

Fig. 2 Ramachandran plot of TaIDS3 protein model

TaIds3 gene shared a close evolutionary relationship with *HvIds3*, while *OsIds2* and *OsIds3* shared the relationship with *HvIds2*.

Physical mapping of *TaIds3* on 7AS chromosome

To confirm the chromosomal location of *TaIds3* in hexaploid wheat genome, the genomic DNA of the cytogenetic stocks was amplified with a pair of 7AS specific primers of *TaIds3*

Table 1 Summary of quality factors of TaIDS3 protein structure generated using SAVES and PSVS1.5

	Mean score	SD	Z-score ^b
Procheck G-factor ^a (phi/psi only)	−0.57	N/A	−1.93
Procheck G-factor ^a (all dihedral angles)	−0.28	N/A	−1.66
Verify3D	0.35	0.0000	−0.77
ProsaII (−ve)	0.53	0.0000	−0.50
MolProbity clashscore	16.62	0.0000	−1.33
Ramachandran plot summary from Procheck			
Most favoured regions	82.9%		
Additionally allowed regions	15.2%		
Generously allowed regions	1.9%		
Disallowed regions	0.0%		
Ramachandran plot statistics from Richardson's lab			
Most favoured regions	87.7%		
Allowed regions	10.3%		
Disallowed regions	2%		

Selected residue ranges: 4A–257A

^aResidues selected based on: Dihedral angle order parameter, with $S(\phi) + S(\psi) \geq 1.8$

^bWith respect to mean and standard deviation for a set of 252 X-ray structures <500 residues, of resolution ≤ 1.80 Å, R-factor ≤ 0.25 and R-free ≤ 0.28 ; a positive value indicates a 'better' score

Fig. 3 Neighbour-joining phylogenetic tree of the *Ids3* and *Ids2* members; the tree was constructed with the amino acid sequences of *Ids3* and *Ids2* proteins from barley (Hv), rice (Os), and wheat (Ta) using the neighbour-joining method in MEGA 6.0 software. A bootstrap with 1000 repetitions was included**Fig. 4** Polyacrylamide gel electrophoresis analysis of the PCR products of *TaIds3* amplified using nullisomic–tetrasomic and ditelosomic lines of *T. aestivum* cv. Chinese Spring

gene. The PCR products of 22 NT lines and di-telocentric lines of 7A are illustrated in Fig. 4. A single fragment of *TaIds3* was amplified in 20NT lines, while there was no amplification in N7A-T7B and N7A-T7D. The amplification of the fragment in all 20 NT lines and absence in N7A-T7B and N7A-T7D indicates the presence of this fragment somewhere on chromosome 7A (Fig. 5). Furthermore, to localize the fragment on the specific arm of 7A chromosome, the genomic DNA of Dt7AS and Dt7AL was amplified with the same primers. The PAGE analysis of the PCR product resolved that there was no amplification in Dt7AL while Dt7AS line gave the amplification with 7AS-specific primers (Fig. 4). These results indicate the location of the gene on short arm of 7A chromosome. Furthermore, the genomic DNA of deletion lines of 7AS did not give any amplification. Therefore, it was confirmed that *TaIds3* is located in the telomeric region of 7AS. On the basis of the above results, the location of *TaIds3* gene is illustrated in Fig. 4.

Expression analysis under Fe-starvation

The results of real-time RT-PCR showed that the detectable expression of *TaIds3* was started on 5th day (2.5-fold) after Fe-starvation. In response to Fe deficiency, the relative expression was increased gradually up to 15th day (maximum 33.5-fold), and thereafter, the expression was decreased on 25th and 35th day. However, under Fe-sufficient condition, the expression of *TaIds3* was detectable but almost constant at all stages (Fig. 6). The relative expression analysis reflected that Fe deficiency directly regulates the induction of *TaIds3* expression in the roots. The expression of *TaIds3* in Fe-deficient roots was much greater than Fe-sufficient roots at all stages (Fig. 6).

Molecular dynamics simulations

The molecular dynamics simulation concerned with stability and fluctuations in protein model of IDS3 by analyzing

Fig. 5 Physical map of Chinese Spring chromosome 7A; the identification of deletion line fraction arm length (FL) values breakpoints is indicated on the right in parentheses. The telomeric bin of 7AS shows the putative location of the *TaIds3* gene

20 ns simulation trajectories. RMSD plot in Fig. 7b shows protein structure has RMSD between 1.3 and 2.7 Å. From the starting point (1 ns) of simulation, RMSD shows higher value between 3.4 and 3.7 Å, because protein structure has large loop region residues 4–46 that is why it shows conformational changes between 1 and 3 ns. After 3 ns, structure conformations show stable RMSD between 2.2 and 2.7 Å (highlighted in red line) in 4–13 ns simulation time. After 13 ns, RMSD shows some conformational changes due to a second loop region 173–179 in protein and RMSD decrease 2.7–1.6 Å for 2 ns (13–14 ns). After 15 ns, RMSD remains constant and stable between 1.4 and 1.6 Å until the end of the simulation time (highlighted in red line).

Fluctuations in IDS3 protein residues were analyzed by backbone atoms motions and local changes in secondary structure elements. RMSF plot in Fig. 7c shows acceptable fluctuations under 2.4 Å and compares to beta factor (0.5–0.8 Å) aspect one large fluctuations higher than 3 Å between 173 and 179 residue ranges. This range comes in loop region of protein that is why this region fluctuated during 13–14 ns simulation. Total 31.57% protein contributed to secondary structure (alpha helices = 19.52% and beta strands = 12.05%). SSE elements confirm that protein structure has stable conformation state aspect small loop (residues 173–179) that shows conformational changes during simulation. Based on acceptable RMSD around 1.5–2.7 Å, fluctuations between 1.0 and 2.4 Å confirm that protein has stable state during whole simulation time.

Fig. 6 Relative expression level (change in fold) of *TaIds3* gene in roots of *T. aestivum* cv. Chinese Spring; under Fe-sufficient and deficient conditions at 5, 10, 15, 25, and 35 days after transplantation of seedlings in hydroponic condition. The means of three independent experiments are shown. Error bars indicate standard deviation

Fig. 7 Molecular dynamics simulation results of HvIDS3 protein model; **a** RMSD plot of TaIDS3 backbone atoms, **b** RMSF plot with beta factor (experimental X-ray B-factor) of TaIDS3 backbone atoms and **c** secondary structure elements of TaIDS3 protein model during simulation

Discussion

In the present study, we reported that *TaIds3* gene in hexaploid wheat, which encodes a dioxygenase that converts DMA to MA or MA to epi-HMA. These results are contrary to a number of earlier reports showing the absence of the *Ids3* gene(s) or the presence of a mere pseudo gene in hexaploid wheat and its wild relatives (Nakanishi et al. 2000; Mori et al. 1990; Ma and Nomoto 1994). The bioinformatics analysis indicated that the functional wheat ortholog of *Ids3* gene might be present at chromosome 7AS, which is supported by the results obtained from physical mapping of the wheat ortholog in the telomeric region of chromosome 7AS. In the constructed phylogenetic tree, the close evolutionary relationship of *TaIds3_7AS* with *HvIds3* also supports the above results (Fig. 1). Our results are also supported by the location of most of the QTLs of high grain iron and zinc contents on chromosome 7A of different *Triticum* species (Tiwari et al. 2009; Peleg et al. 2009). The *Ids3* gene has been mapped on the chromosome arm 4HL of barley and on chromosome arm 5RL of rye using wheat–barley addition lines and wheat–rye addition lines, respectively (Ma and Nomoto 1994; Mori and Nishizawa 1989; Ma et al. 1999).

We used a novel real-time RT-PCR approach to examine the expression of *Ids3* gene expression over a period of 35 days coinciding with important developmental stages following under Fe deprivation. The key finding of our study was that the expression of *TaIds3*-like gene was significantly induced under Fe deprivation, whereas its expression under Fe-sufficient condition was not as high as under Fe deprivation and it remained constant throughout. These changes in expression of this gene could translate into significant changes in protein activity and may thus have true physiological importance under Fe-deficient condition. These results show that the regulation of expression of *HvIds3*-like gene in wheat is Fe dependent, which might be associated with iron utilization by plant. An earlier study in barley showed that under Fe-starvation conditions, the expression of *Ids3* gene was induced after 3 days, which gradually increased to a maximum level on 7th day, while the expression of *Ids3* gene was not at all observed in hexaploid wheat (Nakanishi et al. 2000). This is in contrary with our results in wheat, which showed high expression of *Ids3* after 5 days of Fe-starvation, which increased up to 15 days and declined thereafter. These results indicate that the *Ids3* expression seemed to be more sensitive to Fe deficiency in barley than in wheat. Another gene responsible for encoding

hydroxylated phytosiderophore, i.e., *Ids2* followed the similar expression pattern whose expression was hardly detectable at least for the first 7 days after Fe starvation in barley (Okumura et al. 1994), while the expression of its ortholog in tobacco was clearly detectable within 3 days of Fe-starvation (Kobayashi et al. 2007). These studies show that the expression pattern of genes and their orthologs vary in different plant systems. In another study done on the release of higher amount of phytosiderophores under Fe- and Zn deficiency by Tolay et al. (2001) suggests the important role of the presence of all three genomes as in case of hexaploid wheat than tetraploid and diploid species. This might be because the genes responsible for the synthesis of phytosiderophores are expressed predominantly and effectively when three genomes are present together.

The induction of *Ids3* in barley is dependent on the availability of Fe and it is negatively correlated with the induction of NAS, NAAT, and IDS3 enzymes and subsequently with secretion of MAs (Kobayashi and Nishizawa 2012; Higuchi et al. 1996; Kanazawa et al. 1998). These results strongly support the results of present investigation that the deficiency of Fe directly regulates the induction of *Ids3* expression in the roots of hexaploid wheat. Nakanishi et al. (1993) also reported that the *Ids3* gene and its product detected only in Fe-deficient barley roots.

On the basis of “Draft genome of the wheat A-genome progenitor *Triticum urartu*” (Ling et al. 2013), homology analysis of the *H. vulgare* IDS3 protein as a query with BLASTp exhibited 65% identity with *T. urartu* (Accession no. EMS 480341) and predicts the molecular function exactly similar as HvIDS3 protein, i.e., dioxygenase and oxidoreductase activity (<http://www.uniprot.org/uniprot/M7ZIY2>). These results suggest the presence of IDS3 protein ortholog in diploid progenitor of cultivated wheat also, which might be responsible for higher secretion of phytosiderophores in Fe- and Zn-deficient conditions. The good correlation between the protein and RNA data demonstrates that regulation of gene expression by concentration of Fe is at the level of transcription or RNA stability and not at the level of translation, as in the case of Fe-regulated genes in other systems. In spite of the presence of *Ids3* gene and its mRNA transcripts in hexaploid wheat, the less RNA stability might be the reason to produce the optimum amount of IDS3 peptide to be detectable in the root exudates.

In the present study, the *HvIds3* gene sequence was aligned against wheat survey sequence. BLASTn results showed 74% sequence similarity with IWGSC_chr7AS_ab_k71_contigs of chromosome 7AS in wheat genome having same conserved domain. Results indicate the presence of putative ortholog of *HvIds3* on the short arm of chromosome 7A of hexaploid wheat genome. Furthermore, results of structural alignment of proteins between *HvIds3* and *TaIds3* ortholog by FATCAT server showing 57.25%

similarity of *HvIds3* with putative wheat ortholog of *Ids3* indicated significant structural similarity in the proteins of both the genes which is in agreement with the observations of Domingues et al. (2000). According to Domingues et al. (2000), if a protein has more than 40% sequence identity to another protein, whose biochemical function is known and if the functionally important residue (for example, those in the active site of an enzyme) is conserved between the two protein, a reasonable working assumption can be made that the two proteins have a similar biochemical function. ProFunc analysis also identified the functional motifs of TaIDS3 and showed the close relationships of TaIDS3 to functionally characterized HvIDS3 protein. Even the sub-cellular locations of the gene in both wheat and barley were found in cytoplasm and suggest that the presence of putative barley ortholog in wheat. Crystal structure of cereals IDS3 protein is not available in PDB yet. Moreover, high-throughput protein structure methods, such as yeast X-ray crystallography and NMR, cannot be easily applied due to their high cost. Therefore, homology modeling for predicting TaIDS3 protein structure and function could provide alternative solutions. Homology modeling referred to as comparative or knowledge-based modeling method relies on detectable significant similarity between the query amino acid sequences and protein of known 3D structure (resolved by X-ray crystallography and NMR) and can be used to model 3D structure of all the members of a protein family using a single representative 3D structure as an initiating point (Kumar et al. 2013, 2016a). The TaIDS3 sequence alignment with template is needed, because Swiss-model algorithm can discover structural information based on sequence. This alignment information is applied to simulation of target structure. Sequence alignment quality of target and template is important to predict a good homology model. Swiss-model algorithm predicted the 3D structure of TaIDS3 with good stereochemical properties.

After protein structure modeling of TaIDS3, we went for stability and local changes in structure analysis through molecular dynamics (MD) simulation for small time scale. Predicted 3D model of TaIDS3 was placed into cytoplasmic condition under TIP3P water molecules (Fig. 7a). MD results suggest that predicted TaIDS3 protein model has stable state during simulation between 1.4 and 2.7 Å average RMSD (Fig. 7b). Root-mean square fluctuations value between 1.4 and 2.4 Å shows stable fluctuations in protein compatible with experimental beta factor. One large fluctuation in TYR177 residues that comes in loop region is reported between 3.2 and 5.7 Å RMSF. This large fluctuation does not affect the local changes in secondary structure conformations. The percentage of secondary structure elements (31%) also show that predicted model has stable conformation during simulation (Fig. 7c).

Conclusions

The present study first time reported the *HvIds3* ortholog in the hexaploid wheat genome and physically mapped on telomeric region of chromosome 7AS. The expression analysis reflected that Fe deficiency directly regulates the induction of *Ids3* gene in Chinese Spring roots. Modeling and MD simulations results of HvIDS3 revealed that identified TaIDS3 is stable in cell during Fe deficiency. Identified *TaIds3* being utilized by us in ongoing marker assisted program (MAS) to develop the Fe efficient wheat cultivar and to boost up the biofortification program. However, extensive characterization and functional validation of *Ids3* gene in other cereals is also necessary to further explore their biological roles using different reverse genetics approaches like RNAi and VIGS.

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Author contributions SK and HSD designed and supervised the study. PM, UK, and AK designed experiment and analyzed the data as a whole and wrote the manuscript. SM, PM, and NK collected samples for the analysis. SK and AK performed the molecular dynamics analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest whatsoever.

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