Indian Journal of Agricultural Sciences **86** (5): 621–7, May 2016/Article
https://doi.org/10.56093/ijas.v86i5.58299

In-silico characterisation and comparative mapping of yellow stripe like transporters in five grass species

M G MALLIKARJUNA[1], T NEPOLEAN[2], SHIKHA MITTAL[3], FIROZ HOSSAIN[4], J S BHAT[5], K M MANJAIAH[6], S S MARLA[7], AMITHA CR MITHRA[8], P K AGRAWAL[9], A R RAO[10] and H S GUPTA[11]

ICAR-Indian Agricultural Research Institute, New Delhi 110 012

Received: 6 January 2016; Accepted: 14 January 2016

ABSTRACT

Iron (Fe) and Zinc (Zn) are the key elements required for many of the biological process in plants and animals. Transporter proteins are essential for uptake, transport and accumulation for Fe and Zn in plants. The present investigation was undertaken to study and compare the structural and functional diversity and evolutionary significance of the *yellow stripe-like* (YSL) transporters through *in-silico* tools in five species (barley, *Brachypodium*, foxtail millet, maize and rice) of Poaceae. One hundred and two YSL transporters collected from public databases were used in the analysis. All YSL transporters possessed PF03169 domain which belongs to the oligo peptide transporters (OPT) super family. Molecular weight of YSL proteins ranged from 11.10 to 84.70 kDa while *p*I values ranged from 4.99 to 11.64. Scondary structure analysis identified that, alpha helix and random coils were the most common structures of the YSL proteins. Phylogenetic analysis revealed that the YSL transporters are highly conserved in these five grass species. Comparative mapping of genes of YSL transporters showed maximum synteny between *Brachypodium* and barley (30%) followed by *Brachypodium* and rice (25%). Neutrality test has in fact revealed the positive or Darwinian selection on YSL transporters. The results of the present investigation provided a significant understanding of the structural and biological role of YSL transporters as well as the evolutionary pattern in Poaceae family.

Key words: *In-silico* analysis, Iron and zinc transporters, Selection pressure, Synteny, Yellow stripe like

Mineral nutrition plays a vital role in growth, development and yield of crop plants. Among various minerals, iron (Fe) and zinc (Zn) are the important essential minerals required for key metabolic process such as, photosynthesis and respiration (Kobayashi and Nishizawa 2012). Fe and Zn also plays prominent role in animal nutrition including humans. Although Fe is a fourth major component available in the earth's crust and contributes 5% of the lithosphere (Schmidt 1999), plants do poses Fe deficiency due to various reasons, viz. low solubility, complex form, low genetic potential of plants for uptake and transportation. Compared to Fe, the share of Zn in the earth crust is very low (0.0078%), however, the bioavailability of Zn is high (25%) as compared to Fe (5%) (Bouis and Welch 2010).

Members of Poaceae predominately follow chelation-based strategy (strategy-II) for Fe uptake (Kobayashi and Nishizawa 2012), in which plant releases high Fe-affinity-chelators into rhizosphere to bind and transport Fe ions. These Fe chelator-complexes are recognized and transported into inner root cells by the action of specific transporters. Phytosiderophores, members of mugineic acid (MA) family chelators, possess high affinity to Fe^{3+} ions and lead to formation of Fe^{3+}-MAs complexes, which are later absorbed into root by yellow stripe-like (YSL) transporters. Generally, Zn is predominantly absorbed in free ionic form but grass plants also possess chelation-based strategies in uptake of Zn. Several previous reports also suggested the role of YSL transporters in Zn homeostasis. *Yellow Stripe 1* (YS1) is the

---

[1] Scientist (e mail: mgrpatil@gmail.com), Division of Genetics, [2] Senior Scientist (e mail: tnepolean@gmail.com), [3] Senior Research Fellow (e mail: shikhamittal259@gmail.com), [4] Senior Scientist (e mail: fh_gpb@yahoo.com); [5] Senior Scientist (e mail: jsbhat73@gmail.com), RRS-IARI, Dharwad, [6] Principal Scientist (e mail: manjaiah.math@gmail.com), Division of Soil sciences and Agricultural Chemistry; [7] Principal Scientist (e mail: ssmarl@yahoo.com), National Bureau of Plant Genetic Resources, New Delhi; [8] Scientist (e mail: amithamithra.nrcpb@gmail.com), National Research Centre on Plant Biotechnology, New Delhi 110 012; [9] Principal Scientist (e mail: pawankagrawal@hotmail.com), Vivekananda Parvatiya Krishi Anusandhan Sansthan, Almora, Present address: Assistant Director General, ICAR, New Delhi; [10] Principal Scientist (e mail: rao.cshl.work@gmail.com), Centre for Agricultural Bioinformatics, IASRI, [11] Former Director (e mail: h.s.gupta@cgiar.org), Indian Agricultural Research Institute, New Delhi, Present address: Director General, Borlaug Institute for South Asia, New Delhi

first Fe^{3+}-MAs transporter gene isolated from plants (Curie *et al*. 2001) and presently YSL transporter genes were extensively studied and cloned in various plant species such as *Arabidopsis* (Chu *et al*. 2010, DiDonato *et al*. 2004, Jean *et al*. 2005), rice (Aoyama *et al*. 2009; Koike *et al.* 2004) and barley (Harada *et al.* 2007).

Cereals are the most important crops for meeting the nutritional requirement of world's growing population, presently contributing more than 60% of total energy requirement (www.fao.org). Generally cereals are rich source of starch, but poor suppliers of many micronutrient including Fe and Zn (Shahzad *et al*. 2014). YSL transporters play a potential role in Fe and Zn homeostasis in cereals (Kobayashi and Nishizawa 2012, Koike *et al*. 2004). *In-silico* analysis of YSL transporters in cereals and millets provides an insight on the sequence and functional diversity and helps in understanding of structural and evolutionary pattern of YSL transporters. Although appreciable research efforts have been diverted towards studying the evolutionary relationship of gene families related to Fe and Zn homeostasis in plants, such research reports on YSL genes specific to cereals and millets are meagre. Hence, we performed a genome-wide *in-silico* analysis of YSL transporters in five different grass species (barley, *Brachypodium*, foxtail millet, maize and rice) belonging to different tribes of Poaceae family in order to understand structural, functional and evolutionary relationship of YSL transporter genes.

MATERIALS AND METHODS

YSL transporter protein sequences for all the five species under study were retrieved from the Uniport protein (http://www.uniprot.org; Sep 2014) and Aramemnon plant membrane protein database (http://www.aramemnon.uni-koeln.de; Sep 2014). The gene sequences for maize were collected from the maize database (http://www.maizegdb.org) and for other four species from the Ensembl database (http://plants.ensembl.org). The redundant sequences are removed manually. The Blast2GO (Conesa *et al*. 2008) functional annotation suite was employed to annotate the remaining 150 and non-redundant sequences.

ProtParam tool (ExPASy) was employed to predict the molecular weight, isoelectric point, grand average of hydropathy (GRAVY) and instability index (Gasteiger *et al.* 2005) using amino acid sequences of YSL transporters of all the five species. Genes for each species were selected on the basis of maximum sequence length to predict the secondary structure of proteins. GORIV and SOPMA methods were employed to predict the secondary structure of protein sequence (Garnier *et al.* 1978).

The domains of the protein sequences were identified using pfam (http://pfam.xfam.org) with an E-value of 1 (Punta *et al*. 2012). The MEME suite 4.10.2 (http://meme-suite.org) was used to assess the functional motifs of annotated amino acid sequences by setting up the E-value of 0.01 and motif length from 20 to 60.

The gene structures of YSL transporters were constructed by comparing the coding sequences and full genomic sequences of the genes. During the analysis, intron phases in between the exon-intron junction were obtained by using Gene Structure Display Server (http://gsds.cbi.pku.edu.cn). The selected sequences were subjected to multiple alignments using clustalW (Higgins *et al*. 1994). An initial tree was built using the Neighbor-Joining method (Saitou and Nei 1987) with 1 000 rapid bootstrap replicates. MEGA v6.06 was used to visualize the resulted phylogenetic tree (Tamura *et al*. 2013).

The YSL transporters from *Brachypodium* species were BLASTN searched against genome sequences of barley, foxtail millet, maize, and rice (www.phytozome.net) to study the synteny among the chromosomes of *Brachypodium* and four other grass species. A cut-off bit score of 72 and E value of <1e-05 were considered as optimum for BLASTN analysis. The syntenic relationships among selected species were finally visualized using Circos v0.63 (http://circos.ca) (Krzywinski *et al*. 2009). The results of neutrality tests performed for each of the transporter families by using coding sequences alignments to decipher the direction and magnitude of natural selection acting on YSL transporter genes. Selective pressure (dN/dS) is an indicator used in genetics, to estimate the ratio of the non-synonymous substitution per non-synonymous site (dN), to the number of synonymous substitution per synonymous site (dS). Selective pressure acting on YSL transporters were computed for the sequence positions with ≥ 80% site coverage by determining the non-synonymous (dN) to synonymous (dS) nucleotide substitution (dN-dS) indicated as δ. The average nucleotide diversity (π) of the YSL transporters in each species was computed with Nei-Gojobori method and single likelihood ancestor counting (SLAC) method and *p*-value of rejecting null hypothesis of strict neutrality and in favour of alternative hypothesis (positive or negative selection). An evolutionary fingerprint analysis was carried out using the SLAC algorithm to depict the proportion of sites under selection using Datamonkey server (www.datamonkey. org).

RESULTS AND DISCUSSION

In-silico mining of YSL transporters

Using the available enormous genomics data for various cereals and millets in the public databases such as Uniport (www.uniprot.org) and Aramemnon (www.aramemnon.uni-koeln.de), it was possible to mine out the protein sequences of YSL transporters. A total of 102 protein sequences were collected from five species of Poaceae falling under five different tribes namely Triticeae (barley), Brachypodieae (*Brachypodium*), Paniceae (foxtail millet), Andropogoneae (maize) and Oryzeae (rice). Barley had the maximum number of sequences (29) followed by *Brachypodium* (19) and foxtail millet (19). A few of YSL transporters (HvYS1, OsYSL2, ZmYS1 etc.) are well characterized in the crop plants (maize, rice, barley etc.) for their activity under mineral stresses (Curie *et al*. 2000, 2001; Gurerinot 2000).

Physico-chemical properties of YSL transporters

Molecular weight, isoelectric point, GRAVY and instability index of all the retrieved 102 proteins were estimated using ProtParam tool ExPASy (Gasteiger *et al*. 2005) (Supplementary file 1). Molecular weight ranged from 11.10 to 84.70 kDa with a mean value of 65.89 kDa. Isoelectric point (*p*I) is a pH at which the net charge of the protein is zero and at this point the protein is least soluble. The mean *p*I of YSL transporters was 8.46 with a minimum of 4.99 (rice: YSL5; LOC_Os04g32060) and maximum of 11.64 (maize: GRMZM2G35805 1). In *Arbidopsis*, AtYSL3 transporter showed the *p*I value of 8.87 (www.arabidopsis.org). GRAVY gives information about polar and non-polar nature of proteins. GRAVY is calculated as the sum of the hydropathy values (Kyte and Doolittle 1982) for all the amino acids in a protein divided by the total number of amino acids residue in it. Negative GRAVY value indicates the non-polar and positive value indicates the polar nature of a protein. Our analysis showed positive GRAVY value for all the YSL transporters except for GRMZM2G358051 from maize (-0.901) and MLOC_37227 from barley (-0.062). Similar nature of YSL transporters were also reported in poplar and grape (Cao *et al*. 2011). Stability of the YSL transporters was estimated using instability index. Protein whose instability index is <40 is predicted as stable one, and >40 as unstable protein. In the present investigation, majority of transporters were found stable in nature. However, 11.8% YSL transporters were found to be unstable (Table 1; Supplementary file 1). Further, secondary structures of the selected proteins were predicted for a member with largest sequence length in each of the crop species (Table 2). The results of GOR IV and SOPMA showed that four YSL transporter members mainly composed of alpha helices, random coils and followed by extended strands. In barley, the iron-specific *HvYS1* transporter protein had 40% of α-helix structure (Murata 2010).

Domain and motif identification

The domain is the sequential feature and acts as a distinct structural and functional unit of a protein. The prediction of domain organization through the InterPro domain analysis tool revealed the presence of PF03169 domain in YSL transporter family. MEME suite identified the best five motifs across all the five species (Table 3). Motif 1 and 5 were more repetitive and found to be conserved. YSL transporters from barley M0V028_HORVD,

Table 1 Summary of physico-chemical properties of YSL transporters in the five species of Poaceae

Species	Molecular weight (kDa)			Isoelectric point (*p*I)			GRAVY			Instability index		
	Min.	Max.	Avg.	Min.	Max.	Avg.	Min.	Max.	Avg.	Min.	Max.	Avg.
Barley	11.10	76.77	48.41	5.14	11.17	7.98	-0.06	6.63	0.72	28.23	52.42	35.27
Brachypodium	62.56	79.03	74.25	5.52	9.29	8.54	0.38	0.66	0.49	25.63	42.03	32.65
Foxtail millet	38.81	84.70	72.12	6.33	9.46	8.65	0.30	0.65	0.46	29.42	42.61	35.43
Maize	26.54	79.15	70.99	6.53	11.64	8.76	-0.90	0.61	0.39	28.32	78.32	36.78
Rice	46.35	78.77	73.85	4.99	9.76	8.65	0.41	0.72	0.50	28.86	38.67	33.25
Across the species	11.10	84.70	65.89	4.99	11.64	8.46	-0.90	6.63	0.54	25.63	78.32	34.71

Table 2 Secondary structure prediction of YSL transporters in five species of Poaceae by GOR IV and SOPMA*

Secondary structure parameter (%)	Barley		*Brachypodium*		Foxtail millet		Maize		Rice	
	MLOC_52343		BRADI5G16160		Si009402m.g		GRMZM2G400570		LOC_Os02g42220	
	GOR IV	SOPMA	GOR IV	SOPMA	GOR IV	SOPMA	GOR IV	SOPMA	GOR IV	SOPMA
Sequence length (bp)	704	704	724	724	786	786	737	737	727	727
Alpha helix (Hh)	30.40	33.24	32.04	37.29	20.99	32.32	29.58	38.67	30.67	41.68
Extended strand (Ee)	23.30	22.59	20.72	20.30	26.21	21.12	22.52	19.54	25.45	21.32
Beta turn (Tt)		9.38		10.91		7.51		8.96		12.38
Random coil (Cc)	46.31	34.80	47.24	31.49	52.80	39.06	47.90	32.84	43.88	24.62

*Parameters 310 helix (Gg), Pi helix (Ii), Beta bridge (Bb), Bend region (Ss), Ambiguous and other states shown 0.00%.

Table 3 Identified best five protein motifs of YSL transporters across the five species

Motif	Best possible match	E value	Sites	Width
Motif 1	GGVIAGLAACGVMMSIVSTAADLMQDFKTGYLTLTSPRSMFVSQVIGTAMGCVINPCTFW	4.7e-3779	89	60
Motif 2	FPTFGLEAYKNRFYFDFSPTYVGVGMICPHIVNCSMLLGAIISWGIMWPYIEKKKGDWY	2.5e-3686	83	60
Motif 3	PLRKVMIIDYKLTYPSGTATAHLINSFHTPQGAKQAKKQVRCLGKYFSISFLWSFFQWFY	2.5e-3511	78	60
Motif 4	PLPMAMAIPFYIGPYFAIDMCVGSVILFVWERMDRKEAEDFAPAVASGLICGDGIWTLPQ	1.7e-3347	86	60
Motif 5	YVVAPVLAFCNAYGTGLTDWNMAYTYGK	7.3e-1716	89	28

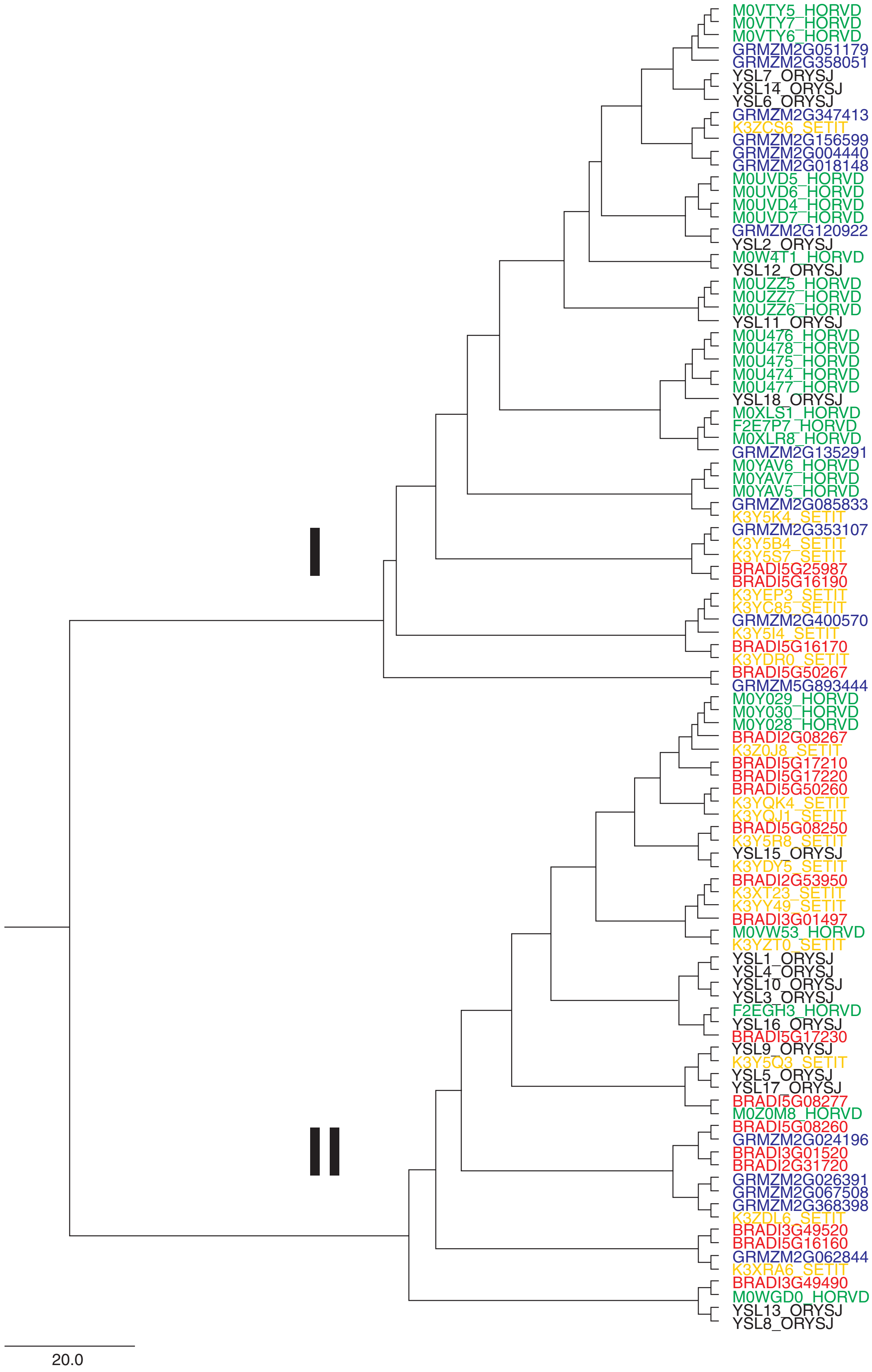

Fig 1 Phylogenetic tree of YSL transporters in five grass species (green text: barley; red text: *Brachypodium*; golden yellow text: foxtail millet; blue text: maize and black text: rice)

and M0UG77_HORVD showed only motif 4 and motif 1, respectively. However, Bradi3g49490.1 transporter from *Brachypodium* showed both motif 4 and motif 5. Further to understand the conserved nature of these motifs across the crop species, a BLASTP search was performed with cut-off bit score of 85 and E value of <1e-05 against the non-redundant protein database of NCBI. BLASTP results revealed that all these motifs are conserved in YSL transporter proteins of cereals (rice, maize), pulses (*Vigna* sp., Phaseolus *vulgaris*), oilseeds (*Brassica* sp., Soybean), vegetables (*Cucumis* sp., *Solanum* sp.), fruit crops (grape, citrus, *Musa* sp.) etc. indicating that identified motifs could possess important functional role in the YSL transporters.

Prediction of gene structure and phylogenetic analysis

Gene structure prediction showed the arrangement and number of introns and exons in the iron and zinc transporter genes. The YSL transporter gene structure analysis showed that five-intronic regions were the most common (31%) followed by six-intronic regions (21%) (Supplementary file 1). These exon/intron variations might have occurred during the development of orthologous genes (Rogozin *et al.* 2005) and could be related with species specific functional diversities. Additionally, the structural diversity of transporter genes might be involved in transporting a variety of other cations such as manganese and cadmium (Belouchi *et al.* 1997). The length as well as the number of introns may also contribute to the structural diversity. Largest intron and exon was observed in YSL transporter of maize (GRMZM2G347413; ~16kb) and rice (YSL11_ORYSJ: ~3kb). Shorter exons and longer introns increase the potential for alternative splicing and genomic complexity. However, alternative splicing doesn't always correlate with species complexity (Koralewski and Kurtovsky 2011).

Phylogenetic analysis for each of the iron and zinc transporters families showed two clades (clade I to clade II). Clad I had 53 genes and clade II had 49 genes (Fig 1). Phylogenetic analysis of the transproters also revealed the absence of species based grouping, since YSL transporters in each clades of the phylogenetic trees had the representation from all the five species. These results suggested the conserved nature of YSL transporters in the grass family lineage. Non-divergence YSL transporters was also found among monocots and dicots plants (Victoria *et al.* 2012).

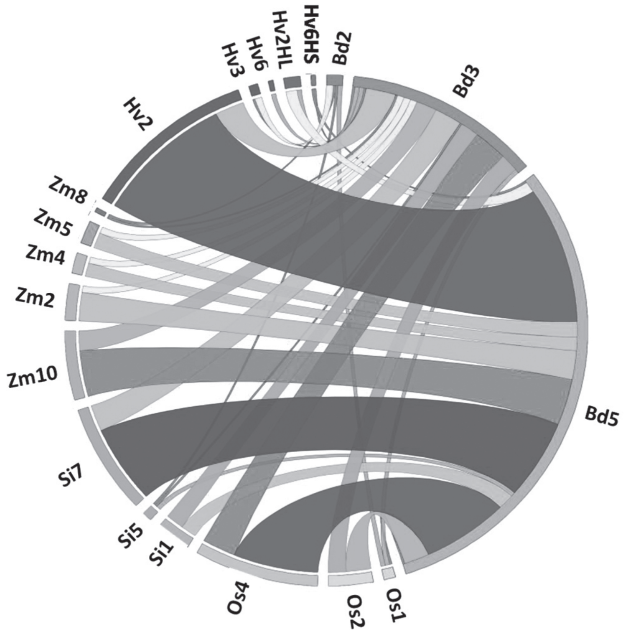

Fig 2 Comparative map showing orthologous gene positions of YSL genes among the different chromosomes of barley (Hv), *Brachypodium* (Bd), foxtail millet (Se), maize (Zm), and rice (Os)

Synteny analysis and selective pressure on YSL transporters

All the sequences of YSL transporters of cereal model species *Brachypodium* was used as query sequences to blast search against the databases of maize, rice, barley and foxtail millet to identify the respective orthologs with more than 72% similarity and E value of <1e-05. In the present investigation, *Brachypodium* genome showed maximum synteny with barley (90 genes) followed by rice (77 genes), foxtail millet (67 genes) and maize (67 genes). In *Brachypodium,* out of 301 genes, 294 genes of the YSL transporters were localized on chromosome 3 and 5 and both of these chromosomes are found syntenic with maize chromosome 2, 4, 5 and 10 (65 genes) , barley chromosome 2, 2HL, 6HS (84 genes), rice chromosome 1, 2 and 4 (75 genes) and foxtail millet chromosome 1, 5 and 7 (66 genes) (Fig 2). The syntenic association among the grass family was also confirmed by their evolutionary relationship. The maximum synteny of the *Brachypodium*-barley followed by barley-maize could be due to the recent divergence between Brachypodieae and Triticeae (32 to 39 million years) as compared to Brachypodieae and Andropogoneae (45 to 60 million years) (Bennetzen *et al.* 2012; www.cerealsdb.uk.net/cerealgenomics/WheatBP/Documents/DOC_ Evolution.php; Sep 2015). Further, comparative map for YSL transporters of the selected grass species showed the possible nested chromosomal fusion in the grass genomes (Murat *et al.* 2010). The comparative analysis could be useful in choosing the candidate YSL transporter genes for functional validation. The results of the neutrality tests for each of the species are presented in the Table 4. The average δ (dN-dS)

Table 4 Estimation of non-synonymous and synonymous substitutions mean dissimilarity for all the five species of Poaceae

Species	Sequences	π	Nei-gojobori method		SLAC method
			δ (dN-dS)	p-Value	ω (dN/dS)
Barley	66	0.67	6.80	0.03	6.75
Brachypodium	19	0.71	8.40	0.03	1.10
Foxtail millet	29	0.70	7.35	0.04	1.51
Maize	17	0.68	7.86	0.03	1.36
Rice	19	0.74	7.39	0.05	7.37

and ω (dN/dS) values ranged from 1.10 (*Brachypodium*) to 7.37 (rice) and Barley (6.80) to 8.40 (*Brachypodium*), respectively, suggesting the positive or Darwinian selection on YSL transporter genes. Positive selection in OPT super family including YSL transporters also reported in poplar and grape plants (Cao *et al*. 2011).

In the present study, we did comparative analysis and *in-silico* characterization of YSL transporters in five species of the Poaceae. The findings will be useful in understanding the characters of individual YSL genes and their functional utility in Fe and Zn transport across plant species. The information generated from our study will help in breeding iron and zinc bio-fortified cultivars in cereal and millet crops.

ACKNOWLEDGEMENT

First author is thankful to the Department of Science and Technology, Government of India, for the INSPIRE Fellowship (DST/INSPIRE/2011/IF110504) to carry out the doctoral degree programme. This work was supported by the ICAR-NPTC on Functional Genomics (Maize component: 21–22).

REFERENCES

Aoyama T, Kobayashi T, Takahashi M, Nagasaka S, Usuda K, Kakei Y, Ishimaru Y, Nakanishi H, Mori S and Nishizawa N K. 2009. OsYSL18 is a rice iron (III)–deoxymugineic acid transporter specifically expressed in reproductive organs and phloem of lamina joints. *Plant Molecular Biology* **70:** 681–92.

Belouchi A, Kwan T and Gros P. 1997. Cloning and characterization of the OsNramp family from *Oryza sativa*, a new family of membrane proteins possibly implicated in the transport of metal ions. *Plant Molecular Biology* **33:** 1 085–92.

Bennetzen J L, Schmutz J, Wang H, Percifield R, Hawkins J, Pontaroli A C, Estep M, Feng L, Vaughn J N, Grimwood J and Jenkins J. 2012. Reference genome sequence of the model plant *Setaria*. *Nature Biotechnology* **30:** 555–61.

Bouis H E and Welch R M. 2010. Biofortification-a sustainable agricultural strategy for reducing micronutrient malnutrition in the global south. *Crop Science* **50:** S20.

Cao J, Huang J, Yang Y and Hu X. 2011. Analyses of the oligopeptide transporter gene family in poplar and grape. *BMC Genomics* **12:** 465.

Chu H H, Chiecko J, Punshon T, Lanzirotti A, Lahner B, Salt D E and Walker E L. 2010. Successful reproduction requires the function of Arabidopsis Yellow Stripe-Like1 and Yellow Stripe-Like3 metal-nicotianamine transporters in both vegetative and reproductive structures. *Plant Physiology* **154:** 197–210.

Conesa A and Goetz S. 2008. Blast2GO: A comprehensive suite for functional analysis in plant genomics. *International Journal of Plant Genomics* **619:** 832–43.

Curie C, Alonso J M, Jean L M, Ecker J R and Briat J F. 2000. Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. *Biochemical Journal* **347:** 749–55.

Curie C, Panaviene Z, Loulergue C, Dellaporta S L, Briat J F and Walker E L. 2001. Maize yellow stripe1 encodes a membrane protein directly involved in Fe (III) uptake. *Nature* **409:** 346–9.

DiDonato R J, Roberts L A, Sanderson T, Eisley R B and Walker E L. 2004. Arabidopsis Yellow Stripe Like2 (YSL2): a metal regulated gene encoding a plasma membrane transporter of nicotianamine–metal complexes. *Plant Journal* **39:** 403–14.

Garnier J, Osguthorpe D J and Robson B. 1978. Analysis of accuracy and implications of simple methods for predicting secondary structure of globular proteins. *Journal of Molecular Biology* **120:** 97–120

Gasteiger E, Hoogland C, Gattiker A, Wilkins M R, Appel R D and Bairoch A. 2005. Protein identification and analysis tools on the ExPASy server. *The Proteomics Protocols Handbook*, pp571-607. Walker J M (Ed). Humana Press.

Guerinot M L. 2000. The ZIP family of metal transporters. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1 465:** 190–8.

Harada E, Sugase K, Namba K, Iwashita T and Murata Y. 2007. Structural element responsible for the Fe (III)–phytosiderophore specific transport by HvYS1 transporter in barley. *FEBS Letters* **581:** 4 298–302.

Higgins D, Thompson J, Gibson T, Thompson J D, Higgins D G and Gibson T J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4 673–80.

Jean M L, Schikora A, Mari S, Briat J F and Curie C. 2005. A loss of function mutation in AtYSL1 reveals its role in iron and nicotianamine seed loading. *Plant Journal* **44:** 769–82.

Kobayashi T and Nishizawa N K. 2012. Iron uptake, translocation, and regulation in higher plants. *Annual Review of Plant Biology* **63:** 131–52.

Koike S, Inoue H, Mizuno D, Takahashi M, Nakanishi H, Mori S and Nishizawa N K. 2004. OsYSL2 is a rice metal nicotianamine transporter that is regulated by iron and expressed in the phloem. *Plant Journal* **39:** 415–24.

Koralewski T E and Krutovsky K V. 2011. Evolution of exon-intron structure and alternative splicing. *PLoS One* **6:** e18055.

Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones S J and Marra M A. 2009. Circos: an information aesthetic for comparative genomics. *Genome Research* **19:**1 639–45.

Kyte J and Doolittle R F. 1982. A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157:** 105–32.

Murat F, Xu J H, Tannier E, Abrouk M, Guilhot N, Pont C, Messing J and Salse J. 2010. Ancestral grass karyotype reconstruction unravels new mechanisms of genome shuffling as a source of plant evolution. *Genome Research* **20:** 1 545–57.

Punta M, Coggill P C, Eberhardt R Y, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J, Heger A, Holm L, Sonnhammer E L L, Eddy S R, Bateman A and Finn R D. 2012. The Pfam protein families database. *Nucleic Acids Research* **40:** D290–D301.

Rogozin I B, Sverdlov A V, Babenko V N and Koonin E V. 2005. Analysis of evolution of exon-intron structure of eukaryotic genes. *Briefings in Bioinformatics* **6:** 118–34.

Saitou, N and Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4:** 406–25.

Schmidt W. 1999. Mechanisms and regulation of reduction based iron uptake in plants. *New Phytologist* **141:** 1–26.

Shahzad Z, Rouached H and Rakha A. 2014. Combating mineral malnutrition through Iron and Zinc biofortification of cereals. *Comprehensive Reviews in Food Science and Food Safety* **13:**

329–46.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* **30:** 2 725–9.

Victoria F D C, Bervald C M P, Da Maia L C, De Sousa R O, Panaud O and De Oliveira A C. 2012. Phylogenetic relationships and selective pressure on gene families related to iron homeostasis in land plants. *Genome* **55:** 883–900.