

Assessing the Allelic Diversity of *OsNRAMP5* among Sri Lankan Traditional Rice Varieties by *in silico* Analysis

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Abstract *OsNRAMP5* encodes a root expressed transporter that can significantly affect the Cd content in rice grains. An *in silico* analysis on haplotypes of *OsNRAMP5* was performed on selected traditional Sri Lankan rice varieties in order to identify potential low Cd accumulators among them. *OsNRAMP5* sequences of 47 Sri Lankan traditional rice varieties from the 3000 genome project repository in the Rice SNP seek database were selected for this study. A total of 14 genic haplotypes were obtained of which *Pannithi* and *Godawel* showed similarities with the low Cd accumulator Nipponbare. The results did not show significant polymorphic variation in the coding region (4.88%) of *OsNRAMP5*. Haplotype analysis of the promoter region of *OsNRAMP5* in the selected rice varieties has revealed 10 haplotypes. In the promoter region, *Pannithi* and *Godawel* differ by one SNP position compared to Nipponbare. Transcription Factor binding sites were not detected in the corresponding SNP position and hence, this mutation is unlikely to affect the level of *OsNRAMP5* expression in *Pannithi* and *Godawel*. Therefore, there is a potential to use *Pannithi* and *Godawel* varieties in the breeding programs aimed at reducing grain Cd accumulation.

Keywords: cadmium, transporters, haplotype, indica rice

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1. Introduction

Cadmium (Cd) is a soft, ductile and silver white heavy metal that belongs to the group II-B of the periodic table [1]. It has been identified as a class I carcinogen by the International Agency for Research on Cancer [2,3,4]. This widespread environmental pollutant can threaten human health resulting in Cd-related diseases such as renal tubular dysfunction, bone diseases (osteomalacia, osteoporosis, bone demineralization), lung damage and even cancers in the lungs and pancreas [1,3,5,6]. Human exposure to cadmium is mainly through the consumption of contaminated food crops [1,6,7]. Crop plants uptake Cd from polluted soils and the heavy metal transported into edible parts may be ingested by animals and human [5,7]. The long term irreversible accumulation of Cd through food chains and its long biological half-life of about 10-30 years results toxic effects on the human body [5,7].

Out of all crops, cereal grains contribute to a large proportion of dietary intake of Cd [5,7]. Rice, among all cereal crops is the staple food for half of the world population [4]. It accounts for a larger portion of the total dietary intake of Cd in Asian populations, including 40% and 56% dietary Cd intake in Japan and China respectively [7]. The tragic incident of "Itai- itai" disease that was

reported in Japan around 1960s was a result of long term consumption of Cd contaminated rice [1,6]. According to some studies, in Asian countries such as China, Bangladesh and Sri Lanka somewhat higher grain Cd concentration was reported in rice [2,6]. A survey conducted by Meharg *et al.* (2013) has shown that the mean Cd content (mg/ kg) in grain in Sri Lanka is second only to Bangladesh rice where it is 0.80 mg/kg implicating ~10µg weekly intake of Cd per kg body weight in Sri Lanka. However, a research conducted on rice samples from widely cultivated areas in North central province in Sri Lanka has shown that the Cd levels are below the JEFCA (FAO/WHO Expert Committee on Food Additives) permissible level of 400 µg/kg [8]. Another Sri Lankan study has revealed that traditional rice varieties possess an average Cd content of 33 µg/kg, while newly improved rice varieties show an average of 85 µg/kg [9].

Anthropogenic activities such as rapid industrialization, the use of super phosphate fertilizers, Cd containing fungicides and the use of irrigation water contaminated with Cd are the main sources of paddy soil contamination by this toxic heavy metal [2,7,10]. In Sri Lanka, among the fertilizers analyzed, TSP (Tri-super Phosphate) exceeded the maximum permissible Cd level (10µg/g) recommended by the Sri Lanka Standard Institute [11]. The largest amount of TSP is used in intensive farming in dry zone areas in Sri Lanka. Research suggests that there

is a possibility of TSP contributing to elevated Cd amounts in water reservoirs leading to high levels of Cd in plants and fish obtained from such water [12].

The uptake and translocation of heavy metal contaminants in plants vary greatly not only between plant species, but also between cultivars within the same species [13]. In 2015, Arjum and his research team have mentioned the differences in the absorption and translocation of Cd between cultivars in maize (*Zea mays* L.). It was observed that Wan Dan 13 accumulated more Cd in roots, leaves and stem compared to Run Nong 35 [13]. In a study conducted in China, it is mentioned that some rice cultivars accumulated higher Cd concentrations in the roots, but lower concentrations in the aerial parts, such as the variety Nong Ken 57. IR 36 on the other hand showed a lower concentration of Cd in roots but higher concentration in aerial parts [14]. In rice, *qCd7* which is an incomplete-dominant major QTL contributes to variation in grain Cd accumulation [15,54]. Further the allele from Nipponbare has shown reduced Cd content in the grain [54]. The study highlights the identification of the allelic genotype of *qCd7* for the prediction of grain Cd content [54].

In recent years, a series of genes related to Cd transport in cereals have been identified. In plants, natural resistance-associated macrophage proteins (*NRAMPs*) are a family of metal transporters that are integral components of membranes and play an important role in the uptake, translocation, and intracellular transport of metals [16]. The transportation mechanism of Cd into rice begins with absorption from soil through the roots [3,4]. Cd absorbed into roots is translocated into shoots and subsequently accumulated in grains [3,4]. The uptake of Cd from soil into the root system is primarily regulated by the Mn and Fe transporter *OsNRAMP5* [16,17,18].

OsNRAMP1 is another transporter that supports the uptake of Cd from soil into roots. However the contribution of *OsNRAMP1* is minor compared to *OsNRAMP5* [3,6,19]. In addition to the *NRAMP* family, several members of the zinc/iron-regulated transporter-like protein (ZIP) family, the major zinc and iron transporters in plants, are also involved in Cd uptake. Some Fe transporters participate in the uptake of Cd by the plant [20]. Nakanishi *et al.*, [53] found that *OsIRT1* and *OsIRT2* play an important role in Cd uptake. Overexpression of *OsIRT1* resulted in increased accumulation of Cd. P1B-ATPases, also called heavy metal ATPases (*HMA*s), play direct roles in the plant transmembrane transport of heavy metals. OsHMA2, a Zn and Cd transporter, localizes to the plasma membrane and mediates xylem Zn/Cd loading and intervacular transport to grains [21,22]. *HvHMA3* has been identified as a candidate gene for Cd accumulation in sprouts and grains of barley [23]. In durum wheat, the *HMA3-B1* gene has been found to perfectly match a major *Cdu1* locus, which may explain 80% of the variation in Cd accumulation in the grain.

At present several different methods are used to reduce the Cd content in grains including chemical and physical methods, phytoremediation, water and fertilizer management and screening for low Cd accumulator varieties [4,24]. Along with that, biotechnology is also becoming a popular solution to mitigate Cd related issues in crops [3,7,10].

It has been shown that *OsNRAMP5* knockout mutants contain significantly lower Cd concentrations both in roots and shoots compared to wild type, indicating the involvement of *OsNRAMP5* in the uptake of Cd [18]. Another study based on *OsNRAMP5* mutants obtained using CRISPR technology has shown that mutations in *OsNRAMP5* result in significantly reduced grain Cd [4]. In addition to that, Isikawa *et al.*, 2012 has shown that non transgenic *Osnramp5* null mutant greatly decreases Cd uptake by the roots.

Considering the world scenario, different rice varieties have been identified as high or low Cd accumulators. Rice cultivars such as Anjana Dhan, Cho-ko-koku, Jarjan have been found to accumulate higher levels of Cd in shoots [19,21]. Koshihikari Kan No.1 [25], PA64 [26] have been established as low level Cd accumulators. Sri Lanka has a high diversity of rice with resistance to extreme environmental conditions such as high salinity, drought and water logged conditions [27], and resistance to pest and diseases [28]. Some rice varieties are rich in nutritional and medicinal values [29]. However the genetic potential of these varieties for heavy metal uptake, especially for Cd tolerance has been largely unexplored. Therefore, in the present study, an *in-silico* analysis was done to find different alleles corresponding to the *OsNRAMP5* transporter gene among selected Sri Lankan traditional rice varieties.

2. Method

2.1. Sequence Retrieval and Multiple Sequence Alignment of *OsNRAMP5* Gene of 47 Sri Lankan Varieties with Reference Varieties

In this study, 2000 bp promoter sequence and 7194 bp gene sequence of *OsNRAMP5* (Gene ID:Os07g0257200; synonym: LOC_Os07g15370; at position chr 7:8871643-8878905) were retrieved from the panel of 47 Sri Lankan traditional varieties of the 3000 genome project repository in the Rice SNP seek database (<https://snp-seek.irri.org/>) [30]. The corresponding gene and promoter sequence of *OsNRAMP5* were retrieved from *Oryza sativa* subsp. *japonica* reference line Nipponbare and *Oryza sativa* subsp. *indica* reference Cultivar 93-11 (Gene ID: BGIOGA024510) which are designated as low and high Cd accumulators respectively based on previous studies [31]. The retrieved sequences were aligned using ClustalW with default parameters [32] in MEGA v6.0 [33]. Phylogenetic analysis of the aligned haplotype sequences was performed in MEGA v6.0 using maximum likelihood method and the reliability of the test was confirmed with 1000 bootstrap method. Promoter and genic haplotypes of *OsNRAMP5* among Sri Lankan traditional rice varieties were analyzed separately.

2.2. Haplotype Analysis of *OsNRAMP5* Gene in Sri Lankan Traditional Varieties

The sequences were annotated using *Ensembl* database Rice annotation project (www.ensembl.org). In the

alignment, all variable sites (Single Nucleotide Polymorphism (SNP) and Insertions/Deletions (InDel) with more than 5% bi-allelic frequency have been identified using Geneious v7.1 [34]. Subsequently the accessions with ambiguities in the identified variable sites were removed manually. Haplotype analysis based on the selected polymorphic sites was performed and haplotype diversity was calculated using DnaSP v5 [35]. The gene sequence of varieties clustered along with the reference low Cd accumulator, Nipponbare were compared with the high Cd accumulator, 93-11.

2.3. Haplotype Analysis of Promoter Region of *OsNRAMP5* in Sri Lankan Traditional Varieties

Haplotype analysis for promoter region of *OsNRAMP5* in Sri Lankan traditional varieties was performed in the same manner as for the genic region. A 2000 bp of promoter sequence upstream of the ATG site of Nipponbare and closely clustered sequences were compared with that of 93-11. *Cis*-acting elements were identified in the promoter region using the PlantCARE database (<https://bio.tools/plantcare>).

3. Results

3.1. Haplotype Analysis of *OsNRAMP5*

In the genic haplotype analysis, SNPs in all introns and exons have been considered. Altogether 41 SNPs were taken into account in the haplotype analysis considering their >5% occurrence (Table 1). The variations in the coding region were less compared to the introns. Out of 41 SNP variations found spanning through the genic region, only 2 were found in the coding region (exon 10 and exon 13) with an occurrence frequency of 4.88%. A total of 14 haplotypes for the *OsNRAMP5* was obtained among the varieties studied.

The distribution of the allelic haplotypes of *OsNRAMP5* sequence among the Sri Lankan traditional varieties with a frequency of more than 5% are shown in Table 2. Haplotypes 1- 4 are having a frequency of more than 5% while the rest are putative haplotypes. Varieties Pannithi and Godawel were found in haplotype 1 along with the low Cd accumulator, Nipponbare. Haplotype 2 is represented by the high Cd accumulator, cultivar 93-11 along with other 8 varieties. The highest frequency (25%) of all accessions was found in haplotype 2.

Table 1. Haplotypes of *OsNRAMP5* identified among 47 Sri Lankan traditional rice varieties. Haplotype 1 is represented by *Oryza sativa subsp. japonica* variety Nipponbare and haplotype 2 by *indica subsp. cultivar* 93-11. The position of every SNP is indicated in the first row (SNP frequency>5%). Blue shade represents the SNP variations in the reference variety Nipponbare and any deviations from that are denoted by white shade

	1558	1589	1692	2005	3031	3091	3096	3119	3374	3411	3440	3447	3452	3506	3529	3532	3540	3541	3544	3668	3933	4016	4180	4434	4464	4510	4531	4621	4690	5009	5614	5763	5801	5862	6045	6060	6164	6306	6481	6519	7070	
	Intron 5				Intron 8				Intron 9				Exon10				Intron 10				Intron11				Intron 12				Exon13													
HAP1	A	A	G	T	A	T	T	C	C	A	C	G	G	T	C	C	A	A	T	C	A	C	C	A	C	A	A	C	C	G	T	A	A	T	G	G	G	C	C	T	C	
HAP2	T	A	T	A	A	A	T	T	C	A	T	C	G	C	C	T	T	G	G	T	G	C	T	A	T	G	T	T	G	G	A	G	G	A	T	G	G	C	A	A	C	
HAP3	T	A	T	A	A	A	T	T	T	A	T	C	G	C	C	T	T	G	G	T	G	C	T	A	T	G	T	T	G	G	A	G	G	A	T	G	G	C	A	A	C	
HAP4	A	G	T	T	A	G	T	C	T	T	G	A	C	T	C	A	A	T	C	A	A	C	G	T	G	T	T	G	A	A	G	G	T	G	A	A	C	C	T	T		
HAP5	T	A	G	A	A	A	T	T	C	A	T	C	G	C	C	T	T	G	G	T	G	C	C	A	T	G	T	C	G	G	A	G	G	A	T	G	G	C	A	A	C	
HAP6	T	A	T	A	A	A	T	T	C	A	T	C	G	C	C	T	T	G	G	T	G	C	C	A	T	G	T	T	G	G	A	G	G	A	T	G	G	C	A	A	C	
HAP7	T	A	T	A	A	A	T	T	C	A	T	C	G	C	C	T	T	G	G	T	G	C	T	A	T	G	T	C	G	G	A	G	G	A	T	G	G	C	A	A	C	
HAP8	T	A	T	A	A	A	T	T	C	T	A	T	C	G	C	C	T	T	G	G	T	G	C	T	A	C	G	T	T	G	G	A	G	G	A	T	G	G	C	A	A	C
HAP9	T	A	T	A	A	A	T	T	T	A	T	C	G	C	C	T	T	G	G	T	G	C	T	A	C	A	A	T	G	G	A	G	G	A	T	G	G	C	A	A	C	
HAP10	T	A	T	T	A	A	T	T	C	A	T	C	G	C	C	T	T	G	G	T	G	C	T	A	C	G	T	T	C	G	A	G	A	T	G	G	A	C	C	A	C	
HAP11	T	A	T	T	A	A	T	T	C	A	T	G	G	C	C	C	A	A	T	C	A	A	C	A	T	G	T	T	C	G	A	G	G	A	T	G	G	A	C	C	A	C
HAP12	A	G	G	T	T	A	G	T	C	T	T	G	A	C	T	C	A	A	T	C	A	A	C	G	T	T	G	A	A	G	G	T	G	A	A	A	C	T	T			
HAP13	A	G	G	T	T	A	G	T	C	T	T	G	A	C	C	C	A	A	T	C	A	A	C	G	T	G	A	T	C	A	A	G	G	T	G	A	A	C	C	T	T	
HAP14	A	A	G	T	T	A	G	T	C	T	T	G	A	C	T	C	A	A	T	C	A	A	C	G	T	G	T	T	G	A	A	G	G	T	G	A	A	A	A	C	T	T

Table 2. Relative frequencies of genic haplotypes of *OsNRAMP5* among Sri Lankan traditional rice varieties. Haplotypes having more than 5 % relative frequencies are shown here

Haplotype	Varieties	Frequency (%)
HAP 1	Nipponbare, Pannithi, Godawel	6.25
HAP 2	93-11, Chandina, Honderawala, Puttunellu, H6, Pachchaperumal, Kotteyaran, Nalumoolai Karupan, 3210	25
HAP 3	Samba, Herathbanda, Podiwee, Kaluilankayan, Moddaikarupam, Balasuriya A	18.75
HAP 4	Kurulutudu, Matholuwa, Ranruwan, Sudukarayal, Podiheenati, Kurkaruppan	18.75

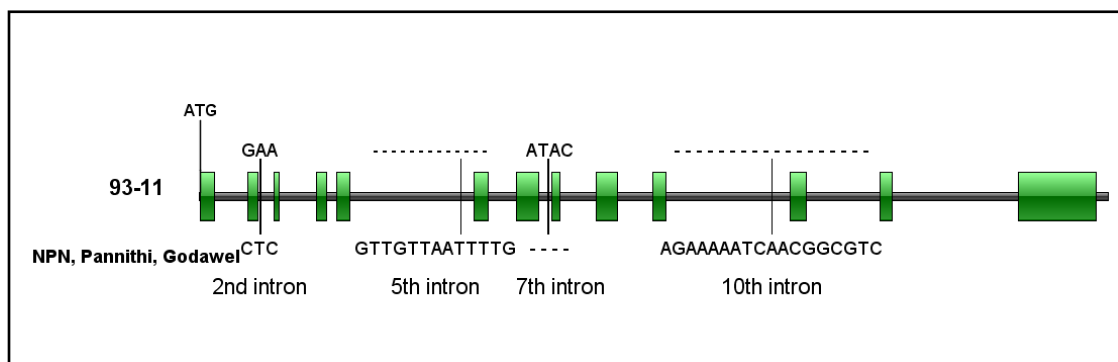


Figure 1. Comparison of gene structure between 93-11 and genic Haplotype 1 (Nipponbare (NPN), Pannithi and Godawel) (The solid horizontal lines represent introns and green boxes represent exons. Dashed lines represent deletions compared to the reference lines)



Figure 2. Phylogenetic tree generated by Maximum likelihood analysis with 1000 bootstrap method performed in MEGA6 of aligned haplotype sequences

The basic sequence differences of *OsNRAMP5* between the two reference varieties; 93-11 and Nipponbare are shown in Figure 1. *Pannithi* and *Godawel* which grouped along with Nipponbare in haplotype 1, share a similar InDel structure.

The phylogenetic relationship between haplotypes with reference varieties is shown in Figure 2, where the haplotypes are clustered into four clades based on the subpopulation they belong to. The first clade consists of haplotype 1 with *japonica* subpopulation varieties *Pannithi*, *Godawel* and Nipponbare. Haplotype 11 representing single variety Kurkaruppan (*indica* subpopulation) forms the second clade. Third clade consists of haplotypes 4, 12, 13 and 14 mainly containing accessions from *aus* subpopulation. The fourth clade represents mainly *indica* subpopulation accessions along with the reference variety 93-11.

3.2. Haplotype Analysis of Promoter of *OsNRAMP5*

Liu *et al.* (2017) identified a high level of sequence diversity in the 2000 bp promoter region of *OsNRAMP5*. Based on the 12 SNP variations identified, rice accessions were grouped into 3 haplotypes. In the promoter haplotype analysis, among the rice varieties studied, 16 polymorphic sites were identified, including the 13 SNP variations and a total of 10 haplotypes (Table 3).

Table 4 shows the relative frequencies of the haplotypes. The haplotype 3 including *indica* subsp. occurs with the highest frequency of 52.27%. Except haplotypes 2, 3 and 5, the rest of the haplotypes are having less than 5% frequency among Sri Lankan traditional rice varieties. It was found that *Pannithi* and *Godawel* which share a similar gene sequence with Nipponbare differed by one SNP at 1214 position upstream of start ATG (Figure 3). This variation placed these two varieties along with WIR 1391 in a different haplotype (HAP2).

The phylogenetic tree obtained for promoter haplotypes (Figure 4) did not clearly divide Sri Lankan haplotypes into haplogroups based on subpopulations like the genic phylogeny. In the promoter region of *OsNRAMP5* of Sri Lankan traditional rice varieties, different *cis*-acting elements responsible for stress response such as ABA responsive element (ABRE), Stress responsive element (STRE), ARE, MYC, MYB, Me-JA responsive motifs, G-box, a salicylic acid responsive element (TCA element), and RY element were identified using PLANTCARE database. RY-element was found only in promoter haplotype 1 and 2 (Figure 5) and absent in all others.

Considering the possibility that even one SNP position can change the gene expression, transcription factor binding sites (TFBS) in the promoter region were analyzed for both HAP1 and 2. It was found that no TFBS were present in the corresponding SNP in either of the haplotypes. It can be hypothesized that the gene expression of the two haplotypes may be similar to each other (Figure 6).

Table 3. Promoter haplotypes of *OsNRAMP5* identified among 47 Sri Lankan traditional rice varieties. HAP 1 is represented by *japonica subsp.* Nipponbare and HAP3 by *indica subsp.* cultivar 93-11. Blue shade represents the SNP variations in reference variety Nipponbare and any deviations from that are denoted by white shade

	-1950	-1866	-1854	-1800	-1725	-1692	-1586	-1385	-1365	-1317	-1214	-930	-832	-465	-454	-50
HAP1	T	G	G	T	A	C	T	C	A	T	C	G	T	A	G	C
HAP2	T	G	G	T	A	C	T	C	A	T	T	G	T	A	G	C
HAP3	C	A	G	A	C	T	C	T	G	T	T	T	C	A	G	-
HAP4	C	A	G	A	C	T	C	T	G	T	T	T	C	A	G	C
HAP5	T	A	A	T	C	T	C	T	G	C	T	T	C	T	T	-
HAP6	T	A	G	T	C	T	C	T	G	T	T	T	C	A	G	-
HAP7	C	A	G	A	C	T	C	T	G	T	T	T	T	A	G	-
HAP8	C	A	G	A	A	C	C	T	G	T	C	T	C	A	G	-
HAP9	C	A	G	A	A	C	C	T	G	T	T	T	C	A	G	C
HAP10	T	A	A	T	C	T	T	T	G	C	T	T	C	T	T	-

Table 4. Relative frequencies of haplotypes of promoter region of *OsNRAMP5* among Sri Lankan varieties with more than 5% relative frequencies

Haplotype	Varieties	Frequency (%)
HAP 2	Pannithi, Godawel, WIR1391	6.81
HAP 3	93-11, Race perumal, Chandina, Sigardis, Murunga, Pokkali, Periyavellai, Puttunellu, H6, Pachchaperumal, Samba, Muttu samba, Herathbanda, Alagusamba, 3210, Podiwee, Kotteyaran, Kaluilankayan, 105, Kahatawee, Nalumoolaikaruppan, Sinnasithira kali, A 69-1, Kulakruppan	52.27
HAP 5	Mudaliga wee, Kurulutudu, Matholuwa, Ranruwan, Hodarawala, Sithayinkottai samba, Sudukarayal, Galwakahandeeran, Karuthaseenati, Podiheenati, Kurkaruppan	25

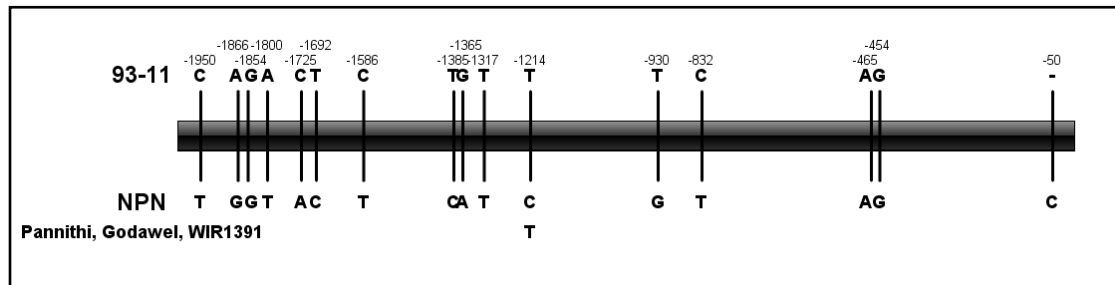


Figure 3. Comparison of promoter sequence 2000bp upstream of ATG between cultivar 93-11 and promoter HAP 1 (Nipponbare) and HAP2 (*Pannithi*, *Godawel* and *WIR1391*)

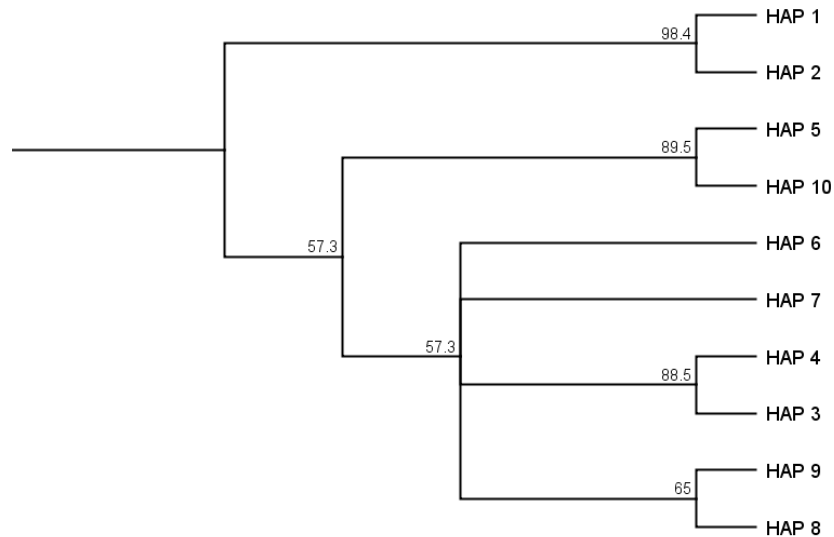


Figure 4. Phylogenetic tree generated by Maximum likelihood analysis with 1000 bootstrap method performed in MEGA6 of aligned promoter haplotype sequences along with reference varieties Nipponbare in HAP1 and 93-11 in HAP 3

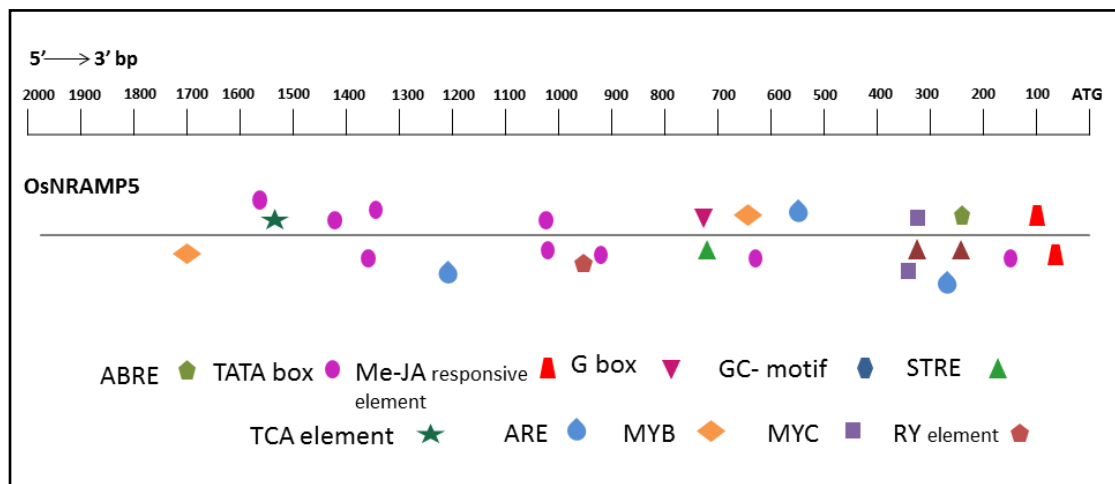


Figure 5. *Cis-* acting elements responsible for stress response identified in the promoter region of *OsNRAMP5* in the reference variety Nipponbare using PLANTCARE database



Figure 6. Transcription factors binding sites (TFBS) in the promoter region of Nipponbare (6a) and Pannithi and Godawel (6b). Colored sites show the TFBS in two haplotypes. Mutation sites are indicated by red box in the uncolored region showing no TFBS are attached to those corresponding sites

4. Discussion

OsNRAMP5 which is mainly expressed in roots, has been reported as the major transporter of Cd. Recently, several molecular breeding technologies have been used in breeding low Cd cultivars such as gene editing, genetic modification and marker assisted selection, targeting *OsNRAMP5* [36]. RNAi silencing of *OsNRAMP5* in rice plants caused less Cd accumulation in roots, but a large amount in shoots [37]. However the knockout of *OsNRAMP5* resulted in decreased Cd content in both shoot and roots in rice plants, but also resulted in large yield reductions [16]. Anyhow promising results were shown by using CRISPR/Cas 9 system in producing low Cd rice cultivars without impairing yield [38]. Different transporters act together in coordination for the uptake and translocation of Cd. The degree of suppression of *OsNRAMP5* expression also determines the degree of decrease in Cd content in shoots. Mutating *OsNRAMP5* has induced the Mn and Fe deficiencies in plants since the same transporter is responsible for the uptake of these trace metals as well [16,17].

Another approach widely used to reduce Cd in rice plants is to screen for the low Cd accumulating varieties. It has been reported that heavy metal accumulation characteristics differ not only among species, but also among cultivars of the same species [39]. Liu *et al.* (2003) showed that cultivars and genotypes differ in Cd absorption, sensitivity to Cd accumulation and grain yield formation. Arao and Ishikawa, 2006, found that *japonica* varieties such as Nipponbare accumulated less Cd in grains compared to *indica* varieties. Chen *et al.* [50]

proved this by showing grain Cd content in *indica* cultivars is 1.8- 4.14 times higher than that of *japonica* cultivars.

It has been estimated that more than 2000 traditional rice varieties have existed earlier in Sri Lanka. However, only few of them have been sequenced and deposited in the databases so far. Few varieties, including Suwadel, Kalu Heenati, Pachchaperumal, Kuruluthudu, Goda Heenati and Pokkali which are known for their nutritional values have been tested for heavy metal tolerance [40,41,42].

In the present study, Sri Lankan traditional rice varieties were screened for low Cd accumulating *OsNRAMP5* alleles by an *in silico* analysis. In a study by Liu *et al.* [51] *OsNRAMP5* allelic sequence of low Cd accumulating accessions were exactly the same as that of Nipponbare. Similarly, we found that two traditional varieties Pannithi and Godawel shared the same sequence as of Nipponbare. This could be due to the fact that these two varieties are coming under tropical *japonica* sub population and are clustered together with Nipponbare. Most of the high yielding varieties are clustered together with cultivar 93-11.

Cis-acting elements in the promoter region of stress responsive genes play an important role in transcriptional regulation by acting as molecular switches. They interact with various transcription factors and initiate transcription of stress responsive genes [43]. In the promoter region of *OsNRAMP5* several stress responsive *cis*-acting elements such as ABRE, STRE, ARE, MYC, MYB, Me-JA responsive motifs, G-box and TCA element were found. It has been reported that MAPKs signaling cascade phosphorylate TFs such as MYB, MYC and change the expression of ABA responsive elements (ABRE) as a

response to heavy metal stress induced ROS production and accumulation [44,45]. Similarly, phytohormone signaling pathways, including auxin, ethylene and JA are found to be disrupted by ROS [46]. A study by Singh and Shah [52] showed that exposure to MeJA alleviated Cd-induced injury in rice plants by switching on JA-biosynthetic pathway.

Among the *cis* elements identified in the promoter region of *OsNRAMP5* in the haplotypes of Sri Lankan traditional varieties, RY/sph element was found only in the *japonica* varieties, Nipponbare, *Pannithi* and *Godawel* while absent in all other haplotypes. RY element is reported to be found mainly in seed-specific promoters and control the embryo maturation. It binds to ABI3/VP-1 like B3-domain proteins to regulate the mid maturation stage, which leads to accumulation of storage compounds [47]. It has been recently reported that OsVP1 controls seed dormancy by binding to *Sdr4* via an ABA signaling pathway [36]. *Sdr4* has been found to be an intermediate regulator of seed dormancy which is responsible for the reduced dormancy in *japonica* (Nipponbare) compared to *indica* (Kasalath) cultivars [48].

Promoter analysis of TFBS for both HAP1 and 2 was done to confirm that no change in gene expression is caused by the single SNP position at 1214 upstream to the start ATG. Since only the variations having more than 5% occurrence were considered in the study, it cannot be neglected that the other variations can also affect gene expression. Other than that several other factors such as post translational mechanisms, epigenetics, including DNA methylation, signal transduction pathways can also affect the gene expression [49]. Therefore, further gene expression analysis along with the reference variety Nipponbare under different Cd concentrations is necessary to confirm the potential of the varieties, *Pannithi* and *Godawel* to be used as low Cd accumulators.

5. Conclusion

In the present study, haplotype analysis for the genic and promoter region of *OsNRAMP5* was performed for the 47 Sri Lankan traditional rice varieties in the 3000 genome project repository. The two varieties *Pannithi* and *Godawel* clustered with the low cadmium accumulating variety Nipponbare. Through the *in silico* analysis, it is speculated that these two varieties may act as low Cd accumulators. Further gene expression and screening experiments are required to confirm the degree of Cd accumulation of these two varieties.

Competing Interests

We have no conflicts of interest to disclose.

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