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# **Genomewide computational analysis of nitrate response elements in rice and** *Arabidopsis*

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**Abstract** Nitrate response element (NRE) was originally reported to be comprised of an Ag/cTCA core sequence motif preceded by a 7-bp AT rich region, based on promoter deletion analyses in nitrate and nitrite reductases from *Arabidopsis thaliana* and birch. In view of hundreds of new nitrate responsive genes discovered recently, we sought to computationally verify whether the above motif indeed qualifies to be the cis-acting NRE for all the responsive genes. We searched for the specific occurrence of at least two copies of the above motif in and around the nitrate responsive genes and elsewhere in the Arabidopsis and rice (*Oryza sativa*) genomes, with respect to their positional, orientational and strand-specific bias. This is the first comprehensive analysis of NREs for 625 nitrate responsive genes of Arabidopsis and their rice homologs, representing dicots and monocots, respectively. We report that the above motifs are present almost randomly throughout these genomes and do not reveal any specificity or bias towards nitrate responsive genes. This also seems to be true for

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smaller subsets of nitrate responsive genes in Arabidopsis, such as the 21 early responsive genes, 261 and 90 genes for root-specific and shoot-specific response, respectively, and 25 housekeeping genes. This necessitates a fresh search for candidate sequences that qualify to be NREs in these and other plants.

**Keywords** Motifs · Nitrate · Promoters · Response elements · Signaling · Transcriptional regulation

#### **Introduction**

Nitrate response at the plant level is mediated by the transcriptional regulation of several hundreds of genes, but the mechanism(s) of nitrate signaling is not known (Raghuram et al.  $2006$ ). The identification of cis-acting nitrate-responsive element (NRE) provides an end point for nitrate signal transduction and paves the way for unraveling the preceding steps of the signaling pathway. Several attempts have been made to delineate the regulatory regions of nitrate and nitrite reductases (NR and NiR) in transgenic plants but none of them identified the minimum consensus sequence specific to nitrate response (Dorbe et al. [1992;](#page-6-1) Vaucheret et al. [1992;](#page-6-2) Neininger et al. [1993;](#page-6-3) Rastogi et al. [1993](#page-6-4); Vincentz et al. [1993;](#page-6-5) Lin et al. [1994](#page-6-6)). Sivasankar et al. [\(1998](#page-6-7)) reported that a 30 bp upstream sequence  $(-230 \text{ to } -200)$ and an untranslated leader sequence  $(+5 \text{ to } +67)$  are responsible for coregulating nitrate induction of spinach NiR, but their relevance to other nitrate responsive genes or other plants remains to be established. Sequence motifs that are targets for GATA family of transcription factors have been reported in the regulatory regions of NR and NiR in higher plants and their possible role in nitrate-stimulated gene expression has been suggested (Jarai et al. [1992](#page-6-8);

Daniel-Vedele and Caboche [1993;](#page-6-9) Rastogi et al. [1997\)](#page-6-10). But GATA-mediation is also known for other signals like light and conclusive evidence for their involvement in nitratespecific response is lacking. Using linker-scanning analy-sis, Hwang et al. [\(1997](#page-6-11)) identified the *cis*-acting sequence elements that are essential for nitrate-dependent transcription of NR in *Arabidopsis thaliana*. These elements are composed of several copies of a core Ag/cTCA sequence motif preceded by a  $\sim$ 7-bp AT-rich sequence present in the 5' flanking regions of NR1 and NR 2 genes. This particular sequence  $[(a/t)_{7}Ac/gTCA]$  motif was also found to be present in the 5' flanking regions of NR and NiR genes from eight other plants at various locations, indicating that it is very well conserved. Using gel-shift assays, it was also demonstrated that this sequence motif is essential for binding to proteins in the nuclear extracts of *Arabidopsis* (Hwang et al. [1997\)](#page-6-11), though nitrate-dependence of the binding could not be established. Subsequently, deletion analyses of birch promoter regions revealed the presence of the same sequence motifs in NR (Hachtel and Strater [2000](#page-6-12)) and NiR (Warning and Hatchel [2000\)](#page-6-13). However, experimental approaches to delineate NREs were so far restricted to NR from *Arabidopsis* and birch, and NiR from birch alone. Also, it has never been demonstrated clearly that the motif was sufficient by itself for nitrate-induced transcription by showing cis-activation of a minimal promoter. Even the sequence comparisons with NR and NiR promoters from other plants were restricted to very few plant species (Hwang et al. [1997\)](#page-6-11). In order to establish the true relevance of the proposed sequence motif to qualify as NRE, its specific occurrence and role needs to be demonstrated in all the nitrate-responsive genes known in plants. The list runs into several hundreds, as revealed by microarray analyses in *Arabidopsis* (Wang et al. [2000,](#page-6-14) [2003,](#page-6-15) [2004](#page-6-16); Scheible et al. [2004\)](#page-6-17) and tomato (Wang et al*.* [2001\)](#page-6-18) and by subtractive hybridization analyses in rice (Wang et al. [2002\)](#page-6-19), making it difficult to validate the NRE in each of them experimentally. We describe here an attempt to validate the relevance of the  $[(a/t)_{7}Ac/gTCA]$  motif as NRE in the post-genomic model plants *Arabidopsis* and rice using a computational approach.

#### **Materials and methods**

#### Compilation of nitrate responsive gene sets

includes 595 genes that responded only to nitrate and not to any of its downstream metabolites, as reported from the microarray analysis on Nitrate reductase null mutant (Wang et al. [2004\)](#page-6-16). Due to the non-availability of exhaustive nitrate-responsive gene expression data in rice, a corresponding list of 283 rice genes was prepared on the basis of their amino acid sequence homology with the 625 nitrate responsive genes in *Arabidopsis* using protein-protein BLAST (blastp) with an *e* value cut off of 0 (Table SII). Even though it would have been possible to obtain a more comprehensive list of rice genes using a higher *e* value, it was more important to have stringent selection criteria and avoid false positives for the purpose of this analysis. Since all genes do not respond uniformly to nitrate in time and space, smaller subsets of nitrate-responsive genes in *Arabidopsis*, comprising of 21 early responsive genes, 261 and 90 genes for root-specific and shoot-specific nitrate response, respectively, and 25 housekeeping controls have also been prepared from the above literature (Table SIII).

#### Sequence retrieval

Two sets of sequences were drawn for both *Arabidopsis* and rice consisting of nitrate responsive and non-nitrate responsive genes. The sequences were extracted from their respective chromosomes according to the annotations downloaded from NCBI in the case of *Arabidopsis* and TIGR in the case of rice, using the program Extractseq from the EMBOSS suite (Rice et al. [2000](#page-6-20)). The annotations for *Arabidopsis* genes (.ptt files) were downloaded from NCBI ftp site [\(ftp://ftp.ncbi.nih.gov/](ftp://ftp.ncbi.nih.gov/)). Since the *.ptt* files for rice were unavailable on the web at the time of this study, they were generated using the annotation information for *Oryza sativa* on the TIGR web site [\(ftp://ftp.tigr.org/pub/](ftp://ftp.tigr.org/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/) [data/Eukaryotic\\_Projects/o\\_sativa/annotation\\_dbs/\)](ftp://ftp.tigr.org/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/). In addition to this, flanking sequences of 1 kb on either side of each of the genes were also extracted similarly. This list was used for all the further analysis.

Determination of the optimum distance between two consecutive Ag/cTCA motifs

For this purpose, 100 random sequences were generated for each of the chromosomes of *Arabidopsis thaliana* and *Oryza sativa* (cv. Japonica) having the same length and base compositions. Using FUZZNUC program from the EMBOSS suite (Rice et al. [2000](#page-6-20)), the occurrence of the Ag/ cTCA motif was searched in all the randomly generated sequences as well as the original chromosomes in all possible orientations. An optimum distance range of 0–35 was calculated on the basis of a plot of the expectation values as a function of the distance between two consecutive motifs (Fig. [1\)](#page-2-0) and used for further analysis in both plants.



<span id="page-2-0"></span>**Fig. 1** Optimum distance between two consecutive Ag/cTCA core motifs. The optimum distance was calculated using the formula  $E(g) = log(P(g)^{Genome}/P(g)^{random})$ , where  $P(g)^{Genome}$  is the probability of finding a pair of Ag/cTCA motifs separated by 'g' bases. P  $(g)$  Genome

Search criteria for the occurrence of the Ag/cTCA core motif

A genome wide search for pair wise occurrence of the Ag/ cTCA motifs was conducted in the regions spanning  $-1,000$  to  $+1,000$  nucleotides of every gene in all 27 possible combinations and orientations on both strands. The raw data thus obtained was further categorized on the basis of position (upstream, downstream or within the ORF), distance from the start site, major and minor grooves of the DNA double helix,  $+/-$  strands and association with upregulated and down-regulated nitrate responsive genes. The statistical significance of these results was tested against randomized sequences by Chi-square tests.

Determination of the AT richness of the region preceding the Ag/cTCA core motif

The flanking sequences of 25 nucleotides on either side of the motif were extracted using the program Extractseq from the EMBOSS suite (Rice et al. [2000\)](#page-6-20) and a pictorial representation of the base composition was generated using WEBLOGO Version 2.8.2 (Crooks et al. [2004\)](#page-6-21).

#### **Results**

A list of 625 unique nitrate-responsive genes was compiled from the published data on *Arabidopsis* and 283 of their rice homologs were generated as described in Materials and methods and considered for the current analysis (Tables SI and SII). In order to search for the distribution of the Ag/ cTCA core motifs of the proposed NRE in multiple copies, it was essential to determine the optimum distance range between adjacent copies. A statistical determination (see Materials and methods) revealed that copies of the core motif separated by a distance of upto 35 nucleotides are



was obtained by calculating the number of pairs of Ag/cTCA separated by 'g' bases and dividing the result by the genome length. P  $(g)^{r}$ was obtained similarly with 100 randomly generated sequences

over represented in the *Arabidopsis* and rice genomes, as compared to randomized genomic sequences (Fig. [1\)](#page-2-0). Accordingly, we searched for pairs of Ag/cTCA motifs separated by 35 nucleotides or less in the genomes of *Arabidopsis* and rice.

Core motifs and preceding sequences around nitrate responsive genes

Initial search for the pair-wise occurrence of the proposed Ag/cTCA core motifs in all possible combinations and orientations within the coding regions and  $1 \text{ kb}$  of flanking regions on either side of the known nitrate responsive genes revealed that the motif occurred in 542 (86.7%) out of the 625 nitrate responsive genes tested in *Arabidopsis*, and 112 (39.58%) out of 283 genes in rice. An analysis of the base composition of 25 nucleotides of flanking sequences on either side of the core motif did not reveal any AT:GC bias in both organisms (Fig. [2](#page-3-0)).

Distribution of the core motif around nitrate responsive genes

The distribution of pairs of the proposed Ag/cTCA core motif was compared in terms of their occurrence either within the coding regions of nitrate responsive genes or their 1,000-bp upstream and downstream regions, with respect to their randomized controls for both *Arabidopsis* and rice. The data (Fig. [3\)](#page-3-1) clearly indicates that the motifs were randomly dispersed throughout and did not show any absolute specificity for either upstream, downstream or the coding regions of the genes in both the organisms.

While there were some differences in the  $%$  distribution between these regions, they were not statistically significant by Chi-square test with respect to their randomized controls in any of the regions either in *Arabidopsis* or in rice. A similar analysis for the distribution of the core motifs in terms



<span id="page-3-0"></span>**Fig. 2** Base composition around the proposed Ag/cTCA core motif. The flanking sequences of 25 nucleotides on either side of the motif around the nitrate responsive genes of *Arabidopsis* and rice were



<span id="page-3-1"></span>**Fig. 3** Positional distribution of the proposed Ag/cTCA core motif. The distribution of the core motif among upstream, downstream or within the coding regions of the nitrate responsive genes was not very distinct from their respective randomized controls for *Arabidopsis* and

of their distance from the translational start site of nitrate responsive genes also revealed more or less uniformly high occurrence over a wide region spanning  $-1$  to 2 kb (Fig. [4](#page-3-2)).

Positional and strand bias evaluation of the core motif

The possibility of strand specific distribution of copies of the Ag/cTCA core motif was also tested with the nitrate

<span id="page-3-2"></span>**Fig. 4** Distribution of the Ag/cTCA core motif around translational start site. The occurence of the core motifs was plotted against their distance, spanning regions of  $-1$  to  $+10$  kb from the translational start site of the nitrate responsive genes in *Arabidopsis*. Note the more or less uniformly high occurrence in the  $-1$  to  $+2$  kb region

Distance from translational start site

Occurrence of the motif

 $80.5$ 

1000

2000

 $3.5$ þ  $2.1$  $\overline{z}$  $1.5$ 



analyzed for their base composition and graphically represented using WEBLOGO



rice. There are no significant differences between % occurrence of the motifs in rice or *Arabidopsis* versus their randomized controls as per Chi-square test ( $P \ge 0.037$  for *Arabidopsis* and  $P \ge 0.02$  for rice; degrees of freedom = 2)

responsive gene set (and their flanking sequences) only in *Arabidopsis*, as the annotations required for this analysis in rice were not available at the time of this analysis. The data presented (Fig. [5\)](#page-4-0) clearly reveals that the motifs were dispersed on both strands and did not show any statistically significant strand bias with respect to their randomized controls by Chi-square test.

The possibility of positional/strand bias in a smaller subset of nitrate responsive genes in *Arabidopsis* was also tested, both with respect to up-regulated and down-regulated genes (Figures S1 and S2, respectively), as well as to their differential expression in time and space, such as the 21 early responsive genes, 261 and 90 genes for root-spe- $\chi$  cific and shoot-specific response, respectively, and 25 housekeeping genes (Figure S3). These analyses were done at all positions viz. upstream, downstream and within the coding regions of the genes. However, there were no significant differences in the strand-wise distribution, either within or between these subsets.

#### Spacing in motif pairs with respect to DNA helicity

An analysis for possible differential spacing between members of a pair of core motifs was also done, in terms of the major and minor grooves of the DNA double helix representing the nitrate responsive genes (and their flanking



<span id="page-4-0"></span>**Fig. 5** Strand-wise distribution of the proposed Ag/cTCA core motif. The occurence of the core motifs on each of the strands of the nitrate responsive genes of *Arabidopsis* was not very distinct from their randomized controls. There are no significant differences between % occurrence of the motifs in the + and - strands of Arabidopsis versus their randomized controls as per Chi-square test ( $P \ge 0.062$ ; degrees of  $freedom = 1)$ 

regions) in *Arabidopsis*. No significant preference for any specific distance between members of the motif pair was found, with respect to their position, either upstream, downstream or within the coding regions of the genes. (Figures S4, S5 and S6, respectively)

Random distribution of the proposed NRE

The absence of even a single pair of the proposed NRE core motif in 83 nitrate responsive genes in *Arabidopsis* and at least 171 such genes in rice as per the search criteria adopted, and the lack of any particular pattern in their distribution raised questions of specificity. This was further verified by a search for pair-wise occurrence of the core motif around genes that are nitrate non-responsive in both the organisms. It was found that the Ag/cTCA core motifs are present in 22,927 out of 29,235 (78.42%) of the nonresponsive gene set in *Arabidopsis* and in 28,324 out of 60,174 (47.07%) in rice (Fig. [6\)](#page-4-1). The randomness of the motif was further confirmed in 25 housekeeping genes of Arabidopsis, which revealed 60 and 80% occurrence of the core motif in the positive and negative strands (Figure S3D). These results indicate that the motif is neither specific to nitrate responsive genes, nor common to all nitrate responsive genes.

### **Discussion**

Response elements can be identified by their essential characteristics such as: specific presence in all responsive genes,



<span id="page-4-1"></span>**Fig. 6** Genomewide distribution of the proposed Ag/cTCA motif. The relative pair wise occurrence values of the core motif in the genomes of *Arabidopsis* and rice were categorized in terms of nitrate responsive or non-responsive genes with or without the motif. Note the lack of specificity in distribution

occurrence in multiple copies (Shinozaki and Shinozaki [2005](#page-6-22)), coexistence with other regulatory elements (Shen and Ho [1995](#page-6-23) and Shen et al. [1996](#page-6-24)) and existence of a clear positional, strand or orientation bias. They may be located either in the region upstream or downstream of the gene or within the gene, especially in introns (Levine and Tjian [2003](#page-6-25); Arnone and Davidson [1997;](#page-6-26) Davidson [2001;](#page-6-27) Carey and Smale [2000;](#page-6-28) Mancini-DiNardo et al. [2003;](#page-6-29) Adlam and Siu [2003](#page-6-30); Lee et al. [2003\)](#page-6-31). The knowledge of the above principles, coupled with the increasing availability of genomic sequences and high throughput gene expression data has made it possible to computationally analyze the promoter regions of co-regulated genes and predict candidate sequences for response element(s). In the case of NRE, which has been experimentally attempted for few of a large set of co-regulated genes in a few plants, validation of the proposed  $[(a/t)<sub>7</sub>Ac/gTCA]$  motif upstream of NR gene in *Arabidopsis* by Hwang et al. ([1997\)](#page-6-11) has not progressed beyond NR and NiR in birch (Hachtel and Strater [2000](#page-6-12); Warning and Hatchel [2000](#page-6-13)). Moreover, it has never been conclusively demonstrated that the motif was

sufficient by itself for nitrate-induced transcription by showing cis-activation of a minimal promoter. The expanding list of nitrate responsive genes (Wang et al. [2000,](#page-6-14) [2001](#page-6-18), [2002,](#page-6-19) [2003](#page-6-15), [2004;](#page-6-16) Scheible et al. [2004\)](#page-6-17) and the increasing availability of their sequences from model plants has prompted the computational re-evaluation of these elements as NREs in the present study. This is the first comprehensive analysis of NREs for 625 nitrate responsive genes of *Arabidopsis* and their rice homologs, representing dicots and monocots, respectively.

*Arabidopsis* has been used as a reference point for the preparation of a nitrate responsive gene list in the present study, as microarray analyses and other published studies in this plant have revealed the largest number of nitrate responsive genes known so far. A unique and confirmed set of 625 genes had been short listed for the present study (Table SI), which includes 595 genes that responded only to nitrate and not to any of its downstream metabolites, as reported from the microarray analysis on a nitrate reductase null mutant of *Arabidopsis* (Wang et al. [2004](#page-6-16)). As complete transcriptome data for nitrate responsive gene set in rice is not yet available and a subtractive hybridization study identified only 37 genes (Wang et al. [2002](#page-6-19)), extrapolation from the *Arabidopsis* nitrate responsive gene set by identifying their corresponding rice sequence homologs was the best option to ensure comprehensive coverage of the rice genes for the current analysis. Using the highest stringency cut off at an *e* value of  $\sim 0$  yielded only 283 rice homologs of *Arabidopsis* nitrate responsive genes (Table SII). However, the compilation and analysis of smaller subsets of nitrate-responsive genes was restricted to *Arabidopsis*, based on the experimental evidence for response types reported in literature, in terms of upregulated/downregulated, or early, shoot-specific and root-specific nitrateresponsive genes (Table SIII).

Based on a statistically determined optimal distance of 35 nucleotides between two members of a pair of Ag/cTCA core sequence motifs in *Arabidopsis* and rice (Fig. [1](#page-2-0)), a genome wide search for pair-wise occurrence of the motifs was conducted in the regions spanning  $-1,000$  to  $+1,000$ nucleotides of every gene. This revealed that the motif occurs in 86.72% (542) of the 625 known nitrate responsive genes in *Arabidopsis*, but also in 78.42% of the non-responsive gene set. Similar distribution was obtained in rice at 39.58 and 47.07%, respectively, indicating that the motif is neither specific to nitrate responsive genes, nor common to all nitrate responsive genes. The higher % occurrence of the core motif in *Arabidopsis* (regardless of nitrate response) is more likely to be due the AT rich nature of its genome (66 %), as compared to that of rice (56.46%). Indeed, this also seems to be the reason why a  $\sim$ 7 bp AT rich sequence preceding the core motif was found to be a necessary component of the experimentally proposed cis-acting sequence

(Hwang et al. [1997](#page-6-11)). However, our analysis of the base composition of the  $-25$  to  $+25$  nucleotides flanking the core  $Ag/cTCA$  motif does not reveal any significant  $AT:GC$ bias among the nitrate responsive genes that contain the motif in both *Arabidopsis* and rice (Fig. [2\)](#page-3-0).

Within the nitrate responsive gene set, there were also no significant positional differences in the occurrence of the core motif, either upstream, downstream or within the coding region (Figs.  $3, 4$  $3, 4$ ). The positional differences in the occurrence of the motif were also analyzed for the *Arabidopsis* gene set with respect to any bias between the positive and negative strands (Fig. [5](#page-4-0)), up-regulated and down-regulated subsets of genes (Figs. S1 and S2), other response subsets such as early, root-specific and shoot-specifc genes (Fig. S3) as well as major and minor grooves (Figs. S4–S6), but no significant differences were observed. The information on translational start sites and strand specifications for rice genes was not available in public domain at the time of the analysis.

Finally, an analysis of the distribution of the core motif throughout the genomes of *Arabidopsis* and rice categorized in terms of nitrate responsive or non-responsive genes with or without the Ag/cTCA core motif was very revealing (Fig. [6\)](#page-4-1). The absence of even a single pair of the core motif in a large number of nitrate responsive genes, as well as its presence in even larger number of nitrate non-responsive genes clearly indicates that the motif is neither specific to nitrate responsive genes, nor common to all nitrate responsive genes and is randomly distributed throughout the genomes in both *Arabidopsis* and rice.

Thus, the occurrence and distribution of the  $[(a/t)_{7}Ac/$ gTCA] motif in *Arabidopsis* and rice is not in accordance with the established common characteristics of known response elements (Shinozaki and Shinozaki [2005](#page-6-22)) and therefore, do not qualify to be cis-acting NREs. However, this does not exclude the possibility that this motif may operate in combination with another sequence motif(s) to qualify as a specific NRE. It is more likely that an entirely new sequence motif will emerge on a more detailed analysis of the nitrate responsive genes for flanking sequences that are specific and conserved. A search for the GATA binding elements in the promoter regions of the 625 nitrate responsive genes and 25 housekeeping genes revealed their lack of specificity in occurrence and distribution (data not shown). We are currently analyzing the results of our motif sampling to identify alternative candidate sequences that may qualify to be NREs for experimental validation.

The robustness of our results is amply indicated by the fact that our computational analysis is based on the 625 nitrate responsive genes (or their smaller subsets) of *Arabidopsis* and 283 genes of rice and their non-nitrate responsive controls, as compared to the experimentally proposed [(a/t)7Ac/gTCA] motif based on one gene in *Arabidopsis*

(Hwang et al. [1997](#page-6-11)) and two genes in birch (Hachtel and Strater [2000](#page-6-12) and Warning and Hatchel [2000](#page-6-13)). While the non-availability of the birch genome does not permit a similar verification of the experimental birch data, it is important to point out that both *Arabidopsis* and birch represent dicots, whereas our analysis includes monocots (rice) to enable generalization across the two phylogenetic groups, using the best characterized post-genomic data available so far.

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**Table SI.** Locus IDs of Arabidopsis Nitrate Responsive Genes used in the present study





**Table SII.** Locus IDs of rice homologs of Arabidopsis Nitrate Responsive Genes used in the present study





### **Table S III: Subsets of nitrate responsive genes in Arabidopsis (Locus IDs)**

### **A. List of 21 Early Responsive Genes in** *Arabidopsis thaliana*



### **B. List of 25 House Keeping Genes in** *Arabidopsis thaliana*



### **C. List of Genes Expressed Only in Roots in** *Arabidopsis thaliana*



# **D. List of Genes Expressed Only in Shoots in** *Arabidopsis thaliana*



### **E. List of Down-regulated genes in** *Arabidopsis thaliana*



## **F. List of Up-regulated Genes in** *Arabidopsis thaliana*



Supplementary Figure S1



Supplementary Figure S2





Supplementary Figure S4



Supplementary Figure S5



Supplementary Figure S6

