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Isolation, molecular characterization and phylogenetic analysis of potential phosphate solubility bacteria from *Oryza sativa*

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Abstract

Uptake and translocation of cationic and anionic nutrients play essential roles in physiological processes including plant growth, nutrition, signal transduction, and development. Approximately 5% of the Oryza sativa genome appears to encode membrane transport proteins. These proteins are classified and several hundred putative transporters have not yet been assigned to families. In this work, we have analyzed the phylogenetic relationships of cation/proton antiporter proteins, CNGC (cyclic nucleotide gated channel) and chloride channels which are anion transporters. This analysis has focused on cation and anion transporter gene families for which initial characterizations have been achieved for individual members, including sodium transporters, calcium antiporters, cyclic nucleotide-gated channels and chloride channels. Phylogenetic tree of each family define the evolutionary relationships of the members to each other. These families contain numerous members, indicating diverse functions in vivo. Closely related isoforms and separate subfamilies exist within many of these gene families, indicating possible redundancies and specialized functions. To facilitate their further study, which include alignment of the analyzed genes and their chromosomal location cation,

Keywords: *Oryza sativa, Cyclic Nucleotide gated channel, alkali cation, Isolation, phylogenetic analysis, potential phosphate*

Introduction:

Transport of alkali cations and anions across plant plasma and organellar membranes is essential for plant growth, development, signal transduction, nutrition, and also for use of plants in toxic metal phytoremediation. In spite of the remarkable advances since the discovery of the essential nutrients (1), until recently we had no idea about the total number or types of transporters required to complete the plant life cycle. Most co transporters depend on the proton electrochemical gradient generated by primary proton pumps and have been classified based on both phylogeny and function as transporters for cation, anion, and C- and N-containing compounds, including sugars, amino acids, drugs, and toxins (2, 3). Within the secondary active transporters, we had found genes predicting proteins that belonged to the monovalent cation/proton antiporter (CPA) super family. The best examples of CPAs in plants are those that extrude excess Ca21 or Na1 from the cytosol either into vacuolar and endomembrane compartments or to the extra cellular space. Eleven members of the CaCA family in Arabidopsis, named CAX1 to CAX11, encode Ca21 or divalent cation exchangers. These transporters, although related to CPA, form a separate clade in phylogenetic analyses (4). Of eight NHX family members in Arabidopsis CPA1, several have been functionally identified as Na1/H1 exchangers after cDNA expression in yeast (Saccharomyces cerevisiae) mutants. The best characterized include AtNHX1 (5) that

sequesters Na1 into vacuoles and the plasma membrane (PM)- localized SOS1/AtNHX7 (6). Ectopic expression of AtNHX1 causes dramatic salt tolerance in Arabidopsis (7). AtNHX1 is localized to plant vacuoles and is highly expressed in all organs.

Its role as a Na1/H1 antiporter was demonstrated by Na1 dissipation of a pH gradient (acid inside) in vacuoles from plants over expressing AtNHX1. SOS1 is primarily expressed in the xylem parenchyma and both transcript level and Na1/H1 antiport activity in PM vesicles are enhanced after plant exposure to high salt (8, 9). Over expression of SOS1 reduces Na content and improves salt tolerance of transgenic Arabidopsis (10). As Na1 is not an essential nutrient for glycophytes, it was surprising to find more than 20 Arabidopsis genes other than NHXs classified as Na1/H1 transporters in the databases. In animals, anion transporters are important for transepithelial transport and regulation of excitability of muscle and nerve, as demonstrated by several diseases, which result from their genetic alteration. In plants, anion transporters contribute to a number of plant specific functions, such as regulation of turgor, stomatal movement, nutrient transport, and metal tolerance (11–13). In contrast to the situation in animals, they are also responsible for the generation of action potentials (14). Being the most abundant anion in higher plants, chloride is important for plant nutrition and osmoregulation.

Materials and methods:

Reference proteins of well-established molecular function, representing each of the protein families investigated, were chosen as query sequences for searches in the rice (O. sativa) genome databases. . These reference proteins were CaCA (At2g38170), CPA1 (At2g01980), CPA2 (At4g23700), NhaD (At1g49810), CNGC3 (At2g46430), CNGC5 (At5g57940), CNGC15 (At2g28260), CNGC19 (At3g17690), CNGC2 (At5g54250), CLC a (CAA96057), CLC b (CAA96058), CLC c (CAA96059), CLC d (CAA96065). Searches were made using the TBLASTN tool (15) against GenBank database non-redundant (NR), with search specifications for *O.sativa*.

The other databases used were Rice Genome Research Program (RGP) (http://rgp.dna. affrc.go.jp/), The Institute of Genome Research (TIGR), rice genome annotation database (http://www. tigr. Org /tdb /e2k1/osa1 /index.shtml) and Universal Protein resource Uniprot (http://www.ebi. uniprot.org/uniprot-srv/protein/ uniProtView). The BLAST server used was that of the National Center for Biotechnology Information (http:// www.ncbi.nlm.nih.gov/BLAST/). As selection criteria of BLAST hits for genomic sequences, a cut off e-value of e- 10 was previously set.

The genomic sequences found were used to predict putative genes contained within them. Whenever possible, genes were predicted on the basis of sequences generated by the IRGSP, since these sequences present a higher degree of accuracy. To that end, a mixed procedure was adopted combining ab initio gene prediction algorithms of genomic sequence alignments with similar sequences from expressed genes (ESTs and cDNAs). The prediction algorithms were GenScan (Burge and Karlin, 1997; http:// genes.mit.edu/ GENSCAN.html),GenomeScan;http://genes.cmit.edu/ genomescan.html), FGENESH [31]; http://www.softberry. - Com/berry.phtml? topic=gfind), GeneMark.hmm (Borodovsky and Lukashin, unpublished; http://opal.biology.gatech.edu/GeneMark/eukhmm.cgi) and GrailEXP; http://compbio.ornl.gov/grailexp/).

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Such expressed sequences were found by BLAST searches against EST and NR databases of GenBank, using the genomic sequence as query. The algorithm of choice for the multiple alignments of protein sequences was ClustalX1.8, available through the BCM Search Launcher server (http://searchlauncher.bcm.tmc.edu/multi-align/multialign. html). The multiple alignments were edited with the help of GENEDOC (Free Software Foundation Inc.). All the proteins with greater than 30% identity, with at least one of the reference proteins used in the searches, were regarded as functionally similar (homologous) to the reference proteins, receiving the same name. Those sequences that did not conform to this criterion were discarded. . Prediction of homology and signature sequences for the putative transporter proteins were carried out with PROSITE (http://www.ebi.ac.uk/interPro Scan /) and Pfam databases. Sequences were included into families based on homology and presence of signature sequences. For topology prediction, HMMTOP was used. RGI gene codes for families were obtained from http:// www.gramene.org/Multi/ blastview and http://tigrblast.tigr.org/euk-blast/index. Protein alignments obtained with ClustalX 1.8 were used as starting points for phylogenetic analysis. Unrooted trees were prepared by the neighbor-joining method using either Clustal, PHYLIP, or and 1000 bootstrap replicates were performed. Bold lines on trees indicate protein sequences that were confirmed by cDNA sequencing. Chromosome locations of the putative transporter genes were estimated in accordance to genetic markers assigned to the BAC/PAC clones. The site used for genetic distance identification was http://rgp.dna.affrc.go.jp/cgibin/statusdb/stattable.plchr=X&lab=RGP chromosomal mapping for all the genes was carried out using MAPINSPECT software.

Results and Discussion:

Genomic overview:

With the availability of the BGI and the IRGSP data in GenBank, it was possible to construct an overview of cation/proton antiporters, CNGC and chloride channels in rice. As a starting point, the protein families in cation/proton antiporters, CNGC and Chloride channels like CaCA, CPA1, CPA2, NhaD, CNGC3, CNGC5, CNGC15, CNGC19, CNGC2, CLC a, CLC b, CLC c, CLC d which have positive molecular implications on cation and anion transport, intracellular targeting and storage in Arabidopsis, were chosen for analysis Taking specific members of these families as query sequences, searches were carried out for orthologous sequences in GenBank, RGP and Uniprot current databases using TBLASTN. After searching the databanks with TBLASTN sequences, clones having genomic sequences to the related family were taken and converted to amino acid sequences. In each family, similar sequences were removed and the sequences were subjected to PROSITE and Pfam databases to see the presence of signature sequences for the corresponding families. After subjecting the sequences to PROSITE Nineteen new putative genes, 11 characterized genes, 13 cation/proton family like genes were predicted in cation/proton antiporters, 14 new putative genes, 9 characterized, 2 CNGC family like genes were predicted in CNGC and 8 new putative genes, 1 characterized genes, 5 chloride channel like genes were predicted in chloride channel families. Together with four previously reported sequences in cation/proton antiporter, five previously reported sequences in CNGC and four previously reported sequences in chloride channels, we analyzed in this study a total of forty-three genes possibly involved with cation/proton antiporter in rice: 11 genes related to the CaCA transporter, 10 genes related to CPA1, 19 sequences related to the CPA2, 3 genes related to NhaD, 24 genes related to CNGC, 14 sequences related to chloride channel. The percent identity for all the sequences was calculated in each family with the corresponding query sequence using GENEDOC. The presence of transmembrane domains was also predicted for all transporter proteins. The result was heterogeneous with majority of proteins presenting 12–15 transmembrane domains (Table 1), which is close to the 12 observed in Arabidopsis. Phylogenetic analysis of the sequences of transporters revealed that the rice transporters were divergent, showing branches in tree view. The phylogentic analysis shows four branches indicating different transporting function to each family. Some of the orthologous sequences are available as full-length cDNA clones. The expressed sequence tags were mentioned as accession numbers for the sequences. All positions in centMorgans (cM) were estimated in accordance to genetic

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markers assigned to the BAC/PAC clones (http://rgp.dna.affrc.go.jp/cgibin/statusdb/stattable.pl?chr=X&lab=RGP) related to the predicted sequences. The proposed gene nomenclature is as follows: OsCaCA for *Oryza sativa* Ca/H antiporter, OsCPA for monovalent cation/proton antiporter, OsNhaD for Na/H antiporter.

Fig. 1: **Chromosome locations of the putative Cation/protonantiporter genes.**

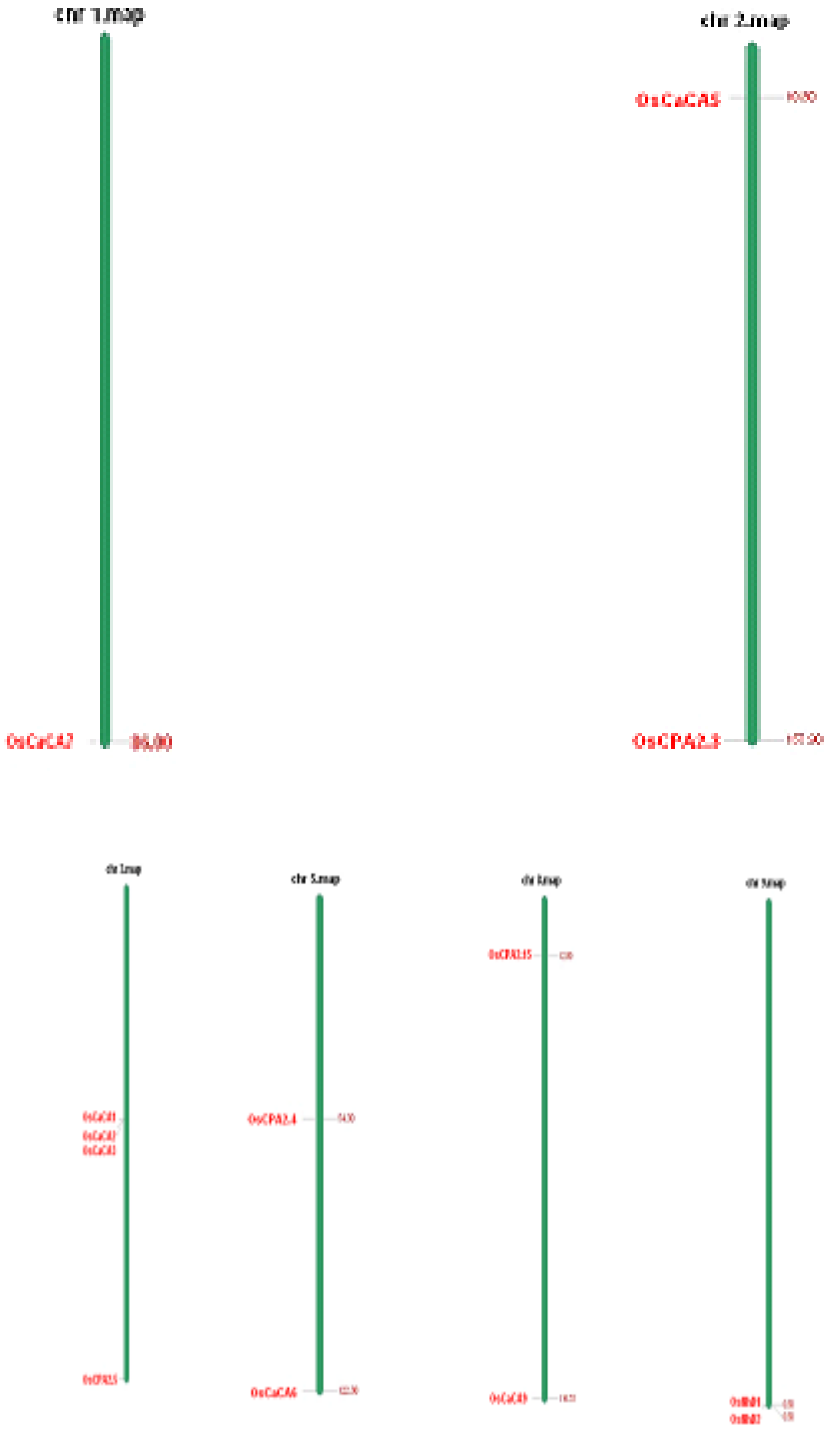
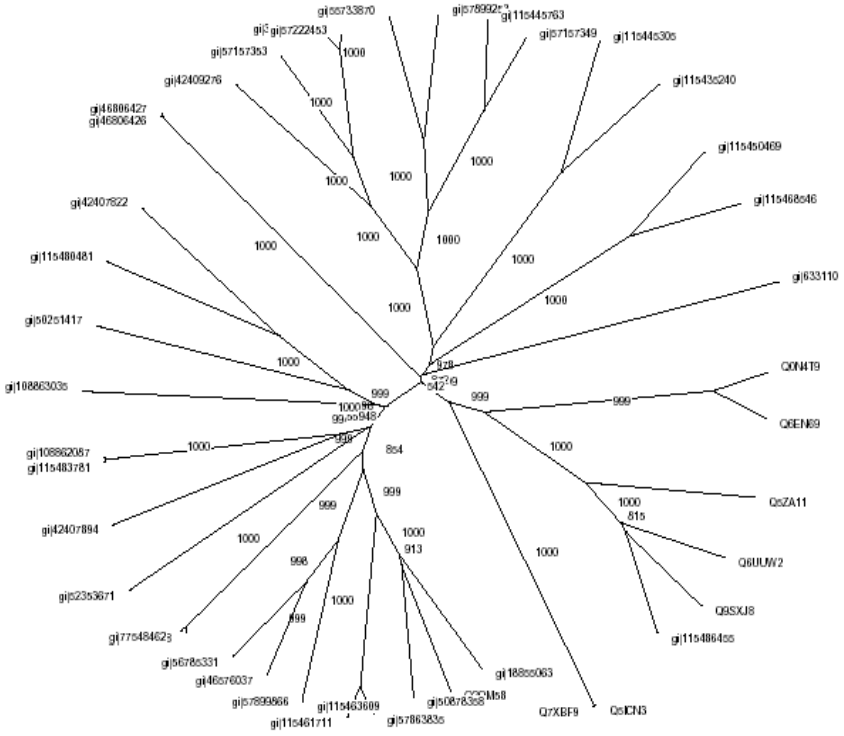


Fig. 2: Overview of rice Cation/protn antipoters. A tree of all Cation/proton antiporters from rice has four major branches: a, CaCA antiporters (11 genes); b, CPA (; 20 genes); c, NhaD (3 genes). Programs used were ClustalX (Thompson *et al*., 1997) for alignments, and graphical output produced by Treeview. Values indicate the number of times of 1000 bootstraps

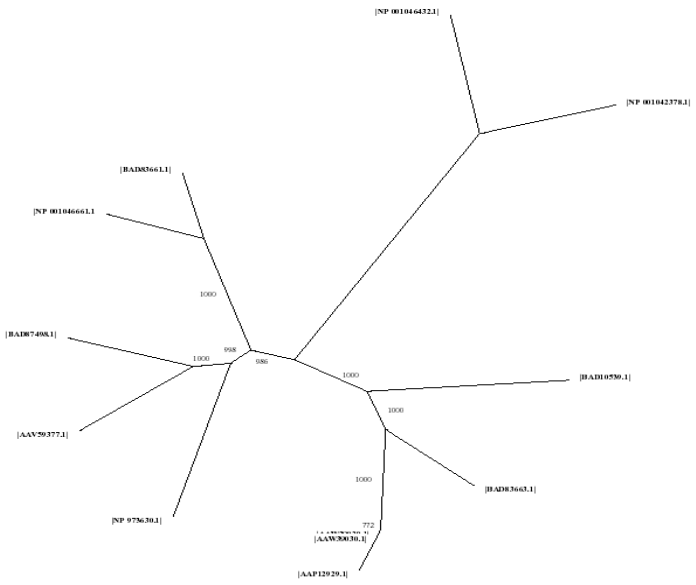
that each branch topology was found during bootstrap analysis. Scale bar corresponds a distance of 10 changes per amino acid positions.



4. SUMMARY:

Cation/proton antiporters:

CaCA family: In this family exchange of proton with calcium takes place. Nearly eleven genes related to CaCA transporters were identified and named as OsCaCA. These are numbered based on their alignment. The genes, which are showing more than 30% identity with query, are said to be homologous sequences.



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CPA family: CPA family is the monovalent cation/proton antiporter, which is the largest gene family. Nearly twenty-nine genes were identified and named, as OsCPA.These is also numbered based on the alignment with reference protein of Arabidopsis .

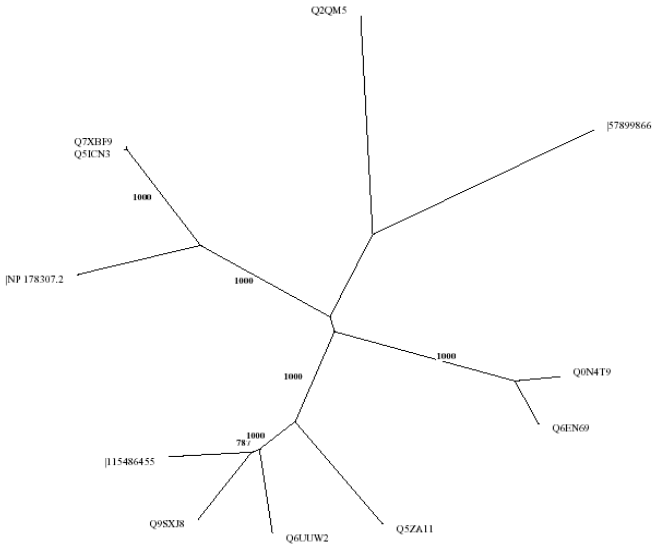
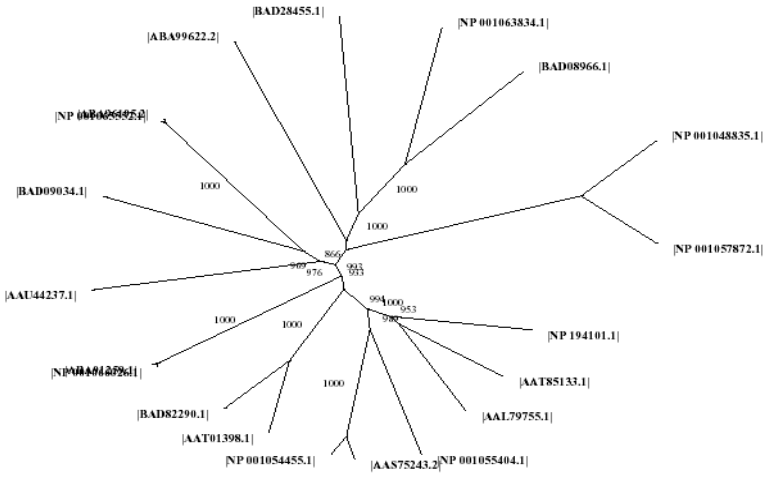


Fig. 3: Phylogenetic tree of rice CPA antiporters. Programs used were ClustalX (Thompson *et al*., 1997) for alignments and Treeview for graphical output.
Values indicate the number of times (in %) that each branch topology was found during bootstrap analysis. Scale bar corresponds a distance of 10 changes per amino acid positions.
Na/H^+ Antiporters: Smallest gene family in the cation/proton antiporter family. Only three genes were identified in this family. The genes, which have more than 30%identity with query, are regarded as homologous sequences.



CNGC family: Cyclic nucleotide gated channel family contains a total of forty-two genes and these are named as OsCNGC. These are numbered based on the alignment of the gene with query. The genes, which are showing more than 30% identity, are homologous sequences.Chloride channels: Chloride

channels are anion channels in which nearly fourteen genes were identified. These are named as OsCLC and numbered based on alignment with query.

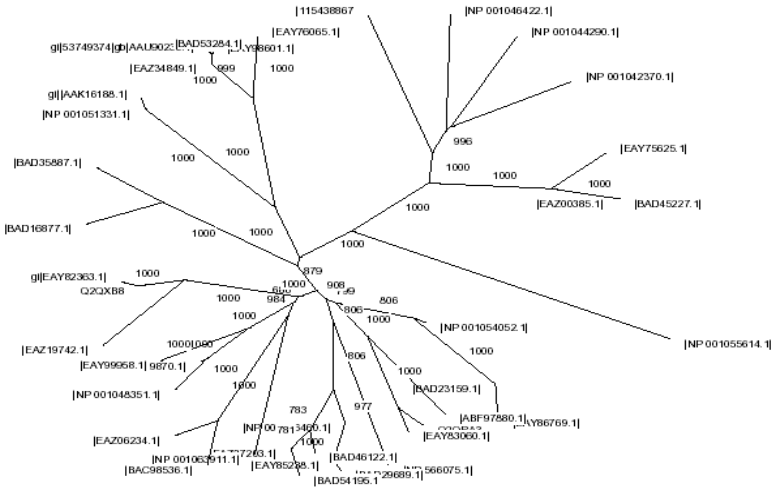


Fig. 4: Alignment of Chloride channel proteins showing conserved regions in rice. Amino acids in putative transmembrane fragments are shadowed, and amino acids, which are conserved in most sequences, are highlighted.

Alignments were made using the ClustalX (Thompson *et al*., 1997) program. Sequence accession numbers: SKT1

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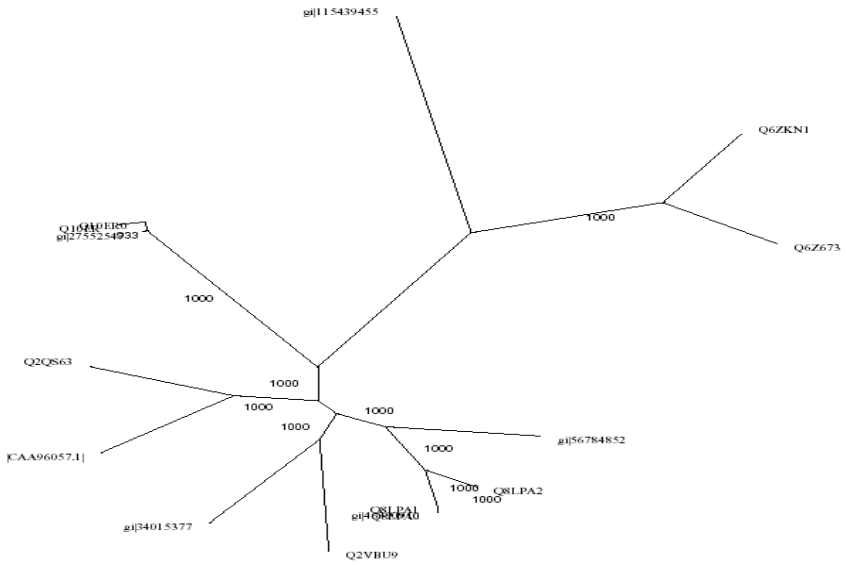

Fig. 5: Phylogenetic tree of rice CHL channels. Programs used were ClustalX (Thompson *et al*., 1997) for alignments and Treeview (Page, 1996) for graphical output. Values indicate the number of times (in

%) that each branch topology was found during bootstrap analysis. Scale bar corresponds a distance of 10 changes per amino acid positions.

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