CLONING, SEQUENCE ANALYSIS AND *In-Silico* MAPPING OF AN ABA-INDUCIBLE GENE CODING FOR ORNITHINE δ-AMINOTRANSFERASE FROM *Vicia villosa*

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Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture economics and result in the deterioration of the environment. Abiotic stresses considered as the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Boyer, 1982; Bray *et al.*, 2000). These stresses have adverse effects not only on crops that are being cultivated, but also are significant barriers to the introduction of crop plants into areas that are not, at this time, being used for agriculture (Joe, 1994).

Salinity is a major abiotic stress affecting plant cultivation and productivity (Pang *et al.*, 2010) and its adverse impacts are getting more serious problem in regions where saline water is used for irrigation (Türkana and Demiral, 2009). Salinity affects all the major processes such as growth, photosynthesis, protein synthesis, and energy and lipid metabolism (Parida and Das, 2005). Since salt stress involves both osmotic and ionic stress (Hagemann and Erdmann, 1997; Hayashi and Murata, 1998), growth suppression is directly related to total concentration of soluble salts or osmotic potential of soil water (Flowers *et al.*, 1977; Greenway and Munns, 1980). The detrimental effect is observed at the whole plant level as death of plants or decrease in productivity. Suppression of growth occurs in all plants, but their tolerance levels and rates of growth reduction at lethal concentrations of salt vary widely among different plant species.

According to FAO (2008) more than 800 million hectares of land throughout the world are salt-affected (including both saline and sodic soils),

equating to more than 6% of the world's total land area. The increased salinization of arable land showed that it is expected to have devastating global effects, resulting in 30% land loss within the next 25 years, and up to 50% by the year 2050 (Wang *et al.*, 2003). Egypt is a part of an arid belt that extending from the Atlantic coast to central Asia with more than 33 % of the irrigated land area affected by salinity (Ghassemi *et al.*, 1995). FAO (1986) reported that the major problem for Egyptian soils was the rising of soil salinity. It was stated that a decline of 30% of the soil productivity is attributed to this unfavorable process. The area which suffers from primary or secondary salinity in Egypt was estimated to about 800,000 ha representing approximately a third of the entire area of the arable land in the country.

One of the most common stress responses in plants is overproduction of different types of compatible organic solutes (Serraj and Sinclair, 2002). Proline accumulation is one of the most frequently reported modifications induced by water and salt stresses in plants and is often considered to be involved in stress resistance mechanisms, although its precise role still remains a controversial subject. Cytoplasmic accumulation of this amino acid is thought to be involved in osmotic adjustment of stressed tissues (Delauney and Verma 1993, Kavi Kishor *et al.*, 1995).

Ornithine δ-aminotransferase (δ-OAT) is an important enzyme in proline biosynthetic pathway and is implicated in salt tolerance in higher plants. OAT transaminates ornithine to pyrroline-5-carboxylate, which is further catalyzed to proline by pyrroline-5-carboxylate reductase (Sekhar *et al.*, 2007). It was reported that the activity of δ-OAT as well as the proline contents in plants increased when treated with cold (Charest and Phan, 1990), salt (Roosens *et al.*, 1998), dehydration (Hervieu *et al.*, 1994) and ABA (Yang *et al.*, 2000).

There are two potential tasks, which elucidate the significance of studying OAT: (i) genetic engineering of plants for increased production of the osmoprotectant proline; (ii) considering human OAT as a potential target for development of new therapeutic drugs (Stránská *et al.*, 2008). In the present study, *Vicia villosa* δ-OAT (VvOAT) gene was isolated and its sequence analysis and genomic location were investigated.

MATERIALS AND METHODS

Plant material and abscisic acid treatment

Vicia villosa seeds were sterilized and then germinated on MS medium (Duchefa Biochemie, Netherlands) for 7-8 days. For ABA treatment, seedlings were transferred into solution containing 100 μM abscisic acid (Sigma, USA) for 3 hrs (Rabbani *et al.*, 2003). The treated seedlings were harvested and immerged in liquid nitrogen and stored at -80°C until RNA extraction.

Isolation of Total RNA and RT-PCR

The total RNA was extracted using the TriPure isolation reagent (Roche Molecular Biochemicals, Germany) from the shoots of treated plants. The full-length coding sequence cDNA of *Vicia villosa* δ-OAT gene (VvOAT) was isolated using a RT-PCR based approach. The primers, 5' ATG GCT GCC ACA CGA CAA G 3' and 5' TTA ACC ATA CAC GAG TCG ACC AC 3' were synthesized and used to perform target RT-PCR amplification using ImProm-IITM reverse transcription system (Promega, Cat. No. A3800) according to manufacturer's instructions, another control reactions were employed by using 18s rRNA primers as a positive control also a negative control and experimental control were employed to ensure that PCR product amplified only from *villosa* cDNA. The PCR conditions were a predenaturation of 2 min at 94°C, 40 cycles, each 50 sec at 94°C, 1 min at 61°C, 2 min at 72°C; and a final extension cycle for 10 min at 72°C. Reaction products were size fractionated in a 1.5% agarose gel in 1 X TAE buffer as described by (Sambrook *et al.*, 1989), GeneRuler™ 1 kb marker Fermentas SM0311 was used in this study in DNA electrophoresis analysis.

Purification and Cloning of PCR Product

Obtained OAT band in the PCR product was excised from agarose gel and purified for the subsequent steps of ligation and cloning procedures. The purification completed using Axy-Prep™ DNA Gel Extraction Kit AP-GX-50. The purified Band were cloned in pGEM-T Easy plasmid (Promega, Madison, WI) and transformed into *E. coli DH5α*. White colonies were picked and plasmid DNA was isolated.

Sequencing and bioinformatics analysis

The chain termination procedure (Sanger *et al.*, 1977) was performed with ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, USA) with T7 and SP6 primers in conjunction with ABI PRISM (310 Genetic Analyzer).

Protein homology search was performed using BLASTX against the NCBI protein database (http://www.ncbi.nlm.nih.gov). Sequences of plant δ-OAT genes that showed similarity to the δ-OAT gene were obtained from the NCBI nonredundant and dbEST data sets using BLASTX or BLASTP (ver. 2.0.10) (Altschul *et al.*, 1997). The full amino acid sequences of the proteins were aligned using CLUSTAL W (ver. 1.8) (Thompson *et al.*, 1994) and subjected to phylogenetic analysis. Phylogenic trees were constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987) with parsimony and heuristic search criteria and 1000 bootstrap replications to assess branching confidence.

The VvOAT sequence was compared to barrel medic BAC/PAC sequences using BLAST (with an e-value

threshold of 1e-10) (http://www.tigr.org). The barrel medic BAC/PAC that matches the query was used to identify anchored barrel medic markers from the barrel medic genetic linkage map (http://www.medicago.org/genome/). The results obtained from this stage were used to construct a comparative map between barrel medic, lotus japonicus and soybean to identify the tentative chromosomal location of VvOAT in barrel medic, lotus japonicus and soybean using (http://www.comparative-legumes.org) comparative mapping strategy (Diab *et al.*, 2007).

RESULTS AND DISCUSSION

Total RNA extracted from ABA treated shoots leaves was used in RT-PCR; a candidate band of 1410 bp was obtained (Fig. 1). The purified candidate δ-OAT PCR fragment was ligated into pGEM-T easy vector and transformed into *E. coli* DH5α competent cells. Plasmids were isolated from candidate colonies obtained from transformation.

Isolated fragment was sequenced using ABI PRISM (310 Genetic Analyzer). The sequence data (Fig. 2) was utilized to run a homology search using blast tool provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The results of the homology research revealed that the isolated fragment was designated as δ-OAT which displayed a different degree of similarities to many δ-OAT genes.

In plants, it has been established that transamination of ornithin can happen via either α-OAT or δ-OAT (Mestichelli *et al.*, 1979). However, until now only δ forms of OAT have been cloned in plants such as moth bean *Vigna aconitifolia* (Delauney *et al.*, 1993), human (Inana *et al.*, 1986), and yeast *Saccharomyces cerevisiae* (Degols, 1987), and *Bacillus subtilis* (Gardan *et al.*, 1995).

Full length coding sequence of *Vicia villosa* δ-OAT (VvOAT) gene was successfully submitted to GenBank under accession number AB548891 for DNA sequence and BAI81979 for protein sequence. The complete coding sequence DNA is 1410 bp and its protein is 470 aa with predicted molecular masses of 51.3 KDa (pI 8.5). The *V. villosa* OAT is a δ-OAT. The possibility that this clone would be an α form has to be discarded because the sequence of the region involved in the interaction with the pyridoxal phosphate has no significant homology with the α -aminotransferase (Schultz and Coruzzi, 1995). Alignment of the predicted amino acid sequence of VvOAT with OAT proteins from other species identified several conserved regions (Fig. 3). The most important domain is the one catalyze ω-transamination which corresponds to the putative pyridoxal phosphate-binding site (Roosens *et al.*, 1998), and δ-amino acids involved in the interaction with pyridoxal phosphate (Heimberg *et al.*, 1990).

To determine the evolutionary relatedness of VvOAT to Ornithine δ-aminotransferase proteins isolated from other species, the neighborjoining method

(NJ) was used to generate a gene tree based on amino acid sequence homology of the Ornithine δ-aminotransferase regions of isolated genes to those of VvOAT proteins (Fig. 4). The tree showed that VvOAT protein forms a distinct clade on phylogenetic trees derived from various OAT sequences isolated from other dicot plants. VvOAT showed a different degree of similarity with the other Ornithine δ-aminotransferase genes.

The *Vicia villosa* OAT was 94% identical to the *Pisum sativum* enzyme, 89% to *Medicago truncatula*, 82% to *Glycine max*, 72% to *Ricinus communis*, 71% to *Populus maximowiczii* x *Populus nigra* and *Populus trichocarpa* and 70% to *Vitis vinifera*, *Brassica rapa*, *Brassica napus*, and *Arabidopsis thaliana*. Those confirm the fact that the enzyme maintained a fairly high degree of conservation across a wide evolutionary spectrum (Gafan *et al.*, 2001).

In-silico mapping of the VvOAT revealed that VvOAT gene is located on barrel medic chromosome 3, lotus japonicus chromosome 1 and soybean chromosome 4 (Fig 5). The barrel medic BAC/PAC clone (mth2-27e7) was found to be matching with the sequence of VvOAT (http://www.tigr.org), (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The marker Dk473L (AC122172) is mapped to the BAC/PAC clone (mth2-27e7) on barrel medic chromosome 3 at 61.5 cM (http://www.medicago.org/genome/). Comparative mapping showed that the marker PRTS on soybean-Gm JGI 8X sequence assembly was found to be closely linked (0 cM) to the locus Dk473L on barrel medic chromosome (almost share the same locus) (http://www.comparative-legumes.org) also the marker 1433P on lotus-Lotus genome Kazusa (2009) was found to be closely linked (8.8 cM) to the locus Dk473L on barrel medic chromosome (http://www.comparative-legumes.org).

The results obtained from this comparative map between barrel medic, lotus japonicus and soybean indicated that this gene is located on barrel medic chromosome 3, lotus japonicus chromosome 1 and soybean chromosome 4. These results are supported by Weeden *et al.* (1992) and Menancio-Hautea *et al.* (1993) that the members of the Papilionoideae subfamily exhibited extensive genome conservation based on comparative genetic mapping.

These studies, however, also revealed many exceptions to the conserved synteny, with frequent local genic rearrangements including gene inversion, duplication, translocation, and insertion/deletion. Although the degeneracy of local genome microstructure has been widely documented, it is less clear the extent to which such alterations to genome microstructure contribute to the divergence of genome function (Zhu *et al.*, 2005).

Previous studies have found that the expression of the δ-OAT gene is dramatically induced by drought, salinity and ABA stresses suggesting that the regulation at transcriptional level is very impor-

tant in the activation of the δ-OAT gene under stress conditions (Armengaud *et al.*, 2004). Schmid *et al.* (1993) cloned and identified an OAT gene in *P. falciparum* Honduras, while screening plasmodial cDNAs for glucose transporters. However, functional enzyme was neither expressed nor characterized. Roosens *et al.* (1998) isolated the gene from *Arabidopsis thaliana* and studied the effect of salt stress on its expression. Liangqi *et al.* (2003) transferred Arabidopsis δ -OAT gene into rice and obtained an enhancement of salt and drought tolerance and an increase in rice yield. As our laboratory has been interested in abiotic stress tolerance genes, particularly those involved in salt tolerance, we have cloned VvOAT, so we can deliver it to our economically important crops in order to gain salt tolerance beside the availability of cloned gene encoding the key enzyme in both the ornithin and glutamate pathways will enable us to directly investigate the potential in controlling this pathways in proline biosynthesis under salinity stress.

SUMMARY

Proline is the most common organic solutes that play an active role in the mechanism of plant salt tolerance. Ornithine δ-aminotransferase (δ-OAT) is a key enzyme in proline biosynthetic pathway and is implicated in salt tolerance in higher plants. A full-length of coding sequence encoding the Ornithine δ-aminotransferase enzyme was isolated from legume Vicia villosa by RT-PCR approach. Sequence designated as VvOAT was found to exhibit variation from another sequences of δ-OAT registered in NCBI Database. VvOAT protein is composed of 470 amino acids with predicted molecular masses of 51.3 KDa (pI 8.5). Alignment of the deduced polypeptide of VvOAT with proteins from other dicots revealed several homologous regions, in particular the conserved domains of putative pyridoxal phosphate-binding site. The evolutionary relatedness of VvOAT revealed that VvOAT protein forms a distinct clade on phylogenetic tree derived from various VvOAT sequences isolated from dicots. In silico mapping of VvOAT revealed that this gene is located on Barrel medic chromosome 3, lotus japonicus chromosome 1 and soybean chromosome 4.

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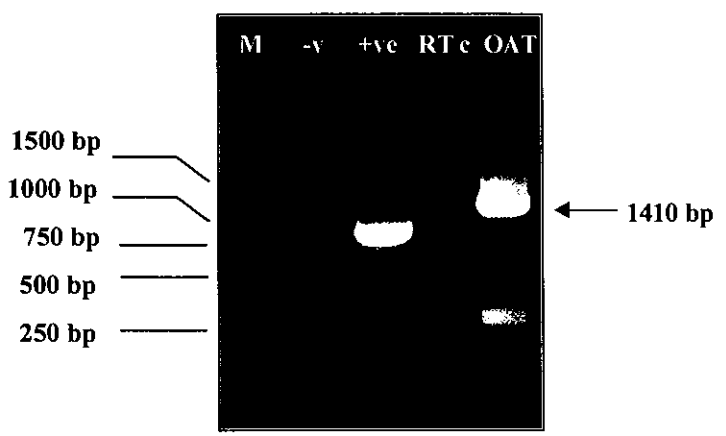

Fig. (1): 1.5 % agarose gel electrophoresis showing RT-PCR of δ-OAT. (M) 1 kb marker (Fermentas), (–ve) negative control, (+ve) Positive control, 18s rRNA gene 1000 bp, (RTc) RT-PCR control without reverse transcriptase and (OAT) RT-PCR product of the gene 1410 bp.

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> VvOAT 1410 bp complete cds        (A)
ATGGCTGCCACACGACAAGTTCACTGTTTGATGCGAACAGTTTCTAGAGGTACTAGGTTCTTCG
CTGTTGCCACACAATCCAACGCTTCTTCCTCTTCCCAAACCATCATCGACAAAGAATACCAACA
CAGTGCCCACAACTATCATCCACTTCCCATTGTTTTTGCTCATGCTAAGGGTTCATCTGTGTG
GGATCCAGAGGGAAACAAATATATTGATTTTCTGTCTGGATATTCTGCGGTTAATCAGGGACAC
TGTCATCCTAAAACTCTGAAAGCCTTACATGATCAGGCGGATAGGTTGACGGTGAGCTCTCGTG
CGTTCTATAATGATCGGTTTCCGGTTTTTGCTGAGTATTTGACAGCCTTGTTTGGTTATGATAT
GGTGCTTCCTATGGATACTGGTGCTGAAGGTGTGGAAACTGCTTTGAAATTGGCAAGAAAGTG
GGGTTATGAAAAGAAAAAAATTCCTAATGATGAAGCTCTTATTGTCTCATGCTGTGGCTGCTTC
AATGGTCGTACATGGGGGGTCATAACTATGAGTTGTGACAATGAAGCTACCCGCGGTTTTGGA
CCTTTAAAGCCAGGCCAACTTAAAGTTGATTTGGGGGATCAAAAACCCATTGAACGGATTTTCA
AAGAAAAAGGAAACCGCATACCG
GCTTTTATTTTAGAACCTATCCAAGGGGAAGCCGGGGTGGTAATTCCCCCAGATGGTTATTT
GAAAGCTGTTAGAGATCTATGCTCCAAATACAACGTGTTGATGATTGCTGATGAAATCCAAACT
GGATTAGCAAGAACCGGGAAAATGTTGGCTTGTGAGTGGGAAGATGTTCGTCCAGATGTAGTGA
TTCTAGGGAAAGCATTAGGTGGAGGAATTATACCAGTTAGTGCAGTTCTTGCCGACAAAGATGT
GATGCTTTGTATTAAACCTGGACAGCATGGAAGTACTTTTGGAGGAAATCCATTGGCTAGTGCA
GTTGCCATTGCTTCACTCGAAGTGATCAAAGAAGAGAGACTTACCGAGAGATCTACCAAAATGG
GAGGCGAGCTTCTCGGTCTGCTACATAAGATTCAGAAGAAACACCCGGACCACGTAAAGGAGGT
ACGGGGAAGAGGTTTGTTTATTGGAG
TGGAGCTTAACAGCGAAAGCTTGTCTCCCGTATCAGGCTTTGAGTTAAGCGAAAAATTGAAAGA
GAGAGGAGTTCTTGCCAAGTCAACACACGACACAATTATTCGCTTCACCCCCCCACTCTGC
ATAAGCGCGGATGAGATTCAACAAGGTTCTAAGGCATTG GCTGATGTGCTGGAAATTGATCTA
CCATTGCTGAAGAAGATGAAGCCGAAAGACGCTGTTCCTCCAGCTGGGCCTAGTCCATGTGA
TCGTTGTGGTCGACTCGTGTATGGTTAA

>VvOAT 470 aa protein              (B)
MAATRQVHCLMRTVSRGTRFFAVATQSNASSSSQTIIDKEYQHSAHNYHPLPIVFAHAKG
SSVWDPEGNKYIDFLSGYSAVNQGHCHPKTLKALHDQADRLTVSSRAFYNDRFPVFAEYL
TALFGYDMVLPMDTGAEGVETALKLARKWGYEKKKIPNDEALIVSCCGCFNGRTWGVITM
SCDNEATRGFGPLKPGQLKVDLGDQKPIERIFKEKGNRIPAFILEPIQGEAGVVIPPDGY
LKAVRDLCSKYNVLMIADEIQTGLARTGKMLACEWEDVRPDVVILGKALGGGIIPVSAVL
ADKDVMLCIKPGQHGSTFGGNPLASAVAIASLEVIKEERLTERSTKMGGELLGLLHKIQK
KHPDHVKEVRGRGLFIGVELNSESLSPVSGFELSEKLKERGVLAKSTHDTIIRFTPPLCI
SADEIQQGSKALADVLEIDLPLLKKMKPKDAVPPAGPSPCDRCGRLVYG*
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Fig. (2): (A): Nucleotide sequence of the isolated VvOAT gene representing 1410 bp as obtained from the ABI PRISM 310 DNA sequencer. (B): The deduced 470 amino acids residues from the only open reading frame (ORF).

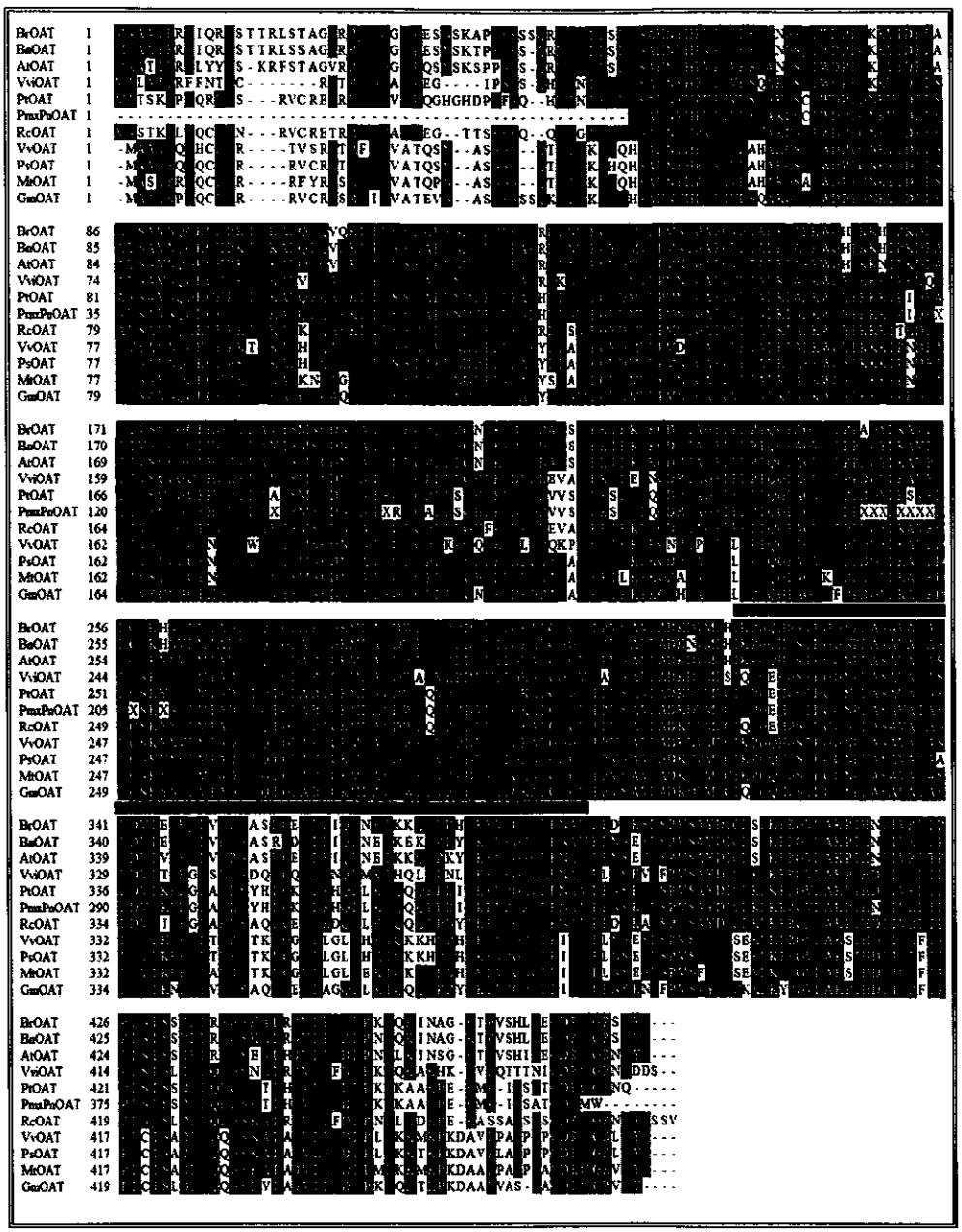

Fig. (3): Alignment of the predicted amino acid sequence of *Vicia villosa* (VvOAT) and those of several other plants including *Pisum sativum* (PsOAT), *Medicago truncatula* (MtOAT), *Glycine max* (GmOAT), *Ricinus communis* (RcOAT), *Populus trichocarpa* (PtOAT), *Populus maximowiczii* x *Populus nigra* (PmxPnOAT), *Vitis vinifera* (VviOAT), *Brassica rapa* (BrOAT), *Brassica napus* (BnOAT), *Arabidopsis thaliana* (AtOAT). Shaded boxes indicate conserved amino acid residues. The domain corresponding to the putative pyridoxal phosphate-binding site is underlined. δ -amino acids involved in the interaction with pyridoxal phosphate are marked with ( ).

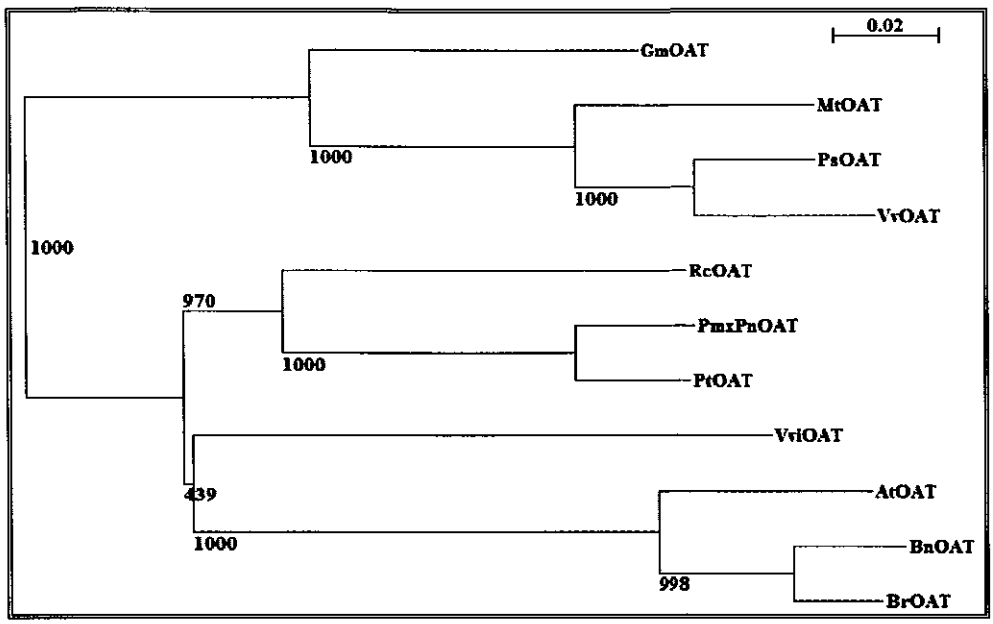

Fig. (4): Phylogenetic analysis of OATs. (Vv, *Vicia villosa*, BAI67827 - Ps, *Pisum sativum*, ABZ10818 – Mt, *Medicago truncatula*, CAC82185 - Gm, *Glycine max*, ABA86965 - Rc, *Ricinus communis*, XP_002519647 – Pt, *Populus trichocarpa* XP_002317282 - PmxPn, *Populus maximowiczii* x *Populus nigra*, ACQ66335 - Vvi, *Vitis vinifera*, XP_002278418 - Br, *Brassica rapa*, BAF81516 - Bn, *Brassica napus*, ACA63476 - At, *Arabidopsis thaliana*, NP_199430).

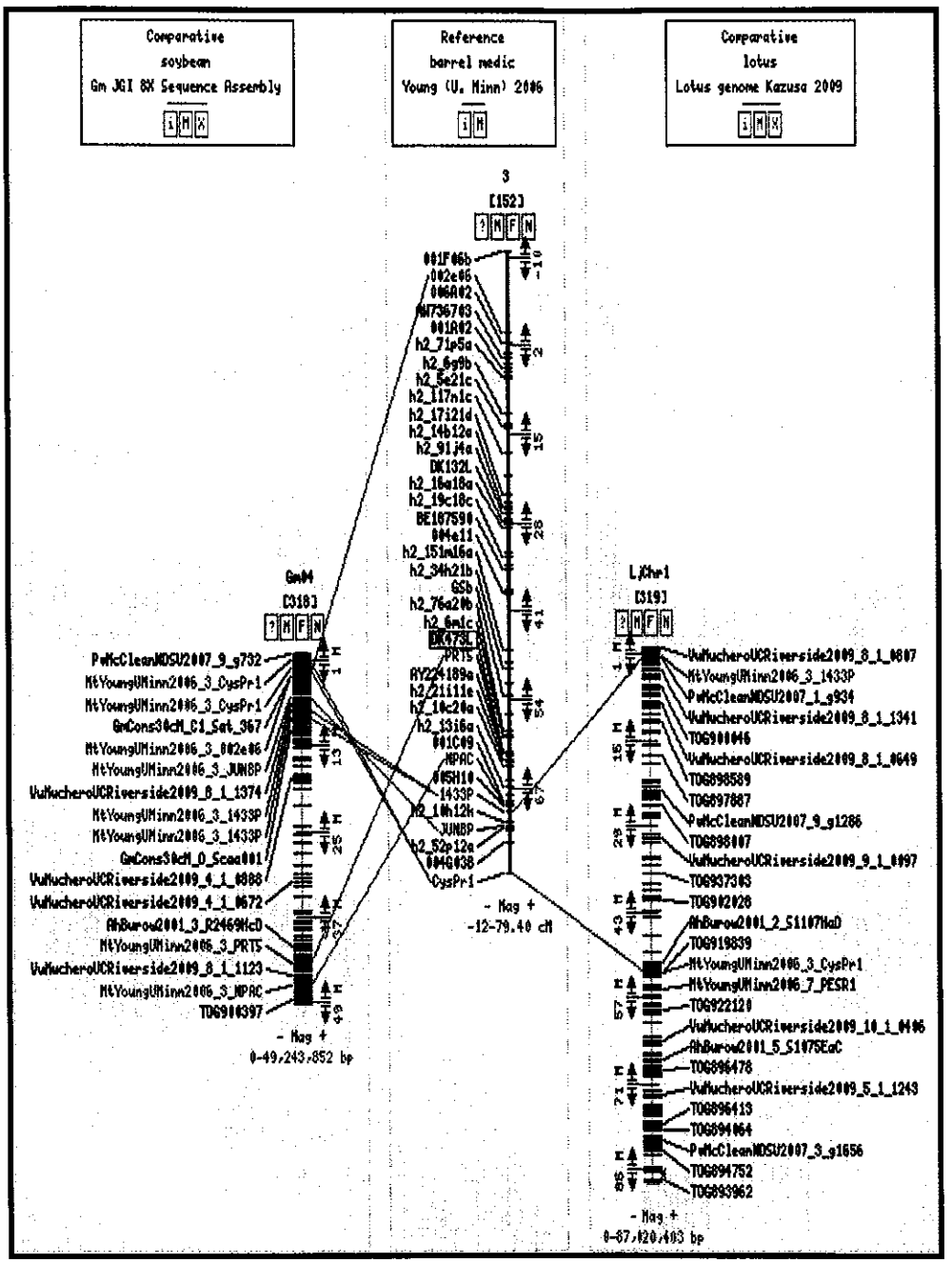

Fig. (5): Comparative map showing the location of the marker PRTS on soybean Gm4 chromosome. This marker is closely linked (0 cM) to the locus DK473L on barrel medic chromosome also the marker 1433P on lotus japonicus chromosome 1 is closely linked (8.8 cM) to the locus DK473L on barrel medic chromosome.