Molecular Cloning and Characterization of a cDNA Encoding Sedoheptulose-1,7-bisphosphatase from Mulberry (*Morus alba* var. multicaulis)

By X. Ji[1], Y. Gai[2], J. Ma[1], C. Zheng[2] and Z. Mu[1],[*]

Abstract

A full-length cDNA encoding sedoheptulose-1,7-bisphosphatase (SBPase; EC 3.1.3.37) was cloned from mulberry (*Morus alba* var. multicaulis) by rapid amplification of cDNA ends (RACE). The cDNA consisted of 1,527 nucleotides with an open reading frame (ORF) of 1,179 nucleotides encoding a 393 amino acid protein of approximately 42.6 kDa. Sequence comparison analysis showed that mulberry SBPase (MSBPase) had high homology to other plant counterparts. Phylogenetic and molecular evolutionary analysis revealed that MSBPase fell into plant SBPase group. Moreover, SBPase and fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) shared 28–32 % identical residues, suggesting that the two enzymes originated from the same evolution branch. Molecular modeling indicated that each subunit of MSBPase was composed of α-helices and β-sheets joined by turns and loops, and folded into a structure of hexahedron shape which was very similar to FBPase.

Key words: sedoheptulose-1,7-bisphosphatase, fructose-1,6-bisphosphatase, cDNA cloning, mulberry.

Introduction

Sedoheptulose-1,7-bisphosphatase (SBPase) is a key enzyme in the regenerative phase of Calvin cycle, which is the primary pathway of carbon fixation in the chloroplast stroma (ANJA et al., 2002). SBPase catalyses the irreversible dephosphorylation of sedoheptulose-1,7-bisphosphate to sedoheptulose-7-phosphate (CHRISTINE et al., 1999). This reaction is essentially irreversible and commits intermediates to the regenerative part of the Calvin cycle, so as to it competes for the substrates with the starch synthetic pathway. Two enzymes, SBPase and FBPase, catalyse the irreversible reactions in the branched region where the intermediates may leave the cycle, and for this reason may have particular roles in regulating carbon portioning to sucrose and starch (WOODROW and BERRY, 1988; GEIGER and SERVAITES, 1995). It is proposed that SBPase plays an important role in regulating the flow of intermediates through the Calvin cycle (PETTERSON and RYDE-PETTERSON, 1989; POOLMAN et al., 2000). SBPase is a nuclear-encoded enzyme synthesized as a precursor of 393 amino acids with a transit peptide which directs the enzyme to the chloroplast. Biochemical studies including the purification of the SBPase protein from a number of species show that the SBPase is dimeric and comprises two identical subunits of molecular weight 35–38 kDa (CADET et al., 1987; CHRISTINE et al., 1999).

Mulberry (*Morus alba* L.) is a perennial woody plant of considerable economic importance because of its foliage, which constitutes the chief food for the mulberry silkworm (*Bombyx mori* L.) (JAIN et al., 2000). We are interested in mulberry SBPase because of its pivotal role in the photosynthesis which products are used to synthesize a variety of metabolites beneficial to silkworm growth and development. Here, we described the isolation and nucleotide sequence analysis of the MSBPase cDNA from mulberry and compared its structure with SBPases from other plant species.

Materials and Methods

Plant materials and chemicals

Mulberry (*Morus alba* var. multicaulis) leaves were frozen immediately in liquid nitrogen and stored at

[1] College of Forestry, Shandong Agricultural University, Taian, Shandong, 271018, People's Republic of China.

[2] State Key Laboratory of Crop Biology, Shandong Agricultural University, Taian, Shangdong, 271018, People's Republic of China.

[*] Corresponding Author: ZHIMEI MU, College of Forestry, Shandong Agricultural University, Taian, Shandong, 271018, People's Republic of China. Tel: +86-538-8241315, Fax: +86-538-8249131. E-mail: gyp@sdau.edu.cn

Table 1. – Oligo nucleotide primers used for RT-PCR and RACE-PCR.

Name	Sequence(5'-----3')
T15	GACTCTAGACGACATCGA(T)$_{15}$
ST	GACTCTAGACGACATCGA
SBP-1	TTY GGN GAY GAR CAR YTN GC
SBP-2	TG RTT NAC RTC NGG NAC CAT
3SW	TCCTGGAAACTTGAGAGCCACT
3SN	CTTTGAGATACACTGGAGGAAG
G15	GGCCACGCGTCGACTAGTAC (G)$_{16}$
GT	GGCCACGCGTCGACTAGTAC
5SW	AGC CAC ACT GAA TCC ACC TT
5SN	GGA ACT TCC TCG GAA CAA GCA

–80 °C. Ex Taq™, pMD18-T vector, T4 DNA ligase, DNA Gel Extraction Kit, 100 bp DNA Ladder, Reverse Transcriptase M-MLV, and Terminal Deoxynucleotidyl Transferase were all from Takara Biotechnology Co., Ltd. (Dalian, China). The degenerate and specific primers used in PCR were synthesized by Shanghai Invitrogen Biological Engineering Technology and Service Co., Ltd. (Shanghai, China). The primers used in this study were all listed in *Table 1*.

RNA isolation and cDNA synthesis

Mulberry leaves were ground to fine powder in liquid nitrogen and total RNA was extracted by means of the hexadecyltrimethylammonium bromide (CTAB) method (CHANG et al., 1993). To synthesize the first strand cDNA, 2 μg of total RNA mixed with 20 pmol oligo (dT)$_{15}$ primer T15 was treated at 70 °C for 10 min and then cooled on ice for 2 min. After adding 2 μl of 10 x buffer, 20 nmol dNTP mixture, 0.2 μmol DTT, 40 units placental RNase inhibitor, and 100 units reverse transcriptase M-MLV, the reaction system was made up to 20 μl volume in the end using DEPC-ddH$_2$O. Then it was incubated at 42 °C for 1 h, and the reaction was terminated by incubation at 95 °C for 5 min.

Amplification of MSBPase cDNA fragments

The degenerated PCR primers (forward primer: SBP-1, reverse primer: SBP-2) were designed according to the high conserved domain of the amino acid sequences of SBPases in GenBank. The total volume of the PCR reaction mixture was 25 μl, containing 1 μl of the first strand cDNA, 0.5 μM of each primer, 2.5 μl of 10 x PCR buffer, 0.4 mM dNTPs and 1 unit of Taq DNA polymerase. The reaction was denatured at 94 °C for 5 min, and then followed by 35 cycles of 1 min at 94 °C, 1 min at 56 °C and 1 min at 72 °C, finally extended for 10 min at 72 °C. The PCR products were separated on a 1% agarose gel. The bands of the expected size were purified using DNA Gel Extraction Kit.

Rapid amplification of the 3´-and 5´-end of MSBPase cDNAs

RACE-PCR was used to amplify the 3´-and 5´-end SBPase cDNAs. On the basis of the obtained sequence information, specific primers were designed and nested PCR was performed for 3´-and 5´-RACE. For 3´-RACE, the first strand cDNA synthesized by primer T15 was used as template. For 5´-RACE, the first strand cDNA synthesized by T15 was further tailed poly (C) at the 3´-end with terminal deoxynucleotidyl transferase according to the manufacturer instructions, and the poly (C)-tailed cDNA was used as template. The first round PCR was carried out using a forward specific primer 3SW designed against obtained sequence information by RT-PCR and a reverse adapter primer T15 for 3´-RACE, and using primer G16 and the reverse specific primer 5SW for 5´-RACE. The second round PCR was performed using a forward specific primer 3SN and the adapter primer ST for 3´-RACE, and using primer GT and the reverse specific primer 5SN for 5´-RACE, with the first round PCR products as templates, respectively. The two round amplification reactions were carried out as described in RT-PCR. The bands of the expected size were purified using DNA Gel Extraction Kit.

Cloning and sequencing

All the DNA fragments obtained from RT-PCR and RACE-PCR were subcloned individually into the pMD18-T vector. After transformation, the positive clones were selected and further sequenced by Shanghai Invitrogen Biological Engineering Technology and Service Co., Ltd.

Deducing of the Full-length MSBPase cDNA

By comparing the overlapping sequences of the partial cDNA fragments, the full-length cDNA sequence was deduced and cloned by RT-PCR.

Molecular information analysis

The deduced amino acid sequence of MSBPase was aligned with other plant SBPases using DNAMAN multiple alignments program. Homology analysis was performed with BLASTP programs on NCBI (http://www.ncbi.nlm.nih.gov/BLAST). The molecular weight and isoelectric point were predicted with Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). Prediction of the structure of a N-terminal extension in MSBPase was performed by using TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP). The structure of MSBPase domain was analyzed with NCBI database (http://www.ncbi.nlm.nih.gov/Structure).

Results and Discussion

Cloning of the Full-length cDNA of MSBPase

Based on the primer designed for the amplification of the partial cDNA fragment of MSBPase, an expected 510 bp cDNA fragment was amplified (*Fig. 1 A*). The
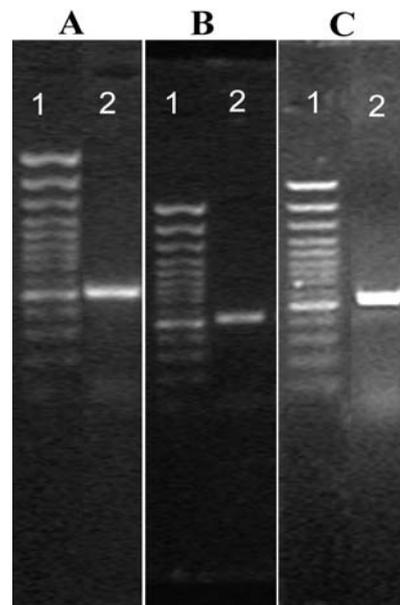
Figure 1 A. – PCR amplification of the fragment of MSBPase cDNA; B: PCR amplification of 3´-terminal sequence of MSBPase cDNA; C: PCR amplification of 5´-terminal sequence of MSBPase cDNA; 1: DNA marker 100 bp; 2: PCR amplification band.

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nucleotide sequence of this fragment showed a high similarity with the previous cloned SBPase genes from other plant species. By two rounds of nested PCR, a 540 bp DNA fragment of the 3´-RACE (*Fig. 1 B*) and a 600 bp DNA fragment of the 5´-RACE (*Fig. 1 C*) were amplified, respectively. By comparing the overlapping sequences of the partial cDNA fragments, the full-length cDNA sequence of MSBPase was obtained (GenBank accession number DQ995346). The 1,527 bp full-length cDNA contained a 1,179 bp ORF beginning with the initiation codon ATG at nucleotide 133 and terminating with stop codon TAA at nucleotide 1309 (*Fig. 2*). The ORF of MSBPase included 393 amino acids, whose predicted molecular weight was 42.6 kDa and isoelectric point was 5.85 (*Fig. 2*).

Nucleotide and deduced amino acid sequence analysis of MSBPase

To obtain more information about the nucleotide and deduced amino acid sequence of MSBPase, the sequence

comparisons as well as the multiple alignment analysis were carried out via blast search in GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). The results revealed that there was high homology between the MSBPase and SBPases in many other plant species. MSBPase deduced amino acid sequence shared 83%, 81%, 75% and 77% identity with its counterparts from *Arabidopsis thaliana*, *Spinacia oleracea*, *Triticum aestivum* and *Oryza sativa*, respectively (*Fig. 3*). The highly conserved amino acid region of SBPase implies that these amino acids may play an important role. Though limited homology exists in the N-terminal, the general features of chloroplast transit peptide could be observed based on rich Ser and absent acidic residues (FUJIMORI et al., 1998; VON-HEIJINE et al., 1989). Moreover, the structure prediction of the N-terminal of MSBPase also indicated that it contained the putative chloroplast transit peptide of 55 amino acids, which directs the protein translocation from cytosol to chloroplast. Therefore, after the putative transit peptide removed, the molecu-

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1      AAACCAATTACTAGTAGTCTAGTAGTACAAGCAAAGTAAGCAAATAGAGCTTTTGAAGAA
61     ATCAAAGCTCTTCCTTGGTGTTTTTTTTTTTGGTGTTAATTTGAGGAAAAAATTTTCGA
121    CATAATTTCATTATGGAGACAAGTATCACTTGCTACACTCGTGGAGCTTTCCTTCCTAAT
1              M   E   T   S   I   T   C   Y   T   R   G   A   F   L   P   N
181    ATCTCGTCTTCGCATTCCATGTCTTTTGTGTCTCCCTCATCCATCTCTCCATCTTTCAAC
17     I   S   S   S   H   S   M   S   F   V   S   P   S   S   I   S   P   S   F   N
241    TCCAAGATTCTGAAATCGAGCTCGCTGTTCGGGGAATCATTACGCACGGTGCCAAAATCT
37     S   K   I   L   K   S   S   S   L   F   G   E   S   L   R   T   V   P   K   S
301    TCTCTAAAGGTTTCAAAATCGAAGAACGCTTTGCCAGTGACGAAATGCGAGATAGGCGAA
57     S   L   K   V   S   K   S   K   N   A   L   P   V   T   K   C   E   I   G   E
361    AGCTTGGAGGAATTCCTTATAAAGGCGACGCCTGATAAAGGACTGAGGGCATTGCTGATC
77     S   L   E   E   F   L   I   K   A   T   P   D   K   G   L   R   A   L   L   I
421    TCGATGGGAGAAGCATTGAGGACCATTGCCTTTAAAGTGAGAACAGCTTCTTGTGGTGGA
97     S   M   G   E   A   L   R   T   I   A   F   K   V   R   T   A   S   C   G   G
481    ACGGCTTGTGTCAATTCCTTTGGTGACGAACAGCTGGCAGTTGATATGCTTGCAAACAAC
117    T   A   C   V   N   S   F   G   D   E   Q   L   A   V   D   M   L   A   N   N
541    CTTCTTTTTGAGGCCCTAAGGTACTCCCATTTCTGCAAGTATGCTTGTTCCGAGGAAGTT
137    L   L   F   E   A   L   R   Y   S   H   F   C   K   Y   A   C   S   E   E   V
601    CCTGAGCTCCAAGACATGGGAGGCCCAGTTGAAGGTGGATTCAGTGTGGCTTTCGATCCC
157    P   E   L   Q   D   M   G   G   P   V   E   G   G   F   S   V   A   F   D   P
661    CTCGACGGTTCTAGCATCGTCGACACAAATTTCACGGTGGGGACAATTTTTGGAGTGTGG
177    L   D   G   S   S   I   V   D   T   N   F   T   V   G   T   I   F   G   V   W
721    CCAGGAGATAAGTTGACTGGAGTAACAGGAAGAGACCAAGTTGCTGCTGCCATGGGGATT
197    P   G   D   K   L   T   G   V   T   G   R   D   Q   V   A   A   A   M   G   I
781    TATGGCCCTAGAACTACATATGTTCTTGCTCTTAAAGACGTTCCCGGGACACACGAATTC
217    Y   G   P   R   T   T   Y   V   L   A   L   K   D   V   P   G   T   H   E   F
841    CTTCTTTTGGATGAAGGAAAATGGCAGCACGTCAAGGAGACAACAGAAATTGGTGAGGGA
237    L   L   L   D   E   G   K   W   Q   H   V   K   E   T   T   E   I   G   E   G
901    AAGCTTTTCTCTCCTGGAAACTTGAGAGCCACTTTTGATAATCCGGAATACAACAAGCTA
257    K   L   F   S   P   G   N   L   R   A   T   F   D   N   P   E   Y   N   K   L
961    ATTGATTACTATGTGAAAGAGAAGTATACACTTTGAGATACACTGGAGGAAGGGTACCCGAC
277    I   D   Y   Y   V   K   E   K   Y   T   L   R   Y   T   G   G   R   V   P   D
1021   GTCAACCAGATTATTGTGAAAGAGAAGGGTATTTTCACCAATGTCTTATCCCCATCAGCA
297    V   N   Q   I   I   V   K   E   K   G   I   F   T   N   V   L   S   P   S   A
1081   AAGGCCAAGCTAAGACTGTTGTTTGAGGTGGCCCCACTTGGTTTCTTGATTGAGAAGGCA
317    K   A   K   L   R   L   L   F   E   V   A   P   L   G   F   L   I   E   K   A
1141   GGAGGGTTCAGTAGTGATGGCCATCAGTCTGTGCTAGAAAAGGTGATTGAGAACCTTGAT
337    G   G   F   S   S   D   G   H   Q   S   V   L   E   K   V   I   E   N   L   D
1201   GATAGAACTCAGGTTGCGTACGGATCGAAAAACGAGATTATCCGATTCGAAGAAACTCTA
357    D   R   T   Q   V   A   Y   G   S   K   N   E   I   I   R   F   E   E   T   L
1261   TACGGATCTTCCAGGCTCAAGGGAGCAGTGCCTGTTGGAGCTGCTGCATAAATATTACAG
377    Y   G   S   S   R   L   K   G   A   V   P   V   G   A   A   A   *
1321   AACTACATGTGATTCTTACTTATGTTTGTAGCTCCTTTCTTTTGTTTTTGTTTTCACTTG
1381   GTTTTCTCCTCAAGTAAAAATTTAAAAAATGCTTGATATTCATAGTCTGGCGAGCTGTGT
1441   ATCATAGTGCATAATGGGAGGCTTGAAATTAAGACTTTTGAATCATCACAAGGTTGAATC
1501   TTTCTCTGCTCTAAAAAAAAAAAAAAAA
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Figure 2. – Nucleotide sequence of MSBPase cDNA clone and deduced amino acid sequence. The amino acid sequence of the polypeptide is given in a three-letter code for each residue. Potential codons for initiation (ATG) and termination (TAA) of translation are boxed.

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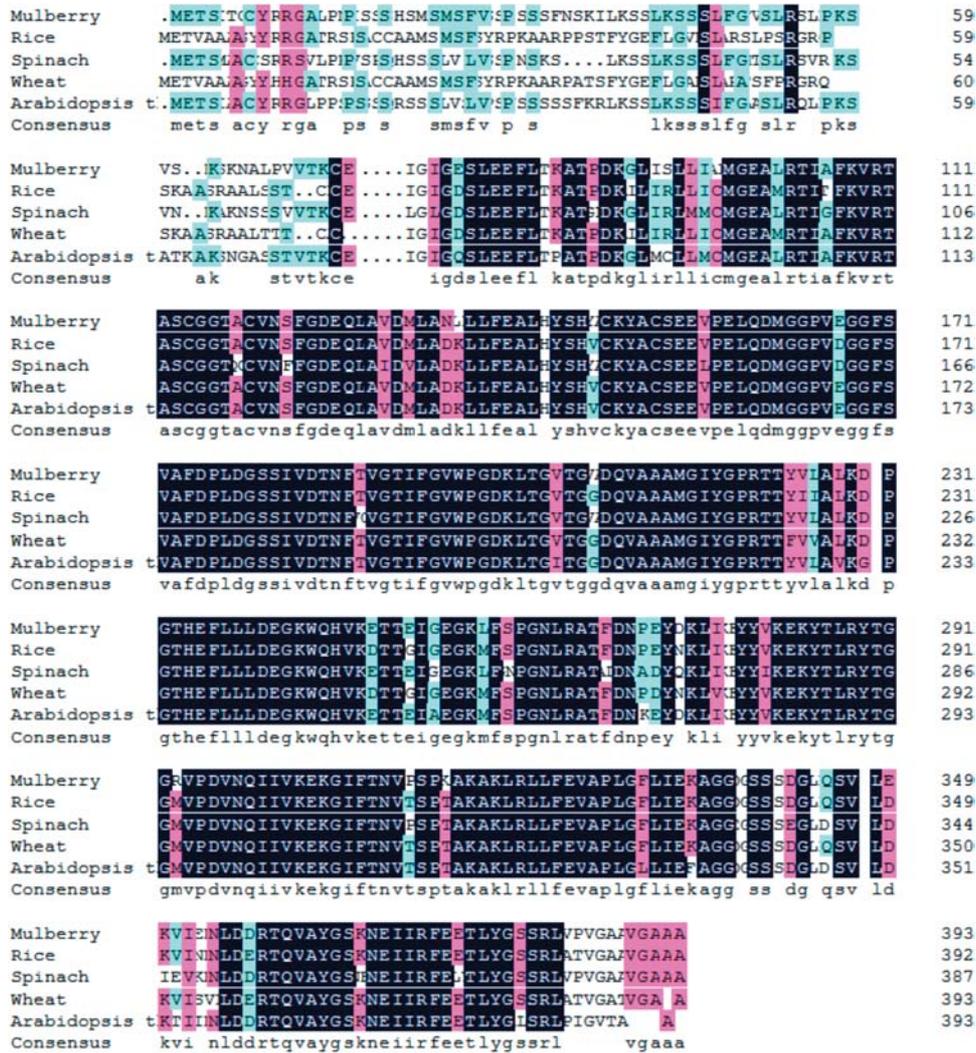
Figure 3. – Alignment of the amino acid sequence of MSBPase with that of *Arabidopsis thaliana* (AAM91137), spinach (*Spinacia oleracea*) (AAB81104), wheat (*Triticum aestivum*) (CAA46507) and rice (*Oryza sativa*) (AA022559) SBPases. Amino acid sequences were downloaded from the NCBI database and the alignments were performed using the DNAMAN program. Identical amino acids were black shaded and similar amino acids were gray shaded.

lar weight of predicted mature protein is in the range of 35–36 kDa which is in good agreement with earlier biochemical studies about SBPase (NISHIZAWA and BUCHANAN, 1981; CADET et al., 1987).

Phylogenetic and molecular evolutionary analysis

To investigate the molecular evolutionary relationships among different species, a phylogenetic tree was constructed based on the amino acid sequences of MSBPase, SBPase and FBPase from various organisms including plants, fungi, insects and animals by performing blast search in GenBank database (http://www.ncbi. nlm.nih.gov/BLAST/). As expected, the result showed that the MSBPase gene fell into the plant group (*Fig. 4*). Interestingly, all the SBPase genes were derived from a common ancestor in evolution, suggesting that they shared a common evolutionary origin. In addition, the amino acid sequences of SBPases and FBPases from various species shared 28–32% identical residues, indicating that the two enzymes may share a common catalytic mechanism and evolution origin, and acquire substrate specificity following divergence from a bifunctional enzyme (CHRISTINE et al., 1992; HANNAERT et al., 2003). Recently, it is reported that the evolutionary histories of the SBPase and FBPase gene families are very complex, including extensive paralogy, lateral transfer, and retargeting between cellular compartments (ROGERS and KEELING, 2004)

The 3-D structure of MSBPase

The 3-D structure of MSBPase was determined by aligning the amino acid sequences obtained from the NCBI database. It was clear from initial sequence comparisons of SBPases and FBPases from various sources that these two enzymes were structurally related. The structural similarities between SBPase and FBPase highlighted by these amino acid sequence comparisons made it possible to gain an insight into the structure of SBPase by the 3-D structure model of FBPase (HANNAERT et al., 2003). The model (*Fig. 5*) showed that

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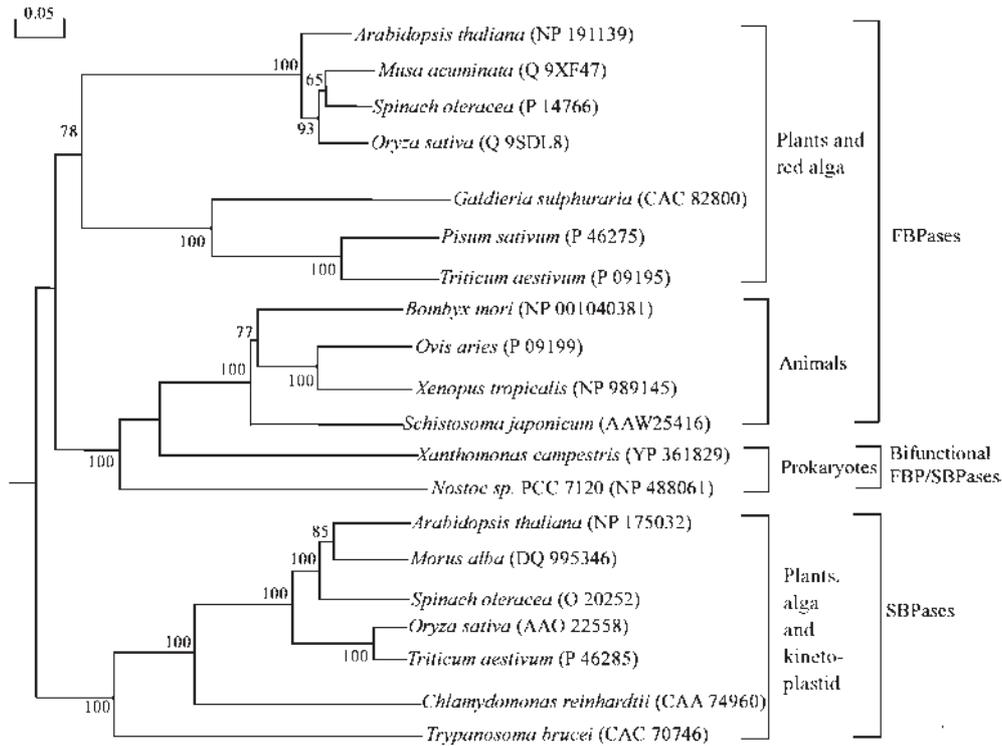
Figure 4. – Neighbor-joining tree of SBPases and FBPases. The SBPase and FBPase sequences were compared by performing blast search in GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

MSBPase contained two subunits and each subunit was composed of four α-helices and six β-sheets joined by turns and loops and folded into a hexahedron shape. There are four cysteine residues apart in a predicted loop between two α-helices, two of which probably form a disulphide bond when oxidized analogous to the allosteric regulation of FBPase by adenosine monophosphate in terms of the resulting structural changes. Active sites are located at the interface between pairs of subunits, with each subunit contributing to the active site of its partner (DUNFORD et al., 1998).

In conclusion, we have isolated and characterized a new SBPase cDNA clone from mulberry. Evolution analysis revealed that the MSBPase gene had high homology with other plant SBPases. SBPases and FBPases share a common evolution origin and are structurally related. To our knowledge, this is the first report of the molecular studies of SBPase from mulberry. Further investigation should be carried out focusing on the biochemical and physiological function of MSBPase.
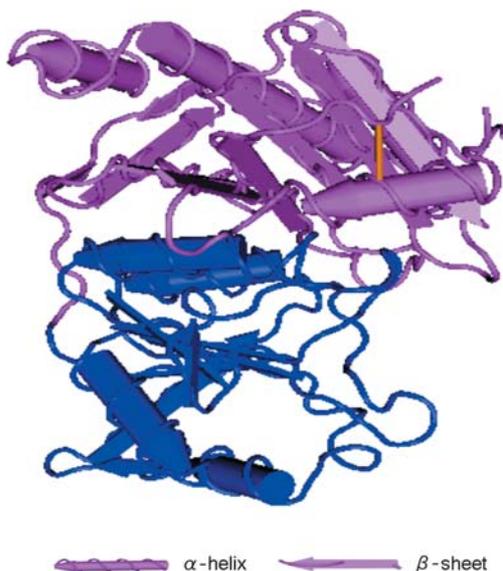
Figure 5. – Predicted tridimensional structure of MSBPase. The homodimer pair of subunits which constitute MSBPase are marked in red and blur colors.

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Genetic Diversity and Population Structure of Apricot (*Prunus armeniaca* L.) from Northern Pakistan using Simple Sequence Repeats

By M. ALI KHAN[1], F. MAGHULY[2], E. G. BORROTO-FERNANDEZ[2], A. PEDRYC[3], H. KATINGER[2] and M. LAIMER[2],[4]

Abstract

A collection of 44 *P. armeniaca* accessions and three related species, from diverse geographic areas, covering the entire Northern hemisphere with emphasis on Pakistani apricots, was screened with 10 SSR primer pairs developed in apricot, to characterize the cultivars and establish their genetic relationship. Given the fact that the Central Asian region is considered a center of origin of apricot, particular attention was devoted to accessions from the Hunza region of Pakistan. The primers correctly amplified a repeatable polymorphic pattern, which unequivocally distinguished all genotypes under study. Altogether 123 alleles were identified with an average of 12.30 alleles per locus. The observed heterozygosity for individual loci ranged from 0.28 to 0.77 with an average of 0.64. A neighbour joining method identified four groups from: (A) Central Asia, (B) Irano-Caucasia, (C) Continental Europe and (D) North America. The dendrogram confirms the historic dissemination pathways of apricot from its centre of origin in Asia to the West. Apricot cultivars from the Hunza region (Northern Pakistan) revealed a high variability, as genetic diversity is still conserved due to the traditional practice of planting seeds from the best trees.

Key words: Genetic variation, microsatellites, eco-geographic groups, Hunza, center of origin.

[1] Institute of Applied Biotechnology, GC University, Lahore, Pakistan.

[2] Plant Biotechnology Unit, Institute of Applied Microbiology BOKU, Muthgasse 18, A-1190 Vienna, Austria.

[3] Corvinus University, Villányi ut 29-43, 1118 Budapest, Hungary.

[4] Corresponding author: MARGIT LAIMER. University of Natural Resources and Applied Life Sciences, Institute of Applied Microbiology, Plant Biotechnology Unit, Nussdorfer Lände 11, 1190 Vienna, Austria. Tel. +43-1-36006 6560, Fax +43-1-36 97 615, Email: m.laimer@iam.boku.ac.at

Introduction

Botanists distinguish the following apricot species: *P. ansu*, *P. armeniaca*, *P. brigantina*, *P. mandshurica*, *P. x dasycarpa*, *P. holosericea*, *P. mume* and *P. siberica* (OECD, 2002). Most cultivated apricots, belong to the species *P. armeniaca* L., are native to Asia and Caucasus