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Cloning and characterization of anthocyanin biosynthetic regulatory gene of purple sweet potato (*Ipomoea batatas* L.)

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ABSTRACT

Sweet potato has diverse colored phenotypes of storage tuber, yet very little research reflects the biochemical background of this diversity. Our present study was devoted to characterize the regulatory and structural genes in some Indian orange and purple fleshed sweet potato cultivars. cDNA was synthesized from cultivars like 362-7, S-1221, SV -98, S-61, DOP-92-120 and RT-PCR was done using gene specific primers of regulatory genes like IT1, IT4 (from purple fleshed variety, DOP-92-120) and structural gene like 3GT (3 –O-glucosyl transferase). The amplified fragments of the respective genes were cloned and sequenced. IT1 showed no significant homology whereas IT4 (HE980452) showed homology with mRNA for sporamin A precursor molecule. The IT666 (HE980451) gene of MYB gene family showed high similarity with R2R3 type regulatory factor for anthocyanin biosynthesis. The structural gene (HE978836) showed 95% homology with Ipomoea trifida isolate UDP flavonoid: 3-glucosyl transferase (UFGT) gene, partial sequence (EU852747). The comparative analysis of sequences of IT4 (HE980452) and IT666 (HE980451) with published gene sequences of IbMYB2 gene family confirmed the function of these genes for biosynthesis of anthocyanin pigments in the storage root of sweet potato. Furthermore, IT666 was cloned and sequenced. Our result indicated that IbMYB1 alone was sufficient for induction of structural genes and anthocyanin accumulation in tuberous roots.

***Keywords*:** Anthocyanin, IT-gene family, 3-O-glucosyl-transferase, purple fleshed sweetpotato, regulatory and structural genes

Sweet potato [*Ipomoea batatas* (L.) Lam.] is considered as an important crop in more than 100 countries and used as a major source of food, animal feed and industrial raw material. Tuber crops are the most important food crops of man after cereals and grain legumes and thus find an inevitable niche in socioeconomics in farmers of India (Sinha and Tarafdar 2014). Root crops are the only potential supplementary food crops as they provide more energy per unit area basis and a cheap source of energy also (Jha G. 2011). As reported by the earlier workers, major coloring constituents in sweet potato, specifically in purple-fleshed varieties, have been identified as acylated anthocyanins (Imbert *et al.*, 1966; Zulin *et al.*, 1992; Terahara *et al.*, 1999). In recent research, attention is being focused on anthocyanin due to its therapeutic uses. Anthocyanins are the important plant pigments for the coloring of plant organs and belong to the widespread class of phenolic compounds collectively named flavonoids. They can act as antioxidants, phytoalexins or as antibacterial agents (Jin *et al.*, 2003). Recent research on nutraceutical properties of purple fleshed sweet potato indicated that the extracted anthocyanins exhibits strong free radical scavenging activity, anti-mutagenic activity, and significantly reduces high blood pressure and liver injury (Kano *et al.*, 2005; Suda *et al.*, 2008; Zhang 2009). Other beneficial properties of anthocyanins include anti-inflammatory activity, antimicrobial activity, protection from ultraviolet light, and reduction in memory impairment (Suda *et al.*, 2003; Wu, 2008). Anthocyanin along with other flavonoids plays an important role in plants also. They impart resistance of plants to insect attacks (Harborne 1988). Anthocyanins are the most important flavonoid pigments in red and purple fruits and vegetables and are naturally occurring water soluble pigments (Pazmino-Duran *et al.*, 2001).

Although the role of anthocyanins in roots is not clear, the fact that many sweet potato cultivars having purple flesh color and other varieties such as orange, yellow, or white fleshed sweet potatoes retain anthocyanins in the skin of their tuberous roots suggests that they play an important function (Mano *et al.*, 2007). They also reported that R2R3-type IbMYB gene IbMYB1 predominantly expressed in tuberous roots of purple fleshed sweet potato cultivars and this is the sole gene which results in development of purple pigmentation in tuberous roots.

The regulatory genes in aerial parts of plants, such as flowers, leaves, seeds, and fruits have been identified whereas little is known about their regulation in tuberous roots. Unlike other plants there are few witnesses on the information of the gene structures of the sweet potato, which produces colored underground tuberous roots under the soil. This investigation is an attempt to identify the genes of anthocyanin regulatory pathway system in some Indian purple fleshed and orange fleshed sweet potato and characterization of the major structural gene and regulatory genes of MYB gene family in anthocyanin biosynthesis.

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MATERIALS AND METHODS

Preparation and storage of plant-tissue

Indian sweet potato cultivars (Fig.1) with orange and purple flesh were grown in the experimental field of ICAR-All India Coordinated Research Project on Tuber Crops (AICRP), Kalyani Centre, BCKV. The storage tubers were collected after harvesting for the study.

Isolation of total RNA and synthesis of cDNA

100mg of fresh tissue from the tuber of each variety was excised and used for total RNA extraction. The total plant RNA was isolated by using *SIGMA-ALDRICH's "Spectrum Plant Total RNA Kit" (Catalog no. STRN50)* following user's manual. The quality and concentration of the RNA was checked by 1.3% agarose gel electrophoresis and spectrophotometer analyses and the RNA samples were stored in a -70°C ultra low temperature refrigerator prior to RT-PCR.

The cDNA was synthesized from freshly prepared RNA using *Fermentas's "RevertAid First Strand cDNA Synthesis Kit "(Catalog no. #K1622)* following user's manual. As per instruction, for cDNA synthesis 1µg template RNA, oligo $(dT)_{18}$ primer and DEPC treated water was added to make the final volume of the mix 12µl. The mix was incubated at 65°C for 5 min and then chilled in ice, spin down and again placed on ice. To this mix 5x reaction buffer, *RiboLockRNase inhibitor,* 10mM dNTP mix and *RevertAid M-MuLV Reverse Transcriptase* were added and the volume was made upto 20µl followed by incubation for 60 min at 42°C. For termination of the reaction the mix was placed at 70°C for 5 min. The cDNA was stored at 70°C prior to gene amplification.

Amplification of specific genes

Amplification of anthocyanin biosynthetic genes IT4, IT666 of MYB gene family and the structural gene 3GT, were done by using the gene specific primers as listed in table 1. The cocktail was of 25 µl volume containing 2.5 µl of 10X KCl Buffer, 2.0 µl of 25 mM $MgCl_2$, 0.5 µl 10 mM dNTPs Mix, 1.0 µl of each forward and reverse primer of 10 µM concentration, 0.25 µl of Taq Polymerase (5u/µl) and 1.0 µl of template cDNA. The reaction was carried out in Eppendorf Mastercycler. The themal cycle was set as denaturation of the DNA at 94°C for 5 min, which was followed by 35 cycles of amplification (94°C for 45 s, 52-55°C for 45 s and 72°C for 1 min) and by final extension at 72°C for 7 min. PCR products were checked by running the amplified products on 1% agarose gel. The experiments were repeated three times on independently isolated cDNA preparation.

Purification of the amplified product by high-throughput method

PCR products were purified using *SIGMA-ALDRICH's GenElute PCR Clean-Up Kit (Catalog no. 1020)* following the instructions and further checked the integrity on 1% agarose gel using 1kb ladder (Biolab, England). Amplified fragments of all three partial genes (IT4, IT666 and 3GT), which were reproducible over two amplifications, were photographed under gel documentation system (Vilber Lambert). Then the products were then lyophilized and sent for sequencing to Eurofins, Bangalore.

Data analysis

Maximum homology of the sequences were found using NCBI Blastn by using the default parameters and multiple alignment was done using Bioedit. The sequences showing maximum homology with our gene sequences by following NCBI Blastn were selected on the basis of E-value and percentage homology. By using the homologous sequences pairwise distance matrix and the evolutionary tree was constructed using the UPGMA method (Sneath and Sokal, 1973) in Mega7 software (Kumar et al., 2016).

Primer designing for IT666 full gene

After sequencing the partial sequence obtained was aligned to the complete cds for Ipomoea batatas IbMYB1 gene for transcription factor IbMYB1 available in genebank, accession numbers AB576765 and AB576766 to design the forward and reverse primers for amplification of full gene (Table 1).

Cloning of IT 666

Cloning of IT666 was carried out using DH5á strain of E. coli following Chung *et al.* (1989) using 2X TSS solution (LB broth containing 20% (w/v) polyethylene glycol, 10% dimethyl sulfoxide and 1M $MgCl_2$ at pH 6.5). For preparation of competent cells, the overnight grown bacterial culture was diluted to 1:50 in LB Broth and incubated at 37p C until the cells reach the log phage (O.D. at 600nm is 0.4). 1ml aliquots of early log phage of bacterial culture were prepared and centrifuged at 4p C for 1-2 min. The supernatant was discarded and pellet was suspended in 1X TSS solution and stored at -70p C and used for transformation. The ligation procedure was done using TA Cloning Kit (Invitrogen Cat. no: 45-0030) following the user's manual. Frozen TSS-competent cells were thawed slowly on ice and the ligation mix (100pg -10 ng of DNA) was added to the tube of competent cells. The tubes were flicked to mix the cells and DNA and the cells were incubated on ice for 10 minutes. The tubes were then transferred to room temperature and incubated for 10 minutes. The tubes were again

transferred to ice and incubated for an additional 10 minutes. 1 ml of LB broth was then added and the cells were incubated at 37°C for up to 1 hr with shaking (at 200 rpm). The cells were then plated onto the L.B Agar plates and incubated overnight at 37°C with Ampicillin (50mg/ml). For blue-white colony screening X-Gal and IPTG were used.

Colony PCR

Colony PCR was done with T7 and SP6 primers confirmed the transformed bacterial cells. The PCR product was then lyophilized and then sent for sequencing to Xcelris Labs Pvt. Ltd., Ahmedabad.

RESULTS AND DISCUSSION

Blast results of reported genes

After purification, the amplicons of IT4 (234bp), IT666 (479bp), 3GT (498bp) and IT666 (1003bp) after cloning were sent for sequencing to Xcelris Labs Pvt. Ltd., Ahmedabad. After sequencing the consensus sequence was generated using Bioedit and submitted for accession number. The gene bank accession number for IT4 is HE980452, IT666 is HE980451, 3GT is HE978836 and that for IT666 (cloned) is HF937132.

The NCBI blast was performed to observe highest homology of each gene sequences of IT4 (HE980452), IT666 (HE980451), 3GT (HE978836) and the cloned gene of IT666 (HF937132). Based on percentage homology and E-value 16 gene sequences were selected for each IT4 and IT666 and 17 sequences for 3GT. Similarly, 10 reported gene sequences were selected for the cloned gene IT666 (HF937132). Among 16 sequences, 234 bp amplicon of IT4 gene of DOP-92-120 (Accession no. HE980452) of *Ipomoea batatas* partial mRNA for sporamin A precursor shows 96% homology with DQ195774, DQ195772, DQ195765, U17333, X15091, DQ195766, U17335, DQ195761, EU250004, DQ195760, DQ195767, DQ195764 and 95% homology with DQ195777, DQ195776, DQ195762 and DQ195760 (Table 2). The gene belongs to MYB gene family which comprises candidates for regulators of anthocyanin biosynthesis. They do not directly act in anthocyanin biosynthetic pathway but regulates the mechanism by altering other gene activity.

The amplicon of IT666 (Accession no. HE980451) gene was obtained from tuberous tissue of Indian orange fleshed sweet potato S-61 and the sequence data encodes partial mRNA for transcription factor for IbMYB1 gene. It reveals 95% homology with Ipomoea batatas IbMYB1-2a gene for transcription factor IbMYB1, complete cds, cultivar: Ayamurasaki (AB576766) and Ipomoea batatas IbMYB1-1 gene for transcription factor IbMYB1, complete cds, cultivar: AYM96 (AB576765). The gene sequence HE980451 is 95% identical to the different pseudogenes for IbMYB1 from various cultivars like Elegant summer, Kyushu-121, Naruto Kintoki, Tamaotome, Simon-1, Suiou, Hamakomachi, Ayakomachi, Kokei-14 and Tanegashimamurasaki (Table 3).

The structural gene 3GT (3–O-Glucosyl-transferase) was isolated from orange fleshed sweet potato variety, 362-7 with the band size 479bp and accession no. HE978836 encodes partial mRNA for the enzyme 3-o-glucosyltransferase and reveals 95% identity with Ipomoea trifida isolate V431 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds (EU852747). The gene sequence is 94 per cent identical to various gene sequences taken into account for construction of phylogenetic tree. The accession numbers for different isolates encoding Ipomoea trifida UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene were shown in table 4. The enzyme is involved in the last step of anthocyanin biosynthetic pathway, adding sugar residues to unstable anthocyanidins resulting in formation of stable anthocyanins. Anthocyanidins are initially 3-glucosylated by the enzymatic activity of UDP-glucose:flavonoid (or anthocyanidin) 3GT.

As revealed from table 5, the cloned sequence of IT666 (HF937132) shows 97 per cent homology with Ipomoea batatas IbMYB2-4 gene for R2R3 MYB related transcription factor, complete cds (AB258989), Ipomoea batatas IbMYB2-1 gene for R2R3 MYB related transcription factor, complete cds (AB258986) and Ipomoea batatas R2R3 MYB transcription factor (MYB1) mRNA, complete cds (JQ337861). This sequence is 94 per cent identical with Ipomoea batatas IbMYB2-3 gene for R2R3 MYB related transcription factor (AB258988) and Ipomoea batatas IbMYB2-2 gene for R2R3 MYB related transcription factor (AB258987). We also observed that Ipomoea batatas IbMYB1 pseudogene for IbMYB1of cultivar Koganesengan (AB444409) and cultivar Beniazuma (AB444401) showed 94 per cent homology with HF937132. The Ipomoea batatas IbMYB1-2b gene cultivar Ayamurasaki (AB576767), Ipomoea batatas IbMYB1-2a gene of cultivar Ayamurasaki (AB576766) and Ipomoea batatas IbMYB1-1 gene of cultivar AYM96 (AB576765) revealed 94 per cent identity with the sequence reported from Kalyani, India (HF937132).

Role of regulatory and structural genes in anthocyanin biosynthesis

The structural genes involved in different steps of anthocyanin biosynthesis are CHS, CHI, F3H, DFR, ANS, and 3GT (encoding chalcone synthase, chalcone isomerase, flavanone-3-hydroxylase, dihydroflavonol 4-reductase, anthocyanidin synthase, and flavonoid 3-glucosyl-transferase, respectively) and are shown in

the fig. 6. The results of the present study on the characterization of anthocyanin biosynthesis genes from flesh and skin tissue of different cultivars revealed that the genes for anthocyanin biosynthesis markedly express in the fresh tissue as compared to stored tissue.

Multiple alignment and construction of pairwise distance matrix

For each gene, IT4 (HE980452), IT666 (HE980451), 3GT (HE978836) and cloned gene sequence of IT666 (HF937132), the DNA sequences were aligned along with the other selected sequences as shown in table 2, 3, 4 and 5 respectively using Mega 7 software. The translated protein sequences were also aligned and compared. As it is observed for all accession numbers that there is not any conserved region. There is large variation among all protein sequences taken (Fig. 2a, 3a, 4a, 5a). It is evident from the pairwise distance matrix of all the accession numbers that the distance of Ipomoea batatas partial mRNA for sporamin A precursor (IT4 gene), cultivar DOP-92-120 reported from Kalyani is highest (4.174) with U17333 submitted by Chen et al., (1997) from National Taiwan University, Taiwan. The distance of HE98452 is least (2.228) with U17335 encodes a tuber storage protein with trypsin inhibitory activity (Fig. 2b). As per the distance matrix of IT666 (HE980451), the highest distance is from Daucus carota (AJ006780) which is 2.472 and least (1.774) is from Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Ayakomachi (AB444403) and Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Kokei-14 (AB444402) (Fig. 3b). The pairwise distance matrix of the structural gene reveals largest distance (4.069) from Ipomoea trifida isolate G4822 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene (EU852760) submitted by Rausher et al., (2008) from Duke University, USA. The flavonoid gene shows least distance (2.970) from petunia anthocyanin gene (AF260918) (Fig 4b). Fig. 5b displays the distance matrix of IT666 gene obtained after cloning. The greatest distance for HF937132 is 4.518 with Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Koganesengan (AB444409) and the least distance is 3.070 with Ipomoea batatas R2R3 MYB transcription factor (MYB1) mRNA, (JQ337861).

Phylogenetic tree construction

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 10.65599607 is shown. The evolutionary distances were computed using the Poisson correction method (Zuckerkandt and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 65 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016). The phylogenetic tree was constructed for evaluation of evolutionary relationship among taxa. A phylogenetic analysis derived from nucleotide sequences of different varieties of sweet potatoes and petunia (AF260918) showed two major clusters A and B. The cluster A is further subdivided into two sub-groups (Fig 2c). The first subgroup constituted 13 reported sporaminA precursor mRNA from sweet potato cultivars *viz.*, DQ195770, DQ195767, DQ195761, DQ195765, DQ195772, DQ195764, U17333, DQ195777, EU250004, DQ195762, DQ195774, X15091, DQ195776. The second sub-group contains two accession numbers, DQ195766 and U17335. The second major cluster B bears IT4 (HE980452) from sweet potato cultivar which was found most closely related to petunia (AF260918) sporaminA precursor mRNA which supports the similar results reported from Netherlands (Cornelis *et al.,* 2000). It was reported that *an1* (anthocyanin1), required for pigmentation of all tissues, including the petal limb in petunia (Cornelis *et al.,* 2000).

The phylogenetic tree (Fig. 3c) for IT666 (HE980451) is as per the distance matrix which reveals two major clusters, A and B. Cluster A contains nine reported genes for transcription factor IbMYB1 in anthocyanin biosynthetic pathway (AB444403, AB444402, AB444404, AB576765, AB258985, AB444398, HE980451, AB444400 and AB444397). The second cluster bears eight gene sequences, AJ006780, AB576766, AB444411, AB444408, AB444413, AB444410, AB444407 and AB444406. The present study closely indicated that the transcription factor of the purple fleshed sweet potato of Indian cultivar (IT666) is very close to the reported genes under IbMYB functional gene family of sweet potato varieties in Japan. Also, the dendrogram of structural gene shows two major clusters A and B with all the accessions for gene sequences falling in cluster A and HE978836 being outside the cluster. It shows close resemblance to anthocyanin gene of petunia (AF260918) (Fig. 4c). Likewise our findings *IbANS* had a high similarity to other plant ANSs and the tissue expression profiles of *IbANS* indicated that it could be expressed in all tissues but at different levels (Xiaoqiang *et al.,* 2010). Previous workers also found that the structural gene flavonoid 3-*O*-glucosyltransferase (UFGT) gene plays an important role in the anthocyanin accumulation in litchi as well as pericarp coloration of a given cultivar (Yong-Zan *et al.,* 2011).

The phylogenetic tree for the cloned gene IT666 (accession no. HF937132) was constructed by selecting

Table 1: Primers with their annealing temperature used in RT-PCR experiments

Gene	Forward primer	Reverse primer	Annealing temperature
IT 4	5'CCATACCAGCTCGGATTTGT3'	5' TGGATGCCAACCTTAACTCC3'	55
IT 666	5' GCGAATTTAGTCCCGATGAA3'	5' CGGTGTTTTCCGTGATTTCT3'	52
3GT	5' AAGTATCGATCGGCGAAATG3'	5' CACGATATGGCCTCCAGAGT3'	55

Table 2: NCBI BLAST result of HE980452 (IT4, 234bp)

Sl.No.	Description	E value	Identity	Accession No.
1.	Ipomoea batatas partial mRNA for sporamin A precursor (IT4 gene), cultivar DOP-92-120	1e-117	100%	HE980452
2.	Ipomoea batatas isolate pTrip1Ex2-16 sporamin A precursor, mRNA, complete cds	1e-97	96%	DQ195774
3.	Ipomoea batatas isolate pTrip1Ex2-14 sporamin A precursor, mRNA, complete cds	1e-97	96%	DQ195772
4.	Ipomoea batatas isolate pTrip1Ex2-7 sporamin A precursor, mRNA, complete cds	1e-97	96%	DQ195765
5.	Ipomoea batatas clone PGEM-TIA sporamin precursor mRNA, complete cds	1e-97	96%	U17333
6.	Sweet potato mRNA for sporamin A tuberous root storage protein (clone pIM0335)	5e-96	96%	X15091
7.	Ipomoea batatas isolate pTrip1Ex2-8 sporamin A precursor, mRNA, complete cds	5e-96	96%	DQ195766
8.	Ipomoea batatas clone PGEM-TID sporamin mRNA, partial cds	5e-96	96%	U17335
9.	Ipomoea batatas isolate pTrip1Ex2-3 sporamin A precursor-like mRNA, complete sequence	2e-95	96%	DQ195761
10.	Ipomoea batatas sporamin A precursor, mRNA, omplete cds	3e-94	96%	EU250004
11.	Ipomoea batatas isolate pTrip1Ex2-12 sporamin A precursor, mRNA, complete cds	3e-94	96%	DQ195760
12.	Ipomoea batatas isolate pTrip1Ex2-9 sporamin A precursor, mRNA, complete cds	3e-94	96%	DQ195767
13.	Ipomoea batatas isolate pTrip1Ex2-6 sporamin A precursor-like mRNA, complete sequence	3e-94	96%	DQ195764
14.	Ipomoea batatas isolate pTrip1Ex2-19 sporamin A precursor, mRNA, complete cds	5e-91	95%	DQ195777
15.	Ipomoea batatas isolate pTrip1Ex2-18 sporamin A precursor, mRNA, complete cds	5e-91	95%	DQ195776
16.	Ipomoea batatas isolate pTrip1Ex2-4 sporamin A precursor-like mRNA, complete sequence	5e-91	95%	DQ195762
17.	Ipomoea batatas isolate pTrip1Ex2-2 sporamin A precursor-like mRNA, complete sequence	5e-91	95%	DQ195760

Table 3: NCBI BLAST result of HE980451 (IT666, 479bp)

Sl.No.	Description	E value	Identity	Accession No.
1.	Ipomoea batatas partial mRNA for transcription factor IbMYB1 (IT666 gene), cultivar S-61	0.0	100%	HE980451
2.	Ipomoea batatas IbMYB1-2a gene for transcription factor IbMYB1, complete cds, cultivar: Ayamurasaki	0.0	95%	AB576766
3.	Ipomoea batatas IbMYB1-1 gene for transcription factor IbMYB1, complete cds, cultivar: AYM96	0.0	95%	AB576765
4.	Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Elegant Summer	0.0	95%	AB444413
5.	Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Kyushu-121	0.0	95%	AB444411
6.	Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Naruto Kintoki	0.0	95%	AB444410
7.	Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Tamaotome	0.0	95%	AB444408
8.	Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Simon-1	0.0	95%	AB444407
9.	Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Suiou	0.0	95%	AB444406
10.	Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Hamakomachi	0.0	95%	AB444404
11.	Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Ayakomachi	0.0	95%	AB444403
12.	Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Kokei-14	0.0	95%	AB444402
13.	Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Tanegashimamurasaki	0.0	95%	AB444400
14.	Ipomoea batatas IbMYB1 gene for transcription factor IbMYB1, complete cds, cultivar: Murasakimasari	0.0	95%	AB444398
15.	Ipomoea batatas IbMYB1 gene for transcription factor IbMYB1, complete cds, cultivar: Ayamurasaki	0.0	95%	AB444397
16.	Ipomoea batatas IbMYB1 gene for R2R3 MYB related transcription factor, complete cds	0.0	95%	AB258985
17.	Ipomoea batatas IbMYB1-2b gene for transcription factor IbMYB1, complete cds, cultivar: Ayamurasaki	0.0	95%	AB576767

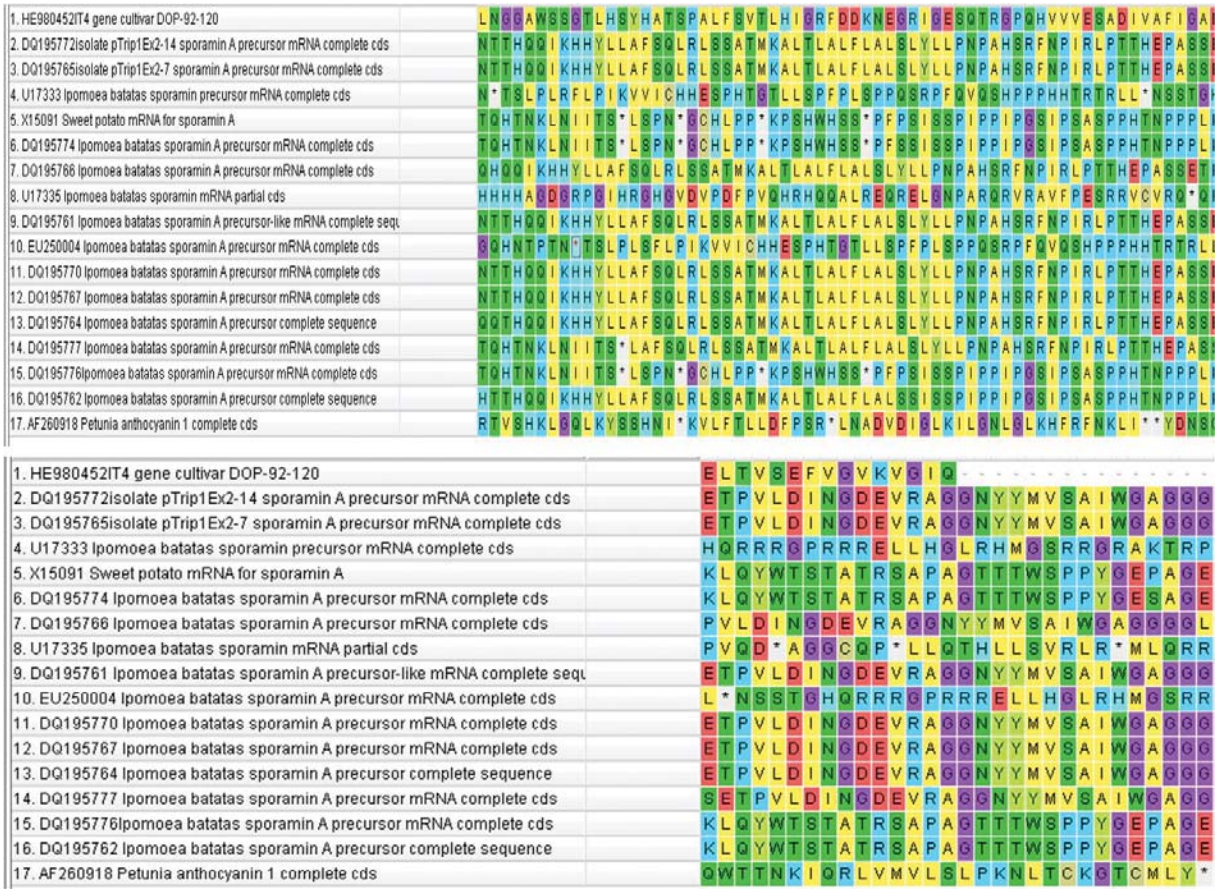
Table 4: NCBI BLAST result of 3GT (498bp)

Sl.No.	Description	E value	Identity	Accession No.
1.	Ipomoea batatas partial mRNA for 3-glucosyl transferase (3GT gene)	0.0	100%	HE978836.1
2.	Ipomoea trifida isolate V431 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	2e-128	95%	EU852747.1
3.	Ipomoea trifida isolate G481 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	9e-127	94%	EU852759
4.	Ipomoea trifida isolate CL153 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	4e-125	94%	EU852739
5.	Ipomoea trifida isolate M619823 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	2e-123	94%	EU852764
6.	Ipomoea trifida isolate M619813 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	2e-123	94%	EU852763
7.	Ipomoea trifida isolate G473 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	2e-123	94%	EU852758
8.	Ipomoea trifida isolate CR284 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	2e-123	94%	EU852756
9.	Ipomoea trifida isolate CR282 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	2e-123	94%	EU852755
10.	Ipomoea trifida isolate CR182 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	2e-123	94%	EU852753
11.	Ipomoea trifida isolate V442 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	2e-123	94%	EU852749
12.	Ipomoea trifida isolate V434 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	2e-123	94%	EU852748
13.	Ipomoea trifida isolate G4822 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	7e-123	94%	EU852760
14.	Ipomoea trifida isolate CR183 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	9e-122	94%	EU852754
15.	Ipomoea trifida isolate V444 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	9e-122	94%	EU852750
16.	Ipomoea trifida isolate CL301 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	9e-122	94%	EU852745
17.	Ipomoea trifida isolate CL182 UDP flavonoid: 3-O-glucosyltransferase (UFGT) gene, partial cds	9e-122	94%	EU852742
18.	Ipomoea batatas flavonoid 3-O-glucocyltransferase 2 mRNA, complete cds	4e-120	94%	KF056329

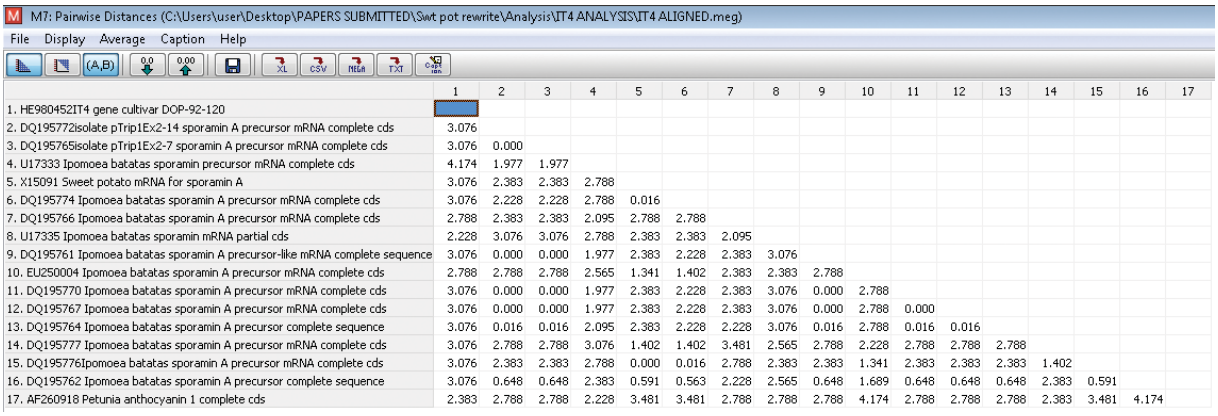
Table 5: NCBI BLAST result of (IT666; 1003bp)

Sl.No.	Description	E value	Identity	Accession No.
1.	Ipomoea batatas partial myb2 gene for R2R3 type transcription factor, cultivar DOP-93	0.0	100%	HF937132.1
2.	Ipomoea batatas IbMYB2-4 gene for R2R3 MYB related transcription factor, complete cds	0.0	97%	AB258989.1
3.	Ipomoea batatas IbMYB2-1 gene for R2R3 MYB related transcription factor, complete cds	0.0	97%	AB258986.1
4.	Ipomoea batatas IbMYB2-3 gene for R2R3 MYB related transcription factor, complete cds	0.0	94%	AB258988.1
5.	Ipomoea batatas IbMYB2-2 gene for R2R3 MYB related transcription factor, complete cds	0.0	94%	AB258987.1
6.	Ipomoea batatas R2R3 MYB transcription factor (MYB1) mRNA, complete cds	0.0	97%	JQ337861.1
7.	Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Koganesengan	0.0	94%	AB444409.1
8.	Ipomoea batatas IbMYB1 pseudogene for IbMYB1, cultivar: Beniazuma	0.0	94%	AB444401.1
9.	Ipomoea batatas IbMYB1-2b gene for transcription factor IbMYB1, complete cds, cultivar: Ayamurasaki	0.0	94%	AB576767.1
10.	Ipomoea batatas IbMYB1-2a gene for transcription factor IbMYB1, complete cds, cultivar: Ayamurasaki	0.0	94%	AB576766.1
11.	Ipomoea batatas IbMYB1-1 gene for transcription factor IbMYB1, complete cds, cultivar: AYM96	0.0	94%	AB576765.1

Fig. 1: Showing different orange and purple fleshed sweet potato varieties

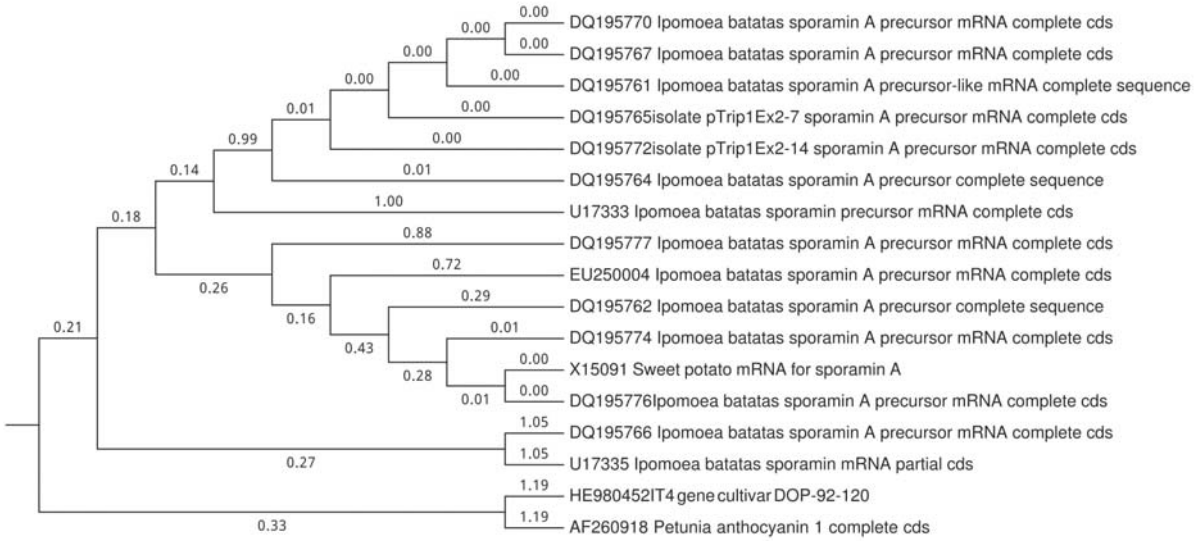


(a)



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. HE980452IT4 gene cultivar DOP-92-120																	
2. DQ195772isolate pTrip1Ex2-14 sporamin A precursor mRNA complete cds	3.076																
3. DQ195765isolate pTrip1Ex2-7 sporamin A precursor mRNA complete cds	3.076	0.000															
4. U17333 Ipomoea batatas sporamin precursor mRNA complete cds	4.174	1.977	1.977														
5. X15091 Sweet potato mRNA for sporamin A	3.076	2.383	2.383	2.788													
6. DQ195774 Ipomoea batatas sporamin A precursor mRNA complete cds	3.076	2.228	2.228	2.788	0.016												
7. DQ195766 Ipomoea batatas sporamin A precursor mRNA complete cds	2.788	2.383	2.383	2.095	2.788	2.788											
8. U17335 Ipomoea batatas sporamin mRNA partial cds	2.228	3.076	3.076	2.788	2.383	2.383	2.095										
9. DQ195761 Ipomoea batatas sporamin A precursor-like mRNA complete sequence	3.076	0.000	0.000	1.977	2.383	2.228	2.383	3.076									
10. EU250004 Ipomoea batatas sporamin A precursor mRNA complete cds	2.788	2.788	2.788	2.565	1.341	1.402	2.383	2.383	2.788								
11. DQ195770 Ipomoea batatas sporamin A precursor mRNA complete cds	3.076	0.000	0.000	1.977	2.383	2.228	2.383	3.076	0.000	2.788							
12. DQ195767 Ipomoea batatas sporamin A precursor mRNA complete cds	3.076	0.000	0.000	1.977	2.383	2.228	2.383	3.076	0.000	2.788	0.000						
13. DQ195764 Ipomoea batatas sporamin A precursor complete sequence	3.076	0.016	0.016	2.095	2.383	2.228	2.228	3.076	0.016	2.788	0.016	0.016					
14. DQ195777 Ipomoea batatas sporamin A precursor mRNA complete cds	3.076	2.788	2.788	3.076	1.402	1.402	3.481	2.565	2.788	2.228	2.788	2.788	2.788				
15. DQ195776Ipomoea batatas sporamin A precursor mRNA complete cds	3.076	2.383	2.383	2.788	0.000	0.016	2.788	2.383	2.383	1.341	2.383	2.383	2.383	1.402			
16. DQ195762 Ipomoea batatas sporamin A precursor complete sequence	3.076	0.648	0.648	2.383	0.591	0.563	2.228	2.565	0.648	1.689	0.648	0.648	0.648	2.383	0.591		
17. AF260918 Petunia anthocyanin 1 complete cds	2.383	2.788	2.788	2.228	3.481	3.481	2.788	2.788	2.788	4.174	2.788	2.788	2.788	2.383	3.481	4.174	

(b)



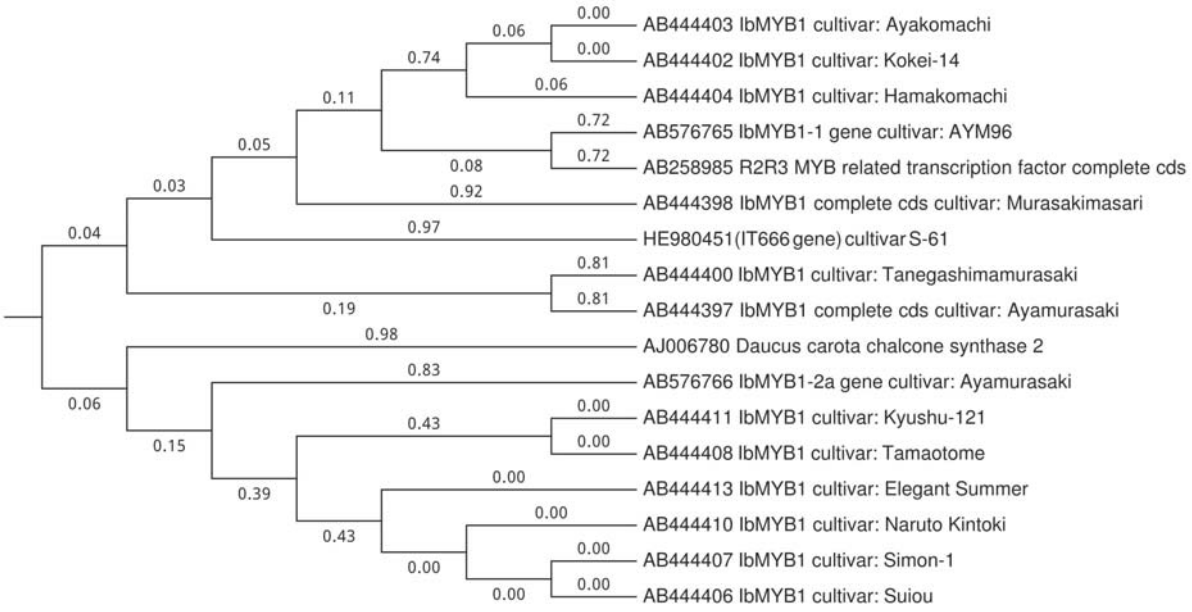
(c)

Fig. 2. (a), (b), (c): Aligned translated protein sequences for IT4 (HE980452), pairwise distance matrix, Phylogenetic tree: UPGMA method

(a)

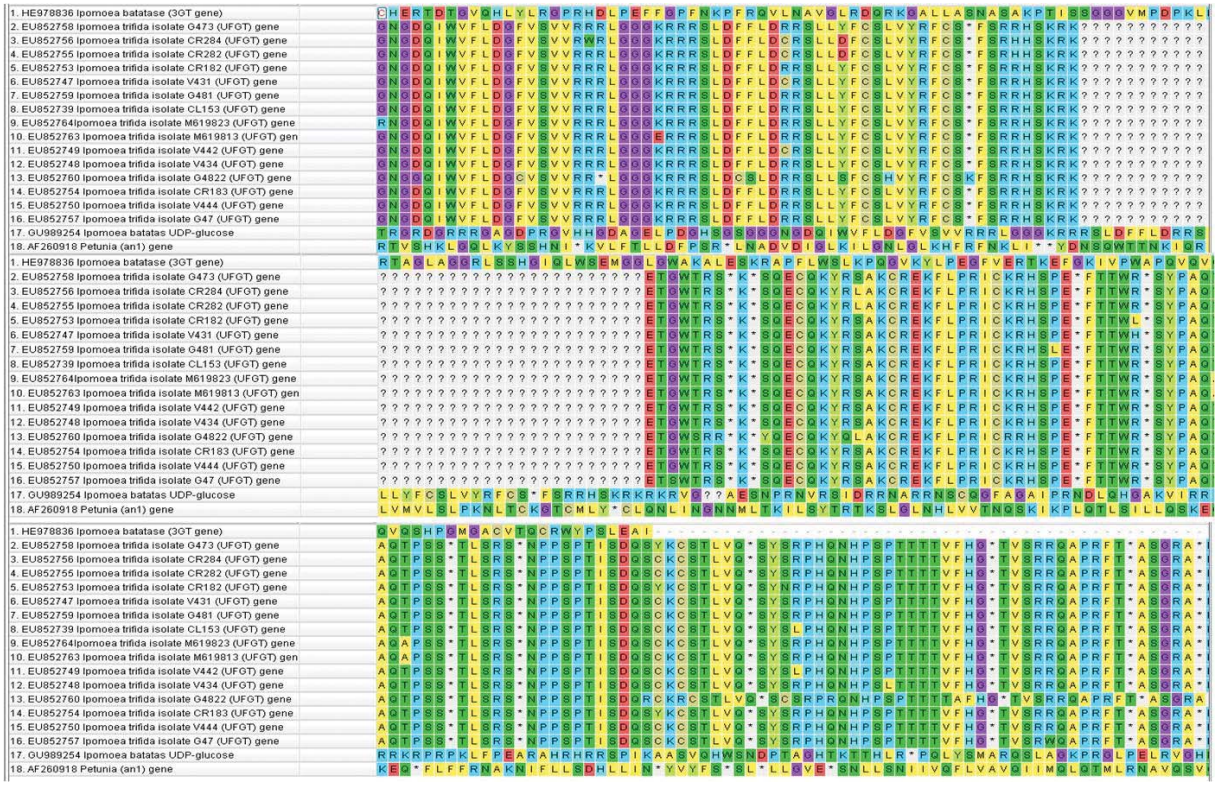
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. HE980451(IT666 gene) cultivar S-61																	
2. AB576766 IbMYB1-2a gene cultivar: Ayamurasaki	1.778																
3. AB576765 IbMYB1-1 gene cultivar: AYM96	2.017	1.815															
4. AB444413 IbMYB1 cultivar: Elegant Summer	2.404	1.629	1.651														
5. AB444411 IbMYB1 cultivar: Kyushu-121	2.245	1.712	1.727	0.868													
6. AB444410 IbMYB1 cultivar: Naruto Kintoki	2.404	1.629	1.651	0.000	0.868												
7. AB444408 IbMYB1 cultivar: Tamaotome	2.245	1.712	1.727	0.868	0.000	0.868											
8. AB444407 IbMYB1 cultivar: Simon-1	2.404	1.629	1.651	0.000	0.868	0.000	0.868										
9. AB444406 IbMYB1 cultivar: Suiou	2.404	1.629	1.651	0.000	0.868	0.000	0.868	0.000									
10. AB444404 IbMYB1 cultivar: Hamakomachi	2.032	1.999	1.725	2.271	2.035	2.271	2.035	2.271	2.271								
11. AB444403 IbMYB1 cultivar: Ayakomachi	1.774	2.034	1.511	2.325	1.849	2.325	1.849	2.325	2.325	0.129							
12. AB444402 IbMYB1 cultivar: Kokei-14	1.774	2.034	1.511	2.325	1.849	2.325	1.849	2.325	2.325	0.129	0.000						
13. AB444400 IbMYB1 cultivar: Tanegashimamurasaki	2.174	2.012	2.372	1.774	1.907	1.774	1.907	1.774	1.774	2.053	1.922	1.922					
14. AB444398 IbMYB1 complete cds cultivar: Murasakimasari	1.938	2.223	1.691	1.818	1.791	1.818	1.791	1.818	1.818	1.782	1.939	1.939	1.868				
15. AB444397 IbMYB1 complete cds cultivar: Ayamurasaki	2.026	2.239	1.599	1.963	2.478	1.963	2.478	1.963	1.963	1.891	2.049	2.049	1.611	2.322			
16. AB258985 R2R3 MYB related transcription factor complete cds	2.088	2.335	1.448	1.966	2.147	1.966	2.147	1.966	1.966	1.742	1.563	1.563	1.863	1.805	1.713		
17. AJ006780 Daucus carota chalcone synthase 2	2.472	1.951	2.428	2.134	1.594	2.134	1.594	2.134	2.134	2.035	2.054	2.054	2.291	2.452	2.231	2.226	

(b)



(c)

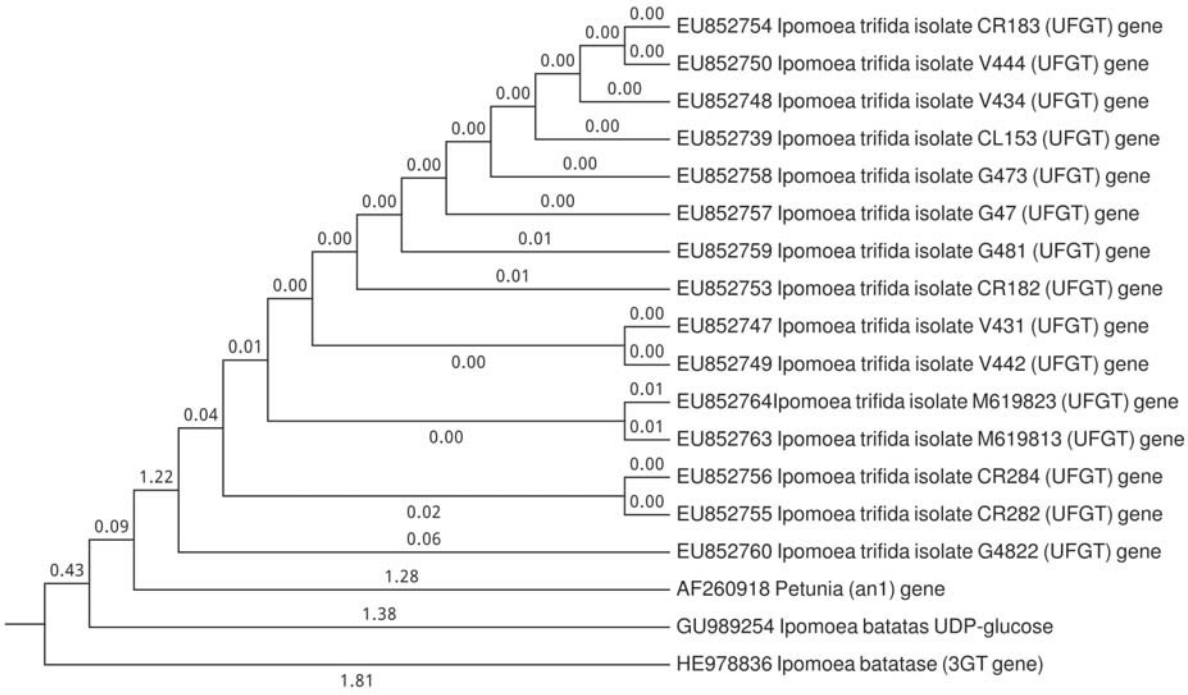
Fig. 3 (a), (b), (c): Aligned translated protein sequences for IT666 (HE980451), pairwise distance matrix, Phylogenetic tree: UPGMA method



(a)

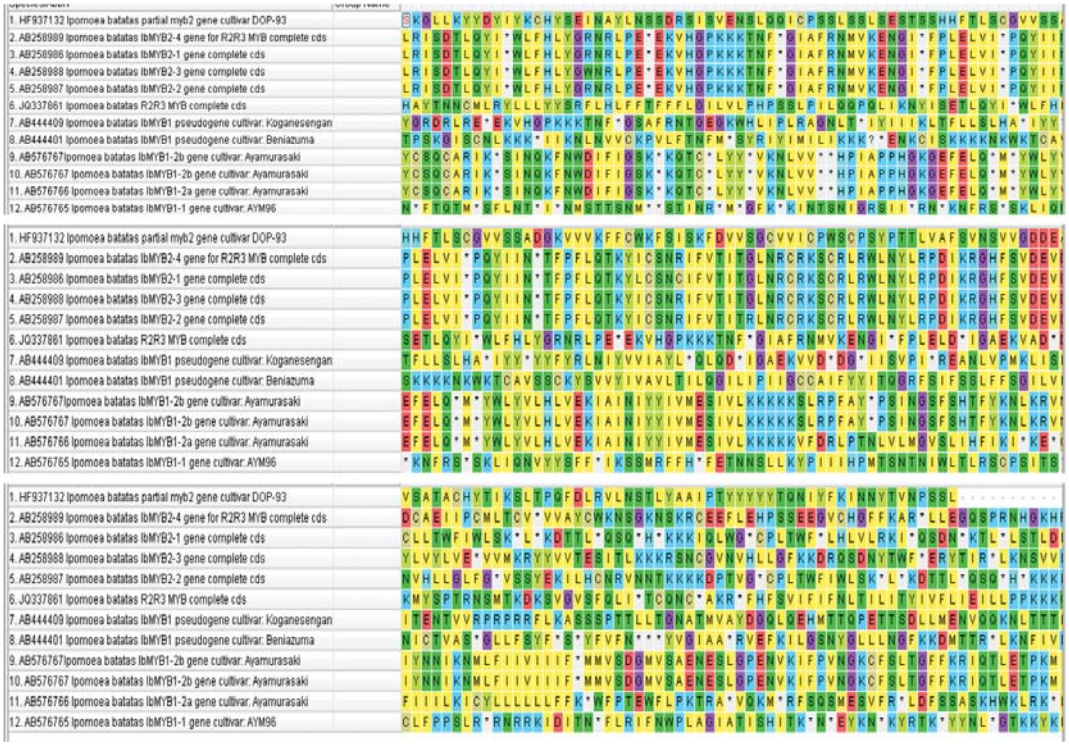
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. HE978836 Ipomoea batatase (3GT gene)																		
2. EU852758 Ipomoea trifida isolate G473 (UFGT) gene	3.664																	
3. EU852756 Ipomoea trifida isolate CR284 (UFGT) gene	3.664	0.044																
4. EU852755 Ipomoea trifida isolate CR282 (UFGT) gene	3.664	0.035	0.009															
5. EU852753 Ipomoea trifida isolate CR182 (UFGT) gene	3.664	0.009	0.053	0.044														
6. EU852747 Ipomoea trifida isolate V431 (UFGT) gene	3.664	0.017	0.044	0.035	0.017													
7. EU852759 Ipomoea trifida isolate G481 (UFGT) gene	3.664	0.009	0.053	0.044	0.017	0.026												
8. EU852739 Ipomoea trifida isolate CL153 (UFGT) gene	3.664	0.000	0.044	0.035	0.009	0.017	0.009											
9. EU852764Ipomoea trifida isolate M619823 (UFGT) gene	3.664	0.017	0.062	0.053	0.026	0.035	0.026	0.017										
10. EU852763 Ipomoea trifida isolate M619813 (UFGT) gene	3.664	0.017	0.062	0.053	0.026	0.035	0.026	0.017	0.017									
11. EU852749 Ipomoea trifida isolate V442 (UFGT) gene	3.664	0.009	0.035	0.026	0.017	0.009	0.017	0.009	0.026	0.026								
12. EU852748 Ipomoea trifida isolate V434 (UFGT) gene	3.664	0.000	0.044	0.035	0.009	0.017	0.009	0.000	0.017	0.017	0.009							
13. EU852760 Ipomoea trifida isolate G4822 (UFGT) gene	4.069	0.108	0.127	0.118	0.118	0.127	0.118	0.108	0.127	0.127	0.118	0.108						
14. EU852754 Ipomoea trifida isolate CR183 (UFGT) gene	3.664	0.000	0.044	0.035	0.009	0.017	0.009	0.000	0.017	0.017	0.009	0.000	0.108					
15. EU852750 Ipomoea trifida isolate V444 (UFGT) gene	3.664	0.000	0.044	0.035	0.009	0.017	0.009	0.000	0.017	0.017	0.009	0.000	0.108	0.000				
16. EU852757 Ipomoea trifida isolate G47 (UFGT) gene	3.664	0.009	0.053	0.044	0.017	0.026	0.017	0.009	0.026	0.026	0.017	0.009	0.118	0.009	0.009			
17. GU989254 Ipomoea batatas UDP-glucose	3.153	2.683	2.683	2.683	2.683	2.683	2.816	2.683	2.683	2.683	2.683	2.683	2.683	2.683	2.683	2.683		
18. AF260918 Petunia (an1) gene	2.970	2.565	2.565	2.565	2.565	2.565	2.565	2.565	2.460	2.565	2.565	2.565	2.683	2.565	2.565	2.565	3.664	

(b)



(c)

Fig. 4 (a), (b), (c): Aligned translated protein sequences for 3GT (HE978836), pairwise distance matrix, Phylogenetic tree: UPGMA method



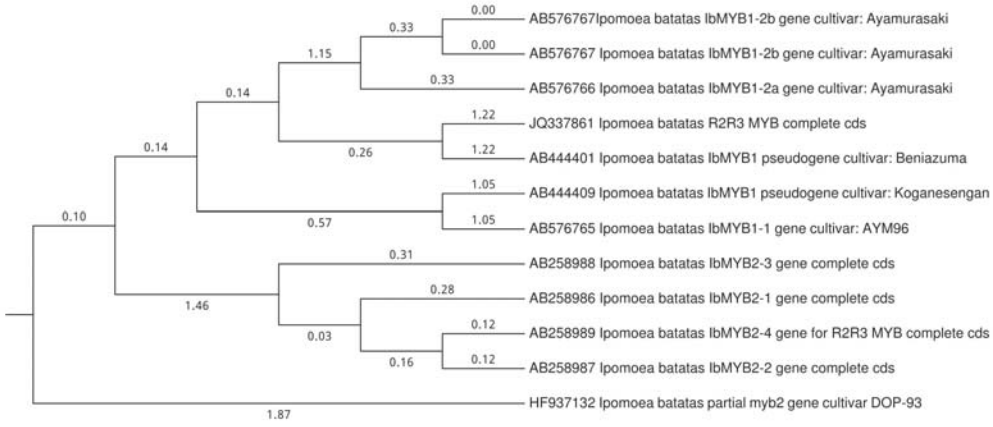
(a)

M7: Pairwise Distances (C:\Users\user\Desktop\PAPERS SUBMITTED\Swt pot rewrite\Analysis\IT666 _Full gene\DNA SEQ ALIGNMENT.meg)

File Display Average Caption Help

	1	2	3	4	5	6	7	8	9	10	11	12
1. HF937132 Ipomoea batatas partial myb2 gene cultivar DOP-93												
2. AB258989 Ipomoea batatas IbMYB2-4 gene for R2R3 MYB complete cds	3.838											
3. AB258986 Ipomoea batatas IbMYB2-1 gene complete cds	3.635	0.553										
4. AB258988 Ipomoea batatas IbMYB2-3 gene complete cds	3.666	0.618	0.618									
5. AB258987 Ipomoea batatas IbMYB2-2 gene complete cds	4.006	0.237	0.547	0.602								
6. JQ337861 Ipomoea batatas R2R3 MYB complete cds	3.070	4.111	3.544	3.098	3.591							
7. AB444409 Ipomoea batatas IbMYB1 pseudogene cultivar: Koganesengan	4.518	3.892	4.642	4.513	4.716	4.690						
8. AB444401 Ipomoea batatas IbMYB1 pseudogene cultivar: Beniazuma	3.451	2.735	2.984	3.152	2.497	2.444	3.185					
9. AB576767Ipomoea batatas IbMYB1-2b gene cultivar: Ayamurasaki	3.932	3.447	4.042	3.619	3.512	3.347	3.152	2.470				
10. AB576767 Ipomoea batatas IbMYB1-2b gene cultivar: Ayamurasaki	3.932	3.447	4.042	3.619	3.512	3.347	3.152	2.470	0.000			
11. AB576766 Ipomoea batatas IbMYB1-2a gene cultivar: Ayamurasaki	3.878	3.675	3.666	4.035	3.708	3.330	3.023	2.854	0.667	0.667		
12. AB576765 Ipomoea batatas IbMYB1-1 gene cultivar: AYM96	3.108	2.754	2.740	2.732	2.680	3.618	2.103	2.607	2.867	2.867	3.251	

(b)



(c)

Fig. 5 (a), (b), (c): Aligned translated protein sequences for cloned IT666 (HF937132), pairwise distance matrix, Phylogenetic tree : UPGMA method

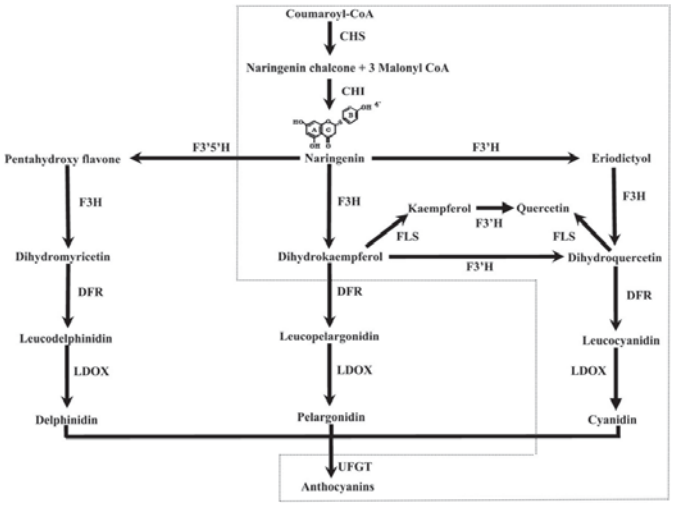


Fig. 6: Involvement of different structural and functional genes in various steps of anthocyanin biosynthetic pathway

eleven different gene sequences. The tree (Fig. 5c) shows two major clusters with all eleven gene sequences in Cluster A and our sequence in the other cluster singly.

It shows that IT666 (HF937132) is a novel gene and evolutionary different with that of Japan, China and USA origin of sweet potatoes.Recent work has also been done in *Brassica rapa* of cruciferae family, which promotes understanding of the roles of genes involved in mechanism of anthocyanin biosynthesis as well as help the improvement of nutritional quality of *Bassica rapa* through the cultivation of high anthocyanin content varieties (Guo *et al.,* 2014). Our present findings also support the previous findings that regulation of anthocyanin biosynthesis and other related gene families are tissue specific as observed in sweetpotato.

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