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**Original Article**

# Identification, Cloning and Structural Analysis of Major Genes from *Portulaca oleracea* L. Hairy Roots that Involved in the Biosynthesis of Dopamine

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## Abstract

Dopamine is one of the important medications of *Portulaca oleracea* L. To optimize the production of dopamine, one of the methods is the identification and engineering of metabolite pathways. To investigate the *tyrosine decarboxylase* (TDC) and *tyrosinase*, which seem to be the most important genes in dopamine synthesis pathway, hairy roots were produced from *Portulaca oleracea* using *Agrobacterium rhizogenes* and total RNA was extracted from hairy roots. A cDNA library was synthesized using RT-PCR. Then, the two genes were amplified, isolated and cloned in a pTG 19-T vector. Bioinformatics' databases were used to predict the details of the structural, functional and biological characteristic of these genes. Nucleotide sequence analysis revealed that the cloned cDNAs expressed TDC and tyrosinase, and contained a single open reading frame of 1800 bp and 1750 bp, respectively. TDC has the most similarity with TDC of *Arabidopsis thaliana* (L.) Heynh., but *tyrosinase* has 98% similarity with tyrosinase of *Agaricus bisporus*. Because of More negatively charged amino acids the TDC has hydrophobic properties, therefore affinity and hydrophilic chromatography can be used for purification of TDC. But tyrosinase has hydrophilic properties and hydrophobicity chromatography can be used for its purification. There were two peroxisomal signal peptide (KLAKEFEQL) and (KIEGRPLHL) in the TDC and tyrosinase, respectively. Therefore, they are biologically active in the peroxisomes, and included in biosynthesis dopamine through the transformation of L-lysine to L-dopa and finally to the dopamine. In conclusion, increasing the expression of TDC and tyrosinase through the genetic engineering can increase dopamine production in the Portolaca.

**Keywords:** Cloning, *Portulaca oleracea*, Sequence analysis, Tyrosinase, Tyrosine decarboxylase

## Introduction

Many plant-derived compounds have been used as drugs, either in their original or semi-synthetic form. Plant secondary metabolites can also serve as drug precursors, drug prototypes, and pharmacological probes [1,2]. Examples of important drugs obtained from plants are morphine and codeine from *Papaver somniferum* L., vincristrine and vinblastine from *Catharanthus roseus* (L.) G.Don, digoxin from *Digitalis lanata* Ehrh. and quinine and quinidine from *Cinchona sp*. [3,4]. Moreover, there are many plant-derived anticancer agents such as vinblastine, irinotecan, topotecan, oposide, and paclitaxel [5].

*Portulaca oleracea* L. is a medicinal plant found in Europe and Asia. Iran is one of the origins of the plant. The importance of this plant is in the treatment of urinary, digestive problems, and cardiovascular diseases [6]. *P. oleracea* has a variety of pharmacological activities, including analgesic, anti-inflammatory, antifungal, wound healing and hypoglycemic [7,8]. It contains plenty of bioconstituents, including catecholamines,

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lnoradrenalin, dopamine, α-amyrin, β-amyrin, and portuloside. Dopamine (4-(2-aminoethyl)-1, 2-benzenediol), stimulates the nervous system and is used for treatment of Parkinson's disease, congestive heart failure, and myocardial dysfunction [9-11].

The main challenge of medicinal plant is purification of secondary metabolites because of low concentration of these substances. Metabolite engineering has been suggested as a potential tool for the increasing and production of useful secondary metabolites [12].

Three things are required for successful engineering of a plant metabolic pathway for improved human health outcomes: (a) information on which compound to produce and in what quantity, (b) knowledge of which pathway gene to target, and (c) a means to alter the activity of that gene in the plant. Identifying a target gene usually requires knowledge of the DNA sequences encoding the key biosynthetic enzymes or regulatory factors for the metabolite pathway and detailed computational models of metabolism [13].

Dopamine, sold under the brand name Intropin among others, is a medication most commonly used in the treatment of very low blood pressure, a slow heart rate that is causing symptoms, and, if epinephrine is not available, cardiac arrest [14]. It is known that tyrosine decarboxylase (TDC) and tyrosinase are major enzymes in the metabolism of dopamine in human body. Tyrosine decarboxylase (TDC, EC 4.1.1.25) is classified into Group II and shares high identity with L-3,4-dihydroxy-L-phenylalanine (DOPA) decarboxylase (DDC), glutamic acid decarboxylase (GAD), and histidine decarboxylase (HDC). TDC could catalyze the decarboxylation of L-tyrosine and L-DOPA to tyramine and dopamine [15]. Moreover, tyrosinase (EC 1.14.18.1) is an oxidase that is the rate-limiting enzyme for controlling the production of dopamine. This study was designed to identify, cloning and predict the structure and activities of TDC and tyrosinase for using the engineering of dopamine metabolic pathways.

## Material and Methods

Seeds of *Portulaca oleracea* L. were collected from the Medicinal Plant Garden of the Hamedan city, Iran. Seeds were surface-sterilized in 70% ethanol for 45 seconds, and then washed with sterile water several times. Afterwards, seeds were transferred to 2% sodium hypochlorite solution in a sealed bottle under sterile condition, gently agitated for 10 minutes and then rinsed three times with sterile distilled water. Next, sterilized seeds were transferred to the sterilized petri dish containing wet filter paper soaked in the 1/2 MS [16] liquid. Two weeks after seedling establishment in a medium containing wet filter paper soaked in the 1/2 MS liquid, different seedling parts including roots, stems and cotyledon leaves were isolated from *in vitro* growing seedlings and were cut (length 3 cm for roots and stem and complete cotyledon leaves with some petiole) into explants, then pre-cultured on solid, growth regulator-free 1/2 MS medium for 24 hours. Explants were kept on 1/2 MS solid medium containing 300 mg $L^{-1}$ cefotaxime in an air-conditioned chamber at 24°C, under 16 h $day^{-1}$ light to induce hairy roots. After 7 days of co-cultivation with *A. rhizogenes* strain ATCC15834, roots with a length 4-5 cm were excised from the incision site and placed on 1/2 MS medium for further growth.

Genes Identification and Amplification

The RNA was extracted from 100 mg hairy roots by RNXPlus (Cinnagen, Iran) according to the manufacturer's recommendation. To discard secondary metabolites, 100 mL polyethylene glycol (PEG) 20% was added to reaction. The cDNA was synthesized on 100 ng RNA by 0.2 mg random examer primers, 20U RNase Inhibitor (Fermentas, Litany) and 200U RevertAid M-MuLV reverse transcriptase (Fermentas, Litany) at 42 °C for 60min.

**Table 1** Applied primers for amplification of TDC and tyrosinase.

| Primer type | sequence |
|---|---|
| TDC forward primer | 3' GGATCCCTGAGACATAAACATC 5' |
| TDC reverse primer | 3' GAGCTCTAATGGTGGTGGTGATGATGAACCACAAAATAACCC 5' |
| Tyrosinase forward primer | 5' GGATCCCTTACCTAGGAATGTCGCTG -'3 |
| Tyrosinase reverse primer | 3' GAGCTCTAATGGTGGTGGTGATGATGAGAGAATACAACTTCC 5' |

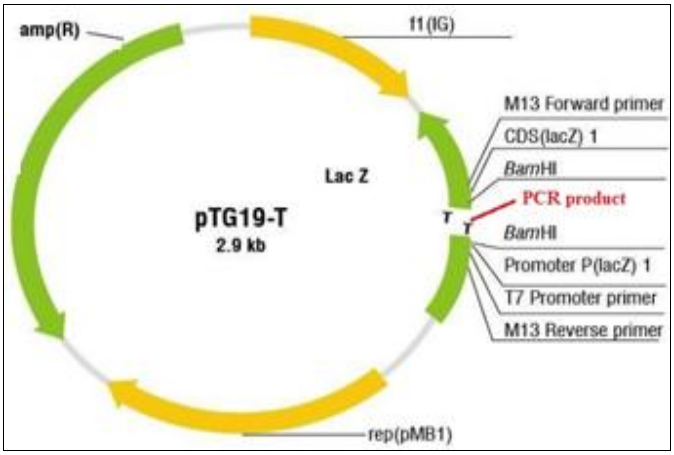



**Fig. 1** Genetic map of pTG 19-T vector. Cloning site of PCR products which has T overhang is indicated by red line.

The primers were designed using Vector NTI and *Arabidopsis thaliana*'s TDC (AJ011049) and *Agaricus Bosporus*'s tyrosinase (AJ223816.1) used as templates. Primers were so designed that have initiation codon, stop codon, his tag and restriction sites of *BamHI* and *SacI* (Table 1).

The PCR conditions for TDC amplification as follows: 25 cycles of 60 s at 98 °C for denaturation, 60 s at 60 °C for annealing, and 120 s at 72 °C for extension, followed by 5min at 72 °C for final extension. These conditions were used to amplify tyrosinase, but annealing was 62 °C for 60 s. PCR product was extracted from 1% agarosegel using Sinaclone extracting kit according to manufacture structure.

Cloning of the Amplified Genes in pTG 19-T

The Vivntis pTG19-T vector (T/A vector) with 3'-dA overhangs (Fig. 1) was used for rapid and efficient cloning of PCR products according to the manufacture structure. The ligation mixes were transformed into competent *Escherichia coli* (*E. coli*) DH5α cells and transferred to X gal-ampicillin containing LB-agar plates [17, 18]. After overnight incubation colonies were screened by PCR, the PCR conditions for TDC as follows: 35 cycles of 60 s at 98 °C for denaturation, 60 s at 60 °C for annealing, and 120 s at 72 °C for extension, followed by 5 min at 72 °C for final extension. This thermal conditions were used to amplify the tyrosinase as follows: 35 cycles of 60 s at 98 °C for denaturation, 60 s at 62 °C for annealing, and 120 s at 72 °C for extension, followed by 5 min at 72 °C for final extension. Plasmids from the insert positive colonies were extracted and sequenced at GATC Biotech AG, Konstanz, Germany.

Analysis of Genes Structure

Gene structure was analyzed using software and bioinformatics databases. DNA sequences were analyzed by BLAST, RefSeq gene, GenBank, ClastalW. Databases of Conserved Domain Database (CDD) ،BLASTX ،Cn3D ،ProSplign ,UniProtKB were used to investigate proteins structure. Kegg database resource was used for understanding high-level functions and utilities of the TDC and tyrosinase metabolite pathways.

## Result and Discussion

Inoculation of cotyledons with *A. rhizogenes* strain ATCC15834 resulted in formation of hairy roots (Fig. 2). No root emerged from the control treatment and explants treated with a scalpel.

The morphological identification of the transformed hairy roots was confirmed by PCR using *rol* B gene specific primers. *A. rhizogenes* used as a positive control and DNA from the non-transformed seedling roots as a negative control. All transformants showed the presence of the 780 bp *rol* B amplified product. No *rol* B gene was found in the control tissue (Fig. 2).

Identification and Cloning of the Genes

After RNA extraction and cDNA synthesis, identification of TDC and tyrosinase in the genome of hairy roots was amplified by PCR. The results of RT-PCR demonstrated that 1800bp and 1750 bp bands were amplified for TDC and tyrosinase, respectively (Fig. 3).

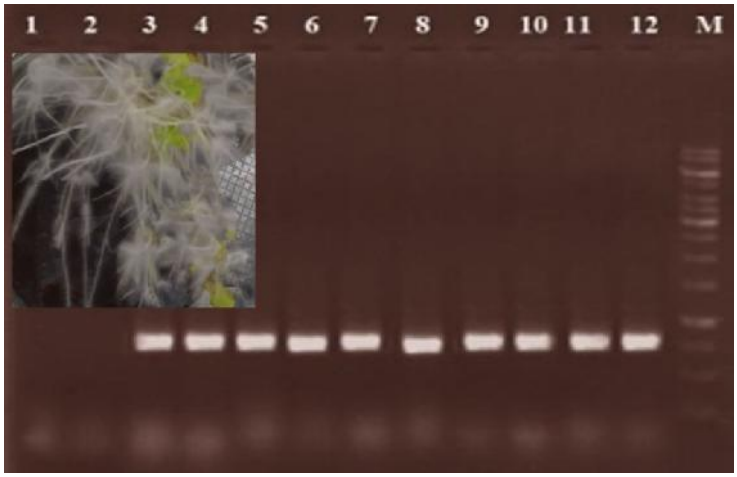



**Fig. 2** Results of PCR on the hairy root clones. Line M: 1kb molecular ladder, Line 1 and 2: Non-transformed roots (negative control); Lanes 3 and 4: *A. rhizogenes* DNA (positive control), line 5-12: hairy root clones.

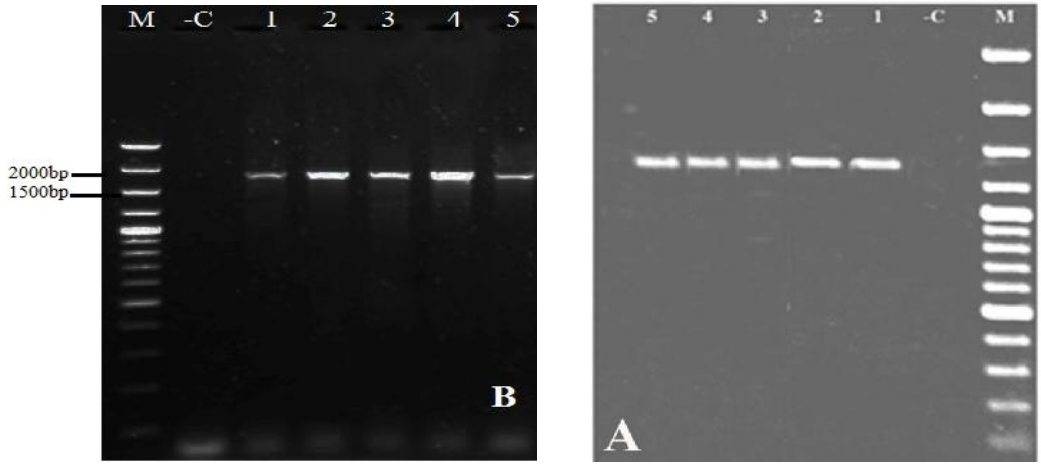



**Fig. 3** Identification and amplification of genes by RT-PCR. A) Amplification of TDC. M: 100 Kb DNA marker, -C: negative control, lines 1-5 hairy roots clones. B) Tyrosinase amplification. M: 100 Kb DNA marker, -C: negative control, lines 1-5 hairy roots clones.

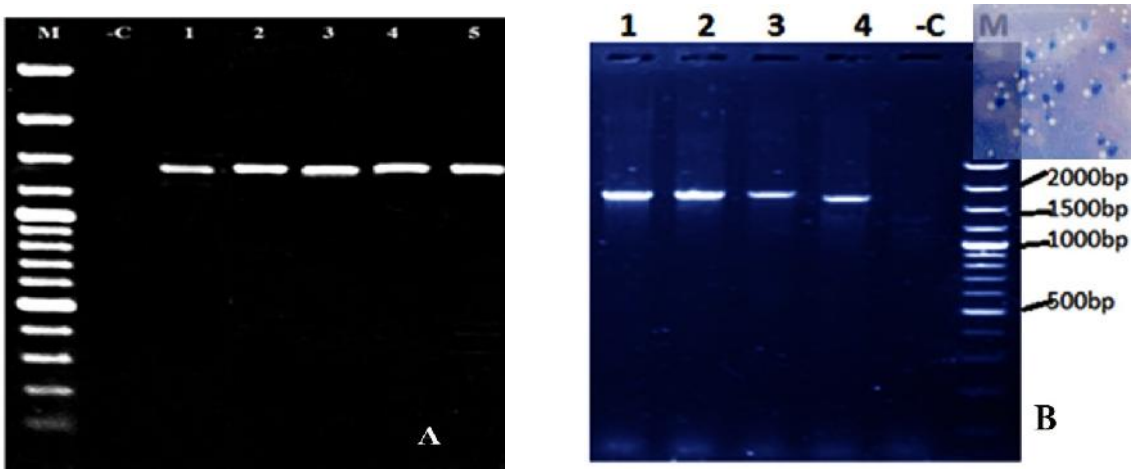



**Fig. 4** Results of PCR cloning. A) PCR product of TDC. M: 100 Kb DNA marker, -C: blue clone as a negative control, lines 1-5 white clones. B) PCR product of tyrosinase. M: 100 Kb DNA marker, -C: blue clone as a negative control, lines 1-4 white clones

Amplified genes were extracted from agarose gel, cloned in T/A vector and transformed to *E.coli*. Blue/white screening of clones on a selective medium indicated that the genes were cloned into T/A vector (Fig. 4B). Moreover, PCR cloning on the white clones confirmed the presence of 1800 and 1750 bp bands corresponding to TDC (Fig. 4A) and tyrosinase, respectively (Fig. 4B).

Structural Analysis of the TDC and Tyrosinase

After sequencing of the TDC and tyrosinase**,** homology search for sequenced genes was done using the BLASTN. Results of the BLAST database revealed that cloned TDC of *P. oleracea* has the highest cover (98%) and identity (97%) with TDC of *A. thaliana* (AC: AJ011049.1). Multiple alignment of the query genes by Clustalw software indicated that the main differences between the query and subject is two gaps in the regions of 1528-1540 and 1758-1802 (Fig. 5).

*Portulaca oleracea* L. tyrosinase has the highest cover (97%) and identity (97%) with *A. bisporu*s tyrosinase (AC: AJ223816.1) and other phenol oxidases. Multiple alignment indicated that there are some mismatch and gaps (region 1-10 and 174-182) between the query and subject (Fig. 6).

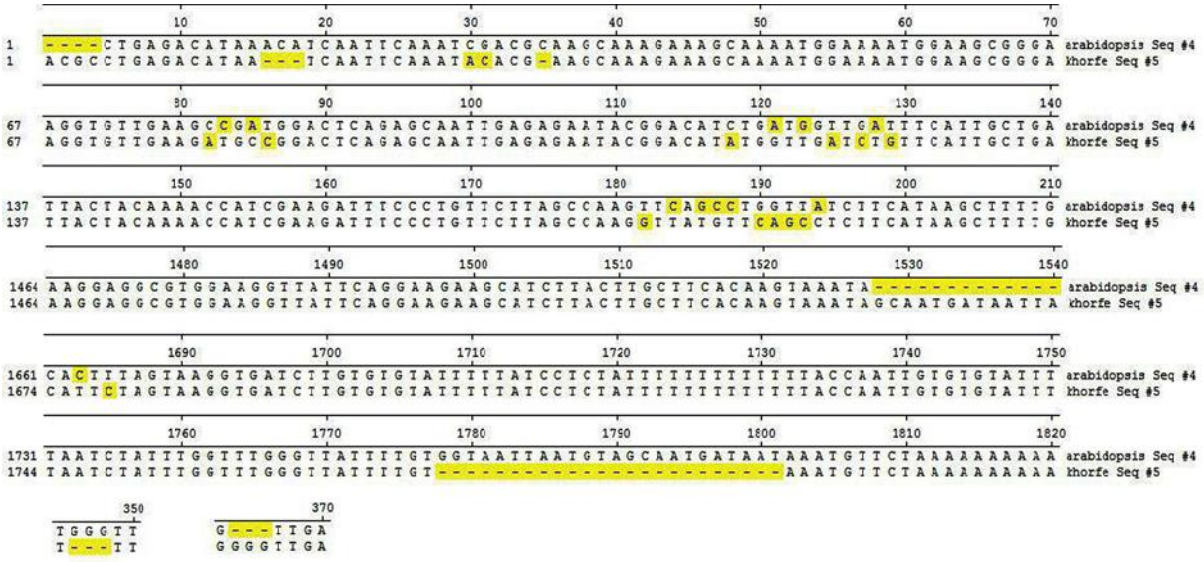



**Fig. 5** Multiple alignment of cloned TDC of *Portulaca oleracea* L. with the A. *thaliana* TDC. Differences between the two genes are highlighted.

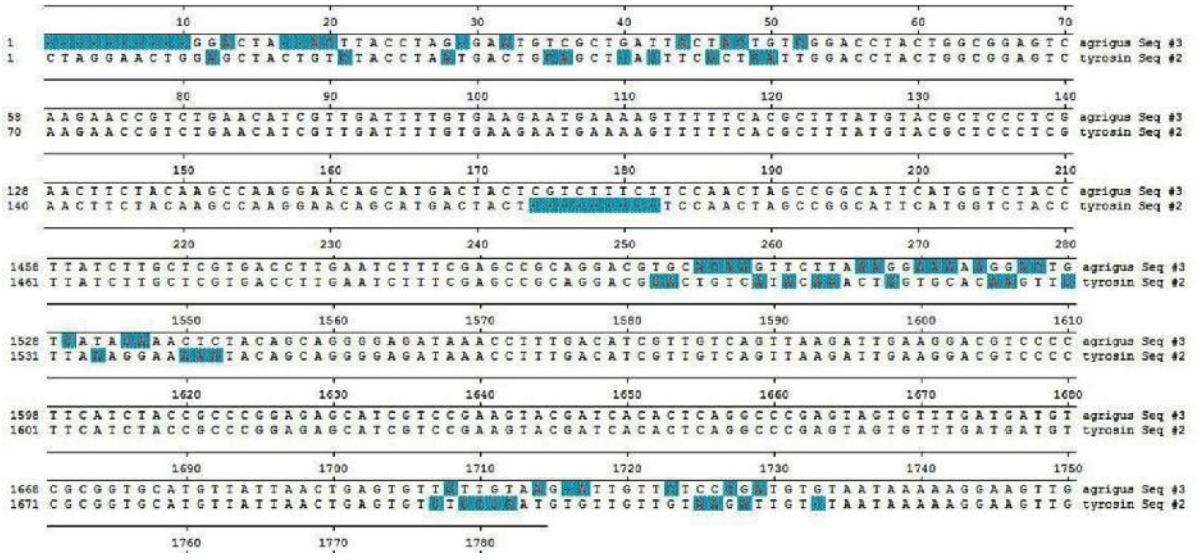
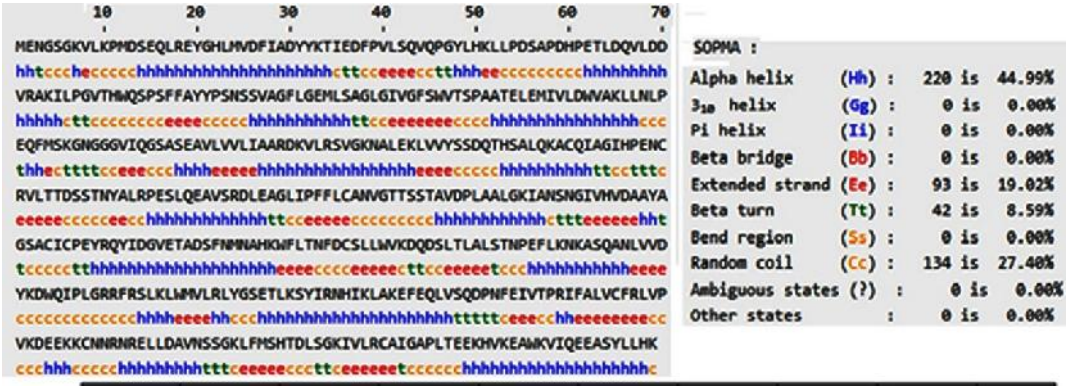



**Fig. 6** Multiple alignment of cloned tyrosinase of *Portulaca oleracea* L. with the *A. bisporu* tyrosinase. Differences between the two genes are highlighted.




SOPMA :

| | | | |
|---|---|---|---|
| Alpha helix | (Hh) : | 220 is | 44.99% |
| $3_{10}$ helix | (Gg) : | 0 is | 0.00% |
| Pi helix | (Ii) : | 0 is | 0.00% |
| Beta bridge | (Bb) : | 0 is | 0.00% |
| Extended strand | (Ee) : | 93 is | 19.02% |
| Beta turn | (Tt) : | 42 is | 8.59% |
| Bend region | (Ss) : | 0 is | 0.00% |
| Random coil | (Cc) : | 134 is | 27.40% |
| Ambiguous states (?) | : | 0 is | 0.00% |
| Other states | : | 0 is | 0.00% |

**Fig. 7** Prediction of the secondary structure of the TDC by SOPMA. Hh: alpha helix, Ee: extended strand, Tt: beta strand and Cc is random coil.

Sequence alignment is crucial in any analyses of evolutionary relationships, in extracting functional and even tertiary structure information from a protein amino acid sequence. Since evolutionary relationships assume that a certain number of the amino acid residues in a protein sequence are conserved, the simplest way to assess the relationships between two sequences would be to count the numbers of identical and similar amino acids. A gap (marked by a dash in the sequence) in one of the sequences simply means that one or more amino acid residues have been deleted from the sequence, or we could also say that there is an insertion in the sequence.

The secondary structure of proteins was predicted by the self-optimized prediction method (SOPMA). The TDC secondary structure includes extended strand 19.02%, alpha helix 44.9%, beta turn 9%, and random coil 27.4% (Fig. 7).

Secondary structure of the tyrosinase includes extended strand 19%, alpha helix 38%, beta turn 10%, and random coil 33% (Fig. 8). Tyrosinase is an oxido-redoctase enzyme class and known as monophenol monooxygenase, phenolase, monophenol oxidase and tyrosine-dopa oxidase.

Both protein and nucleic acid secondary structures can be used to aid in multiple sequence alignment. These alignments can be made more accurate by the inclusion of secondary structure information in addition to simple sequence information [19]. This is sometimes less useful in RNA because base pairing is much more highly conserved than sequence. Distant relationships between proteins whose primary structures are unalignable can sometimes be found by secondary structure [20]. The large-scale characteristics of proteins are consistent with their secondary structures. Proteins can be either fibrous (derived from fibers) or globular (meaning, like a globe). Fibrous proteins are usually important in forming biological structures. For example, collagen forms part of the matrix upon which cells are arranged in animal tissues.

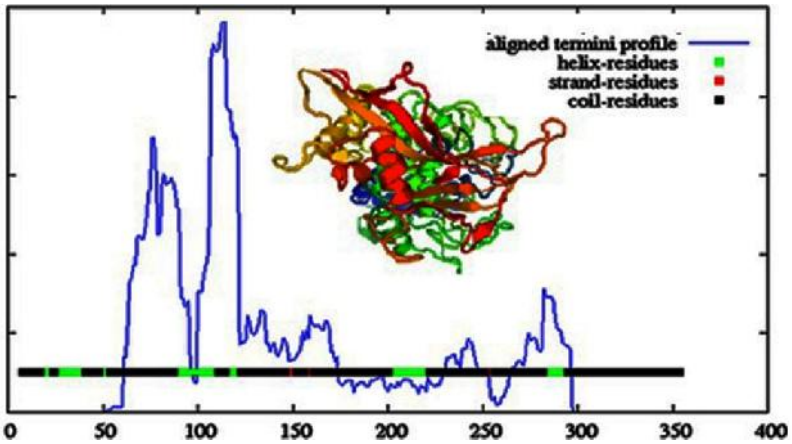



**Fig. 8** Prediction of the secondary structure of TDC by SOPMA. Green region: helix residues, Black: coil residues: Red region: strand residues.

Globular proteins, such as most enzymes, usually consist of a combination of the two secondary structures-with important exceptions. For example, hemoglobin is almost entirely alpha-helical, and antibodies are composed almost entirely of beta structures [21].

Expasy ProtParam tool predicted that tyrosinase has 64 kDa molecular weight ($C_{2891}H_{4369}N_{779}O_{842}S_{13}$), point of isoelectric 5.9, aliphatic index 75.59, Grand average of hydropathicity (GRAVY) -0.544, instability index (II)30.64, and Abs 0.1% (=1 g/l) 1.549. These indexes for TDC was molecular weight 54 kDa ($C_{2434}H_{3823}N_{645}O_{720}S_{18}$), point of isoelectric 5.79, aliphatic index: 96, Grand average of hydropathicity (GRAVY) -0.85, instability index II)37.99, and Abs 0.1% (=1 g/l)1.224.

The aliphatic index of a protein is a measure of the relative volume occupied by aliphatic side chain of the following amino acids: alanine, valine, leucine and isoleucine. An increase in the aliphatic index increases the thermo stability of globular proteins. The index is calculated by the following formula:

Aliphatic index = X(Ala)+a * X(Val)+b * [X(Ile)+X(Leu)]

Where X(Ala), X(Val), X(Ile), and X(Leu) are mole percent (100 X mole fraction) of alanine, valine, isoleucine, and leucine. The coefficients a and b are the relative volume of valine side chain (a = 2.9) and of Leu/Ile side chains (b = 3.9) to the side chain of alanine.

Aliphatic and instability indexes classified the cloned tyrosinase and TDC as stable proteins.

Hydrophobicity index was calculated using Proscale and Hphob. /Kyte & Doolittle method. Because of having more negatively charged amino acids (reductive properties), TDC is a hydrophobic protein (Fig. 9).

But, due to more positively charged amino acids (oxidative properties) tyrosinase is a hydrophilic protein. According to this hydrophobicity index, affinity and hydrophobic chromatography (2-mercaptobenzothiazol and tropolone as ligand) can be applied for purification of tyrosinase.

Based on the UniProtKB database, main post-translational modification in the TDC is lysine 309 which a N6-pirodoxal phosphate is attached. The main interaction of this decarboxylase is with the copper-amino oxidases. Tyrosinase sequence contains a peroxisomal signal peptide (KIEGRPLHL), and its biological activity mainly located in the peroxisomes. Because of no endoplasmic reticulum signal, it seems that this protein has no post translational modification such as disulfid bands, addition and processing of carbohydrates, and assembly into multimeric proteins. In the mature tyrosinase a part of terminal amino acids (SKPSSGARNTAFDLLADFKGITKEHKEDLKM YDWTIHVAFKKFELKESFSLLFYFASDGGDY DQENCFVGSINAFRGTAPETCANCQDNENLIQ EGFIHLNHYLARDLESFEPQDVHKFLKEKGLS YKLYSRGDKPLTSLSVKIEGRPLHLPPGEHRP KYDHTQARVVFDDVAVHVIN) deleted from pre-protein through a specific protease digestion.

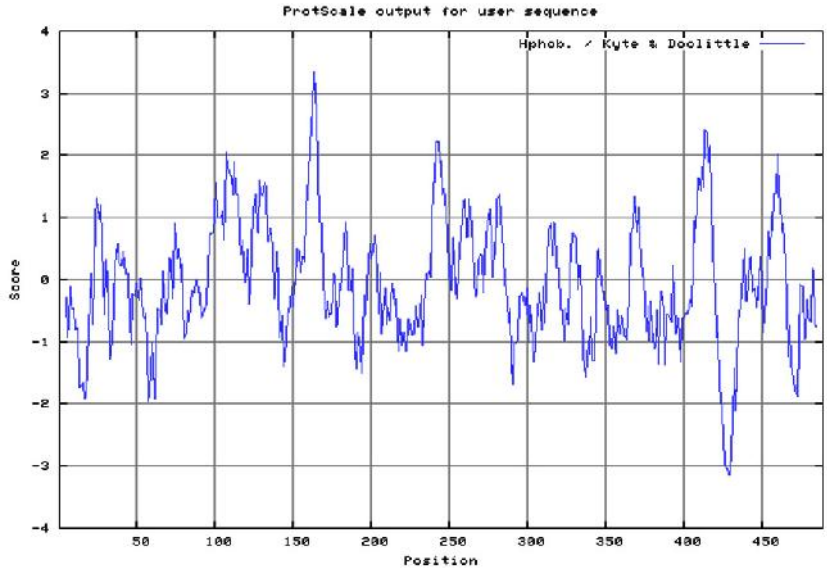



**Fig. 9** ProtScale database results. Number of negative charged amino acids are higher than positive ones in the TDC, therefore TDC categorized as a hydrophobic protein.

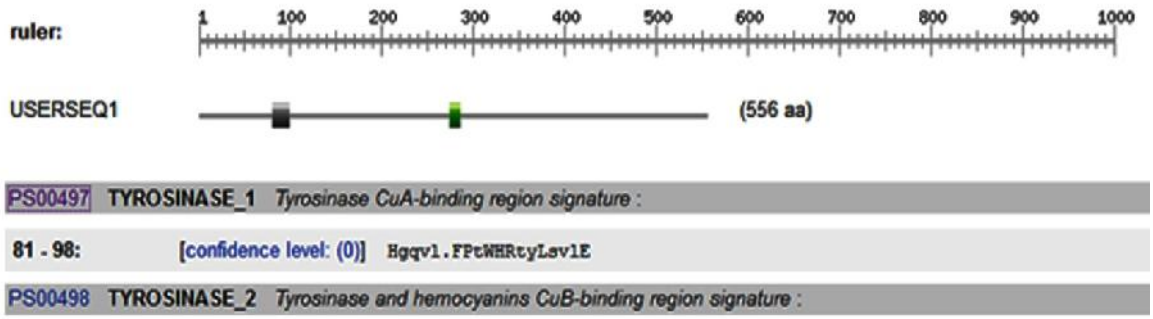



**Fig. 10** Analysis of tyrosinase by ScanProsite. Copper A binding domain and Copper B binding domain are located in region of 90-100 and 280, respectively.

Results of ScanProsite indicated that tyrosinase contain the two Copper A binding region (HGQVLFPTWHRTYLSVLE) and Copper B binding region (DPIFWLHHTNVD), which have key role in the enzyme activity (Fig. 10). Tyrosinase domain is conserved and located in the 50-300 region (LIATVGPTGGVKNRLNIVDFVKNEKFFTLYV RSLELLQAKEQHDYSSFFQLAGIHGLPFTEWA KERPSMNLYKAGYCTHGQVLFPTWHRTYLS VLEQILQGAAIEVAKKFTSNQTDWVQAAQDL RQPYWDWGFELMPPDEVIKNEEVNITNYDG KKISVKNPILRYHFHPIDPSFKPYGDFATWRT TVRNPDRNRREDIPGLIKKMRLEEGQIREKTY NMLKFNDAWERFSNHGISDDQHANSLESVH DDIHVMVGYGKIEGHMDHPFFAAFDPIFWLH HTNVDRLLSLWKAINPDVWVTSGRNRDGTM GIAPNAQINSETPLEPFYQSGDKVWTSASLAD TARLGYSYPDFDKLVGGTKELIRDAIDDLIDE RYG).

TDC contains a peroxisomal signal peptide (KLAKEFEQL) and a pyrophosphate binding region with conserved pattern (S-[LIVMFYW]-x-{KG}-x(3)-K-[LIVMFYWGH]-[LIVMFYWG]-x-{R}-x-[LIVMFYW]-{V}-[CA]-x(2)-[LIVMFYWQ]-{K}-x-[RK]), which there is in the other carboxylases such as glutamate decarboxylase (Fig. 11 ).

Based on the Kegg resource tyrosinase and TDC are the major enzymes in the biosynthesis pathway of isoquinoline alkaloids such as dopamine (Fig. 12). In this enzymatic cascade pathway L-lysine transformed to L-dopa by tyrosinase and TDC transforms L-dopa to the dopamine. Moreover, in the other pathway tyramine transformed to the dopamine by tyrosinase and L-dopa oxidoreductase. Pyridoxal 5'-phosphate is a cofactor of the TDC, therefore addition of this substrate to the hairy root medium culture as an elicitor can increase the dopamine synthesis.

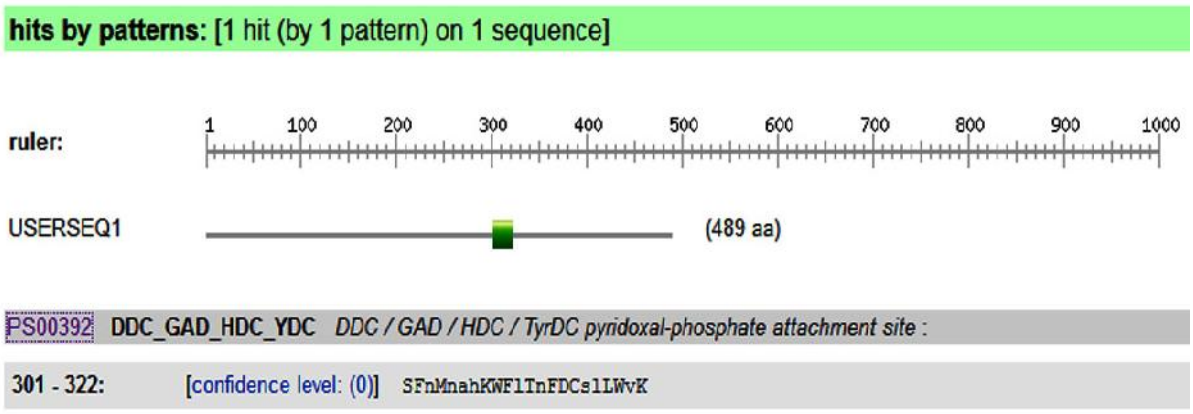



**Fig. 11** Analysis of TDC by ScanProsite. Piridoxal phosphate binding domain is located in region of 300-322.

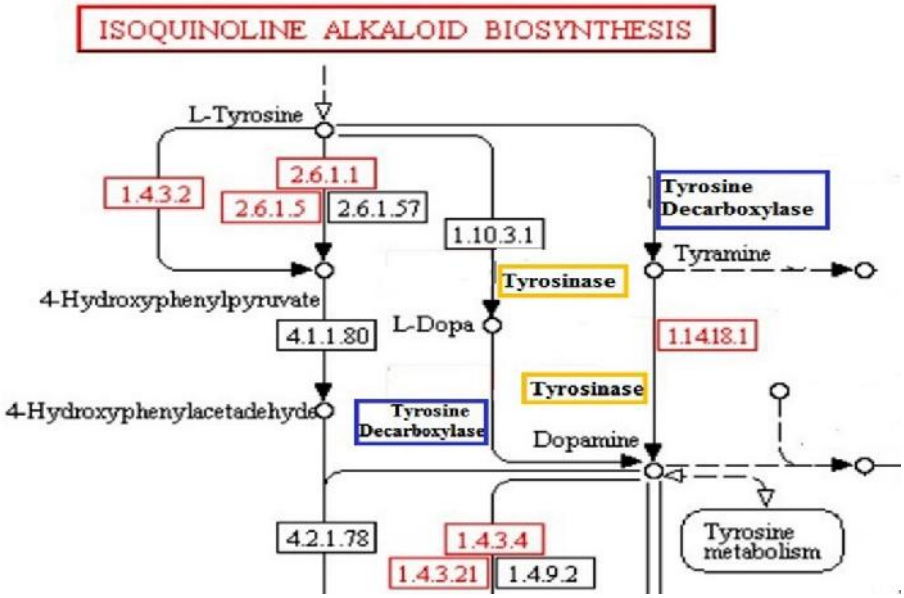



**Fig. 12** Analysis of isoquinoline alkaloid biosynthesis pathway by Kegg database. Process of dopamine synthesis is mainly catalyzed by tyrosine and TDC.

## Conclusion

Medicinal plants from thousands of years ago are as one of the most important source of medicine. Transgenic hairy root are the ideal systems for research on metabolic engineering and production of secondary metabolites. Moreover, hairy root cultures due to their rapid growth, biochemical stability, and relatively high production of secondary metabolites are widely used to produce useful compounds [22,23].

Because of low concentration of secondary metabolites, their purification from medicinal plants is not economic [24]. Biotechnology, using strategies such as cell culture, genetic engineering and molecular markers capable of increasing efficiency and productivity of medicinal plants as renewable resources for the production of bio-drugs. Genetic engineering of metabolite pathways play a significant role in the identification and genetic manipulation of enzymes involved in the biosynthesis of secondary metabolites to increase metabolite concentration.

In the present study, transgenic hairy root of *P. oleracea* were established using *A. rhizogenes* ATCC15834. For engineering of dopamine biosynthesis pathway, TDC and tyrosinase as the major genes of this pathway was Identified, cloned and characterized. Results of sequencing confirmed the colned genes. TDC of *P. oleracea* has the highest similarity with TDC of Arabidopsis. Tyrosinase of *P. oleracea* has the highest similarity with the tyrosinase of *A. bisporus.* The homology results indicated the evolutionary pathway.

Structural analysis deciphered some characteristics of TDC and tyrosinase such as thermostability, post translational modification, site and biological activity. There were two peroxisomal signal peptide in the TDC and tyrosinase, therefor they are biologically active in the peroxisomes, and included in biosynthesis of dopamine. Therefore, increasing the expression of TDC and tyrosinase through the genetic engineering can increase the dopamine production in the Portolaca.

## Acknowledgement

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