Research Article

Tryptophan decarboxylase (TDC) in sweet pepper (*Capsicum annuum* L.): Gene expression analysis during fruit ripening and after nitric oxide exposure

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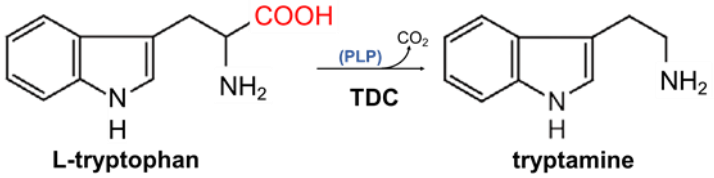
ABSTRACT

Tryptophan decarboxylase (TDC) catalyzes the conversion of L-tryptophan (Trp) to tryptamine, a first step in the biosynthesis of serotonin and melatonin in plants. Pepper (*Capsicum annuum* L.) fruit, a globally popular horticultural product has great nutritional and economic values. In addition to that pepper fruit undergoes phenotypical changes during ripening, many other alterations also occur at the transcriptomic, proteomic, biochemical, and metabolic levels. However, little information is known on how many genes encoding for TDC in pepper plants and their expression levels during the ripening of sweet pepper fruit. In the current study, based on a data-mining approach on the pepper genome and transcriptome (RNA-seq), five putative *CaTDC* genes were identified. They are designated as 1 to 5 based on their localizations in chromosomes and also their previous biochemical data. Among them, *CaTDC3* and *CaTDC4* encode proteins with tryptophan decarboxylase activity; however, *CaTDC1*, *CaTDC2* and *CaTDC5* encode either tyrosine decarboxylase (TYDC) or aromatic aldehyde synthase (AAS), although CaTDC5 shares some degree TDC homology. Therefore, they are considered as the putative CaTDCs until their activity is corroborated. The *CaTDC4* and putative *CaTDC5* are expressed in pepper fruit. The time-course analysis of these genes during fruit ripening (green immature, breaking point, and red ripe) showed that they were differentially expressed, i.e., *CaTDC4* was upregulated, and putative *CaTDC5* was downregulated. *CaTDC4* was positively modulated by two light-responsive elements, Box4 and TCT-motif, while *CaTDC5* was influenced by GT1-motif and G-Box. The protein sequence analysis also allowed identifying the Trp-substrate-binding pocket which is a characteristic of the TDC proteins. Exogenous NO (a signaling molecule) treatment triggered the downregulation of *CaTDC4* but not putative *CaTDC5*. These data provide a novel insight on the potential functions involved in the secondary metabolism of TDCs in fleshy fruits. In the identified three new *CaTDC* genes, two (*CaTDC4* and *CaTDC5*) expressed in pepper fruits are modulated by exogenous NO treatment during ripening.

Key words: Tryptophan decarboxylase (TDC), cis-regulatory element, intron/exon, fruit ripening, melatonin, nitric oxide, pepper, serotonin

1. INTRODUCTION

Tryptophan (Trp) is an essential aromatic amino acid. It is generated via the shikimate/chorismate pathway in plants and is the starting point that allows the biosynthesis of multiple secondary metabolites with signaling properties such as phytoalexins, indole glucosinolates, the plant hormone auxin, serotonin and melatonin (1, 2). Tryptophan decarboxylase (TDC, EC 4.1.1.28) catalyzes the decarboxylation of tryptophan to generate tryptamine using the cofactor pyridoxal-5'-phosphate (PLP) (Scheme 1)



Scheme 1. The conversion of L-tryptophan to tryptamine.

TDC: Tryptophan decarboxylase, PLP: pyridoxal-5'-phosphate.

TDC is the first enzyme of the serotonin and melatonin biosynthesis pathway in higher plants (3, 4). Serotonin and melatonin exert signaling functions both in physiological processes (5–9) and in response to environmental stress which usually are associated with oxidative stress. Interestingly, melatonin also exhibits antioxidant activity in plants (10-12). The functions of plant melatonin, also known as phytomelatonin, have been extensively studied for its potential applications in food chemistry, human and animal nutrition, or medicine, etc. (13, 14).

Nitric oxide (NO) is a free radical gas endogenously generated in plant cells (15). Some of its derivatives are referred as reactive nitrogen species (RNS). NO is also a signaling molecule since it exerts posttranslational modifications (PTMs) of proteins via tyrosine nitration and *S*-nitrosation (16, 17). In addition, RNS can interact with other molecules including Trp or melatonin to generate their 6-nitrotryptophan or N-nitromelatonin and 1-nitrosomelatonin, respectively (11). As the signal molecule, NO regulates a variety of biological processes in plants including seed germination, regulation of root architecture, growth and development, senescence, and fruit development and ripening (18–20). Accumulating evidence also supports the beneficial effects of exogenous NO application on plants to protect against different environmental stresses including salinity (21), drought (19, 22), heavy metals (23, 24), high temperature (25), and to promote the quality and shelf time of the postharvest fruits (26). One of the main beneficial effects of NO on plants is to stimulate antioxidant enzymes both at the level of their activity and their gene expressions (27, 28).

Pepper (*Capsicum annuum* L.) plant is an important agricultural crop with high economic value since its fruits are very popular for consumes worldwide. From the nutritional point of view, pepper fruits have a high level of compounds with antioxidant properties. For example, their vitamin C content is even higher than that in citrus or kiwi fruits (29). They also contain other vitamins including A and E (30). During ripening, pepper fruits experience dramatical phenotypical modifications including color change and emission of the volatiles. These modifications are a consequence of alterations at the transcriptomic, biochemical, and metabolic levels and they involve in the degradation of chlorophylls and the biosynthesis of carotenoids. But this transition also involves structural changes at the subcellular level, i.e., the dismantlement of chloroplasts and the formation of *de novo* chromoplasts. During ripening the fruits have the elevated production of reactive oxygen species (ROS) generated by NADPH oxidases, and their antioxidant enzymes including catalase, superoxide dismutase, ascorbate peroxidases, and lipoxygenases also undergo drastic changes (31-36). Likewise, the exogenous application of NO delays the ripening of the pepper fruits and increases the content of vitamin

C up to 40% due to an upregulation of the activity and expression of the mitochondrial galactono-1,4-lactone dehydrogenase, the enzyme involved in the last step of ascorbate biosynthesis (37).

Considering the importance of serotonin and melatonin biosynthesis in plants and the lack of information on the roles of TDC during the ripening of pepper fruits, in the current study, by using a data-mining approach on the pepper genome and transcriptome, we have identified several genes that code for the TDC in pepper plants. We have further identified which of these genes are present in pepper fruits and whether exogenous NO application can modify their expressions during the ripening of the fruit.

2. MATERIALS AND METHODS

2.1. Plant material and exogenous nitric oxide (NO) treatment.

The plant material used and the NO treatment were carried out as described by González-Gordo *et al*. (33). Briefly, sweet pepper (*Capsicum annuum* L. cultivar Melchor, California-type) fruits were collected from plants grown in commercial plastic-covered greenhouses. The healthy fruits without any external injury were selected at three different developmental stages: green immature (G), breaking point (BP1), and red ripe (R). The harvested fruits were placed in black plastic bags, transported to the laboratory at room temperature, washed with distilled water, and kept at a low temperature (about 7 °C ± 1 °C) for 24 h. To assess the influence of the exogenous NO treatment, two additional groups were included: fruits treated with 5 ppm NO for 1 h (BP2 + NO) and a control group without NO treatment (BP2 − NO). NO treatment was carried out in a hermetic methacrylate box with fruits inside. The NO was generated by the reaction of HNO_3 with solid copper and pumped into the chamber until reaching a concentration of 5 ppm as indicated by the NO detector (38). After 3 days of treatment at room temperature, all fruits were chopped into small cubes (5 mm/edge), frozen under liquid nitrogen, and stored at -80 °C until use. The details of experimental designs including the representative phenotypes of the sweet pepper fruits at different ripening stages and subjected to NO treatment (39) were summarized in Supplementary Figure 1 (S1).

2.2. Phylogenetic and conserved motif analyses of TDC sequences.

The identified TDC sequences in 18 plant species (Supplementary Table 1) were used to construct a phylogenetic tree. The alignment of TDCs was performed using the CLUSTALW method (40). Then, the aligned sequences were subjected to MEGA11 (41) to perform an unrooted maximum likelihood phylogenetic tree with default parameters. Finally, the resulting phylogenetic tree was modified using the online tool Evolview v3 (42). Conserved motifs of CaTDCs were analyzed using the MEME tool (43) and visualized using TB tools software v1.108 (44). The protein localization based on their amino acid sequences was predicted using WoLF PSORT (45). The molecular weight and theoretical isoelectric point of CaTDCs were calculated using the Compute pI/Mw tool which is hosted on the Expasy server (46).

2.3. Intron-exon structure and cis-regulatory elements analysis of the *CaTDC* genes.

To predict putative promoter sequences of the identified *CaTDC* genes obtained from the NCBI Nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide/; accessed on February 10, 2023), 1,500 bp upstream from the transcription start point of each gene were considered. These sequences were analyzed to identify the intron-exon organization and searched for possible cis-acting regulatory elements using the PantCARE tool (47). These results were

manually processed and visualized using the 'HeatMap' function of TBtools v1.108 software (44).

2.4. Identification of the *TDC* genes in pepper, chromosomal location and synteny analysis.

To ascertain the different TDC-encoding genes, pepper proteome was downloaded from the NCBI database (Assembly UCD10Xv1.1; BioProject PRJNA814299; accessed on February 10, 2023). The amino acid sequences of the TDCs described in rice (*Oryza sativa* subsp. japonica), OsTDC1 (Q6ZJK7) and OsTDC2 (Q94EE9) were downloaded from the UnirProtKB database (accessed on February 10, 2023). These sequences were used as a means to search for TDCs in the complete pepper proteome using the BLASTP tool. Candidate TDCs in pepper were selected at ≥97% query coverage and ≥52% identity using e-value ≥ e-30 as a threshold. Additionally, we confirmed the presence of the conserved pyridoxal-dependent decarboxylase domain (PF00282) in the candidate proteins using InterProScan software (45). Finally, redundant sequences and proteins lacking the pyridoxal 5'-phosphate (PLP) binding site (IPR021115) were manually checked and rejected.

Location coordinates of the identified *CaTDCs* in the pepper genome were obtained from the NCBI database. This information was used to draw a genomic map using the MG2C v2.1 tool (46).

2.5. Library preparation and RNA-sequencing.

All procedures were performed as previously described by Matsufuji *et al.* (30) with minor modifications. Briefly, libraries were prepared using an Illumina protocol and were sequenced on an Illumina NextSeq550 platform using 2 × 75 bp paired-end reads. These reads were preprocessed to remove low-quality sequences. Useful reads were mapped against the set of transcripts available for *Capsicum annuum* species in the NCBI database (assembly UCD10Xv1.1; accessed on February 10, 2023) using Bowtie2 (47). Transcript counts were obtained using Samtools (48).

Differential expression analyses were done using DEgenes-Hunter (49). This R pipeline examined the relative change in expression between the different samples using different algorithms (EdgeR, DESeq2, Limma, and NOISeq) which apply their own normalization and statistical tests to validate the whole experiment. On the other hand, an analysis of the time course of *CaTDC* genes expression was performed considering as reference of the expression levels in green fruits (G). Raw data are accessible at the Sequence Read Archive (SRA) repository under the accession PRJNA668052. This reference pepper fruit transcriptome and differentially expressed (DE) genes among the analyzed ripening stages and the NO treatment involved the analysis of twenty-four biological replicates corresponding to five replicates of each stage (G, BP1, BP2, and R), except for green fruits that involved four replicates.

2.6. Protein modeling of TDCs.

The three-dimensional (3D) structure of the CaTDCs was predicted using the artificial intelligence (AI) program AlphaFold (50). Amino acids involved in tryptophan binding were highlighted and visualized using YASARA software (51).

2.7. Statistical analyses.

Post hoc comparisons of means were made by using a Tukey honestly significant difference (HSD) test. Statistical significance was considered at the conventional 5% level ($p < 0.05$). All calculations were performed using R Studio.

3. RESULTS

3.1. *TDC* Genes from Pepper: Sequence, cis-regulatory elements and intron-exon organization.

TDCs in higher plants belong to the group of aromatic L-amino acid decarboxylase (AADC) that catalyzes the PLP-dependent decarboxylation of aromatic amino acids including tryptophan and tyrosine designated as TDC and L-tyrosine decarboxylase (TYDC; EC 4.1.1.25), respectively. All *AADC* genes share extensive similarity and the encoded proteins have amino acid identities over 50% (52). Consequently, the BLAST search in the databank is not robust enough to differentiate *TDC* from other *AADC* gene family.

The data-mining of the pepper genome using the available information on the *TDC* genes in other plant species, particularly tomato (*Solanum lycopersicum*) and rice (*Oryza sativa* subs. japonica), has allowed us to identify five potential *TDC* genes designated as *CaTDC1* to *CaTDC5* based on their chromosomal location. These genes were localized in 12 pepper chromosomes (Table 1). However, in the transcriptome obtained in sweet pepper fruits (33), only two *CaTDC* genes, *CaTDC4* and *CaTDC5*, were identified. Table 1 outlines some properties of these genes and the properties of their encoded proteins including the number of amino acids (aa), subunit molecular mass (kDa), theoretical pI, and their subcellular localization. To facilitate the nomenclature indicated in Table 1, the abbreviation CaTDC, either gene or protein, will be used generically in the rest of the manuscript.

Table 1. **Summary of the properties of *TDC* genes identified in the pepper genome**

Gene Name	Gene ID	Chromosome	Protein ID	Length (aa)	kDa	pI	Subcellular location
Putative *CaTDC1*	107866061	3	XP_016567730.1	517	57,85	7.16	Cytosol
Putative *CaTDC2*	107868087	4	XP_016570150.1	510	57,34	6.78	Cytoskeleton
CaTDC3	107877290	7	NP_001312016.1	503	56,39	5.63	Cytosol
CaTDC4	107878308	7	XP_016580730.1	516	57,95	6.03	Endoplasmic reticulum
Putative *CaTDC5*	107842494	9	XP_016541857.1	487	54,40	6.03	Plastid/Nucleus

Aa: amino acids, kDa: subunit molecular mass. The genes specifically detected in the sweet pepper fruit transcriptome are highlighted in red.

Figure 1 displays the Heatmap analysis of 26 cis-regulatory elements identified in 1500 bp upstream regions from the transcription start point of the potential *CaTDC* genes. Thus, several families of elements were recognized that are involved in various processes including (i) DNA, regulation, and cell cycle; (ii) light-responsive; (iii) stress; and (iv) phytohormones. However, the cis-regulatory elements which exert the most remarkable effects were G-Box as part of the light-responsive family and ABRE (ACGT-containing abscisic acid response element) which is involved in the abscisic acid (ABA) responsiveness. Both cis-regulatory elements modulate

positively and predominantly the *CaTDC1* gene. On the other hand, the light-responsive Box4 positively affects *CaTDC2* and *CaTDC3*. Focusing on the genes that are expressed in fruits, *CaTDC4* is positively modulated by two light-responsive elements, Box4 and TCT-motif, and *CaTDC5* was influenced by GT1-motif and G-Box.

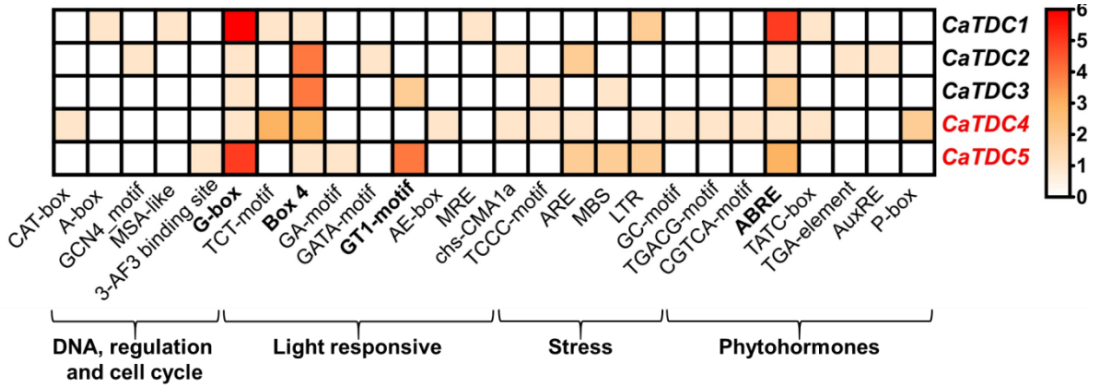


Fig. 1. Heat map of cis-regulatory elements corresponding to the 1500 bp upstream region from the transcription start point of *CaTDC* genes in pepper.

The genes were classified according to their functions. The cis-regulatory elements were identified in the PlantCARE database. The genes specifically detected in the sweet pepper fruit transcriptome are highlighted in red.

The five *CaTDC* genes contain a dissimilar number of exons and introns spanning a length ranging from around 4,000 nucleotides in *CaTDC1* to over 15,100 nucleotides in *CaTDC5* (Figure 2). Thus, *CaTDC1* has 3 exons, *CaTDC5* contains 12 exons, whereas *CaTDC2* to *CaTDC4* have a single exon.

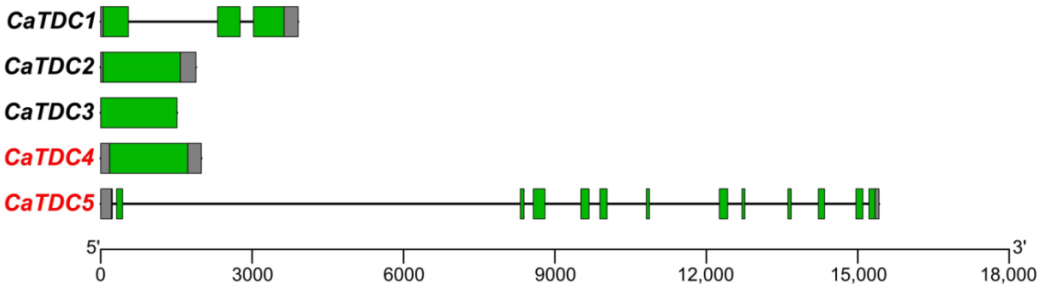


Fig. 2. Genomic organization of the pepper *CaTDC* gene family.

The structure of the genes is shown with exons indicated by green boxes and introns shown as black lines. Untranslated regions are shown by grey boxes. Exon–intron regions are drawn at scale. The genes specifically detected in the sweet pepper fruit transcriptome are highlighted in red.

3.2. TDC proteins from pepper: sequence and phylogenetic analysis

The protein analysis of the identified CaTDCs (Table 1) had a mean subunit molecular mass of 56.7 kDa and was distributed in the cytosol, cytoskeleton, endoplasmic reticulum, and plastid/nucleus. The analysis of the primary structure of these CaTDCs and their alignment allowed the identification of ten amino acid motifs. Figure 3a showed the distribution of these motifs in the different CaTDCs and Figure 3b illustrated the sequence of amino acids motifs where the height of each amino acid symbol is proportional to the degree of conservation in the consensus sequences. The sequence of the pyridoxal-5'-phosphate (PLP) binding site was located in motif 1.

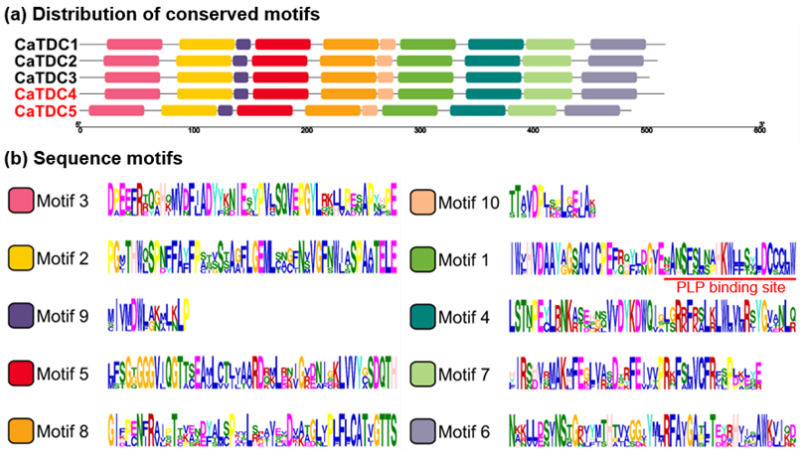


Fig. 3. Identification and position of consensus amino acid motifs for pepper CaTDCs.

(a) Amino acids motifs. Ten amino acid motifs with various widths were identified. The height of each amino acid symbol is proportional to the degree of conservation in the consensus sequences depicted in the ten motifs. (b) Distribution of conserved motifs. The distribution of conserved motifs, numbers 1–10, of these CaTDCs are represented by boxes of different colors. Pyridoxal-5'-phosphate (PLP) binding site is underlined in red. Sequence logos of conserved motifs were created by MEME.

The phylogenetic comparative analysis among the TDCs from 18 different plant species (Supplementary Table 1) allowed the identification of three main TDC groups/subgroups, designated as Ia-Ie, IIa-IIc, IIIa-IIIb and depicted with different colors (Figure 4). It is noteworthy to indicate that the TDCs of pepper are very close to that of tomato (*Solanum lycopersicum*) in subgroups Ib, IIa and IIc, both being Solanaceae.

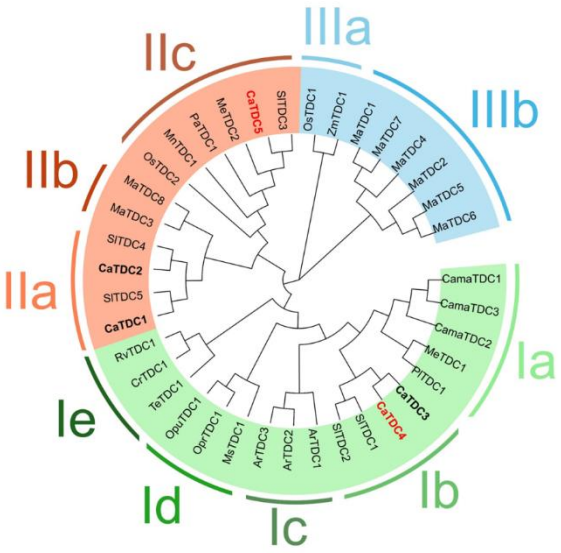


Fig. 4. Phylogenetic relationships between TDCs from different plant species.

Clusters (I-III) are displayed using different colors. Species abbreviations: Ca (Capsicum annuum L.), Os (Oryza sativa subs. japonica), Zm (Zea mays), Me (Manihot esculenta), Ar (Actaea racemosa), Cama (Camptotheca acuminata), Cr (Catharanthus roseus), Ms (Mitragyna speciosa), Opr (Ophiorrhiza prostrata), Opu (Ophiorrhiza pumila), Rv (Rauvolfia verticillata), Te (Tabernaemontana elegans), Vv (Vitis vinifera), Pa (Prunus avium), Mn (Morus nigra), Sl (Solanum lycopersicum), Pl (Paeonia lactiflora Pall.), Ma (Musa spp.). CaTDCs detected in pepper are in bold letters and those identified in the transcriptome of pepper fruit are highlighted in red.

Recently, the analysis of the X-ray crystal structures of *Catharanthus roseus* TDC (CrTDC) in complex with L-Trp has allowed the identification of residues which constitute the Trp-substrate-binding pocket. They are constituted by eight conserved aromatic and hydrophobic residues including Trp92, Phe100, Phe101, Pro102, Val122, Phe124, His318, and Leu325 plus another three variable residues Ala103, Thr369, and Gly370 (51). The protein alignment of the CrTDC with the five CaTDCs corroborated the presence of these residues in the newly identified pepper TDCs suggesting their catalytic activity to L-Trp (Figure 5).

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CrTDC       MGSIDSTNV--AMSNSPVGEFKPLEAEEFRKQAHRMVDFIADYYKNVETYPVLSEVEPGY     58
CaTDC3      MGSLDSNNS--TQTQSNVTKFNPLDPEEFRTQAHQMVDFIADYYKNIESYPVLSQVEPGY     58
CaTDC4      MGTLDSNSS--TQTHSSVTEFNPLDPEEFRTQAHQMVDFIADYYKNIESYPVLSQVEPGY     58
CaTDC5      ----------------MEGELKPMDAEQLREYGHKMVDFIADYYKNIETLPVLSQVEPGY     44
CaTDC1      MGTVKINREDVFDGQFSCNTSTLLDPEEFRRQGHIMVDFLADYFNDIEKYPVRSQVEPGY     60
CaTDC2      MGTLNINPE--IDDQF-FNSINPLDPEEFRRQGHKIVNFLADYYQNIEQYPVCSQVNPGY     57
                                . :: *::*  .* :*:*:***::::*  ** *:*:***

CrTDC       LRKRIPETAPYLPEPLDDIMKDIQKDIIPGMTNWMSPNFYAFFPATVSSAAFLGEMLSTA     118
CaTDC3      LRNHLPENAPYLPESLDTIMKDVEKHIIPGMTHWLSPNFFAFFPATVSSAAFLGEMLCNC     118
CaTDC4      LRSHLPENAPYFPESLDTIMKDVQNHIVPGMTHWLSPNFFAFFPATVSSAAFLGEMLCNC     118
CaTDC5      LRKLLPETAPAHSETLQNVLEDVQTKILPGVTHWQSPDYFAYFPSNSSVAGFLGEMLSAG     104
CaTDC1      LKKLLADSAPNQPESIEKILQNVRKDIFPGLTHWQSPNFFAYFPCISSTAGILGEMLTAG     120
CaTDC2      LQKLVPNSAPNHPEPLEKILEDVKRDMIPGITHWQSPNFFAYFPSSGSTAGFLGEMLSVG     117
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CrTDC       LNSVGFTWVSSPAATELEMIVMDWLAQILKLPKSFMFSGTGGGVIQNTTSESILCTIIAA     178
CaTDC3      FNSVGFNWLASPAMTELEMIIMDWLANMLKLPECFMFSGTGGGVIQGTTSEAILCTLIAA     178
CaTDC4      FNSVGFNWLASPAMTELEMIVMDWLANILKLPQCFMFSGTGGGVIQGTTSEAILCTLIAA     178
CaTDC5      INMVGFSWITSPAATELEMIVLDWLAKALKLPDEFLSTGQGGGVIQGTASEAVLVVLLAA     164
CaTDC1      LNVVGFNWIASPAATELECIVMDWLGKLINLPKTHLFSGGGGGVIQGTTCEAMLCTIVAA     180
CaTDC2      FNVVGFNWISSPAATELESIVMDWFGKMLNLPHCYLFSGGGGGVLQGTTCEAMLCTIVTV     177
            :* ***.*::*** **** *::**::.: ::**. .: :* ****:*.*:.*::* .::::.

CrTDC       RERALEKLGPDSIGKLVCYGSDQTHTMFPKTCKLAGIYPNNIRLIPTTVETDFGISPQVL     238
CaTDC3      RDRKLENIGVDNIGKLVVYGSDQTHSMYAKACKAAGIFPCNIRAISTCVENDFSLSPAVL     238
CaTDC4      RDRKLENIGVDNIGRLVVYCSDQTHSTYTKACKVAGIFPCNIRAVQTSVESDFALSPVIL     238
CaTDC5      RDKVLRRVGKDAISKLVVYCSDQTHSSLQKACQIGGIHPENFRVLKTDPSRDYALSPDTL     224
CaTDC1      RDQMLDKVGRENIDKLVVYASDQTHFSFQKAVKISGIKLENFRAIPTTKATEYALCPILL     240
CaTDC2      RDQMLRKIGRENFCKLVVYGSDQTHFSLKKAAHIAGIDPENFRVIPTTKANEYTMCPKSL     237
            *:: * .:* : : :** * *****    *: : .**   *:* : *    :: :.*  *

CrTDC       RKMVEDDVAAGYVPLFLCATLGTTSTTATDPVDSLSEIANEFGIWIHVDAAYAGSACICP     298
CaTDC3      RGIVEVDVAAGLVPLFLCATVGTTSTTAIDPISELGELANEFDIWLHVDAAYGGSACICP     298
CaTDC4      RRVIEADVAAGLVPLFLCATVGTTSTTAVDPISQLAELADEFDIWLHVDAAYGGSACICP     298
CaTDC5      SEAVSHDMATDLIPFFFCATIGTTSSTAVDPLLDLGKIAQSNSIWFHVDAAYAGSACICP     284
CaTDC1      SKTIQEDKKTGLLPLFLCATVGTTSTTVVDPLKPLCEIAKEYGIWVHVDAAYAGNACICP     300
CaTDC2      HLAVLNDIKEGNIPLFLCATLGTTSTTSVDPLRPLCEIAKTFGIWVHVDAAYAGSACICP     297
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CrTDC       EFRHYLDGIERVDSLSLSPHKWLLAYLDCTCLWVKQPHLLLRALTTNPEYLKNKQSDLDK     358
CaTDC3      EFRQYLDGIERANSFSLSPHKWLLSYLDCCCMWVKEPSVLVKALSTNPEYLRNKRSEHGS     358
CaTDC4      EFRQYFDGIEQANSLSLSPHKWLLSYLDCCCMWVKEPNVLVKALSTNPEYLRNKRSEHGS     358
CaTDC5      EYRGYINGVEEAHSFNMNAHKWFLTNFDCSALWVKDRSALIQSLSTNPEYLKNKASQGNL     344
CaTDC1      EFQHFLNGVENANSFSLNAHKWLFSVLDCCCLWVKDPNSLTKALSTTPECLRNKATDSKQ     360
CaTDC2      EFQHFLDGVENANSFSLNAHKWFFSTLDCCCLWVKDPSALTKALSTNPECLRNKATELNQ     357
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CrTDC       VVDFKNWQIATGRKFRSLKLWLILRSYGVVNLQSHIRSDVAMAKMFEEWVRSDSRFEIVV     418
CaTDC3      VVDYKDWQIGTGRKFKSLRLWLIMRSYGVANLQSHIRSDVRMAKMFEGLVRSDPYFEVIV     418
CaTDC4      VVDYKDWQIGTGRKFKSLRLWLVMRIYGVANLQSHIRSDVRMAKMFEGFVRSDSKFEIVV     418
CaTDC5      VVDYKDWQVPLGRRFRSLKLWMVLRLYGLEKLQAYIRNHIQLAKLFEKLVAQDQRFEIVT     404
CaTDC1      VVDYKDWQISLSRRFRALKLWLVLRSYGAVNLRNFIRSHVKMAKHFEELVAVDERFEIVA     420
CaTDC2      VIDYKDWQIALSRRFRALKLWLVLRSYGVTNLRNLIRSHVNMAKHFEGLIAMDTRFEIFV     417
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CrTDC       PRNFSLVCFRLKPDVSS---------LHVEEVNKKLLDMLNSTGRVYMTHTIVGGIYMLR     469
CaTDC3      PRRFSLVCFRFNPDKEY-------EPAYTELLNKRLLDNVNSTGRVYMTHTVAGGIYMLR     471
CaTDC4      PRHFSLVCFRFNPDKEY-------EPAYTELLNKKLLDSVNSTGRVYMTHTIAGGIYMLR     471
CaTDC5      PRKFSLVCFRLLPPPS--------NEDYANKLNHNLLDSVNSTGKLFISHTLLSDKYILR     456
CaTDC1      PRNFSMVCFRVSPLALQE-KVIFVEEDQVNKFNAKVLESINSCGNIHMTHAVVGGVYMMR     479
CaTDC2      PRKFAMVCFRISPLVLCRVSTKFDHEEEVNKFNAKLVESINSSGKIYLTHGVVGGTYIIR     477
            **.*::****. *                .: .* .::: :** *.:.::* : .. *::*

CrTDC       LAVGSSLTEEHHVRRVWDLIQKLTDDLLKEA----------500
CaTDC3      FAVGATFTEDRHLICAWKLIKDCADALLRNCQ---------503
CaTDC4      FAVGATFTEDKHVISAWKLIKKSADALLKRSYSYYTTIWGMSLNK516
CaTDC5      FAVGAPLTEERHIVGAWKVLQDEAATLLSKC----------487
CaTDC1      FAIGAPLTDYRHINMAWDVIRNHVNVLLKNLSSVASIG-------517
CaTDC2      FAIGASLTHYWHVDIAWKVIQDHANALLYQGSV---------510
            :*:*: :*.  *:  .*.:::. .  ** .
```

Fig. 5. Sequence comparations between *Catharanthus roseus* TDC (CrTDC, #MG748691) and newly identified *Capsicum annuum* TDCs

*The boxes with red dashed lines indicate the identified amino acids involved in the Trp-substrate-binding pocket in CrTDC which are Trp92, Phe100, Phe101, Pro102, Val122, Phe124, His318, and Leu325 (red arrows) and 3 variables Ala103, Thr369, and Gly370 (53). *: positions which have a single, fully conserved residue. :: conservation between groups of strongly similar properties. .: conservation between groups of weakly similar properties.*

Based on this information, the 3D structures of pepper fruit CaTDC4 (Figure 6, panel a) and CaTDC5 (panel b) were constructed, in which, the conserved hydrophobic residues with the Trp-substrate-binding pocket were obvious.

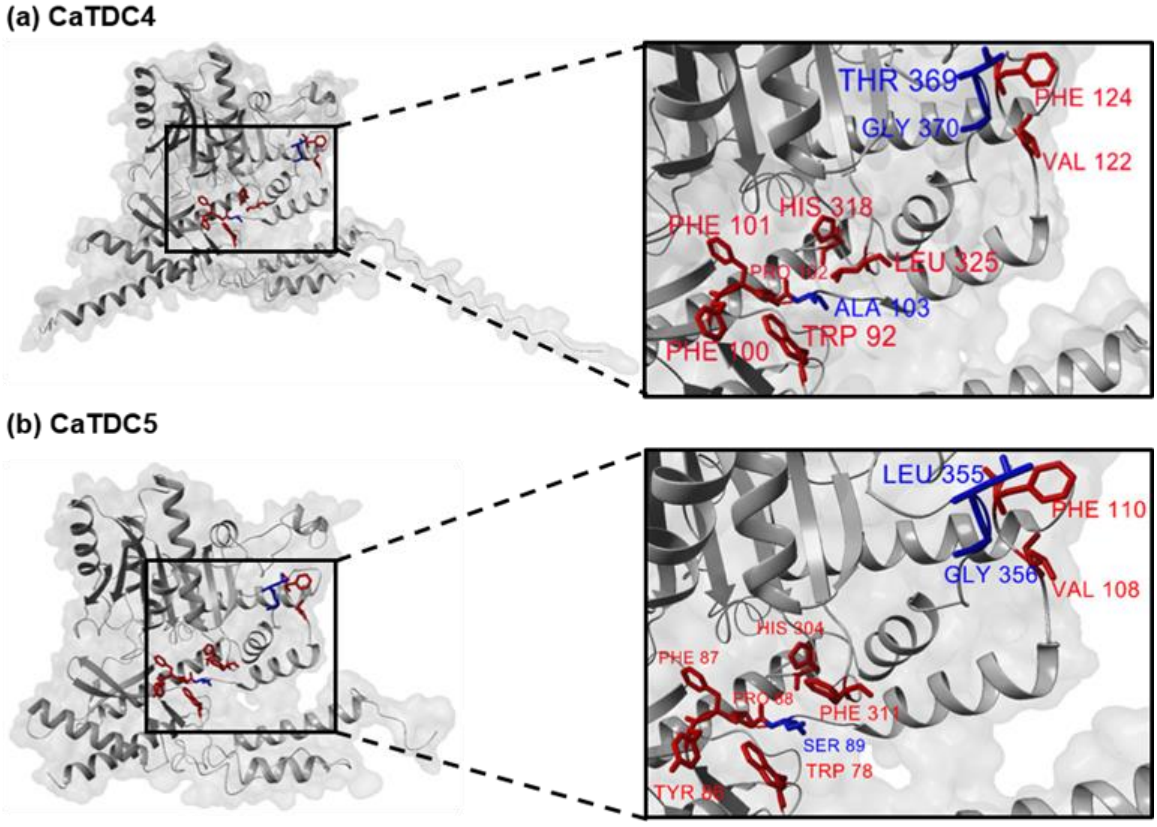

Fig. 6. The three dimensional (3D) structures of CaTDC4 and CaTDC5.

(a) *An overview of the tertiary structure of CaTDC4 (left part) and the Trp-substrate-binding pocket highlighting the conserved and variable amino acids in red and blue, respectively (right part); (b) For the CaTDC5.*

To better understand the dynamic metabolic processes among the five CaTDCs, the potential protein-protein interaction (PPI) network of these proteins was examined. Figure 7 illustrated the predicted PPI network using the STRING database, version 11.0 (https://string-db.org/ accessed on 19 February 2023) (54) which allows for the visualization/evaluation of the functional association of these CaTDCs. This model highlights the interaction of CaTDC5 with other CaTDCs, whereas CaTDC4 does not interact with CaTDC3.

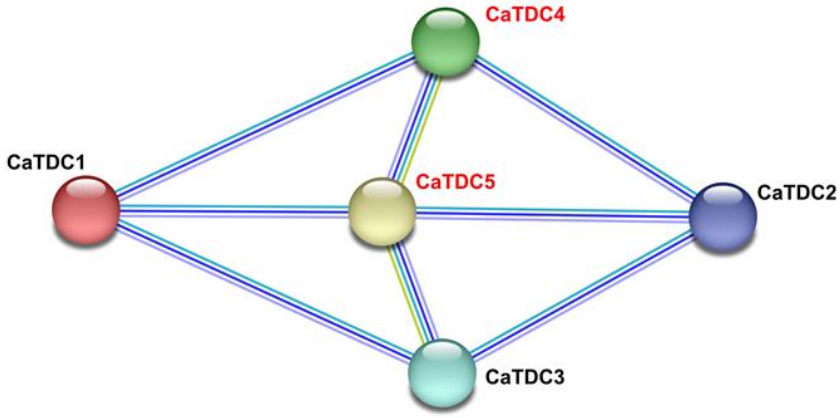

Fig. 7. Predicted computational protein-protein interaction (PPI) network among the five identified CaTDCs.

The color code for depicted lines is as follows: blue, known interactions from curated database evidence; green, neighboring genes; dark blue, gene co-occurrence; and purple, protein homology. The analysis was performed using STRING v11.0 with a minimum required interaction score set in "medium confidence" (0.400).

3.3. Effects of different ripening stages and NO treatment on *CaTDC4* and *CaTDC5* gene expression pepper fruits.

The mining analysis of the *CaTDC* genes from the RNAseq of sweet pepper fruits was conducted at the different ripening stages and after exposure to exogenous NO gas including green immature (G), breaking point (BP1), and red ripe (R) as well as fruits treated with 5 ppm NO for 1 h (BP2 + NO) and its non-treated control (BP2 − NO) group (Figure S1). The results showed that *CaTDC4* was upregulated during ripening whereas *CaTDC5* was downregulated (Figure 8). After NO treatment, *CaTDC4* was slightly downregulated but *CaTDC5* was unaffected (green line versus red one).

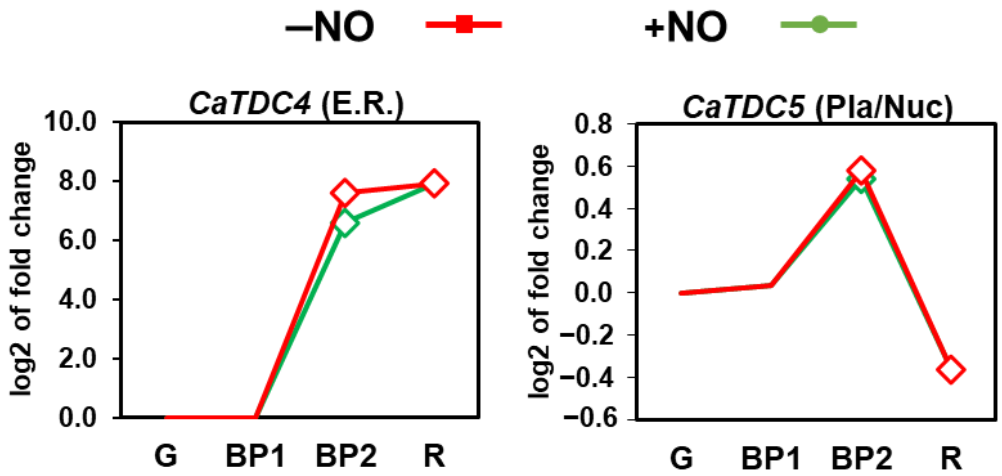

Fig. 8. Effects of different ripening stages and NO treatment on *CaTDC4* and *CaTDC5* gene.

expression of pepper fruits. Immature green (G), breaking point 1 (BP1), breaking point 2 with and without NO treatment (BP2+NO and BP2–NO, respectively) and red (R) were used (see Supp. Fig. S1 for the experimental design). Diamonds indicate statistically significant changes in expression levels ($p < 0.05$) vs G fruits. Green line: BP2 fruits treated with NO. Red line: untreated fruits.

4. DISCUSSION

The pepper plant is an economic and nutritional valuable crop globally. Therefore, this crop has been intensely studied from its biochemical and molecular viewpoints, with a special interest in the processes of its fruit ripening, an event chaperoned by nitro-oxidative stress (35, 36). However, the information on the enzymes/genes involved in the biosynthesis of serotonin/melatonin during the stages of its fruit ripening is limited (55, 56). Serotonin is synthesized in two steps catalyzed by TDC and tryptamine 5-hydroxylase (T5H) enzymes in plants (57). TDC is being considered the rate-limiting step of plant serotonin synthesis (58-60).

This enzyme was first purified and characterized as a dimeric protein with a subunit size of around 57 kDa from the cell cultures of the herbaceous plant bright eyes (*Catharanthus roseus*) (61). Subsequently, the gene that encodes for this protein was also identified in *C. roseus* (62). To our knowledge, this is the first *TDC* gene identified in the higher plants. Thereafter, homologs of *TDC* were reported in other plant species including one in *Ophiorrhiza pumila* (63, 64), seven in rice (*Oryza sativa*) (58), two in hot pepper fruit (55 53), three in the black cohosh (*Actaea racemosa* L.) (65), one in devil pepper (*Rauvolfia verticillata*) (66), one in *Mitragyna speciosa* (67), one in citrus (68-70), two in the tree *Camptotheca acuminata* (71, 72), one in the herbaceous peony (*Paeonia lactiflora* Pall.) (73), three in tomato (*Solanum lycopersicum* L.) (74), and nine in banana (*Musa acuminate*) (75). In the current study, we have identified five *CaTDC* genes in sweet pepper cultivar *Melchor* in pepper designated as *CaTDC*1-5. As mentioned previously, two *TDC* genes were cloned from the hot pepper fruits cultivar *Nokkwang*, i.e., *CanTDC1* and *CanTDC2*. Whereas the *CanTDC1* expression was significantly induced in fruit after treatment with the anthracnose fungus and ethylene, *CanTDC2* was constitutively expressed in all plant organs (55). The analysis of the current five *CaTDC* genes showed that the *CaTDC3* and *CaTDC5* were the homologs of *CanTDC1* and *CanTDC2* respectively (Table 1). Furthermore, it was observed that the expression of *CaTDC3* and *CaTDC5* was differentially expressed since *CaTDC4* was upregulated during ripening whereas the *CaTDC5* was not significantly affected. This is consistent with the fact that *CaTDC5* is considered to be constitutively expressed, and the *in silico* analysis of the interaction among the different CaTDCs suggests that CaTDC5 plays a central role.

The five identified *CaTDC* genes encode for TDC proteins which contain similar molecular mass and the binding site for pyridoxal 5′-phosphate (PLP). PLP is a cofactor necessary for the TDC activity, similar to other enzymes such as lipoxygenases, L-cysteine desulfhydrases, among others (76-78). In higher plants, TDCs and L-tyrosine decarboxylases (TyDCs) are part of the canonical aromatic amino acid decarboxylases which belong to the group of PLP-dependent enzymes (53, 68). TDCs and TyDCs share significant similarities, but it has been suggested that the amino acid specificity is due to key amino acid substitutions in the active site of the enzymes (69). The analysis of the amino acids involved in the Trp-substrate-binding pocket in the five CaTDCs suggests that they seem to have enzymatic functions of TDCs (79). However, biochemical analysis on the TDC activity of them is required.

Information related to whether the cis-responsive elements will affect the TDC gene expression is also limited. It was reported in cassava (*Manihot esculenta*) plants that the WRKY transcription factor regulates melatonin accumulation through W-box which affects some genes involved in its biosynthesis including the *TDC* (80). In pepper fruits, we found that *CaTDC4* was positively modulated by two light-responsive elements, Box4 and TCT-motif, and *CaTDC5* was regulated by GT1-motif and G-Box. This is in good agreement with the mechanism of regulation of melatonin biosynthesis by different light conditions including the

presence/absence of light (81), the intensity of light (82) or circadian rhythms (83), but also when the light is combined with other situations such as the leaf senescence (84) or under cadmium stress (85). Furthermore, it is known that in leaves of Valencia sweet orange (*Citrus sinensis*) the bacterial infection with *Candidatus liberibacter* upregulated the expression of the genes involved in the biosynthesis of melatonin including *CsTDC* as well as increased melatonin content (70).

The genetic study has shown that the degree of protein variation is inversely related to the size of the introns (86-88). An analysis of *TDC* genes from algae, low and high land plants showed that the numbers of intros were variable from none to 10 introns. Our observation on the numbers of introns/exons in the *CaTDCs* is in good agreement with that observed in other species and are remarkably similar to tomato TDCs (89).

On the other hand, we also investigated the potential modulation effects of NO application on TDC gene expression since this information was not available currently. The results indicated that at the fruit level, NO caused a slight decrease in the expression of *CaTDC4*. Actually, when plants were exposed to different types of stressors, the application of either NO or melatonin would impact the levels of each other suggesting crosstalk between them. Nevertheless, how NO or melatonin affect the biosynthesis as well as the metabolism of other pathways such as the ROS or phenolic compounds among others (10, 90-97) is still unknown.

In conclusion, the available information related to the number of genes encoding for TDC enzymes is limited in higher plants and particularly in pepper fruits. The present data provide new insights into this scenario, with the identification of a total of five potential *CaTDC* genes in pepper plants. Furthermore, based on the RNAseq analysis in sweet pepper fruits during ripening, the presence of two *CaTDC* genes (*CaTDC4* and *CaTDC5*) was identified. The amino acid sequence analysis of the proteins encoded by these two genes also allowed identifying the Trp-substrate-binding pocket which is a characteristic of the TDC proteins. *CaTDC4* and *CaTDC5* were differentially modulated during different ripening stages and the *CaTDC4* was slightly downregulated by exogenous NO treatment. Therefore, these data extend our knowledge about TDCs in pepper plants and their expression during fruit ripening and under the influence of NO, suggesting that NO may operate through the signaling cascades influenced by both serotonin and melatonin. Nevertheless, future biochemical and molecular studies are needed to evaluate how NO could affect the other enzymes involved in the biosynthesis of melatonin including tryptamine 5-hydroxylase, serotonin *N*-acetyltransferase and *N*-acetylserotonin methyltransferase which in pepper fruits is also unknown.

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AUTHORSHIP

J.T. and S.G.-G. performed bioinformatics analyses of the genome and transcriptome. F.J.C. and J. M.P. get funding acquisition and drove and coordinated the tasks. F.J.C. designed the

work and wrote the first draft of the manuscript. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

Authors have no conflict of interest to declare

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