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ORIGINAL ARTICLE

First report about the identification and preliminary analysis of a partial sequence of dihydropyrimidine dehydrogenase (NADP+) in *Thermopsis turcica* during floral development using degenerate primers

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Abstract *Thermopsis turcica* Kit Tan, Vural & Küçüködük is an herbaceous perennial, endemic and listed as endangered species by IUCN in Turkey. This plant is noted for its unusual floral structure characterized by a 2–4 carpellary ovary. In this study, a *DPD* (*NADP*+)-like gene was partially sequenced for the first time in *T. turcica*. Since there is no previous molecular genetic information available for *T. turcica*, RT-PCR was performed using degenerate primers targeted to conserved sequences of WUS protein homologues from related legume species. Amplified cDNAs of the expected size were sequenced and analyzed using bioinformatics tools. The analysis strongly suggested that a 283 bp PCR product was part of a dihydropyrimidine dehydrogenase (NADP+)-like coding sequence with a Flavin mononucleotide binding domain. The putative gene was named *Tt-DPD* and the partial CDS submitted to the NCBI database (accession number KT182937). This gene has not been identified previously in *T. turcica*. The DPD (NADP+) enzyme is the rate-limiting step in pyrimidine degradation, which is essential for the biosynthesis of beta-alanine and pantothenates in plants; it has also been shown to be required for normal seed development in *Arabidopsis*. Expression of *Tt-DPD* was monitored by both endpoint and real-time RT-PCR. High expression of the identified gene was observed in the mid-developmental stage of the pistil of *T. turcica*. The findings presented here provide a starting point for understanding the roles of this gene in pyrimidine catabolism in *T. turcica.*

Keywords Degenerate primer · DPD (NADP+) · Pyrimidine catabolism · Gene identification · Sequencing · *Thermopsis turcica*

Abbreviations

Aa	Amino acid(s)
AGE	Agarose gel electrophoresis
BLAST	Basic local alignment search tool
bp	Base pair
C. arietinum	*Cicer arietinum*
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
DPD	Dihydropyrimidine dehydrogenase
5-FU	5-Fluorouracil
L. japonicus	*Lotus japonicus*
M. truncatula	*Medicago truncatula*
NADP	Nicotinamide-adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NGBG	Nezahat Gökyiğit Botanical Garden
ORF	Open reading frame
PCR	Polymerase chain reaction
P. vulgaris	*Phaseolus vulgaris*

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RT-PCR	Reverse transcription polymerase chain reaction
TBE	Tris–boric acid–EDTA buffer
T. turcica	*Thermopsis turcica*

Introduction

Thermopsis turcica Kit Tan, Vural & Küçüködük is a single endemic plant species belonging to the Fabaceae family and found only in Turkey that is marked out by its polycarpellary morphology. It has been announced as a critically endangered (CR status) species in the Red Data Books of Turkish plants, according to IUCN threat categories (Ekim et al. 2000; Özhayat et al. 2005; IUCN 2009). While 25 other species belonging to the genus *Thermopsis* are naturally found mainly in mountainous areas of Asia and North America, *T. turcica* is the only endemic species of this genus in Turkey. *T. turcica* is unique in the Papilionoideae subfamily due to its carpel polymerization (Cenkci et al. 2008, 2009; Ozdemir et al. 2008; Tekdal and Cetiner 2014).

Degenerate PCR primers were designed to amplify genes which govern possible mechanisms of gynoecium multiplication including *WUSCHEL* (*WUS*) and its negative regulators, *CLAVATA* (*CLV*) and *FASCIATA* (*FAS*) (Weigel and Meyerowitz 1994; Bäurle and Laux 2003, 2005; Choob and Sinyushin 2012; Endress 2014), with the aim of providing information on the mechanism of gynoecium polymerization in *T. turcica*.

Degenerate primers consist of a mixture of similar oligonucleotides with variable bases at some positions that are used to amplify unknown DNA sequences. In particular, this method is used to amplify gene-coding sequences, where genomic data are unavailable, based on previously sequenced orthologous genes from different organisms (Rose et al. 1998; Song 2005; Walker 2011). In the same way, we recently used degenerate primers to isolate homologous genes in *T. turcica,* and identified five partial sequences of the target genes *WUS*, *FAS*, and *CLV*; however, they are not the subject of the present study. Herein, we report that unexpected sequences were also amplified using the degenerate primers, due to their potential to hybridize to more than one sequence. In this study, a partial sequence of an oxidoreductase family protein from *T. turcica* was identified. Sequence analysis indicates that the ORF translates into the N-terminal 74 amino acids of a dihydropyrimidine dehydrogenase (DPD) enzyme, so it was labelled *Tt-DPD*. This protein is involved in the beta-alanine biosynthesis pathway (Kafer et al. 2004), and this is the first time that it has been described in *T. turcica.* In other species, DPD is synthesized as a precursor containing a signal peptide and acts with other enzymes in pyrimidine catabolism (Zrenner et al. 2006). Specifically, uracil and thymine are converted to dihydrouracil and dihydrothymine, respectively, by DPD (Raman and Rathinasabapathi 2004). The pyrimidine degradation pathway is well characterized in certain bacterial species [*Escherichia coli* (Hidese et al. 2011); *Alcaligenes eutrophus* (Schmitt et al. 1996)] and animals [pig (Rosenbaum et al. 1997; Hagen et al. 2000); cow (Lu et al. 1993); and rat (Harris et al. 1988; Lu et al. 1993)], but has not been clearly described in plants; however, it is thought to be the reverse function of the *de novo* biosynthetic pathway. The first study of pyrimidine catabolic metabolism in plants was announced in pea seedlings (Mazus and Buchowicz 1968). The intracellular localization of a dihydropyrimidinase (EC 3.5.2.2), the enzyme downstream of DPD, has been studied in tomato cell suspension culture and *Euglena gracilis* (Tintemann et al. 1987), and the secondary products generated by dihydropyrimidinase examined in *Albizia julibrissin* (Brown and Turan 1995; Turan and Sinan 2005). Products of pyrimidine catabolism can be used in the biosynthesis of β-alanine and pantothenates (Zrenner et al. 2006).

Pyrimidine nucleotides are required in a wide range of processes such as pollen tube growth, germination, and flowering (Cooke et al. 2001; Kafer et al. 2004; Raman and Rathinasabapathi 2004). Control of these processes through regulation of cellular free pyrimidine levels is thought to be a major role of the pyrimidine catabolic pathway (Cornelius et al. 2011).

Real-time RT-PCR permits the analysis of multiple genes simultaneously both in specific organs and during development (Song et al. 2011). In this study, qRT-PCR was used to monitor the expression of the isolated partial homologues of *Tt-DPD* in pistils from different developmental stages of *T. turcica* during floral development with the expectation that *Tt-DPD* expression might be increased during flower bud initiation; in fact, *Tt-DPD* expression was greatest in pistils at mid-developmental stages in *T. turcica*, which might indicate involvement in pistil enlargement.

Materials and methods

Plant materials

Thermopsis turcica plants, which are being maintained in Nezahat Gokyigit Botanical Garden (NGBG) in Istanbul, Turkey, were used as the main source of plant material. In addition, monocarpellary *Phaseolus vulgaris* (which belongs to the same taxonomic family), provided by local breeders in Turkey, was used for comparison. Flower buds

were collected from different growth stages for each species (*T. turcica* and *P. vulgaris*). Pistil samples at early-stage (1–3 mm in length), mid-stage (10–15 mm), and late-stage (20–25 mm) were removed and frozen immediately in liquid nitrogen, and stored at −80 °C for molecular analysis.

Degenerate primer design

To find possible orthologs of the *WUS* gene, protein sequences of legumes (*Medicago truncatula, Cicer arietinum,* and *P. vulgaris*) were retrieved from the NCBI database. Degenerate primers of 16–24 nucleotides were designed based on sequences conserved between these species (Table 1). The *WUS* mRNA sequences identified in the selected species were first aligned using Clustal Omega (Sievers et al. 2011) to identify conserved sequence segments, and degenerate primers were designed manually (Fig. 1) predicated on minimum codon degeneracy. The specificity of designed primers was checked using the NCBI's Primer-BLAST tool. The primers designed for *Tt-DPD* sequence and selected for further expression analysis are described in Table 2. Oligonucleotides were synthesized by Sentegen Biotechnology, Ankara, Turkey.

Total RNA isolation

Frozen reproductive tissue (pistils at various ages) (∼500 mg) samples of *T. turcica* and *P. vulgaris* and 3 mm tungsten carbide beads were added to 2 ml tubes and then frozen in liquid nitrogen. For homogenization, frozen plant tissue samples were disrupted by bead beating in a TissueLyser II (Qiagen, Hilden, Germany). The disruption was performed in two steps of 1 min at 30 Hz. Total RNA was extracted using TRI Reagent (PeqLab) as described in Song (2005). The quality and quantity of total RNAs were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). The integrity of isolated RNAs was checked by visualization of ethidium bromide-stained RNA separated on 2% (w/v) agarose gel. DNase I treatment of the isolated RNA samples was implemented in 50 µl reactions containing 4 µl RNA (30–40 ng), 5 µl reaction buffer, 2 µl DNase I, 0.5 µl RNase inhibitor, and 38.5 µl DEPC-H_2O. RNA samples were kept at −80 °C until use.

First-strand cDNA synthesis

The first-strand cDNA was synthesized using the Tetro cDNA Synthesis Kit (Bioline, London, UK) with oligo-dT primers according to the manufacturer's protocol, and used as a template for RT-PCR and quantitative real-time RT-PCR analysis.

RT-PCR analysis, agarose gel electrophoresis, and gel extraction

For endpoint RT-PCR, thermal cycler conditions were as follows: initial denaturation at 95 °C for 10 min followed by 35 cycles of: denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min. These cycles were followed by a final extension step at 72 °C for 7 min.

PCR products were separated and visualized on agarose gels (2%). cDNA samples mixed with loading dye were loaded on the gel, which was run at 100 V for 45 min in 0.5× TBE, and bands were visualized using UV light on a Biorad Imager (Bio-Rad Laboratories, Segrate, Milan, Italy) DNA bands of approximately the expected size were excised from the agarose gel and extracted using a Qiagen Gel Extraction Kit according to the manufacturer's protocol.

Sequencing of strong candidates, evaluation of the results, and sequence analysis

After successful amplification, purified DNA bands from the agarose gel were sent for Sanger sequencing using the same degenerate primers as those used for PCR amplification. Both forward and reverse primers were used in separate sequencing reactions to obtain bi-directional DNA sequences. The sequencing service was commercially provided by MCLAB (Molecular Cloning Laboratories, CA, USA) (http://www.mclab.com/). Raw DNA sequence data were checked manually, and then, forward and reverse complement sequences were aligned using the Pairwise Sequence Alignment (Nucleotide) Tool constructed by EMBL-EBI (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html). The consensus sequence was used to search for similar sequences in GenBank at the National Center for Biotechnology Information (NCBI), Bethesda,

Table 1 *WUS* gene homologues used for mRNA sequences comparison and analysis of *T. turcica* putative genes

Family	mRNA	Species	Accession no.
Fabaceae	*WUSCHEL*-like	*Cicer arietinum*	XM_004512172.1
Fabaceae	*WUSCHEL* (*WUS*)	*Medicago truncatula*	FJ477681.1
Fabaceae	PHAVU_002G109400g	*Phaseolus vulgaris*	XM_007157862.1

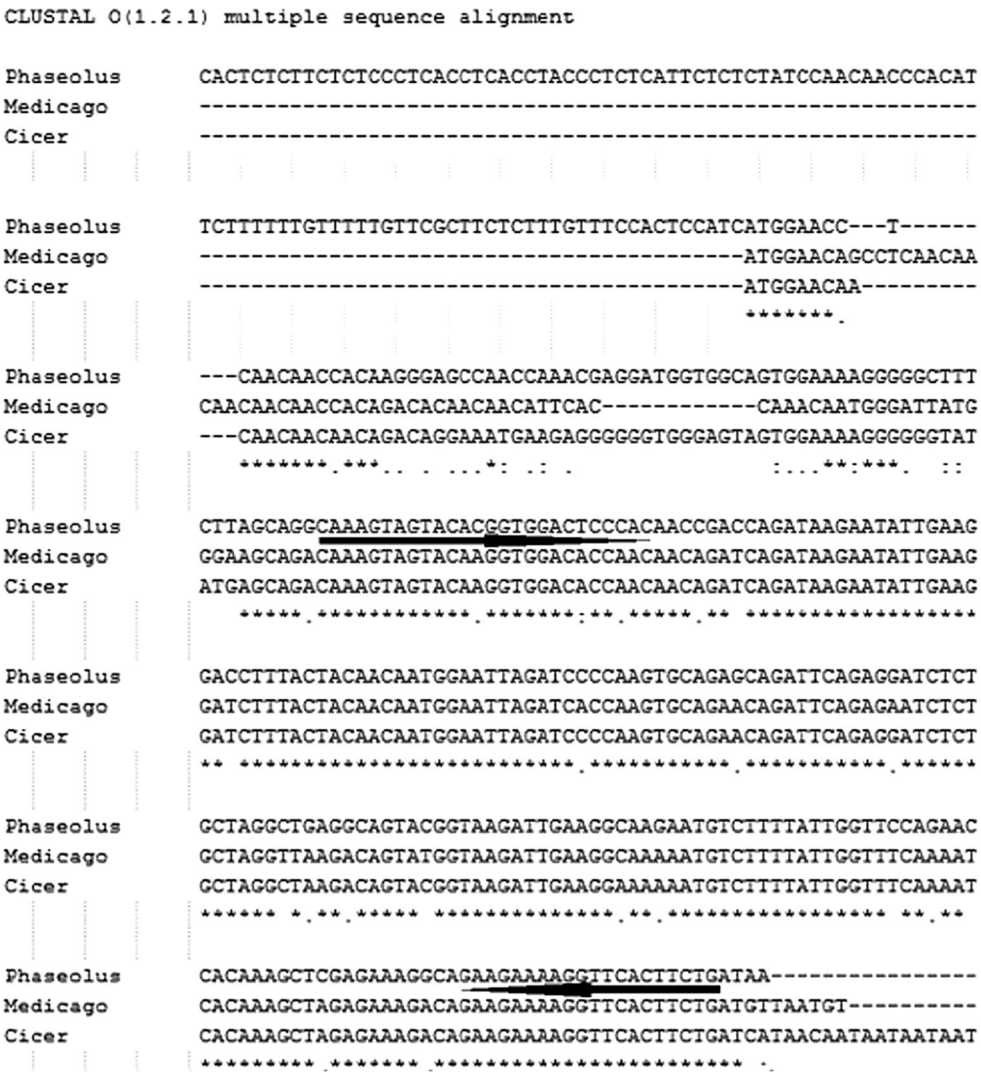

Fig. 1 Multiple alignment of *WUS* orthologues in relative legume species of *T. turcica* (*P. vulgaris*, *M. truncatula*, and *C. arietinum*; Table 1) and designing of degenerate primers based on mostly conserved sequences; *arrows* selected forward and reverse primers, *asterisks* identical amino acid residues, *colons* conserved substitutions, *dots* semi-conserved substitutions

Table 2 List of the designed degenerate primers, control, and housekeeping gene primers employed in this research (F, forward, R, reverse)

Target	Name	Sequence (5′–3′)	Definition	Product size (bp)
WUSCHEL	*WUSF*	CAAAGTAGTACAMGGTGGAC	*WUSF*/sense primer	211-*P. vulgaris*
	WUSR	CAGAAGTGAACCTTTTCTTC	*WUSR*/antisense primer	283-*T. turcica*
18S rRNA	18S-F	TACCGTCCTAGTCTCAACCATAA	18S/sense primer	Reference
	18S-R	CAGAAGTGAACCTTTTCTTC	18S/antisense primer	Song (2005)
P. vulgaris-(internal control)	PV-F	GGAAAAGTGTTGGAGGTG	PV/sense primer	189
	PV-R	GTACGCTTGCTTCTCCTC	PV/antisense primer	
Tt-DPD (NADP+)	Tt-DPD-F	GGACCCGACCCAATAACA	DPD-F/sense primer	162
(Sequence-specific primer)	Tt-DPD-R	CATCTGGGTTTGCTTTGA	DPD-F/antisense primer	

USA, using the BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi) search program (Altschul et al. 1990). Both cDNA and predicted amino acid sequences for each edited sequence were used to search the GenBank database.

Phylogenetic analysis was performed by the TreeDyn 198.3 program using Newick format (Dereeper et al. 2008; http://www.phylogeny.fr/one_task.cgi?task_type=treedyn). Secondary structure predictions of Tt-DPD (NADP+) protein were performed using Jpred4 secondary structure

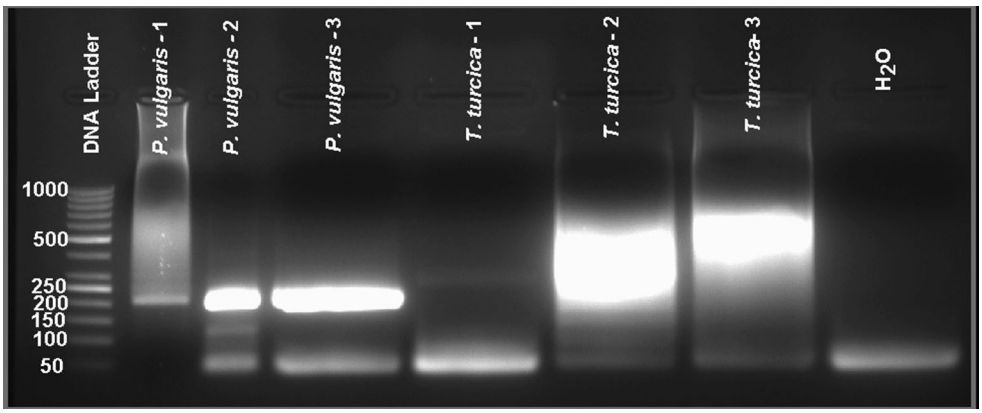

Fig. 2 2% Agarose gel electrophoresis of RT-PCR analysis of isolated pistil samples at various developmental stages of *P. vulgaris* and *T. turcica* using degenerate *WUS* primers; *1* early-stage pistils 1–3 mm in length, *2* mid-stage pistils 10–15 mm in length; late-stage pistils 20–25 mm in length; H_2O is negative control; DNA ladder, Thermo Scientific DNA Ladder SM1133

prediction server (Drozdetskiy et al. 2015; http://www.compbio.dundee.ac.uk/jpred/).

Quantitative real-time (qRT)-PCR

Quantification of cDNA in RT-PCR in combination with SYBR Green DNA dye is based on the monitoring of the enhancing fluorescence intensity after each PCR cycle (Ramakers et al. 2003). Real-time PCR conditions for SYBR Green quantification were based on the recommended protocol for the LightCycler® 480 Instrument (Roche Diagnostics, Mannheim, Germany). Relative quantitative PCR reactions were carried out using SensiFAST™ SYBR® No-ROX Kit (Bioline) according to the manufacturer's instructions. mRNA levels were assessed by qRT-PCR using the sequence-specific primer pair, *Tt-DPD*, as listed in Table 2. Primers derived from the 18S rRNA of Fabaceae (Song 2005) were used as a control for global RNA levels, and the expression was normalized to that of 18S rRNA. Tt-*DPD* reactions were carried out in triplicate in a 96 well plate with a total reaction volume of 20 µl. Experimental data were analyzed according to the method of Pfaffl (2001).

Results and discussion

Total RNA isolation, cDNA synthesis, and RT-PCR

In the present study, cDNAs were used as the template for PCR-based amplification using degenerate primers designed to target certain genes. The identification of unknown homologues of known genes is a valuable tool for understanding biological function in plants. In the literature, previous studies reported that designing degenerate primers to match regions that are well conserved between the sequences of distant relatives could prevent them from amplifying unrelated sequences (Rose et al. 1998; Benedito 2004). In the present study, the primers were used to amplify gene segments from a cDNA template by RT-PCR, to avoid the risk of genomic DNA introns interrupting the primer binding site or causing amplification of non-specific PCR products (Walker 2011). Degenerate primers were designed using conserved regions of *WUS* mRNAs available in the GenBank database (Table 1). Following RT-PCR using these degenerate *WUS* primers, multiple bands of different levels of intensity were produced (Fig. 2). The difference between the more intense bands and the weaker bands is possibly related to the PCR conditions. The same amount of cDNA was used in each reaction, and no differences were found between the sequences identified from pistil samples at different developmental stages. Based on this, this result was not related to alternative splicing. A putative *WUS* homolog with a size close to the expected 283 bp was obtained from *T. turcica*.

Sequence analysis

PCR products were obtained from cDNAs of *T. turcica* pistils harvested at different developmental ages. To identify the ~283 bp PCR product, it was isolated and directly sequenced by the Sanger method. Since degenerate primers may amplify multiple loci simultaneously, causing multiple overlapping peaks in the chromatogram, direct sequencing of the isolated PCR products is not often preferred (Walker 2011). However, in the present study, the direct sequencing of the PCR fragments was deemed to be a viable approach due to the low degeneracy of the designed primers. Only *WUSF* (Table 2) is slightly degenerate with a single 'M' residue, which means that half of the primers will include a C (cytosine) at this position and the other half an A (adenine).

Table 3 Sequence similarities according to the BLAST search against NCBI database based on identified partial sequence in *T. turcica*

Mrna	Species	Accession no.
NAD-dependent dihydropyrimidine dehydrogenase subunit PreA-like (LOC100804632)	*Glycine max*	NM_001252829.1
Unknown	Soybean clone JCVI-FLGm−12G14	BT098323.1
PREDICTED: dihydropyrimidine dehydrogenase (NADP+)	*Cicer arietinum*	XM_004512717.2
Hypothetical protein (PHAVU_004G148000g)	*Phaseolus vulgaris*	XM_007152595.1
PREDICTED: dihydropyrimidine dehydrogenase (NADP+)	*Vigna radiata* var. *radiata*	XM_014662535.1
Tt-dihydropyrimidine dehydrogenase (NADP+)	*Thermopsis turcica*	KT182937

The sequence obtained through RT-PCR was verified to be 283 bp in length; however, rather than *WUS,* the identified sequence had high similarity to dihydropyrimidine dehydrogenase (*DPD*) genes from other legumes. It was named *Tt-dihydropyrimidine dehydrogenase* (*NADP+*), or *Tt-DPD*, and was submitted to the NCBI GenBank database with the following accession number: KT182937. Pairwise sequence alignment of the identified sequence from *T. turcica* with known *WUS* genes indicated that the maximum sequence identity (49.4%) was between *P. vulgaris WUS* and *Tt-DPD* (Table 1) (Fig. S1, Supplementary Data). This was much lower than the similarity of the obtained sequence to other DPD genes. Amino acid sequences of *WUS* genes of tested species were largely similar; however, only nine residues were conserved with the Tt-DPD sequence (Fig. S2, Supplementary Data). Taken together, these results show that a gene that was not a *WUS* homologue was amplified. Using degenerate primers may have allowed the amplification of more than one fragment of the expected size during PCR, which then dominated the following sequencing reaction (Song 2005). To take this into account, at least three independent PCR products amplified using the same primers were sequenced: all of them were identified as *Tt-DPD.* Reasons for the unexpected amplification of a different gene may include codon degeneracy, Tm, PCR cycle, primer length, and the dominance of the primers unused in PCR (Rose et al. 1998; Benedito 2004). Moreover, synonymous mutations in the region to which specific oligonucleotides bind might occur, thereby changing the preferred primer annealing site and resulting PCR product formation (Walker 2011).

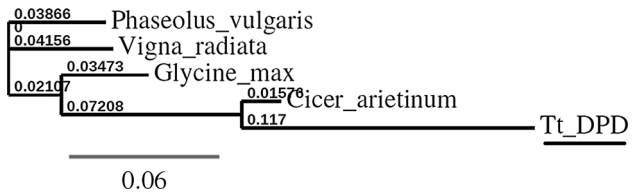

Fig. 3 Comparison of phylogenetic relationship of *DPD* homologues of the species given in Table 3. The gene isolated from *T. turcica* is underlined. Refer to Table 3 for NCBI accession numbers. The tree was produced with TreeDyn Program using the tree file in Newick format. *Numbers* indicate branch lengths. Sequence segments covered by identified *DPD* (*NADP+*) fragment were included for comparison

All positive hits obtained in the BLAST search against the GenBank nucleotide database were dihydropyrimidine dehydrogenase orthologues from a wide range of Eudicots (Table 3). A phylogenetic tree was constructed using the sequences for each orthologous gene identified in the BLAST search. The sequences were first trimmed to cover the same region of the gene isolated in the present work and its homologues from the NCBI database (Figs. S3 and S4, Supplementary Data). The phylogenetic tree showed that *Tt-DPD* placed most closely to its ortholog from *C. arietinum* and was well separated from the other legumes (Fig. 3).

Conceptual translation of the ORFs of the cDNA sequence of *T. turcica* yielded an amino acid sequence of 74 aa (Fig. 4). The amino acid sequence was:

MASLSMTQIRTGNSASGFALNCSKKVHAGPS
RIGFKVFASETQGSAEPALSVTVNGLHMPNPF
VIGSGPPCTTL.

Phylogenetic relationships of the deduced amino acid sequences of the genes from other species (Table 3; Fig. S5, Supplementary Data) also showed that Tt-dihydropyrimidine dehydrogenase (NADP+) isolated from *T. turcica* was most similar (65.7% identity) to the protein from *C. arietinum* (XM_004512717.2) (Fig. 5; Fig. S6, Supplementary Data). During phylogenetic tree construction, only the sections of each homologue sequence from the NCBI database that covered the region of the gene isolated in the present study were used to avoid errors resulting from comparing sequences of different lengths.

In this work, the Jpred (4) server was used to investigate the secondary structure of Tt-DPD in comparison with that of the protein from *C. arietinum* (GI = XM_004512717.2). The structural determinants identified were analyzed to characterize the differences between the two proteins

Fig. 4 Translation of the ORFs of Tt-DPD cDNA sequence of *T. turcica*

```
  1 TCAGAAGTGAACCTT TTCTTCTTCTTCACT TTGCAGAAAGTTGAG ATTTTTCTTTCAACA ATGGCATCTTTGAGC
  1  S  E  V  N  L   F  F  F  F  T   L  Q  K  V  E   I  F  L  S  T   M  A  S  L  S
 76 ATGACCCAGATCAGA ACTGGGAATTCTGCA TCTGGGTTTGCTTTG AATTGTTCTAAGAAG GTTCATGCTGGTCCT
 26  M  T  Q  I  R   T  G  N  S  A   S  G  F  A  L   N  C  S  K  K   V  H  A  G  P
151 AGCAGAATTGGGTTT AAGGTCTTTGCTTCT GAGACTCAGGGTTCT GCAGAACCTGCTCTT AGTGTTACTGTGAAT
 51  S  R  I  G  F   K  V  F  A  S   E  T  Q  G  S   A  E  P  A  L   S  V  T  V  N
226 GGGTTGCACATGCCT AACCCATTTGTTATT GGGTCGGGTCCACCT TGTACTACTTTG
 76  G  L  H  M  P   N  P  F  V  I   G  S  G  P  P   C  T  T  L
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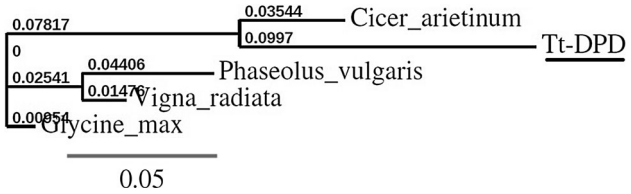

Fig. 5 Phylogenetic tree of representative DPD gene proteins generated using TreeDyn program. The gene isolated from *T. turcica* is underlined. Refer to Table 3 for NCBI accession numbers. The tree was produced with TreeDyn Program using the tree file in Newick format. *Numbers* indicate branch lengths. Sequence segments covered by identified DPD (NADP+) fragment were included for comparison

(Table 3), the ORFs of which were shown to have high homology (85.3%) by Emboss Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/index.html) (Fig. 5; Fig.S6, Supplementary Data).

The secondary structure prediction indicated the presence of one α-helical structure close to the N-terminus of Tt-DPD, whereas two α-helixes were be found in the *C. arietinum* protein (XM_004512717.2). A β-strand was predicted near the middle of the Tt-DPD partial sequence that was not predicted in the *C. arietinum* homologue, while further β-strands were predicted in both sequences towards the C-terminus of the Tt-DPD fragment, with several non-conservative changes between the two putative proteins at the C-terminal end of the obtained sequence (Fig. 6). Structurally conserved patterns identified between these amino acid sequences could be useful for designing specific primers for the further analysis of DPD (NADP+).

The data from the BLAST search against the NCBI database, multiple sequence comparisons, phylogenetic analysis, and protein structure prediction all support the conclusion that the isolated gene fragment from *T. turcica* is a homologue of *DPD* (*NADP+*). This enzyme has been well characterized in mammals by previous studies, owing to its role in degrading the anti-cancer drug 5-fluorouracil (Rosenbaum et al. 1997; Mcleod et al. 1998; Takenoue et al. 2000), but few functional studies have been carried out in plants. The partial CDS of the *DPD* (*NADP+*) gene was isolated in *T. turcica* for the first time in the present study. By comparison with other eukaryotes, plant DPD is believed to be involved in the degradation of uracil and thymine to produce β-alanine, an important precursor for pantothenate biosynthesis (Kafer et al. 2004; Raman and Rathinasabapathi 2004; Chakauya et al. 2006). The Tt-DPD isolated in the present study contained a predicted phosphate binding domain according to the NCBI conserved domain search (Fig. S7, Supplementary Data).

The RT-PCR used in the present study was unable to determine the full-length sequence of the *Tt-DPD* gene in *T. turcica*; however, the data obtained from the sequencing have moved the molecular study of oxidoreductase proteins in *T. turcica* a step forward.

Quantifying relative expression levels of Tt-DPD in pistils at different developmental stages and leaf sample of T. turcica

The expression level of *Tt-DPD* in different developmental stages of the pistil and leaf isolated from *T. turcica* was investigated by qRT-PCR. To avoid any problems based on the degeneracy of the primers used to identify the original fragment, new primers were designed within the partial *Tt-DPD* sequence to hybridize to sequences that were conserved in the orthologs of *DPD* (*NADP+*) in other legumes (Table 2).

Quantitative real-time RT-PCR with SYBRGreen DNA dye is widely performed in expression studies (Lucas et al. 2011; Song et al. 2011). Successful expression analysis depends on RNA quality, optimal primer design, and the removal of genomic DNA contamination (Song 2005; Song et al. 2008). In the present study, cDNAs were synthesized from total RNA isolated from pistil samples at different

```
Tt-DPD          MASLSMTQIRTGNSASGFALNCSKKVHAGPSRIGFKVFASET-QGSAEPALSVTVNGLHMPNPFVIGSGPPCTTL----------
                ------HHH-------------------------EEEEEE-----------EEEEEE-E------EE---------
Cicer_arietinum MASLSMTQMRTMNSASGFSLNCSKKVHALPSRIGFKVFASETSQSSSEPDLSVTVNGLHMPNPFVIGSGPPGTNYTVMKRAFDEGWGAVIAKTVSLDAAKVI
                -----HHHHE-----HHHHHHHHHH-------------------------EEEEEEEEE-----EE---------HHHHHHHHHH---EEEEEE---------
                ********:** ******:********* ************* *.*:** ********************* *.
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Fig. 6 Combination of pairwise sequence alignment of ORFs from KT182937 and XM_004512717.2 and Jpred4 secondary structure predictions for KT182937 and XM_004512717.2. For each residue, the secondary structure class (H, α-helix; E, β-strand; –, coil) is predicted. *Asterisks* identical amino acid residues, *colons* conserved substitutions, *dots* semi-conserved substitutions

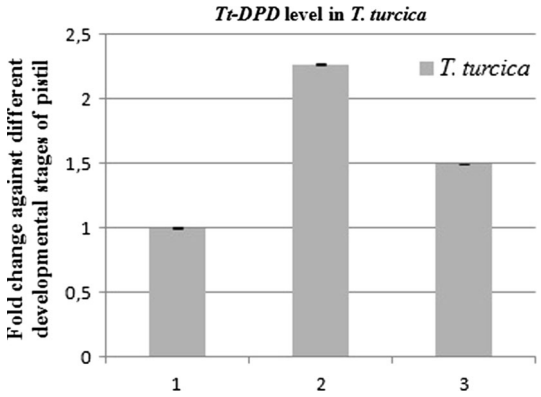

Fig. 7 Comparison of the expression level of cDNA synthesized using designed degenerate primers in different developmental stages of *T. turcica*, performed by amplifying cDNAs generated from same amounts of total RNA for 35 cycles. Values were the mean relative $C_t(C_p)$ values of three replicates. 18S rRNA was used as an internal reference for normalization. *1* Early-stage pistils 1–3 mm in length, *2* mid-stage pistils 10–15 mm in length; late-stage pistils 20–25 mm in length

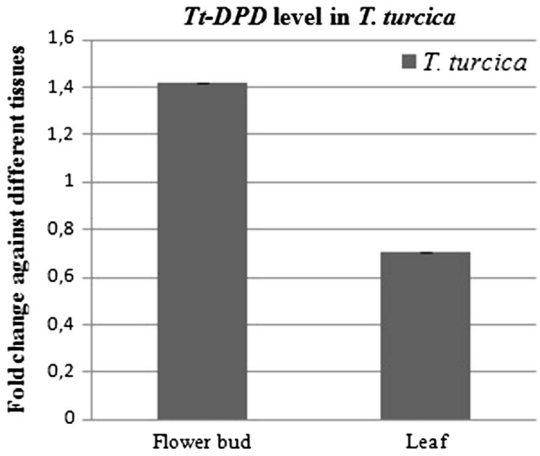

Fig. 8 Evaluation of *Tt-DPD* expression in *T. turcica* tissues, leaf, and flower bud at the late developmental stage. Values were the mean relative $C_t(C_p)$ values of three replicates. 18S rRNA was used as an internal reference for normalization. Leaf *Tt-DPD* expression was the value of 0.7

developmental stages and leaf, treated with DNase I, and sequence-specific primers used for real-time RT-PCR. The expression pattern of *Tt-DPD* in various developmental stages of pistil and leaf may be useful in predicting its function.

Tt-DPD was expressed in all the *T. turcica* samples tested. The expression of the 18S rRNA gene was used as an internal control and was relatively stable in all tested samples. According to the real-time RT-PCR results, expression was low in early-stage pistils, increased by >2-fold in mid-stage pistils, and then reduced slightly in late-stage pistils. It is, therefore, likely that the Tt-DPD enzyme was most active during mid-stage development of the pistil in *T. turcica* (Fig. 7). To determine the expression level of *Tt-DPD* in different tissues of *T. turcica,* the expression level of *Tt-DPD* in leaf and late-stage flower buds was examined. Expression of the gene of interest was low in leaf, nearly twofold less than that of flower bud (Fig. 8). According to previous studies conducted in leguminous species, uracil may have toxic effects on the germination and growth of plants (Turan and Konuk 1999). The germination was inhibited by applying exogenous uracil in *Phaseolus aureus* and *Glycine max* (Turan and Konuk 1999). Knockout of the DPD gene in *Arabidopsis* (Cornelius et al. 2011) resulted in reduced uracil catabolism, and retarded seed and plant development. This suggests a possible explanation for the relatively higher expression of *Tt-DPD* in mid-stage pistils of *T. turcica* than early- or late-stage pistils. Uracil catabolism is probably more active during pistil development to regulate the effects of uracil on growth. Alternatively, pyrimidine catabolism may provide additional biosynthetic precursors to accelerate pistil growth.

Conclusions

In the present study, degenerate primers were designed from multiple aligned protein-coding DNA sequences of target genes from previously sequenced legume species. A partial homologue of *DPD* (*NADP+*) in *T. turcica* was identified using these degenerate primers. The full-length CDS of the target genes was not determined, but data generated in the present study will lead to realising this aim.

In future work, research into the function of the identified putative *DPD* (*NADP+*) ortholog in *T. turcica* will serve as a clue to better understand the mechanism of amino acid biosynthesis in legumes. Since gene identification using degenerate primers only provides partial coding sequences of the target gene, complete gene sequences should then be obtained by other techniques such as inverse PCR, 5′ Rapid amplification of cDNA ends (RACE), 3′ RACE, or circular RACE. Our future project aims include identifying full-length sequences of *Tt-DPD* in *T. turcica* and to studying its function, either in different organ tissues or in different developmental stages of *T. turcica*.

Author contribution statement Conceived and designed the experiments: SC and DT. Performed the experiments: DT. Supervised the study: SC. Analyzed the data with the help of bioinformatics tool: DT and SJL. Wrote the paper:

DT and SJL. Critically revised the manuscript: SC, DT, and SJL.

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Compliance with ethical standards

Conflict of interest We declare that we have no conflict of interest. We certify that all persons who have made substantial contributions to this manuscript. We certify that the submitted original manuscript is not under review by any other journal. There is no financial interest to report.

Studies with human or animal research This article does not contain any studies with human subjects or animals performed by any of the authors.

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