Unveiling the Enigmatic Structure of *TdCMO* Transcripts in Durum Wheat

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Abstract: Durum wheat is one of the oldest and most important edible cereal crops and its cultivation has considerable economic importance in many countries. However, adverse conditions, such as high irradiance and increasing salinity of soils, could lead to a decrease in productivity over the next few decades. Durum wheat plants under salinity are able to accumulate glycine betaine to osmotically balance the cytosol and reduce oxidative stress, especially in young tissues. However, the synthesis of this fundamental osmolyte is inhibited by high light in *T. durum* even under salinity. Choline monooxygenase is the first enzyme involved in the glycine betaine biosynthetic pathway. Thus, to explain the glycine betaine inhibition, we analyzed the effect of both salinity and high light on the putative *TdCMO* gene expression. Thirty-eight *TdCMO* different transcripts were isolated in the young leaves of durum wheat grown in different stress conditions. All translated amino acid sequences, except for the *TdCMO1a6* clone, showed a frame shift caused by insertions or deletions. The presence of different transcripts could depend on the presence of duplicated genes, different allelic forms, and alternative splicing events. *TdCMO1a6* computational modeling of the 3D structure showed that in durum wheat, a putative CMO-like enzyme with a different Rieske type motif, is present and could be responsible for the glycine betaine synthesis.

Keywords: salinity; high light; choline monooxygenase gene; glycine betaine; *Triticum durum* L.

1. Introduction

Plants frequently undergo environmental stresses that are harmful factors that decrease crop growth and productivity [1]. Among the abiotic stresses, salinity is one of the major environmental constraints that modern agriculture has to cope with [2], affecting germination, plant vigor, and crop yield of plants over 800 million ha of land worldwide and 32 million ha of dry land agriculture [3]. Moreover, due to climate change and land and water quality pauperization, salt stress together with drought, will become a hot topic of crop studies [3]. Plant response mechanisms involve the expression/activation of stress-protective proteins and changes in metabolism [4–6]. Particularly, one of the basic strategies ubiquitously adopted in response to salinity stress involves salt sequestration...
into the vacuole, and the synthesis and accumulation of compatible solutes [7]. These latter are small molecules, water soluble, and uniformly neutral with respect to the perturbation of cellular functions, even when present at high concentrations [8,9]. Among the best known compatible solutes, glycine betaine (GB) has been reported to increase greatly under salt and drought stresses [8], and constitutes one of the major metabolites found in durum wheat under salinity, as in other Poaceae [10–13]. It acts not only as an osmoregulator, but also stabilizes protein structures and activities, and maintains the integrity of membranes by preventing stress-related oxidative damages.

In higher plants, GB is synthesized from choline in two oxidation steps, via betaine aldehyde. The first step is catalyzed by choline mono-oxygenase (CMO; EC 1.14.15.7) in the presence of ferredoxin, whereas the second step is catalyzed by betaine aldehyde dehydrogenase (BADH; EC 1.2.1.8) in a NAD$^+$-dependent reaction. Both enzymes are localized in the stroma of chloroplasts. The biosynthesis of GB is stress-inducible [14] and it has been suggested that GB can be derived from salt-stress-induced photorespiratory serine [15]. Particularly, in durum wheat, GB preferentially accumulates in younger tissues after several days of imposed stress [16]. In many halophytes, GB concentration in the leaves contributes significantly to control the ion and water homeostasis maintaining the normal cellular turgor; and since many crop plants do not accumulate GB, crop improvement genetic programs are focused on the analysis of the GB biosynthetic pathway. Therefore, recent research has especially focused on the introduction of choline-oxidizing enzymes such as choline dehydrogenase, choline oxidase, and choline monooxygenase (CMO) in GB non-accumulating plants, or producing GB hyper-accumulating plants [17–19].

After being identified as a unique plant oxygenase containing a Rieske-Type [2Fe-2S] center, CMO was first cloned from spinach by RT-PCR using primers corresponding to amino acid sequences in the iron-sulfur center [20]. Afterwards, it was also cloned from sugar beet [21] Amaranthus tricolor L. [22], and Atriplex prostrat Boucher [23].

Durum wheat is one of the oldest and most important edible cereal crops, of which Italy was the world’s leading producer with a yield of 4.47 million tons in 2017 [24]. Its consistency, together with its high protein content and gluten strength, make it the ideal choice for producing bread, pasta, and bakery products. Durum wheat is well adapted to the Mediterranean environment, but such regions are currently experiencing increasing salt stress problems resulting from seawater intrusion into aquifers [8] and this plant is a salt-sensitive species due to its poor ability to exclude sodium from the leaf blades [10,25]. Therefore, further research on adaptive responses to abiotic stresses is needed.

In a previous study, we found an intriguing inhibition of GB synthesis in durum wheat plants under high light, even in the presence of salt stress, despite the fundamental role of this osmolyte [8]. Subsequently, we discovered that the fine tuning of few specific primary metabolites, such as GABA, amides, minor amino acids, and hexoses, remodeled metabolism and defense processes, playing a key role in the response to salinity and high light [26,27]. However, the reason for GB inhibition in such conditions remained unclear. Therefore, to shed light on the mechanisms underlying the specific inhibition of GB synthesis under high light, we investigated the effects of salinity both in low and high light conditions on the expression of CMO, the first of the two key enzymes involved in its synthesis. From the preliminary results, we had noticed that under high light, no differences were present in BADH expression; therefore, we focused our attention on CMO rather than on BADH. Our study allowed us to isolate thirty-eight TdCMO different transcripts, each one with a unique structural composition. Thirty-seven out of thirty-eight transcripts were non-functional and one, the TdCMO1a6 clone, was in a correct frame of open reading. The deduced amino acid sequence from TdCMO1a6 showed similarity to the Rieske-like iron-sulfur protein.
2. Materials and Methods

2.1. Plant Material and Growth Conditions

Durum wheat (Triticum durum Desf. Cultivar Ofanto genome constitution AABB cod.39) seeds were selected by CRA, Cereal Research Experimental Institute (Foggia, Italy). The growth conditions and the experimental design was performed according to [28,29].

2.2. Isolation of TdCMOTranscripts

Total RNA was extracted from leaf tissue using the RNasy plant RNA extraction kit (Qiagen, Milano, Italy) and poly(A)$^+$. RNA was purified from 700 $\mu$g of leaf RNA using an Oligotex mRNA purification kit (Qiagen). First-strand cDNA was obtained using the Superscript cDNA synthesis kit (Invitrogen, Monza, Italy). RNA samples (2 $\mu$g) were reverse transcribed in a 20 $\mu$L reaction using the Thermo Script RT-PCR system for first-strand cDNA synthesis (Invitrogen, Monza, Italy). Three-microliter aliquots from this reaction were amplified by PCR using the degenerate primers designed for the final 1eson (CMO1 for: $5^{\prime}$-GGCTCGAGCTCGAYCGYGTCT) and final 10 eson (CMO3 rev: $5^{\prime}$-GGCAGTGGAAGTGGTGCATGGC-3) for LeymuschinensisCMO mRNA (ID: GenBank/EU003877), respectively. Primers for actin P1 and P2 were used as the internal control [28]. PCR amplification with Taq DNA polymerase was performed in a 30-cycle reaction under the following conditions: 94 $^\circ$C for 45 s, 56 $^\circ$C for 30 s, 72 $^\circ$C for 60 s. PCR amplification products were purified from the agarose gel and cloned into a pGEM-T Easy Vector system (Promega, Milano, Italy) by mixing 3 $\mu$L of amplified product with 50 ng of pGEM-T Easy Vector, 3U T4 ligase, and 1X ligation buffer in a 10 $\mu$L volume. The ligation product was cleaned with sec-butanol and precipitated with ethanol. The sample was resuspended in 10 $\mu$L of 0.5 $\times$ Tris-EDTA and transformed into Escherichia coli cells. The clones were sequenced using an ABI 377 automated DNA sequencer (Applied Biosystems, Monza, Italy).

2.3. cDNA Sequence Analysis

Sequence data were analyzed and compared to the GenBank-NCBI databases using the BLAST network service [30], ClustalW (http://align.genome.jp/clustalw/) and Mega 6 programs were used to generate most multiple sequence alignments using default settings [31]. All positions containing gaps and missing data were eliminated. The nucleotide sequence data reported are available in the GenBank Data Library under the accession numbers LT627195–LT627232.

2.4. Sequence Analysis and Homology Modeling

Amino acid sequences of Mb from vertebrates were obtained from the Uniprot database (http://www.uniprot.org/). Sequence alignment was performed using the ClustalW tool available on line (http://www.ch.embnet.org/software/ClustalW.html). A model of the 3D structure of common pheasant Mb was obtained using the automated I-TASSER service available at the site https://zhanglab.ccmb.med.umich.edu/I-TASSER/. The online procedure yielded the 3D model on the basis of multiple-threading alignments by LOMETS and iterative TASSER simulations [32].

3. Results

3.1. Choline Monoxygenase Transcripts Isolation

To isolate the putative TdCMO gene transcripts in durum wheat, a RT-PCR analysis was carried out. Total RNA was isolated from young leaves (each sample constituted a pool of 30 leaves) of durum wheat grown in different conditions: control (LLC; 0 mM NaCl, 350 $\mu$mol m$^{-2}$ s$^{-1}$ PAR), high salinity (LLS; 100 mM NaCl, 350 $\mu$mol m$^{-2}$ s$^{-1}$ PAR), high light (HLC; 0 mM NaCl, 900 $\mu$mol m$^{-2}$ s$^{-1}$ PAR), and high light and salinity (HLS; 100 mM NaCl, 900 $\mu$mol m$^{-2}$ s$^{-1}$ PAR). All RT-PCR reactions were performed as triplicates on three independent biological replicates.
Even if the wheat genome has been completely sequenced, both nucleotide sequences and transcripts coding for choline monooxygenase (CMO) protein in *T. durum* genome has never been characterized until now. Therefore, RT-PCR analyses were performed using a degenerate oligonucleotide primer combined with a specific primer. In particular, the CMO1 degenerate primer was designed from the WYTDPGF motif present in the N-terminal region; the CMO3 specific primer was designed on the AMHHFHC motif present in the C-terminus-terminal sequence. The primers were designed on the amino acid sequence alignments belonging to the *Leymus chinensis* Trin. (an. EU003877), *Oryza sativa* L. (an. AJ578494), and *Hordeum vulgare* L. (an. AB434467) species deposited in GenBank. Unexpectedly, the results of the combination of these primers showed two types of transcription products with a different molecular size of 900 bp and 1000 bp, respectively (Figure 1). A primer pair for the β-actin gene was used as the internal control for each RT-PCR reaction. The results of the RT-PCR analysis showed that the 900 bp fragments were strongly expressed in the HLC and HLS samples (Figure 1). Whereas, the 1000 bp fragments were strongly expressed in all samples, with the exception of the LLS sample (Figure 1). The fragments were excised, cloned, and 10 clones for each band were sequenced.

![Figure 1](image-url) Reverse transcription polymerase chain reaction (RT-PCR)-amplified putative choline monooxygenase *(TdCMO)* genes. LLC, cDNA from low light control leaves; LLS, cDNA from low light salt-stressed leaves; HLC, cDNA from high light control leaves; HLS, cDNA from high light salt-stressed leaves. Weight size marker are indicated on the left and the name of the PCR product on the right.

In order to reveal the nature of the isolated transcription products, searches by BLASTN, BLASTX, TBLASTX, and BLASTP were conducted. The results of the transcript analysis showed high homology with the chloroplastic CMO transcripts isolated in different monocots such as *Brachypodium distachyon* L. (LOC100839185), *Sorghum bicolor* L. (LOC8063819), *Panicum hallii* L. (LOC112998400), *Dendrobium catenatum* L. (LOC110093278), *Elaeis guineensis* L. (LOC 105049342), and *Setaria italica* L. (LOC101767591). To examine the *TdCMO* transcript structure and base compositions, a detailed study was done on 80 clones. The obtained nucleotide sequences as well as the deduced amino acid ones were compared by Mega 6. Alignment of nucleotide sequences revealed the presence of 38 different transcripts with a similar molecular size, while the remaining 42 sequences were additional copies of some transcripts (Supplementary Figures S1–S3). Amino acid alignments showed a high conservation of the carboxy-terminal region, unlike the N-terminal region that was highly variable. As a whole, the low conservation observed between the *TdCMO* clones within the N-terminal region was due to the absence of little amino acid stretches within the second exon. In fact, the ExPaSy Proteomics Server (http://www.expasy.ch/tools/dna.html) analysis showed that all *TdCMO* transcripts, except for the *TdCMO1a6* clone, contained premature stop codons in different positions. Stop codons were principally due to the presence of puntiform mutations (deletions, substitutions, insertions) and small insertions/deletions (indel) that changed the reading frame.

In addition, we observed alternative splicing (AS) events in the same transcripts that led to partial exon skipping (ES) and/or intron retention (IR) (Supplementary Figure S1).

### 3.2. Influence of Salt and Light Stresses of the *TdCMO1a6* Expression

To evaluate *TdCMO1a6* expression in LLS, HLC, and HLS compared to LLC, qRT-PCR analysis was performed using a specific primer pair (Figure 2). As shown in Figure 2, LLS *TdCMO1a6* expression was 70% lower than LLC, while HLC expression was about 12-fold higher when compared
to LLC. HLS TdCMO1a6 gene expression was two-fold higher than that of LLC; however, it remained substantially lower than HLC (Figure 2).

**Figure 2.** Relative putative TdCMO1a6 gene expression levels. The experiments were performed on three biological replicates. Transcript levels were normalized using the actin gene. The abbreviations indicate: LLC, cDNA from low light control leaves; LLS, cDNA from low light salt-stressed leaves; HLC, cDNA from high light control leaves; HLS, cDNA from high light salt-stressed leaves.

3.3. Chromosomal Localization and Functional Domain Characterization

In order to determine if putative TdCMO genes were present as orthologous or paralogous in the *T. durum* genome, a search by the Grain Gene (http://wheat.pw.usda.gov/GG3/) and URGI database (https://urgi.versailles.inra.fr/blast/?dbgroup=wheat_all&program=blastn) was performed. Throughout the search, the results showed that putative TdCMO genes were localized on chromosomes of both the A and B sub-genomes. In addition, for the 2AL, 4BL, 6BL, and 7AL chromosomes, putative TdCMO genes were localized on three or more different positions.

Two highly conserved regulatory domains are present within CMO proteins: the iron–sulfur clusters (2Fe–2S) coordinated by two cysteine residues and two histidine residues and mononuclear non-heme iron binding [33]. The analysis of the amino acid conserved domains was conducted by a Motif Finder database search (http://motif.genome.ad.jp).

The alignment of amino acid sequences by Mega 6.0 showed that all 900 bp TdCMO clones lacked the Cys-X-His-(X15-17)-Cys-X-X-His domain because of the deletion of the third exon. The same motif was present only in eight of the 1000 bp TdCMO transcripts (Table 1, Figures S1–S3). Similarly, a mononuclear non-heme Fe binding motif Glu/Asp-X3-4-Asp-X2-His-X4-5-His was conserved in only thirteen of the 1000 bp clones and in seven of the 900 bp ones. Only seven of the 1000 bp clones showed the presence of both conserved domains, however, they were not codified for the presence of stop codons in different positions (Supplementary Figure S1).

In order to obtain structural information on the putative TdCMO1a6 protein, we performed a computational modeling of the 3D structure using the software I-TASSER. The program yielded a 3D model of the protein product with a C-score of 1.01 (Figure 3). The C-score is a confidence score for estimating the quality of predicted models by I-TASSER. The calculation was based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. The C-score is typically in the range of (−5, 2), with higher C-score values for high confidence models and vice-versa. Putative aromatic-ring hydroxylating dioxygenase from *Ruegeria* sp. (pdb code: 3N0Q, 3VCA, 3NQ, 3VCA), cumene dioxygenase from *Pseudomonas fluorescens* (1WQL), and naphthalene 1,2-dioxygenase from *Rhodococcus* sp. (2B1X) were used as structure templates for the I-TASSER algorithm.
Table 1. Clones showing the presence of intact conserved domains. LLC, control plants; LLS, salt stressed plants; HLC, high light stressed plant; HLS, light and salt stressed plants. Amino acids involved in binding domain are underlined.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Clones</th>
<th>Fragments Size</th>
<th>Rieske 2Fe-2S</th>
<th>Non-Heme Fe Binding Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLC</td>
<td>1a2, 1a3</td>
<td>1000 bp</td>
<td>HNVCRHASSLACGSGQ</td>
<td>VFCNYLDGGYH</td>
</tr>
<tr>
<td>LLS</td>
<td>2a1,2a2,2a4,2a5</td>
<td></td>
<td>KTCFOCFPHYGWT</td>
<td>VPYAHGALA</td>
</tr>
<tr>
<td>HLS</td>
<td>4a1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLC</td>
<td>1a2, 1a3, 1a4, 1a5, 1a6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLS</td>
<td>2a1, 2a2, 2a3, 2a5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLC</td>
<td>3a6</td>
<td></td>
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<tr>
<td>HLS</td>
<td>4a6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLC</td>
<td>1b1, 1b5</td>
<td>900 bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLC</td>
<td>3b4</td>
<td></td>
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<tr>
<td>HLS</td>
<td>3b5</td>
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The generated structure (Figure 3) showed that the hypothetical protein belonged mainly to the class characterized by the Rieske-like iron–sulfur domain, responsible for electron transfer [34]. In Figure 3, the specific three-dimensional positions corresponding to the amino acid residues present in mononuclear non-heme iron binding (i.e., Asp187, Asp191, His195, and His195; region A in Figure 3) and the ‘Rieske’ motif (i.e., Val67, Met70, Ala86, and H89; region B in Figure 3) are highlighted. In particular, the amino acid residues necessary for the structure of the non heme-motif, and only the second histidine residue in the Rieske motif were conserved. The 3D model analysis carried out by both the COFACTOR [35] and COACH [36] programs, showed that in the putative TdCMO1a6 protein structure, two ligands were presented. These models deduce protein functions, such as ligand-binding sites, using structure comparison and protein–protein networks. The former contains iron (amino acid residues involved: Asn188, His195, His200, and Asp306) and the latter contains a [Fe2S2] cluster (amino acid residues involved: Cys66, Val68, Ile69, Phe71, Ala86, Tyr88, His89, Gly90, and Trp91).

Figure 3. 3D-molecular model obtained for the putative protein product of TdCMO1a6. In this model, the structure topology is shown. Regions A and B highlight the “non heme-motif” and “Rieske” motif, respectively.
4. Discussion

GB is one of the main osmolytes and protectants present in durum wheat and other cereals during the seedling stage [8], which is, together with germination, the most sensitive phase of durum wheat growth to salinity [37,38]. Its biological functions have been studied extensively in higher plants such as spinach, barley, maize, and durum wheat [8], but little information is available on its synthesis. Several studies have shown no GB accumulation in different plant species. However, only in durum wheat has it been demonstrated that, notwithstanding the unquestioned role of GB as an osmolyte, its synthesis was inhibited under high light, even in the presence of salt stress [8,26].

A preliminary study showed that CMO, but not BADH, expression changed under high light when compared to the control treatment (Supplementary Figure S4); therefore, to shed light on the inhibition of GB under high light, our study focused on the analysis of the TdCMO expression by RT-PCR using degenerate primer pairs.

A total of 38 TdCMO different transcripts were isolated under the different treatments, 37 of which were non-coding for the presence of premature stop codons due to short or long indels. Indels in the TdCMO transcripts were randomly distributed and some of them were conservative or moderately conservative, as previously observed in microsatellite evolution (stepwise mutation model) [39]. It is well known that mutations in the redundant gene copies accelerate the genetic evolution, generating new proteins with different functions [40,41]. Several reports have shown that the transcripts containing premature stop codons can be translated into truncated proteins or small peptides. In plants and animals, small interfering peptides (siPEPs) and micro-proteins (MIPS) have been isolated and described. Functional analysis has shown that they contain specific DNA binding domains that can act positively or negatively on the transcription.

The TdCMO intronic sequence composition was constituted of 70% pirimidine content. In fact, a distinguishing feature is the high composition of UA-rich sequences distributed through the entire length of introns. UA rich sequences are essential for efficient processing, since splicing events occur preferentially between UA-rich introns and GC-rich exons. AS also provides another level of gene regulation by modulating protein productivity, function, and transcript stability. Moreover, about 60% of intron retention occurred in TdCMO transcripts isolated from the HLC or HLS samples, suggesting that alternative splicing could play an important role in modulating the abundance of transcripts in response to individual light stress or combined high light and salinity stresses.

Our results are, thus, consistent with previous studies that showed an increase of alternative splicing with different functions after changes in light intensity [42,43]. In fact, it is well known in higher plants that gene expression may be altered by different transduction signals activated in response to light changes. In addition, light plays an important role in the modulation of chromatin modification and transcriptional activation [44]. Mano et al. [45] reported different light-regulated alternative splicing of two APX protein isoforms located in the stroma and in the thylakoid chloroplast-membrane, suggesting the presence of a new mechanism of mRNA maturation related to peroxisomal transition. Similarly, Wu and co-workers [46] showed a rapid induction of intron retention in chloroplast-encoded ribosomal protein genes in Physcomitrella patens after light stress.

The presence of different transcripts in durum wheat was also due to the presence of duplicated putative TdCMO genes and different allelic forms. In fact, the evolutionary turnover of herbaceous polyploid plants is quite fast. During the course of evolution, upon polyploidization, the TdCMO ancestral sequence could diversify into sub-heterogeneous families, undergoing an independent selective pressure causing the accumulation of different mutations over generations. In addition, the different locus positions confirmed the presence of putative TdCMO genes as orthologous and paralogous in the durum wheat genome. In fact, the comparison of the genomic distribution across the A and B sub-genomes revealed a high number of genes with similar proportions (60.1% to 61.3%), and the average level of synteny for genes located on sub-genome chromosomes was 51% for A and 50% for B [47]. The genetic duplication improves the diversification of gene function as it promotes the accumulation of new mutations on the redundant copy, creating “genome equivalents”
that undergo a lower selective pressure [48]. Several genetic and epigenetic processes are implicated in the regulation of gene duplication, which affects activation, genetic silencing, and loss or new function acquisition deriving from different allelic variants of the same gene. After chromosome duplications, the changes can be stable, or show a gradual and stochastic change [49]. It has been documented by OrthoMCL that for each wheat chromosome, the percentage of genes that has undergone lineage-specific intra-chromosomal duplication is approximately 35%, and for each chromosome, the gene duplicates are on average about 73% [49–51]. The latter phenomenon is due to unequal crossing-over and replication-dependent chromosome breakage or to the activity of transposable elements.

In addition, durum wheat (T. durum, AABB, 2n = 4x = 28) is an allotetraploid formed through hybridization between two separate but related diploid species, T. monococcum or T. urartu (AA, 2n = 14) and an as yet unidentified wild goat grass such as T. searsii or T. speltoides (BB, 2n = 14). Consequently, different allelic variants (isogenes) are present in the durum wheat genome. In fact, one polyploidy consequence is the increase of heterozygosity and the allele frequency changes, which is in contrast to the inbreeding depression. Polyploid genomes produce genotypic and phenotypic ratios deviating from diploid genomes [51], and as a consequence, polyploid plants can evolve more quickly in unexpected directions [51].

Finally, a detailed analysis of the TdCMO amino acid sequences showed that the amino acids involved in the binding domains were present in only a few of the non-codifying clones. In addition, TdCMO1a6, the only transcript present in ORF, lacked the two cysteine residues and one histidine residue involved in the coordination of the Rieske-type [2Fe-2S] cluster. In particular, the first Cys and His were replaced with Val and Met, respectively, while the second Cys was replaced with an Ala.

Hibino and coworkers [52], using site-specific mutations within the CMO protein in spinach, showed that changes of Cys-181 to Ser, Thr, and Ala and His-287 to Gly, Val, and Ala inhibited the accumulation of GB, indicating that Cys-181 and His-287 were essential for the Fe-S cluster and mononuclear Fe-binding, respectively. However, the similarity between the TdCMO1a6 first domain and that of Ruegeria sp., Pseudomonas fluorescens, and Rhodococcus sp. suggests a possible alternative pathway for the production of GB in durum wheat similar to that of bacterial choline oxidase. Sakamoto and Murata [13] reported that plants unable to produce GB, once transformed with bacterial choline oxidase, were able to produce this important compatible osmolyte acquiring resistance to salinity.

Therefore, in our lab, a number of experiments are in progress to isolate the entire transcript codified by this enzyme and verify its catalytic capacity.

**Supplementary Materials:** The following are available online at [http://www.mdpi.com/2073-4395/8/11/270/s1](http://www.mdpi.com/2073-4395/8/11/270/s1), Figure S1: Alignment of the nucleotide sequences of the TdCMO clones, Figure S2: Alignment of the deduced amino acid sequences of the 1000 bp TdCMO clones, Figure S3: Alignment of the deduced amino acid sequences of the 900 bp TdCMO clones, Figure S4: TdCMO and TdBADH gene expression changes and GB accumulation.

**Author Contributions:** P.W., P.C., and L.F.C. conceived and designed the project; P.W., L.F.C., I.K., M.G.A., A.M., and F.I. carried out the experiments; P.W., A.D.M., and L.F.C. analyzed and discussed the data; P.W., L.F.C., P.C., and A.D.M. wrote the manuscript; and A.F. contributed reagents/materials and revised the manuscript.

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**Conflicts of Interest:** The authors declare that they have no conflicts of interest.

**References**


34. Iwata, S.; Saynovits, M.; Link, T.A.; Michel, H. Structure of a water soluble fragment of the ’Rieske’ iron-sulfur protein of the bovine heart mitochondrial cytochrome bc1 complex determined by MAD phasing at 1.5 Å resolution. Structure 1996, 4, 567–579. [CrossRef]


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