J Appl Biotechnol Rep. 2024 December;11(4):1488-1496

doi 10.30491/jabr.2023.407817.1653

Journal of
Applied Biotechnology Reports

Original Article

Sequence Identification and Analysis of the 5′ Untranslated Region of *UGT76G1* gene in *Stevia rebaudiana*

Atefeh Amini Neisiani [1], **Abbas Saidi** [1*], **Masoud Tohidfar** [1*]

[1] Department of Cell & Molecular Biology, Faculty of Life Sciences & Biotechnology, Shahid Beheshti University, Tehran, Iran

Corresponding Author: Masoud Tohidfar, PhD, Professor, Tel: +98-9124627014, E-mail: m_tohidfar@sbu.ac.ir; Abbas Saidi, PhD, Professor, E-mail: a-saidi@sbu.ac.ir. Department of Cell & Molecular Biology, Faculty of Life Sciences & Biotechnology, Shahid Beheshti University, Tehran, Iran.

Received July 19, 2023; **Accepted** October 30, 2023; **Online Published** December 30, 2024

Abstract

Introduction: *Stevia rebaudiana* contains steviol glycosides responsible for their sweet taste. One of the most important enzymes in the steviol glycosides production pathway in *Stevia rebaudiana* is the UGT76G1 enzyme, which is involved in the conversion of stevioside to rebaudioside A. The aim of this study was to identify the 5'-UTR of the *UGT76G1* gene and investigate the cis-elements present in this region.

Materials and Methods: The promoter and gene sequences of this enzyme were obtained from the NCBI database. To identify the 5'-UTR region, a pair of primers was designed. The pure amplified fragment was cloned into the pTG19 vector and transformed into *E. coli* XL1-Blue bacteria. Sequencing data were analyzed, and a fragment with a length of 944 nucleotides was identified as the 5'-UTR. Then, the cis-elements in this sequence were investigated using PlantCARE and PlantPAN.

Results: The 5′UTR sequence has been submitted to the NCBI database with the accession number OP897301. It was found that 65% of the elements in the 5′-UTR are responsible for regulating gene transcription, confirming the regulatory role of this sequence.

Conclusions: The identification of regulatory regions and cis-elements in this region can be used in future research to regulate gene expression.

Keywords: 5' UTR, Cis-Regulatory Elements, Regulatory Sequences, UDP-Glycosyltransferase 76G1

Citation: Amini Neisiani A, Saidi A, Tohidfar M. Sequence Identification and Analysis of the 5′ Untranslated Region of *UGT76G1* gene in *Stevia rebaudiana*. J Appl Biotechnol Rep. 2024;11(4):1488-1496. doi:10.30491/jabr.2023.407817.1653

Introduction

Stevia rebaudiana Bertoni, a dicotyledonous plant from the Asteraceae family, is cultivated in many tropical and subtropical countries.[1-4] Stevia is commonly known as a sweet leaf, sugar leaf, honey leaf, and sweet herb.[5-9] The sweet taste of the leaves is due to the presence of diterpenoid glycosides containing the steviol aglycon core.[10-13] Many enzymes are involved in the metabolic pathway of these sugars' production. One of the most important of these enzymes is UGT76G1 (UDP-glycosyltransferase 76G1), which plays a role in the conversion of stevioside to rebaudioside A.[14,15] Therefore, the regulation of the expression of this gene can play an effective role in the amount of rebaudioside A. Transcriptional regulation, which is controlled by cis-acting elements in the promoters and 5'-UTRs (5'-untranslated region) of genes, plays the largest role in activating and suppressing gene expression.[16] At the transcriptional level, different regulatory sequences are involved in gene expressions, such as enhancers, silencers, insulators, and cis-regulatory elements.[17] Transcriptional regulation depends on the presence and activity of transcription factors, as well as the type, number, position, and combination of existing regulatory elements.[17] Regulation of gene expression at the promoter level is mainly controlled by cis-regulatory elements (CREs) present in the region upstream of the transcription start point. Depending on the distance between the regulatory elements and the central part of the promoter, regulatory proteins and transcription machinery interact physically.[18] Regulatory elements located far from the promoter also influence gene expression. The mechanism of action of these elements involves 3D conformational folding in the DNA structure and chromatin during transcription, bringing the end elements into proximity with the central part of the promoter to modulate gene expression.[19] This means that the promoter and 5'-UTR are sequences located upstream of the gene coding region (before the translation initiating codon) and contain several active cis-elements that serve as specific binding sites for proteins involved in the regulation and initiation of gene expression.[16]

Li et al. (2020) analyzed the MYB transcription factor gene promoter in grapevine. The upstream cis-regulatory elements and their functions were identified, and their results showed that the TCA 1 and 2, ABRE, MYC, and AF1-3 binding site 1 are key CREs and play an important role in

plant biotic/abiotic stress resistance. In another study conducted by Zheng et al. in 2022, the gene promoter involved in the synthesis of Damarenediol synthase, a key enzyme in the saponin synthesis pathway of *Panax notoginseng*, was analyzed. The results of this research showed that DS promoters contain two main types of cis-active elements: one is stress response elements, and the other is growth regulatory elements and light response elements. The 5'UTRs have also been reported to alter gene regulation in prokaryotes and eukaryotes based on changes in temperature, pH, and other metabolites.[21] Utilizing transcriptomic data and bioinformatics analysis, Cho et al (2017) searched for natural cis-regulatory regions in *Zymomonas mobilis*. They identified the potential regulatory role of 5′UTRs and confirmed the responsiveness of 36 5′UTR candidates to acetate, xylose, and ethanol stresses *in vivo* fluorescence-based screening systems. Under ethanol stress, the 5′UTR of the *ZMO0347* gene was found to down-regulate the downstream gene expression.[22] In this research, the 5'UTR region of the *UGT76G1* gene was identified for the first time, and then the cis-elements of this region were investigated as a preliminary step to gene expression regulation in subsequent studies.

Materials and Methods

Disinfection and Seed Planting

In this experiment, Chinese species of *Stevia rebaudiana* seeds received from the Agricultural Biotechnology Research Institute of Iran were used. The seeds were soaked in 70% (v/v) ethanol for 30 seconds and then washed with sterile water. Subsequently, the seeds were immersed in a 2% commercial sodium hypochlorite (NaOCl) solution for 15 minutes. To eliminate any residual sodium hypochlorite, the seeds were rinsed four times with sterile water under a laminar flow. The sterilized seeds were then cultured under controlled conditions in MS medium containing 30 g/L sucrose, 7 g/L agar, and 4.4 g/L MS (Murashige and Skoog) medium (Duchefa, Haarlem, The Netherlands) under controlled environmental conditions of 24 ± 2 ºC, 65% humidity, and a photoperiod of 16/8 hours (16 hours light/8 hours dark).

DNA Extraction

DNA extraction was performed from the leaves of 4-week-old plants using a DNA extraction kit from Dena Zist Asia Company (Iran). Electrophoresis was then carried out to observe the extracted DNA on a 1% agarose gel.

Bioinformatics Analysis

The promoter and target gene sequences were obtained from the NCBI database. The promoter sequence with accession number KM206772.1 (https://www.ncbi.nlm.nih.gov/nuccore/KM206772) and the gene with accession number KC631816 (https://www.ncbi.nlm.nih.gov/nuccore/KC631816) were used. To find the sequence of the 5'-UTR, the promoter and gene sequences were blasted with the whole genome sequence of Stevia (available in contig) at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_SPEC=Assembly&ASSEMBLY_NAME=GCA_009936405.2). After identifying the blasted shotgun sequence with the promoter sequence, approximately 2000 nucleotides downstream of the promoter and about 2000 nucleotides upstream of the gene sequence were saved in FASTA format. The two regions obtained were aligned.

Primer Design

The NCBI sequences related to the promoter and target gene were used to design the primers. The forward primer (P1) and reverse primer (P2) were designed based on 200 nucleotides at the end of the promoter and 200 nucleotides at the beginning of the gene, respectively (Figure 1 and Table 1). Two software programs, Vector NTA and Oligo, were used for the primer design.

Figure 1. Schematic Diagram Showing the Location of the Two PCR Primers.

Table 1. Technical Specifications of the Used Primers

Gene position	Primer name	Primer sequence	size (bp)	Temp PCR (°C)
Between the promoter and the UGT76G1 gene	P1	AACAACCTGGACCACACAAG	1200	62.6
	P2	GGCTAGCTGAAGAATTGGG		63

PCR Condition

Polymerase chain reaction amplifications were performed in a final volume of 25 µl containing 15 µl 2X Master Mix (Ampliqon, Iran), 100 ng of Genomic DNA, and designed primers. The thermal program for the PCR was as follows: an initial denaturation step at 94 °C for 5 min followed by 35 cycles consisting of 94 °C for 1 min (denaturation), 64 °C for 1 min (annealing), 72 °C for 90 s (extension), and a final extension step at 72 °C for 5 min followed by a hold at 4 °C. In order to identify the position and purpose of stripe bands of the fragment, 5 µl PCR amplification products were mixed with 2 µl of 6X loading dye and electrophoresed on a

1% agarose gel.

Cloning and Transfer of Plasmid to Bacteria

Then, the target fragment was isolated from the gel and purified using Roche's purification kit. The purified sample was cloned into a pTG19 vector using the TA cloning kit from Sinaclon Company (Iran). The recombinant vector was then transformed into competent *E. coli* XL1-Blue cells. The recombinant bacterial colony was grown on selective medium containing ampicillin. The recombinant plasmid was extracted using the plasmid extraction kit from Borna Pouyesh Gene Company (Iran). The extracted recombinant pTG19 plasmid was tested by PCR and BamHI restriction enzyme digestion to confirm the transformation and presence of the target genes.

Sequencing and Registration at NCBI

The desired fragment in the recombinant plasmid was sequenced in both directions. Then, the gaps were removed from the sequenced fragment using Mega software, and assembling the sequences and making contigs were done using Vector NTA software and the CAP3 program. The nucleotide fragment was registered in the NCBI database.

Sequenced Fragment Analysis

The sequenced fragment was scanned for the presence of cis-acting regulatory elements based on the online tools PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PlantPAN (http://plantpan.itps.ncku.edu.tw/). The Promo database was used to identify transcription factors that may bind to this sequence.

Results

Seed Planting

The Stevia seed grew well when cultured in the MS medium (Figure 2).

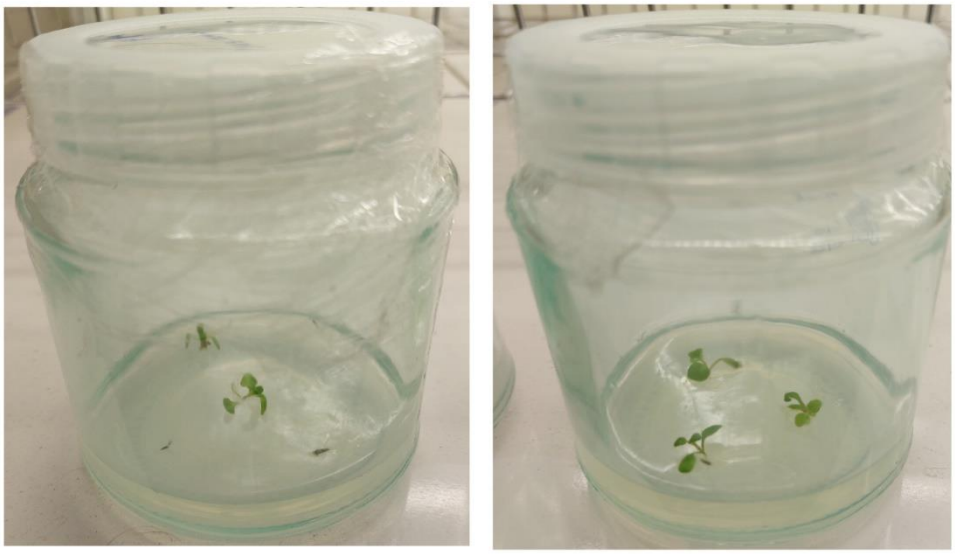

Figure 2. Plants Were Grown in MS Medium (from left to right, 9 and 12 days after seed sowing).

Bioinformatics Analysis

The results of bioinformatics analysis and BLAST of the promoter (1994 nucleotide length) and UGT76G1 gene sequences with the whole genome sequence of the Stevia plant showed that the 5'-UTR region is about 1000 nucleotides in length. The results related to PCR showed a band of about 1200 nucleotides (including 100 nucleotides from the end of the promoter, the 5'-UTR with 1000 nucleotides in length, and 100 nucleotides from the beginning of the gene) (Figure 3). According to Figure 3, there was no amplification in the sample without DNA (control), indicating the absence of contamination in the PCR reaction. However, the presence of 1200 bp nucleotides in the PCR amplification confirmed the expected product from the bioinformatics analysis.

To determine the target fragment sequence, the band was isolated, purified, and cloned. After transferring the recombinant plasmid to the bacteria, the putative recombinant bacteria grown on the culture medium containing ampicillin were selected for further plasmid extraction.

In order to test the transformation accuracy, PCR (using designed primers) and restriction enzyme digestions (*Bam*HI) were performed (Figure 4). After enzymatic digestion of the plasmid extracted from the bacteria, two bands were observed on the gel. Considering that the length of the plasmid was 2880 nucleotides and the length of the desired fragment was about 1200 nucleotides, bands with the same length were observed after enzymatic digestion. The PCR of the extracted vector from the bacteria showed a band that according to the ladder, was about 1200 nucleotides in length,

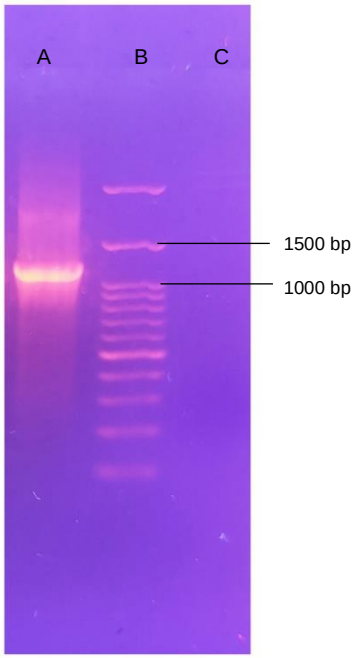

Figure 3. PCR Products with Specific Primers for the 999 5'-UTR Region. The PCR products are visualized on a 1% agarose-TAE gel containing Ethidium bromide. The lanes, from left to right, show: lane A: PCR products showing the amplification of a fragment with a length of about 1200 bp; lane B: DNA ladder (100 –3000 bp) (CinnaGen, Iran); lane C: sample without the DNA template (control).

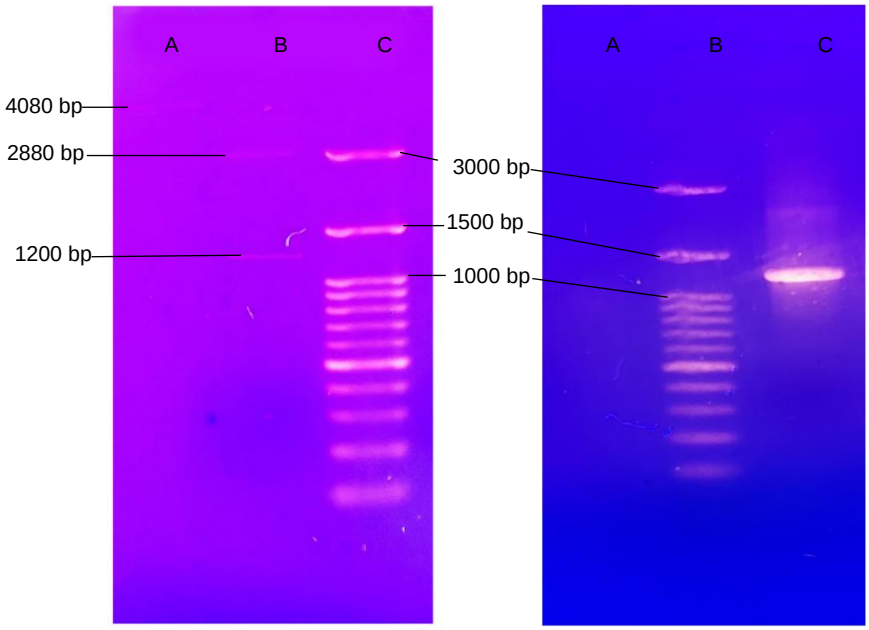

Figure 4. Confirming the Presence of the Desired Fragment in the Extracted Vector**.** Left Figure: The digestion patterns of purified pTG19 recombinant vector using *Bam*HI restriction enzyme on a 1% agarose gel electrophoresis (lane A: uncut pTG19 (about 4080 bp) recombinant vector; lane B: digested plasmid by *Bam*HI including 2880 bp and 1200 bp, and lane C: The DNA ladder (100–3000 bp) (CinnaGen, Iran), respectively). Right Figure: The PCR products in pTG19 transformed recombinant vector and non-recombinant vector (control) on 1% agarose gel electrophoresis (lane A: PCR product of pTG19 transformed recombinant vector, lane B: The DNA ladder (100–3000 bp) (CinnaGen, Iran), and Lane C: PCR product of non-recombinant vector).

confirming the presence of the target fragment in the plasmid. Based on these results, it was shown that the vector was successfully transformed into the host, and the recombinant vector was intended to be produced.

The fragment was sequenced with high quality. The sequence assembly results using Vector NTA software and CAP3 program were the same (data not shown). A fragment with a length of 944 nucleotides was registered in NCBI as

the 5'-UTR of the *UGT76G1* gene with the accession number OP897301.

5'-UTR Analysis

Stevia is a dicotyledonous plant, and according to bioinformatics studies, there is a *UGT76G1* gene sequence on the + strand. In this analysis, only cis-elements present on the + strand and similar to the ones previously reported in dicot plants were selected and are shown in Figure 5 and Table 2.

Table1. The List of Regulatory Elements Predicted on the Upstream Sequence of 5'-UTR of *UGT76G1* Gene Using PlantCARE. This table includes the type, sequence, location, function, and organism containing these cis-elements on the UTR.

Type of cis-element	Sequence	Location	Organism	Function of cis-element
STRE	AGGGG	257	Arabidopsis thaliana	
STRE	AGGGG	355	Arabidopsis thaliana	
MYB-like sequence	TAACCA	854	Arabidopsis thaliana	
MYB	TAACCA	854	Arabidopsis thaliana	
DRE core	GCCGAC	877	Arabidopsis thaliana	
MRE	AACCTAA	283	Petroselinum crispum	MYB binding site involved in light responsiveness
CAAT-box	CAAAT	13	Pisum sativum	common cis-acting element in promoter and enhancer regions
CAAT-box	CAAAT	175	Pisum sativum	common cis-acting element in promoter and enhancer regions
CAAT-box	CAAAT	324	Pisum sativum	common cis-acting element in promoter and enhancer regions
CAAT-box	CAAT	394	Nicotiana glutinosa	
CAAT-box	CAAAT	623	Pisum sativum	common cis-acting element in promoter and enhancer regions
CAAT-box	CAAAT	723	Pisum sativum	common cis-acting element in promoter and enhancer regions
CAAT-box	CAAAT	734	Pisum sativum	common cis-acting element in promoter and enhancer regions
CAAT-box	CCAAT	782	Arabidopsis thaliana	common cis-acting element in promoter and enhancer regions
CAAT-box	CAAT	783	Nicotiana glutinosa	
CAAT-box	CAAT	825	Nicotiana glutinosa	
MYC	CATTTG	239	Arabidopsis thaliana	
AE-box	AGAAACAA	231	Arabidopsis thaliana	part of a module for light response
TATA-box	TATA	86	Arabidopsis thaliana	core promoter element around -30 of transcription start
TATA-box	TATA	141	Arabidopsis thaliana	core promoter element around -30 of transcription start
TATA-box	ATATAT	201	Brassica napus	core promoter element around -30 of transcription start
TATA-box	TATA	202	Arabidopsis thaliana	core promoter element around -30 of transcription start
TATA-box	TATTTAAA	483	Arabidopsis thaliana	core promoter element around -30 of transcription start
TATA-box	TAAAGATT	590	Arabidopsis thaliana	core promoter element around -30 of transcription start
TATA-box	ATTATA	595	Brassica napus	core promoter element around -30 of transcription start
ATC-motif	AGTAATCT	672	Spinacia oleracea	part of a conserved DNA module involved in light responsiveness

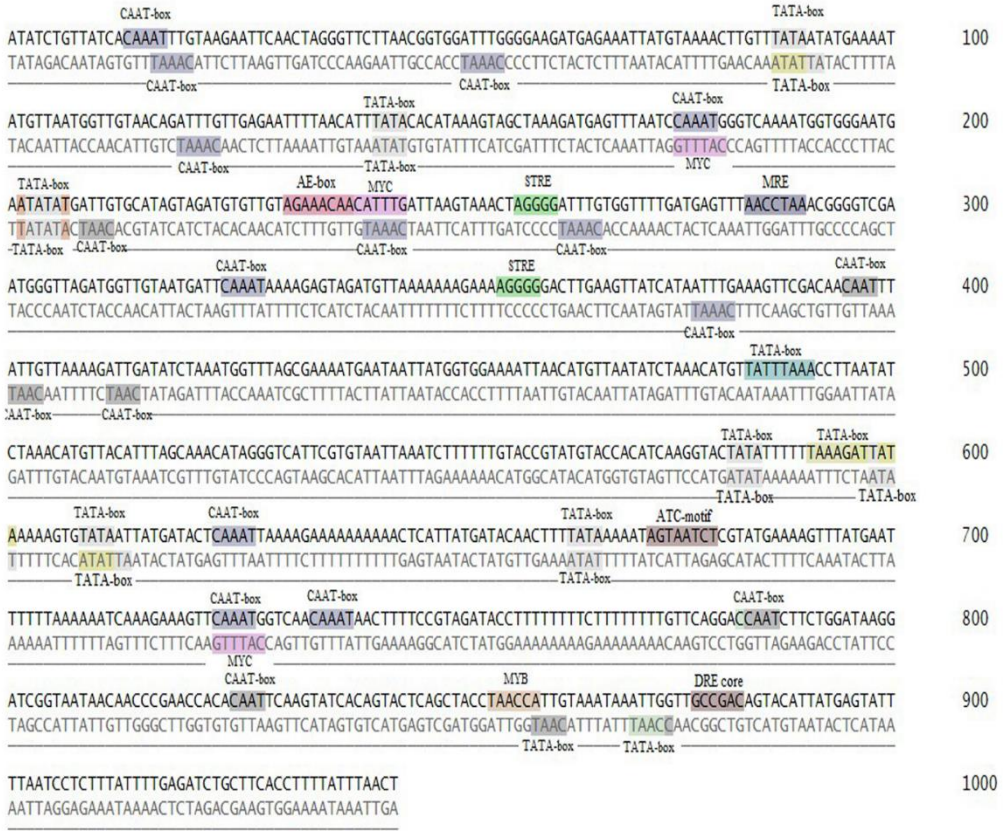

Figure 5. This Figure Shows the Presence of Cis-regulatory Element Regions Obtained from PlantCARE Database.

According to Table 2 and Figure 5, in the 5'-UTR of the UGT76G1 gene, the first TATA box is located at -349 position with the conserved TATA sequence, and the first CAAT box is located at -119 position. The cis-elements identified in the 5'-UTR sequence of our target gene in dicotyledonous plants (on the sense strand) included 2 MYB sequences, 1 MYC sequence, 7 TATA boxes, 10 CAAT box, 2 STRE sequences, 1 DRE core, 1 MER sequence, 1 AE box, and an ATC motif. Based on the results of the analysis of the cis-elements in the PlantPAN site, the highest similarity score in the cis-elements in this sequence was 1. In Figure 6, all cis-elements with a score of 1 are shown.

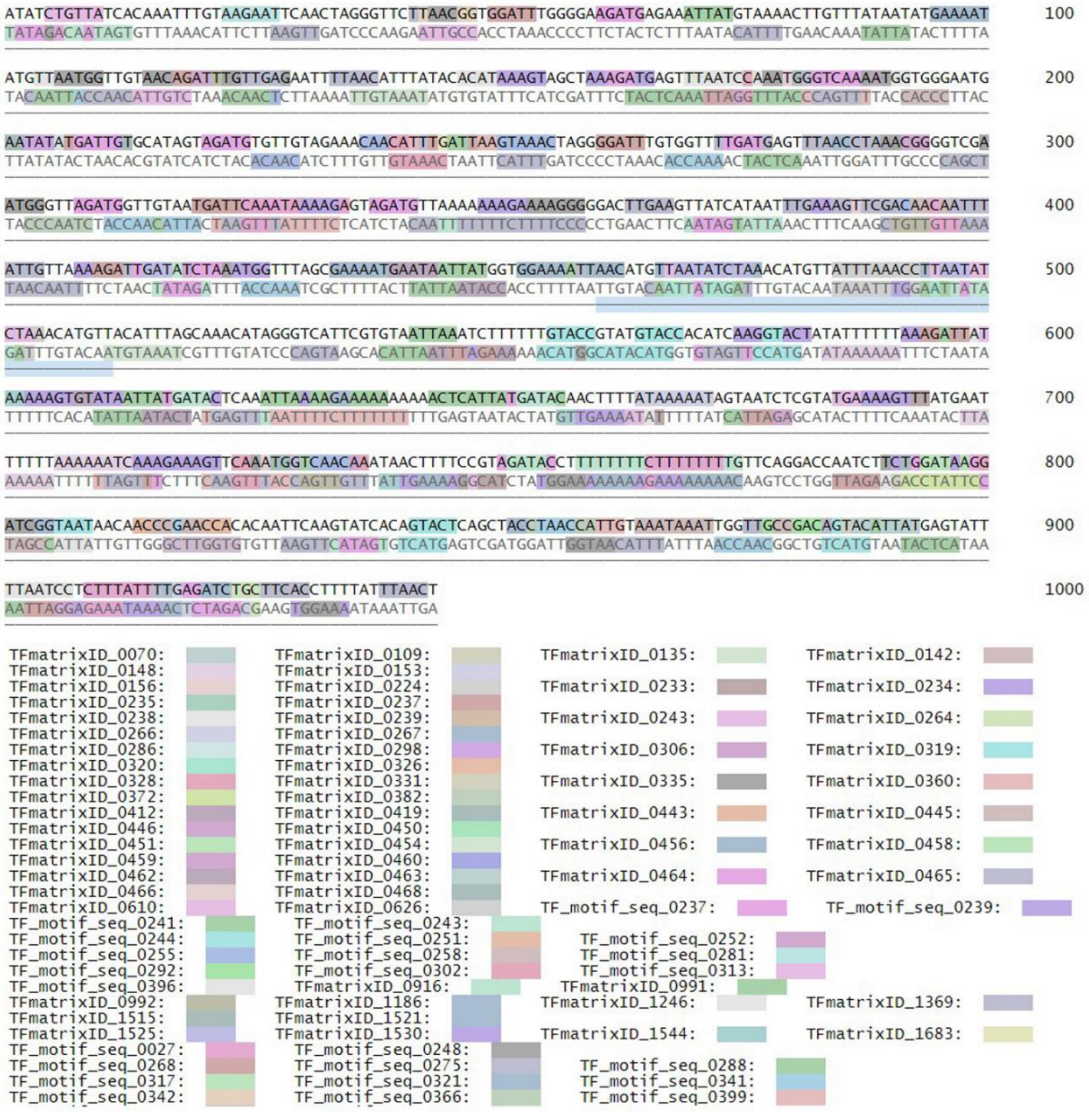

Figure 6. The cis-elements on the UTR sequence with the highest similar score.

Fragment analysis using PlantPAN and plantCARE produced similar cis-elements involved in the 5'-UTR of the UGT76G1 gene. The score range of the available cis-elements was between 0.1 and 1. The most common cis-elements with a score of 1 included MYB (which plays a role in the regulation of growth, development and secondary metabolic biosynthesis pathways,[23,24] WRKY (which plays a role in physiological processes, biotic and abiotic stresses,[25,26] AP2 (involved in the regulation of development and plant growth, defense response, fruit ripening, and metabolism,[27] and DOF (involved in the regulation of metabolism, hormonal regulation, photoperiodic regulation, phytohormone responses, and other aspects of plant development.[28]

Twenty-one plant transcription factors connected to this sequence with less than 15% dissimilarity were identified. Among the identified transcription factors, 33% played a role in plant growth and development. 47% of the transcription factors were involved in biotic and abiotic stresses. Approximately 10% of the transcription factors were involved in secondary metabolite production. Ten percent of the effective transcription factors in response to light were identified. In this sequence, the highest positions

were attributed to MYB2 and HSF1 transcription factors.

Discussion

The UTR sequence is located upstream of the translation start point and can be used to regulate gene expression. This region contains cis-elements that are responsible for binding trans factors. Transcription is initiated by the core promoter, which is composed of the TATA box, the CAAT box, the Inr (initiator region), and the DPE (downstream promoter element).[29] A number of other cis-elements can regulate the transcription of a given gene with spatial, temporal, and stress-triggered factors.[30] The composition of the 5'-UTR in this variety includes 27% guanine-cytosine bases and 73% adenine-thymine bases.

It is possible to engineer transcription factors that are related to cis-regulatory elements to regulate gene expression. The 5'-UTR sequence of the UGT76G1 gene can be used to regulate the expression of our target gene. The presence of a large number of TATA and CAAT box sequences in the identified 5'-UTR, involved in the initiation and enhancement of elements at the beginning of transcription, confirms that this is the regulatory region.[31] The TATA-box acts as cis-elements in the core promoter element around -30 of transcription start,[32,33] and the CAAT-box acts as a regulatory element.[34]

Previous study has shown that translation initiation can be controlled by 5' UTR structures in eukaryotic mRNAs.[34] Additionally, the secondary structures of 5'UTR can affect initiation efficiency at suboptimal start codons. By stalling the scanning 40S subunit near the start codon, a strong stem-loop structure will increase the dwell time, limiting leaky scanning through near-cognates or AUG triplets in a poor context.[35] A 5′-UTR sequence encodes a number of cis-regulatory elements, including a 5′-cap structure, a translation initiation motif, and an upstream AUG (uAUG)[36] which can control gene expression in this way.

A high abundance of TATA-box and CAAT-box in regulatory regions has been reported in other studies.[37] In this study, the identified MYC and MYB in the UTR play important roles in the activation of the ABA-dependent pathway, and many active proteins in this pathway are regulated by members of this family of transcription factors. During this research, it was found that 65% of the elements in the 5'-UTR are responsible for regulating gene transcription, confirming the regularity of this sequence.

According to the results of the analysis of cis-elements using PlantPAN, which showed a high frequency of MYB, DOF, and AP2 regulatory elements in the sequence, it is possible to establish a relationship between this regulatory sequence and the gene involved in the production of secondary metabolites. The MYB2 transcription factor has various functions in different plants, including the regulation of secondary metabolite production, the regulation of dehydration-responsive gene expression, the induction of hypoxia gene expression, the regulation of lignin formation in cell walls, and fruit color change.[38] The HSF1 transcription factor regulates heat shock protein expression, indicating activity under both biotic and abiotic stresses.[39] The analysis of transcription factors has revealed that MYB2 occupies the highest position in this sequence, confirming its effectiveness in regulating secondary metabolites.

The expression of proteins is dependent on the induction of gene transcription, and as such the identification of regulatory factors, regulatory regions, and promoters is very important.[40,41] For the functional description of gene expression, structural analysis of the upstream region of the gene is required.

Conclusion

In this study, for the first time, the 5' UTR region was identified based on the promoter end and the beginning of the *UGT76G1* gene. Examination of the cis-elements in this region and the presence of CAAT-box and TATA-box regions with high frequency confirmed the regulatory nature of this region. Finally, the high frequency of the MYB, DOF, and AP2 cis-elements indicates that this sequence is related to the gene involved in the biosynthetic pathway of secondary metabolites.

Authors' Contributions

Study concept and design by MT and AAN; Analysis and interpretation of data by MT and AAN. Drafting of the manuscript and critical revision of the manuscript for important intellectual content by MT, AS, and AAN.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgment

The authors express their sincere gratitude to Dr. Foad Moradi for providing the *Stevia rebaudiana* seeds.

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