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Identification of a 301 bp promoter core region of the *SrUGT91D2* gene from *Stevia rebaudiana* that contributes to hormone and abiotic stress inducibility

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Abstract

Background The UDP-glucuronosyltransferase 91D2 (*SrUGT91D2*) gene is a crucial element in the biosynthetic pathway of steviol glycosides (SGs) and is responsible for creating 1,2-β-D glucosidic bonds at the C19 and C13 positions. This process plays a vital role in the synthesis of rebaudioside M (RM) and rebaudioside D (RD). The promoter, which regulates gene expression, requires functional analysis to understand gene expression regulation. However, investigations into the function of the promoter of *SrUGT91D2* (*pSrUGT91D2*) have not been reported.

Results The *pSrUGT91D2* was isolated from six *S. rebaudiana* lines, and subsequent multiple sequence comparisons revealed the presence of a 26 bp inDel fragment (*pSrUGT91D2*-B1188 type) in lines GP, GX, 110, 1114, and B1188 but not in the *pSrUGT91D2* of line 023 (*pSrUGT91D2*-023 type). Bioinformatics analysis revealed a prevalence of significant cis-regulatory elements (CREs) within the promoter sequences, including those responsive to abscisic acid, light, anaerobic conditions, auxin, drought, low temperature, and MeJA. To verify the activity of *pSrUGT91D2*, the full-length promoter and a series of 5′ deletion fragments (P1–P7) and a 3′ deletion fragment (P8) from various lines were fused with the reporter β-glucuronidase (GUS) gene to construct the plant expression vector, pCAMBIA1300-pro::GUS. The transcriptional activity of these genes was examined in tobacco leaves through transient transformation. GUS tissue staining analysis and enzyme activity assays demonstrated that both the full-length promoter and truncated *pSrUGT91D2* were capable of initiating GUS expression in tobacco leaves. Interestingly, *P8-pSrUGT91D2*-B1188 (containing the inDel segment, 301 bp) exhibited enhanced activity in driving GUS gene expression. Transient expression studies of *P8-pSrUGT91D2*-B1188 and *P8-pSrUGT91D2*-023 in response to exogenous hormones (abscisic acid and indole-3-acetic acid) and light indicated the necessity of the inDel region for P8 to exhibit transcriptional activity, as it displayed strong responsiveness to abscisic acid (ABA), indole-3-acetic acid (IAA), and light induction.

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Conclusions These findings contribute to a deeper understanding of the regulatory mechanism of the upstream region of the *SrUGT91D2* gene and provide a theoretical basis for future studies on the interaction between CREs of *pSrUGT91D2* and related transcription factors.

Keywords *Stevia rebaudiana* Bertoni, *SrUGT91D2*, Promoter, *GUS*, Exogenous hormones, Light

Background

Stevia rebaudiana Bertoni is an herbaceous perennial plant belonging to the Stevia genus within the Asteraceae family. Known for its naturally sweet-tasting leaves, it has been revered as 'sweet food' by South Americans for centuries and is eaten by various monikers such as 'sweet leaf,' 'honey leaf,' 'sweet herb,' or 'sweet weed' [1]. The primary natural sweetening compounds found in the leaves are steviol glycosides (SGs). With respect to high purity, these products are white, and the sweetness of the mixture is 250–300 times greater than that of sucrose, with a calorie content less than 1/300 of sucrose. Due to its intense sweetness and minimal caloric content, SGs are widely applied in the diet, medicine, daily necessities, and the chemical industry [2–4].

To date, researchers have isolated and identified more than three dozen steviol glycosides, primarily including stevioside (ST), 2 ducosides, 15 rebaudiosides, 2 stevioside monosides, and 3 steviolbiosides [5]. Stevioside is the most abundant compound in stevia leaves, comprising 5–10% of the dry leaf weight, followed by rebaudioside A (RA) at approximately 2–4%, and rebaudioside C (RC) at a lower 1–2%. Moreover, rebaudioside M (RM) and rebaudioside D (RD) are less prevalent, constituting only 0.4–0.5% of the dry leaf weight [6–8]. Both ST and RA glycosides are approximately 250–300 times sweeter than sucrose but possess a more pronounced postbitterness, whereas RC is only 30 times sweeter than sucrose [9, 10]. On the other hand, RD and RM, despite their lower abundance in stevia, are 350 times sweeter than sucrose and exhibit virtually no bitter aftertaste, rendering them the most desirable glycosides in SGs at present [7].

The production of stevia glycosides involves the coglycosylation of four glycosyltransferases. Notably, RD glycoside is exclusively formed by the glycosylation of RA glycoside facilitated by the glucosyltransferase *SrUGT91D2*, or through a sequence of glycosylation processes involving *SrUGT91D2*, *SrUGT76G1*, and *SrUGT74G1* for 13-SMG, or by the glycosylation of rubusoside by *SrUGT91D2* and *SrUGT76G1*. Moreover, RM is formed solely by the glucosyltransferase *SrUGT76G1* via the glycosylation of RD [10]. Notably, the activity of the *SrUGT91D2* gene decreases significantly with an increase in the number of glucose moieties on the glycoside, as documented by Kishore and Wang [11, 12]. Zhang et al. attributed the low production of Reb D and Reb M in native plants to the weak glycosylation of glucose 2-1-R2 by *SrUGT91D2* [13].

Previous research has established a direct correlation between the expression levels of *SrUGT91D2* and the content of RD [14]. Consequently, the genes *SrUGT76G1* and *SrUGT91D2* play a crucial role in the synthesis of RD and RM. Investigating the regulation of gene expression is important for understanding the biosynthesis of RD and RM, as well as for facilitating potential artificial regulation of their synthesis through genetic engineering and other methods. The exploration of gene promoters is a critical field in gene expression regulation and essential for understanding the pivotal role played by the expression regulation of the key genes *SrUGT91D2* and *SrUGT76G1* in the glycoside synthesis process. However, while Yang et al. conducted the cloning and bioinformatic analysis of the promoter of the *S. rebaudiana SrUGT76G1* gene in 2018, cloning and functional studies of the promoter of the *SrUGT91D2* gene (*pSrUGT91D2*) have not been performed [15].

In our research, we designed primers based on the stevia genome sequence [GCA_009936405.1_ASM993640v1_genomic], and we cloned the *pSrUGT91D2* gene, which is pivotal for glycoside synthesis in stevia, using conventional PCR on six stevia lines with varying glycoside compositions and contents, which were all collected and bred by our research groups. We created a series of *pSrUGT91D2* and GUS fusion expression vectors with deletion sequences at their 5′ ends and conducted GUS histochemical staining after Agrobacterium-mediated transient infiltration of tobacco leaves. Furthermore, we measured *pSrUGT91D2* activity through the GUS protease activity. Our initial findings revealed that the *pSrUGT91D2* in the 'B1188' line exhibited the strongest ability to drive the GUS enzyme-encoding gene, while that of the '023' line was the weakest. Additionally, we observed a 26 bp insertion-deletion (inDel) in the promoter sequence of the *SrUGT91D2* gene in the 'B1188' line compared to that in the '023' line. Based on the predicted distribution of CREs in the *pSrUGT91D2* region of 'B1188', we created a series of GUS fusion expression vectors (P1–P8) containing deletion sequences at either the 5′ end or the 3′ end. Through this process, we identified the core region of the promoter as P8, which contained a 26 bp inDel. Subsequently, we applied indole-3-acetic acid (IAA), abscisic acid (ABA), and light using spraying techniques to investigate how P8 responded to hormonal and abiotic stress treatments. This investigation has the

potential to improve the understanding of the expression regulation pattern of the *SrUGT91D2* gene and lay a strong theoretical foundation for the molecular breeding of superior quality stevia.

Materials and methods

Materials

We obtained a collection of six distinct lines of *S. rebaudiana*, namely 1114, 110, GX, 023, B1188, and GP, from a dedicated experimental field at Sichuan Agricultural University. These lines were carefully identified by Prof. Wei Wu. For detailed information on the concentrations or types of SGs in these genotypes, please refer to our previous publication [16]. The B1188 strain exhibited relatively homogeneous contents of various types of glycosides, with RD and RM reaching concentrations of 0.13% and 3.10%, respectively, surpassing those reported in other stevia varieties. Before extracting genomic DNA, all samples were thoroughly rinsed with tap water, followed by distilled water. Our laboratory maintains stocks of *Agrobacterium tumefaciens*, *Escherichia coli*, and *Nicotiana tabacum*.

Tobacco (*Nicotiana benthamiana*) seeds were planted in a phytotron using a substrate mixture containing vermiculite, perlite, and soilrite at a 1:1:2 ratio. These tobacco plants were cultivated under controlled conditions in a phytotron, with a temperature of 25 °C and a 16-hour day length, followed by a temperature of 20 °C during an 8-hour dark photoperiod. The relative humidity was maintained at 60%. The pCAMBIA1300::GUS plasmid, derived from the pCAMBIA1300 plasmid, was obtained from our laboratory and modified accordingly. The recombinant vectors were subsequently cloned and propagated using the DH5α strain of *E. coli*, which was obtained from Shanghai Weidi Biotechnology Co., Ltd. For plant transformation, we used the *A. tumefaciens* strain GV3101, which was also obtained from Shanghai Weidi Biotechnology Co., Ltd.

Cloning and analysis of *the SrUGT91D2* promoter sequence of *S. rebaudiana*

The Trelief® Hi-Pure Plant Genomic DNA Kit (TSINGKE, TSP102-50) was utilized for DNA extraction, which served as the template for subsequent purification and promoter cloning. The *pSrUGT91D2* gene fragment was isolated using a primer pair (*pSrUGT91D2*-F: TAGTTATCAATTTTGTTTCCTTTAATG; *pSrUGT91D2*-R: TGCTTTTGAATTTTGATGATAAGTA) designed based on the reference sequence of *S. rebaudiana* available at https://www.ncbi.nlm.nih.gov/genome/?term=stevia%20rebaudiana. The putative promoter region of *SrUGT91D2* is estimated to span approximately 2000 base pairs upstream from the coding region. The Phanta Max Super-Fidelity DNA Polymerase (Vazyme, China) was used for promoter amplification. Subsequently, the PCR products of *pSrUGT91D2* from six *S. rebaudiana* lines were subjected to sequencing and analyzed using DNAMAN for multiple sequence alignment. PlantCARE [17, 18] (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used for cis-element analysis.

Vector construction of pCAMBIA1300-*pSrUGT91D2*::GUS of six *S. rebaudiana*

To investigate the investigation of the promoter activity of *pSrUGT91D2* in six different *S. rebaudiana* lines (specifically *pSrUGT91D2*-023, *pSrUGT91D2*-110, *pSrUGT91D2*-1114, *pSrUGT91D2*-GP, *pSrUGT91D2*-GX, and *pSrUGT91D2*-B1188) in driving GUS gene expression, modified pCAMBIA1300 vectors were used to fuse the six *pSrUGT91D2* promoters with the GUS reporter gene. The resulting vector constructs were then validated through *BamHI* and *HindIII* digestion. Subsequently, the pCAMBIA1300-*PSrUGT91D2*::GUS constructs were introduced into tobacco leaves using the Agrobacterium-mediated transient expression system. Tobacco leaves infiltrated with the pCAMBIA1300-pro::GUS vector served as the experimental group, while those containing the empty pCAMBIA1300::GUS vector were used as the negative control, and leaves with pCAMBIA1300-35S::GUS were utilized as the positive control.

Cloning of *pSrUGT91D2* deletion fragments and vector construction of the related deletion pCAMBIA1300-pro::GUS

A series of recombinant vectors were constructed to further investigate the activity of *pSrUGT91D2*. The full length of *pSrUGT91D2* in six *S. rebaudiana* lines, as well as seven 5′ deletion fragments of 'B1188' (P1: -1633 bp, P2: -1353 bp, P3: -933 bp, P4: -723 bp, P5: -583 bp, P6: -423 bp, P7: -233 bp, and P8: from -1983 bp to -1683 bp), were amplified using primers containing the *BamHI/HindIII* restriction sites (Table S1). These fragments were then homologously recombined into the pCAMBIA1300::GUS vector using the ClonExpress® II One Step Cloning Kit (Vazyme, China).

Agroinfiltration of tobacco leaves

The recombinant pCAMBIA1300-pro::GUS constructs were subsequently introduced into the *A. tumefaciens* strain GV3101. The promoter assays, which were conducted using Agrobacterium-mediated transient expression, were carried out in accordance with the previous study, with minor modifications [19]. The cultured strain GV3101 containing the recombinant plasmid was grown to an optical density (OD600) of 0.6 – 0.8. The mixture was then incubated in agroinfiltration buffer (composed of 10 mL of 10 mM MES buffer at pH 5.6, 150 μM

acetosyringone, and 10 mM $MgCl_2$) for 2 h. Subsequently, the OD600 was adjusted to 0.6 prior to infiltrated into tobacco leaves. Tobacco plants were grown under 16 h of light at 28 ℃ until they reached 6–7 true leaves, at which point they were ready for agroinfiltration. The tobacco plants were then cultured for 72 h under a light-dark cycle of 16 h–8 h, respectively, at a constant temperature of 28℃. The experimental setup included the establishment of three replicate groups: a negative control group comprising noninfiltrated tobacco, a positive control group comprising tobacco infiltrated with pCAMBIA1300-35S::GUS, an empty plasmid control group comprising tobacco infiltrated with pCAMBIA1300::GUS, and an experimental group comprising tobacco infiltrated with pCAMBIA1300-pro::GUS.

Histochemical GUS staining and fluorometric assay

GUS expression in tobacco leaves was assessed using a GUS staining kit (Coolaber, S7160) according to the manufacturer's instructions. The selected leaves were immersed in the GUS staining solution and incubated overnight at 37 °C to facilitate the staining process. To eliminate background interference, the chlorophyll was completely removed by treating the leaves with 75% ethanol. The results were documented through photography using a camera.

For the determination of GUS enzyme activity, the necessary materials were rapidly frozen in liquid nitrogen and subsequently ground to a powder using a mortar and pestle. The GUS enzyme activity was then evaluated using a GUS gene quantitative detection kit (Coolaber, SL7161) following the manufacturer's protocol. After centrifugation at 4 °C and 12,000 rpm for 10 min, the resulting supernatant was collected as the protein extract. The protein concentration was determined using the Bradford method. Subsequently, the GUS reaction substrate 4-MUG, was added, and the mixture was allowed to react at 37 °C for 30 min. Fluorometric analyses were taken with 455 nm emission light and 365 nm excitation light. To ensure accuracy, three separate biological replicates were conducted. The GUS enzyme activity was then determined by evaluating the relative change in the product over a specific time period.

Hormones and abiotic stress treatments

Hormonal and abiotic stress treatments were assessed using 6-week-old tobacco plants. The leaves were then infested with *A. tumefaciens* containing a recombinant plasmid, and the treated plants were incubated in darkness for 24 h. The right half of the leaves of the light-treated tobacco plants were shaded, and the plants were transferred to light for 48 h of continuous illumination. Light treatment was administered using uniform natural sunlight, with an illumination intensity of approximately 3000 lx [20]. To administer the hormone treatments, the plants were uniformly sprayed with ABA (100 μM) and IAA (100 μM) until all the leaves were fully saturated. After a 48-hour period, both the treatment and control group plants were frozen in liquid nitrogen and stored at -80 °C for subsequent protein extraction and GUS quantification. Each treatment was replicated in three independent biological sets, with a minimum of three tobacco plants selected for each treatment.

Results

Cloning of promoters

In a previous study, researchers examined six different stevia genotypes that contained varying fractions of SG. The focus of the study was to analyze the expression level of *SrUGT91D2* in these genotypes. The results showed considerable variation in the expression level of *SrUGT91D2* among the different genotypes. Additionally, a positive correlation (correlation coefficient=0.91) was observed between the expression level of this gene and RD content [21]. Based on these findings, the researchers hypothesized that the divergent expression levels could be due to differences in the 5'-flanking sequence of *SrUGT91D2*. To test this hypothesis, the researchers cloned the *pSrUGT91D2* sequences of the '023', '110', '1114', 'B1188', 'GP', and 'GX' lines using DNA from *S. rebaudiana* leaves as a template and *pSrUGT91D2*P-F and *pSrUGT91D2*-R primers. The lengths of the fragments obtained were 1952 bp, 1989 bp, 1984 bp, 1983 bp, 1982 bp, and 1985 bp (Fig. S1). By comparing the sequencing results with the genome sequence of *S. rebaudiana*, it was confirmed that the obtained sequences were accurate and properly aligned. Further analysis using DNAMAN software revealed a 26 bp inDel (located from the -1738 to -1713 sites relative to the transcription site TSS) in the *pSrUGT91D2* sequences of the '110', '1114', 'B1188', 'GP', and 'GX' lines compared to the '023' line. Nevertheless, the remaining five lines, exhibiting minimal disparities in promoter sequences along with a few base deletions and substitutions, were categorized as the *pSrUGT91D2*-B1188 type (Fig. S2).

Analysis of CREs of *pSrUGT91D2*

Upon scrutinizing *pSrUGT91D2* using PLACE databases, it was discovered that these promoters contain a substantial abundance of cis-regulatory elements (CREs) with variations in their number and positioning due to sequence disparities (Fig. 1; Table 1). The predicted core promoter elements included the TATA-boxes (found at -30 to -33 bp from the transcription start site, one fewer in the '023' line than in the other lines) and CAAT-boxes (present at various locations in the promoter region). Notable cis-elements associated with hormone response, such as ABRE (abscisic acid response, one less common

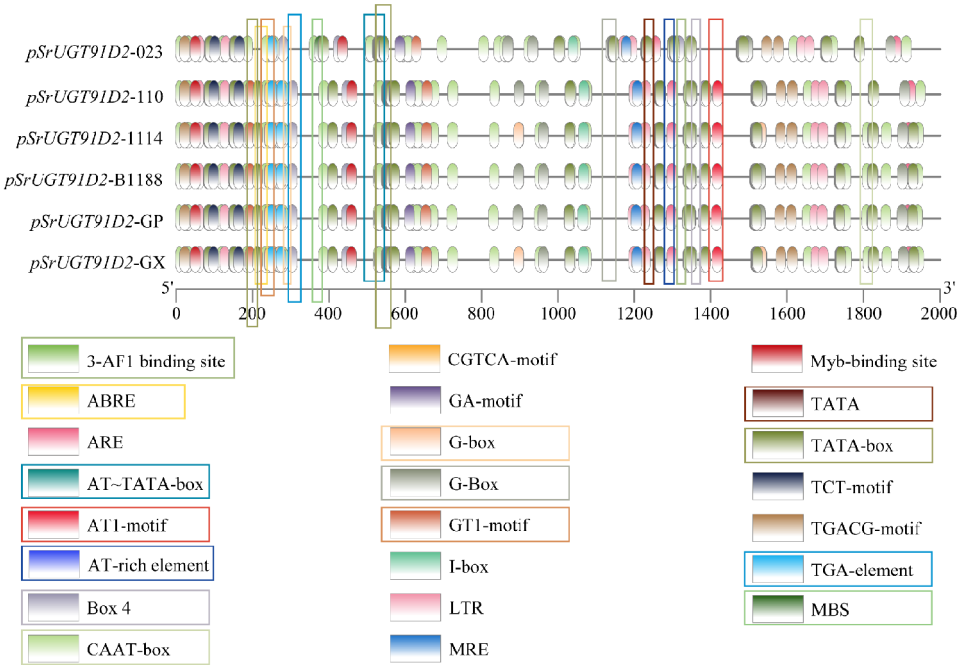

Fig. 1 Potential CREs found in the *pSrUGT91D2* of six *S. rebaudiana lines*. Notes: The names of the CREs are consistent with the names in Table 1, The boxes are labeled with the differential cis-acting elements and their locations

Table 1 Cis-regulatory elements of *pSrUGT91D2* of six *S. rebaudiana*

Cis-acting elements	The number of Cis-acting elements						Sequence	Function
	023	110	1114	B1188	GP	GX		
3-AF1 binding site	-	1	1	1	1	1	TAAGAGAGGAA	light responsive element
ABRE	7	8	8	8	8	8	(C)ACGTG	cis-acting element involved in the abscisic acid responsiveness
ARE	3	3	3	3	3	3	AAACCA	cis-acting regulatory element essential for the anaerobic induction
AT1-motif	-	1	1	1	1	1	AATTATTTTTTATT	part of a light responsive module
AT~TATA-box	11	12	12	12	11	12	TATATA	TATA box
Box 4	6	4	5	4	5	5	ATTAAT	part of a conserved DNA module involved in light responsiveness
CAAT-box	25	25	27	27	27	27	(TG)(C)CA(A)AT	common cis-acting element in promoter and enhancer regions
CGTCA-motif	3	3	3	3	3	3	CGTCA	cis-acting regulatory element involved in the MeJA-responsiveness
G-Box	5	4	4	4	4	4	CACGTG	cis-acting regulatory element involved in light responsiveness
G-box	2	6	6	6	6	6	(C)(C/T)ACGT(G/AA)	cis-acting regulatory element involved in light responsiveness
GA-motif	1	1	1	1	1	1	ATAGATAA	part of a light responsive element
GT1-motif	1	2	2	2	2	2	GGTTAA(T)	light responsive element
I-box	1	1	1	1	1	1	CCATATCCAAT	part of a light responsive element
LTR	6	6	6	6	6	6	CCGAAA	cis-acting element involved in low-temperature responsiveness
MBS	3	2	2	2	2	2	CAACTG	MYB binding site involved in drought-inducibility
MRE	1	1	1	1	1	1	AACCTAA	MYB binding site involved in light responsiveness
TATA	1	2	2	2	2	2	TATAAAAT	TATA-boxs
TATA-box	50	49	51	51	49	51	(A)TATA(T/A)A(AA/TT)	core promoter element around -30 of transcription start
TGA-element	1	2	2	2	2	2	AACGAC	auxin-responsive element
TCT-motif	2	2	2	2	2	2	TCTTAC	part of a light responsive element
TGACG-motif	3	3	3	3	3	3	TGACG	cis-acting regulatory element involved in the MeJA-responsiveness
AT-rich element	1	-	-	-	-	1	ATAGAAATCAA	binding site of AT-rich DNA binding protein (ATBP-1)

Note "-" indicates not predicted

in the '023' variant than others), CGTCA-motif (MeJA response), TGACG-motif (MeJA response), and TGA-element (growth hormone response, one less common in the '023' line compared to others), were identified within their promoters. Furthermore, several light-responsive elements were discerned, including the 3-AF1 binding site (absent in the '023' line), AT1-motif (not present in the '023' line), Box 4 (one more than the other line in "023"), and G-box variations. These light-responsive elements within the *SrUGT91D2* promoter suggest a potential regulatory response to light exposure. Additionally, distinct transcription factor binding sites such as the MYB transcription factor binding site associated with drought response (MBS) and AT-rich element (binding site for the AT-rich DNA binding protein), were exclusively noted in the '023' line. This study also identified stress-related response elements, such as anaerobic induction and low-temperature response elements. Notably, significant differences in sequence (the 26 bp inDel) composition between the *SrUGT91D2*-023 and *SrUGT91D2*-B1188 promoters impacted the presence of crucial cis-acting elements (e.g., ABREs, G-boxes, and TGA elements), as well as the number of MYB binding sites. Specifically, while the *SrUGT91D2*-023-type promoter contained four MYB-binding sites, the *SrUGT91D2*-B1188-type promoter featured only three MYB-binding sites.

Histochemical analysis of GUS expression driven by six *S. rebaudiana* lines of *pSrUGT91D2*

Figure 2 showcases the results of the GUS enzyme activity analysis. Remarkably, the tobacco plants harboring the pCAMBIA1300 vector lacking a promoter for GUS expression (serving as the negative control, Fig. 2) displayed white leaves, indicating the absence of GUS activity. In contrast, GUS staining revealed a range of blue intensities in tobacco leaves infiltrated with different pCAMBIA1300-pro::GUS constructs. This suggests the functional capability of different types of *pSrUGT91D2* in driving the expression of the GUS reporter gene. Notably, the diverse shades of blue observed across the six *S.*

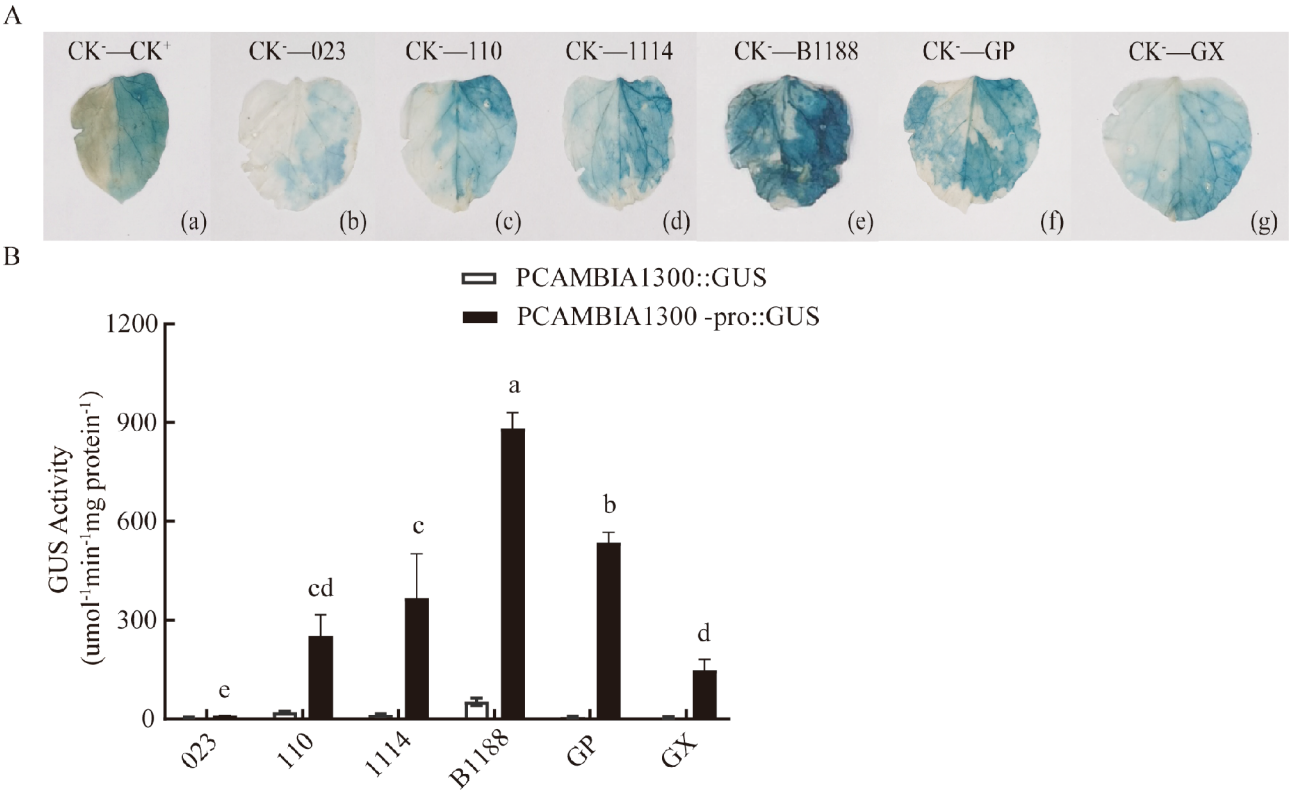

Fig. 2 GUS protein activity in tobacco leaves by the use of several pCAMBIA1300-pro::GUS constructs. (**A**) CK-: tobacco leaves infiltrated with empty pCAMBIA1300::GUS were used as a negative control. CK+: tobacco leaves infiltrated with pCAMBIA1300-35S::GUS were used as a positive control. 023: tobacco leaves infiltrated with the pCAMBIA1300-*pSrUGT91D2*-023::GUS plasmid. 110: tobacco leaves infiltrated with the pCAMBIA1300-*pSrUGT91D2*-110::GUS plasmid. 1114: tobacco leaves infiltrated with the pCAMBIA1300-*pSrUGT91D2*-1114::GUS plasmid. B1188: tobacco leaves infiltrated with the pCAMBIA1300-*pSrUGT91D2*-B1188::GUS plasmid. GP: tobacco leaves infiltrated with the pCAMBIA1300-*pSrUGT91D2*-GP::GUS plasmid. GX: tobacco leaves infiltrated with the pCAMBIA1300-*pSrUGT91D2*-GX::GUS plasmid. (**B**) Fluorogenic quantitative analysis of several lines of *pSrUGT91D2*. The values displayed are the means ± standard deviations of three biologically separate experiments. A one-way ANOVA ($P < 0.05$) indicates a significant difference with distinct letters

rebaudiana lines carrying *pSrUGT91D2* signify varying degrees of effectiveness in regulating gene expression.

Quantitative analysis of *pSrUGT91D2* in different lines by 4-methylumbelliferyl-β-D-glucuronide (MUG) assays

To evaluate the efficacy of *pSrUGT91D2* in different lines and identify those exhibiting robust activity, we conducted an MUG assay to quantify the expression levels of the GUS gene in tobacco plants (Fig. 2). The findings revealed substantial variations in GUS gene expression among the six *S. rebaudiana* lines compared to common tobacco. The hierarchy of driving activities for GUS gene expression was as follows: *pSrUGT91D2*-B1188 > *pSrUGT91D2*-GP > *pSrUGT91D2*-1114 > *pSrUGT91D2*-GP > *pSrUGT91D2*-110 > *pSrUGT91D2*-GX > *pSrUGT91D2*-023. These results suggest a potential correlation between the 26 bp inDel in *pSrUGT91D2* and its activity.

Analysis of the 5′ deletion in the *pSrUGT91D2* of the 'B1188' lines

To investigate the functions of various deletion fragments, a series of fragments (P1, P2, P3, P4, P5, P6, P7, and P8) were cloned and fused with the GUS reporter gene based on the authentic *pSrUGT91D2* sequence of 'B1188' (P0) (Fig. 3A). The GUS staining results revealed distinct levels of blue staining across the deletion fragments P1 to P8 (Fig. 3B). Specifically, P0 and P8 displayed the most intense staining, followed by P2, P3, P4, and P5. Moreover, P1, P6, and P7 exhibited the least intense staining. Additionally, quantitative assessment of GUS activity (Fig. 3C) confirmed that P0 and P8 exhibited the highest GUS activity, whereas the other deletion fragments (P1 to P7) demonstrated lower GUS activity compared to P0 and P8.

Notably, deletion of *pSrUGT91D2* from -1983 bp to -1633 bp resulted in a substantial loss of quantitative GUS activity (representing an 87.60% decrease in *pSrUGT91D2* activity, Fig. 3C). Moreover, the GUS activity of *pSrUGT91D2* from -1983 bp to -1683 bp (P8) was marginally lower than that of P0. These findings suggest that the crucial region of the *pSrUGT91D2* may reside within P8 (301 bp, -1983 to -1683 bp).

Responses of *P8* in two types of *pSrUGT91D2* to abiotic stresses and hormones

Putative abscisic acid responsiveness, light responsiveness, and auxin-responsive elements were predicted in the *pSrUGT91D2*-B1188 type but not in the *pSrUGT91D2*-023 type, suggesting that these two types of promoters may respond differently to light, abscisic acid, and auxin treatments. To investigate this hypothesis, tobacco leaves transiently expressing Agrobacterium-mediated *P8-pSrUGT91D2*-023::GUS and *P8-pSrUGT91D2*-B1188::GUS constructs were subjected to light, ABA, and IAA treatments. Histochemical staining and GUS protein activity assays were performed to assess GUS protein activity in tobacco (*N. benthamiana*) plants. The results indicated that the GUS activities of *P8-pSrUGT91D2*-023::GUS were unaffected by ABA, IAA, and light treatments (Fig. 4). In contrast, the transient expression of *P8-pSrUGT91D2*-B1188::GUS revealed the activation of the GUS protein in tobacco leaves following treatment with ABA, IAA, and light (Fig. 4A, B). The GUS activity increased by 17.79%, 13.49%, and 7.51% under the ABA, IAA, and light treatments, respectively, compared to that under the control (untreated) conditions (Fig. 4B). The primary difference between *P8-pSrUGT91D2*-023 and *P8-pSrUGT91D2*-B1188 is the 26 bp inDel sequence. The 26 bp inDel sequence of *P8-pSrUGT91D2*-B1188 included the ABRE, TGA, and G-box elements, resulting in increased promoter activity under ABA, IAA, and light treatments. These findings suggest that the 26 bp (-1738 bp to -1713 bp) segment is a key sequence for responding to ABA, IAA, and light treatments.

Discussion

The investigation of gene regulation in molecular biology has placed significant emphasis on the structure and function of promoters, which are integral in controlling gene expression [22]. Promoters exert regulatory control by determining the level, location, and manner of gene expression, and this regulation occurs through the complex interplay of multiple factors and levels [23–25]. In-depth exploration of promoters holds great importance in comprehending the processes of organismal development, defense mechanisms, and diseases. However, comprehensive studies on stevia gene promoters remain limited, with the existing literature predominantly focused on *SrUGT76G1*, *SrHDR*, *SrMCT*, *SrCMK*, *SrHDS*, *SrIDI*, *SrGGDPS*, *SrNCYPR*, *SrKAH*, and related genes [15, 26, 27]. Previous studies conducted by this research group revealed a potential link between the differential expression of the glycosyltransferase *SrUGT91D2* gene and variations in glycoside composition and content among six stevia lines [14, 16]. As gene expression initiation and extent are controlled by the respective gene promoter, it was hypothesized that discrepancies may exist within the *pSrUGT91D2* gene among these six stevia lines. Consequently, in this study, the researchers cloned and compared *pSrUGT91D2* sequences measuring 1952 bp, 1989 bp, 1984 bp, 1983 bp, 1982 bp, and 1985 bp from these six lines, observing distinct sequence variations (Fig. S1 and S2). Notably, five lines ('110', '1114', 'B1188', 'GP', and 'GX') exhibited a 26 bp

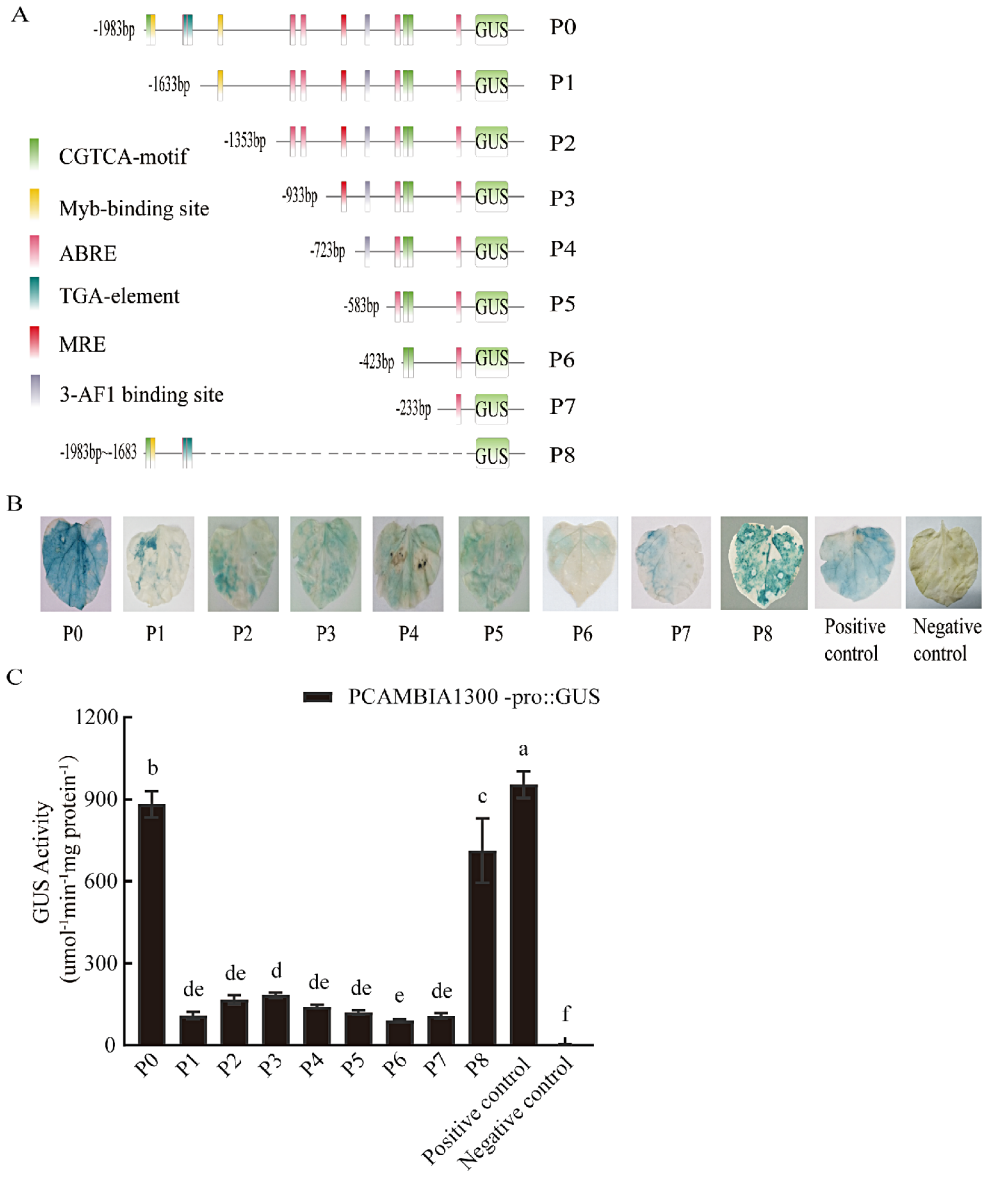

Fig. 3 Analysis of GUS activity for *pSrUGT91D2* and 5′ deletion fragments. (**A**) Schematic map of the *pSrUGT91D2* and 5′ deletion fragments. (**B**) Histochemical assay for *GUS* expression of the *pSrUGT91D2* and 5′ deletion fragment *GUS* expression in tobacco leaves that have undergone temporary transformation. (**C**) Quantitative fluorogenic analysis of the *SrUGT91D2* and 5′ deletion fragments in tobacco leaves that have undergone temporary transformation. The negative control comprises tobacco leaves that were not infiltrated with Agrobacterium. The positive control includes tobacco leaves infiltrated with pCAMBIA1300-35S::GUS. The values displayed are the means ± standard deviations of three biologically separate experiments. Different letters represent a significant difference based on the one-way ANOVA ($P < 0.05$)

indel within *pSrUGT91D2*, while line 023 did not (Fig. S2).

The gene promoter in plants encompasses various regulatory elements, including the CAAT-boxes, TATA-boxes, MYB binding sites, and cis-acting regulatory elements (CREs) related to temperature, light, drought, anaerobic conditions, methyl jasmonate, abscisic acid, and growth hormone responses (Fig. 1; Table 1). The regulation of *SrUGT91D2* gene expression is clearly intricate. For instance, Ceunen et al. demonstrated that a longer photoperiod (16 h) stimulates nutritional growth in *S. rebaudiana*, leading to a significant increase in leaf biomass and steviol glycoside (SG) content [28]. Light stimulation induces changes in plant morphology and biochemical reactions, explaining the peak SG content in stevia leaves during bud emergence, followed by a gradual decline after flowering [4, 29, 30]. Yang et al. studied the transcript levels of 15 genes involved in the SG biosynthetic pathway under different temperatures and found that all genes exhibited the highest transcript levels at 25 °C. However, *SrMDS*, *SrCMK*, *SrDXR*, *SrDXS*, *SrMCT*, *SrIDI*, *SrHDR*, *SrHDS*, *SrCPPS1*, *SrUGT76G1*,

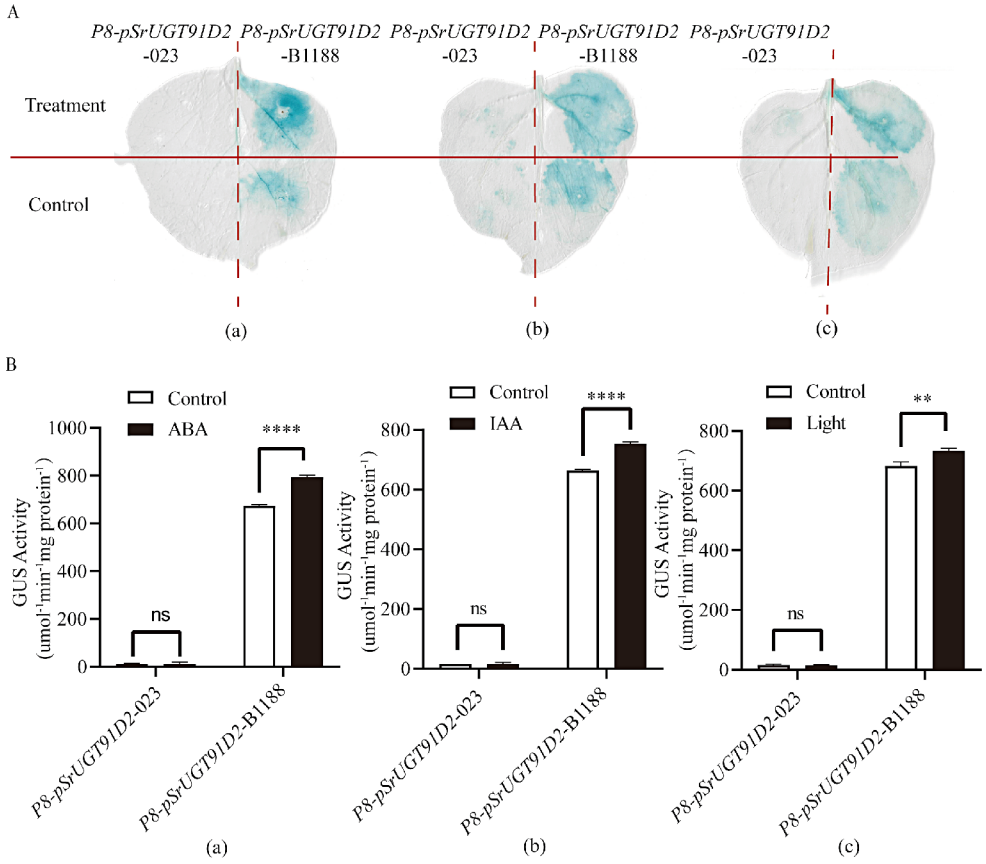

Fig. 4 GUS protein activity in tobacco leaves agroinfiltrated with distinct *P8-pSrUGT91D2*::GUS constructs. The same tobacco leaves on the left and right were injected with the plasmids *P8-pSrUGT91D2*-B1188::GUS and *P8-pSrUGT91D2*-023::GUS. (**A**) Representative images showing GUS immunostaining of leaves of *P8-pSrUGT91D2*::GUS transient transgenic plants after treatment with 100 μM ABA (**a**), 100 μM IAA (**b**), or light (**c**). No treatment was used as the control. (**B**) Activity of GUS driven immunostaining of leaves of *P8-pSrUGT91D2-B1188*::GUS and *P8-pSrUGT91D2-023*::GUS transient transgenic plants after treatment with 100μM ABA (**a**), 100 μM IAA (**b**), or light (**c**). No treatment was used as the control. The standard deviations of three replicates are represented by error bars; $^{**}P < 0.01$, $^{****}P < 0.0001$

SrUGT85C2, and *SrGGDPS* transcription was inhibited at both high (35 °C) and low (15 °C) temperatures [31]. Moreover, Bayraktar et al. discovered that treatment with methyl jasmonate (Me-JA) significantly increased the SG content in vitro plantlets [32]. Predicting cis-elements in the *pSrUGT91D2* promoter is valuable for understanding *SrUGT91D2* gene expression and regulation and for identifying potential targets for gene editing or introduction. These predictions provide essential guidance for developing *S. rebaudiana* varieties with high rebaudioside (RD) and stevioside (RM) contents, representing a crucial research pursuit.

Researchers employed transient infiltration of tobacco leaves by Agrobacterium to efficiently evaluate the activity and driving strength of cloned promoter fragments. This approach was chosen due to the time-consuming and challenging nature of stable genetic transformation of *S. rebaudiana* using Agrobacterium-mediated methods [11]. Their findings indicated that the *pSrUGT91D2*-B1188 type (encompassing sequences from -1738 bp to -1713) demonstrated a robust ability to induce GUS gene expression in tobacco leaves, whereas the *pSrUGT91D2*-023 type exhibited notably weaker potential to drive GUS gene expression (Fig. 2). Researchers have postulated that the presence or absence of the -1738 bp to -1713 bp region of the *pSrUGT91D2* is closely linked to the strength of its promoter activity. Furthermore, a previous study revealed that '023' exhibited the lowest levels of stevioside, stevioside+steviolbioside, and stevioside+steviolbioside+RD and established a meaningful and direct correlation between the expression level of *SrUGT91D2* and the quantity of RD [14, 16]. Additionally, *SrUGT91D2* directly catalyzes the formation of 1,2-β-D glucosidic bonds at the C13 and C19 positions, with a specific emphasis on C13, the first glucose moiety, and serves as a glucosylase directly involved in the biosynthesis of steviolbioside, stevioside, rebaudioside D (RD), and RE, among others [11, 12]. Therefore, it is hypothesized that the deletion of the -1738 bp to -1713 region of *pSrUGT91D2* diminishes its ability to bind to transcription factors, thereby impacting its transcriptional levels

and ultimately leading to reduced synthesis of stevioside, steviolbioside, and RD.

To determine the critical domain responsible for the functionality of *pSrUGT91D2*, we isolated the *pSrUGT91D2* variant from 'B1188', which exhibited the highest level of activity. This variant contained specific cis-regulatory elements (CREs) and an inDel region upstream. Plant expression vectors were constructed for each deletion fragment by selecting fragments of varying lengths that contained essential functional elements at the 5' end of the sequence. Our analysis of these deletion fragments revealed a significant reduction in promoter activity upon removal of the P8 fragment. However, when the P8 fragment, including the -1738 bp to -1713 sequence, was ligated, the promoter activity was nearly equivalent to that of the 1983 bp of *pSrUGT91D2*-B1188 fragment. Previous studies have shown that mutations in the central region connecting the SURE and SP8 elements result in a marked decrease in their binding affinity for specific transcription factors [33, 34]. Thus, we hypothesize that the 26 bp inDel sequence (-1738 bp to -1713) within the *pSrUGT91D2*-B1188 variant is the key fragment responsible for its active transcriptional role. Deletion of the 26 bp inDel sequence in the *pSrUGT91D2*-023 variant weakens its binding activity to regulatory factors, leading to lower expression of *SrUGT91D2* and a decrease in RD glycoside content in 023.

In previous studies, it was commonly observed that the core promoters of most genes were located near the transcription start site (TSS). However, in the case of *pSrUGT91D2*, the region exhibiting transcriptional activity is located at a greater distance from the TSS. This distance can be attributed to chromatin folding within the nucleus, which brings the core promoter and the TSS into spatial proximity. Consequently, downstream gene transcription initiation occurs when the transcription initiation complex, comprising elements from the core promoter, brings various proteins (including TBP proteins, TF II, and polymerase II) in close proximity to the TSS [35]. Typically, biologically significant and functional cis-acting motifs are located within the proximal promoter region, approximately 250–300 bp from the transcription start site [36, 37]. However, our study reveals that a 5' end promoter fragment, approximately 301 bp in length, is crucial for promoter activity. The 5' distal region of this fragment exhibits enhanced activity, potentially indicating the presence of an enhancer element within this region. Enhancers are pivotal in gene expression regulation, and one of their hallmark features is orientation-independent gene activation capability [38]. It is important to note that while our study provides preliminary support for the hypothesis that distal cis-acting motifs may function as enhancers, this still requires validation through further experimental investigation.

By comparing the sequences of *pSrUGT91D2*-023 and type B1188, where the essential cis-regulatory elements (CREs) are consistent across GP, GX, 110, 1114, and B1188, a 26-base pair insertion/deletion (inDel) sequence was found to be closely associated with the transcriptional activity of *pSrUGT91D2*. Additionally, transient infiltration of tobacco leaves with the plant expression vector pCAMBIA1300-pro::GUS, created through Agrobacterium-mediated fusion of a series of 5' deletions (P1–P7) and a 3' deletion (P8) with a GUS reporter gene, further confirmed the crucial role of the 26 bp inDel sequence in the transcriptional activity of *pSrUGT91D2*. Analysis of the CRE prediction results revealed the presence of light-responsive elements, abscisic acid, and growth factor-responsive elements within this inserted fragment. Subsequently, the plant expression vectors *P8-pSrUGT91D2*-023::GUS and *P8-pSrUGT91D2*-B1188::GUS were constructed. Agrobacterium-mediated transformation of tobacco leaves and transient expression were employed to investigate the responsiveness of the P8 fragment to exogenous ABA, IAA, and light signals. The findings indicated that *P8-pSrUGT91D2*-B1188 exhibited responsiveness to external signals such as ABA, IAA, and light, leading to a notable increase in GUS protease activity. Consequently, the presence of CREs within the crucial 26-base pair sequence (-1738 bp to -1713) in *P8-pSrUGT91D2*, which are receptive to abscisic acid, growth hormone, and light signals, exerted a positive regulatory influence on the promoter. Moreover, ABA treatment was shown to impact the accumulation of steviol glycosides (SGs). The content of SGs significantly increased after treatment of severed stevia apical meristematic tissues with exogenous ABA (0, 50, 100, and 150 μM) [39]. Ceunen et al. treated stevia leaves with the growth hormone analog NAA, resulting in a significant up-regulation of the transcript level of UGT85C2, along with a notable increase in stevioside and rebaudioside A. Furthermore, prolonged light exposure was found to promote the trophic growth process of stevia, leading to a significant increase in leaf biomass and SG content [30]. Through the process of transiently inflecting tobacco leaves with *A. tumefaciens*, followed by subsequent induction treatment, it was observed that certain treatments successfully facilitated an increase in transcriptional activity within the core promoter region. The identification of this crucial factor forms the foundation for a thorough examination of the steviol glycoside (SG) synthesis pathway and the screening of possible transcription factors that interact with *pSrUGT91D2*. Cloning the *pSrUGT91D2* gene and analyzing the factors involved in its efficient induction will lay a theoretical foundation for future research on the expression regulation mode of this gene. This will provide new ideas for future research on the molecular breeding of high-quality

stevia so that the *SrUGT91D2* gene can function more economically and efficiently.

Conclusions

In this study, the cloning and sequencing of *pSrUGT91D2* were performed in six different stevia lines. Additionally, an analysis of the upstream sequence of *pSrUGT91D2* was conducted using the Plant CARE online database. The results of this analysis revealed that *pSrUGT91D2* contains not only essential promoter elements such as the TATA-box, CAAT-box, and MYB binding site, but also includes cis-regulatory elements (CREs) involved in the response to various factors such as light, drought, low temperature, and anaerobic conditions. Moreover, it was discovered that the promoter contains CREs involved in the phytohormonal response to growth hormone, abscisic acid, and jasmonic acid methylester. A series of PCAMBIA1300-Pro::GUS fusion expression vectors were generated, and tobacco transient infestation experiments demonstrated that the promoter sequences were all capable of initiating the expression of GUS reporter genes in tobacco leaves.The *pSrUGT91D2*-B1188 was the most potent in driving the expression of GUS reporter genes, with the core region of action being *P8-pSrUGT91D2* (from -1983 bp to -1633 bp), and the 26 bp inDel sequence from -1738 bp to -1713 played a crucial role. Subsequent abscisic acid, growth hormone, and light signaling experiments also showed that the associated cis-acting element in the 26 bp inDel sequence positively regulates the *SrUGT91D2* gene. This study not only provides a theoretical basis for the utilization of the *SrUGT91D2* gene promoter but also lays the groundwork for a comprehensive exploration of the function of the *SrUGT91D2* gene and the glycoside synthesis pathway it governs.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05616-1.

Supplementary Material 1

Supplementary Material 2

Author contributions

J. C.: Conceptualization, Software, Data curation, Writing-original draft preparation. R. L.: Formal analysis, Visualization. C. L.: Formal analysis, Visualization. M. W.: Formal analysis, Visualization. S. L.: Methodology Investigation. M. J.: Methodology Investigation. Y. Z.: Methodology Investigation. D. X.: Writing—review & editing. K. H.: Writing—review & editing. W. W.: Resources, Supervision, Funding acquisition, Writing-review & editing.

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Data availability

Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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