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Identification and Functional Evaluation of Three Polyubiquitin Promoters from *Hevea brasiliensis*

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Abstract: *Hevea brasiliensis* is an economically important tree species that provides the only commercial source of natural rubber. The replacement of the CaMV35S promoter by endogenous polyubiquitin promoters may be a viable way to improve the genetic transformation of this species. However, no endogenous polyubiquitin promoters in *Hevea* have been reported yet. Here, we identified three *Hevea* polyubiquitin genes *HbUBI10.1*, *HbUBI10.2* and *HbUBI10.3*, which encode ubiquitin monomers having nearly identical amino acid sequences to that of *AtUBQ10*. The genomic fragments upstream of these *HbUBI* genes, including the signature leading introns, were amplified as putative *HbUBI* promoters. In silico analysis showed that a number of *cis*-acting elements which are conserved within strong constitutive polyubiquitin promoters were presented in these *HbUBI* promoters. Transcriptomic data revealed that *HbUBI10.1* and *HbUBI10.2* had a constitutive expression in *Hevea* plants. Semi-quantitative RT-PCR showed that these three *HbUBI* genes were expressed higher than the *GUS* gene driven by CaMV35S in transgenic *Hevea* leaves. All three *HbUBI* promoters exhibited the capability to direct *GFP* expression in both transient and stable transformation assays, although they produced lower protoplast transformation efficiencies than the CaMV35S promoter. These *HbUBI* promoters will expand the availability of promoters for driving the transgene expression in *Hevea* genetic engineering.

Keywords: rubber tree; constitutive promoter; *cis*-acting elements; *Agrobacterium*-mediated transformation; protoplast transformation; transgene expression

Citation: Xin, S.; Udayabhanu, J.; Dai, X.; Hua, Y.; Fan, Y.; Huang, H.; Huang, T. Identification and Functional Evaluation of Three Polyubiquitin Promoters from *Hevea brasiliensis*. *Forests* **2022**, *13*, 952. https://doi.org/10.3390/f13060952

Academic Editors: Giovanni Emiliani and Alessio Giovannelli

Received: 11 April 2022
Accepted: 15 June 2022
Published: 17 June 2022

1. Introduction

Hevea brasiliensis, also known as the rubber tree, is an economically important crop that provides the only commercial source of natural rubber. As a cross-pollinated perennial tree species with a long juvenile phase, the genetic improvement of *Hevea* through conventional breeding is troublesome and time-consuming [1]. Genetic engineering is a useful technique to accelerate the trait improvement of this species. Previously, multiple transformation methods have been developed to introduce genes of interest into *Hevea*, thereby improving agronomic traits related to latex yield, disease and stress response, latex quality, etc. [1–5].

In plant transformation systems, the promoter is one of the most important components that determine the expression levels of selectable markers, antibiotic resistance genes and genes of interest, thus affecting the screening and regeneration efficiency of the transgenic system and the performance of transgenic lines. The CaMV35S promoter derived from cauliflower mosaic virus (CaMV) is capable of directing the constitutive expression of adjacent genes in transgenic plants, which makes it the most commonly used promoter both in the stable and transient transformation of *Hevea* [3,6–8]. However, the use of the

CaMV35S promoter still presents some shortcomings that may limit the efficiency and efficacy of *Hevea* transformation. Constitutive promoters isolated from plant pathogens can lead to abnormal conditions in transgenic plants [9]. It has also been extensively reported that, in multiple plant species, the transgenic hosts can recognize and inactivate intrusive DNA by DNA methylation and histone modification within the CaMV35S promoter region, which represses the expression of transgenes, leading to lethality in the selection process [10–13]. Additionally, when repeatedly used in multi-gene constructs, for expressing multiple transgenes of interest, the multicopy events of 35S promoter regions greatly increase the incidence of methylation-associated transgene silencing [14,15], thereby making transgenic products less efficacious. Moreover, the insertion of the viral DNA sequence into the host plant genome may cause regulatory concerns. Therefore, promoters of plant origin are drawing increasing interests in generating transgenic plants.

Ubiquitin is a highly conserved eukaryotic protein that is expressed at considerable levels in diverse plant tissues at different development stages, under both normal and stress conditions [16–18]. The ubiquitin gene promoters are good candidates for driving the expression of transgenes in plants [19]. A soybean polyubiquitin gene promoter Gmubi showed high levels of constitutive expression and represents an alternative to viral promoters for driving gene expression in soybeans [20]. The promoters of two polyubiquitin genes, *PvUbi1* and *PvUbi2*, were previously isolated from switchgrass [21]. More recently, *PvUbi* and maize *Zmubi* promoters were respectively used to constitutively express two transgenes, *xplA* and *xplB*, in transgenic switchgrass, conferring the ability to remove toxic hexahydro-1,3,5-trinitro-1,3,5-triazine in soil [22]. The use of the native combinations of four polyubiquitin gene promoters and corresponding terminators resulted in an up to >3-fold increase in transgene expression in maize [19]. *GUS* expression levels from constructs containing RUBQ1 or RUB2 rice ubiquitin promoters were 8- to 35-fold higher in transgenic rice plants, respectively, as compared with that of the CaMV35S promoter [23]. When used in regulating selectable marker genes in maize, the plant-derived constitutive promoter *ZmUbi1* produced a transformation efficiency of 43.8%, which is significantly higher than those of two viral promoters, CaMV35T (25%) and SCBV (8%) [24]. In multiplexed genome editing system in alfalfa, the replacement of the CaMV35S promoter by the *Arabidopsis* ubiquitin promoter to drive Cas9 expression led to a significant improvement in genome editing efficiency [25]. Ren, et al. [26] identified a novel ubiquitin promoter in grapes, which could improve the expression of Cas9, and thereby significantly increase the editing efficiency.

It is also worth noting that some polyubiquitin promoters have shown contrasting expression levels in different plant species [27]. The ubiquitin promoter of *Gladiolus* was shown to give low levels of transgene expression in freesia, Easter lily, tobacco, rice and rose, but high levels when reintroduced in *Gladiolus* [28], which is probably due to the lack of corresponding factors in heterologous systems [20]. Hernandez-Garcia and Finer [27] suggested that promoters intended for biotechnological applications should be functionally characterized in the same plant species of interest. It is therefore of great value to identify efficient endogenous polyubiquitin promoters in *Hevea*, and use them to improve the genetic engineering of *Hevea*.

In this study, three polyubiquitin promoters were isolated from *Hevea*. Analyses of sequences and expression profiles were performed, and their capabilities of driving transgene expression were evaluated both in transient and stable transformation studies. Our study provides potential tools for improving the genetic modification of *Hevea* via transgenic techniques.

2. Materials and Methods

2.1. Plant Materials

Hevea plant materials used in present study were collected from the "Hainan Innovation Base for production of Natural Rubber New Planting Material" (Danzhou, China). *Hevea* genomic DNA was extracted from the young leaves of *Hevea* clone Reyan7-33-97, and

used for the amplification of promoter fragments. Two- to three-month-old Reyan7-33-97 trees were subjected to dark incubation for the generation of etiolated leaves, which were harvested for the isolation of mesophyll protoplasts. Anther-derived somatic embryos used for *Hevea* transformation were produced following Hua et al. [29].

2.2. Isolation and Bioinformatic Analysis of Polyubiquitin Genes and Their Promoters in Hevea

Polyubiquitin genes are highly conserved across species, and thus the sequence of the *Arabidopsis* polyubiquitin gene *AtUBQ10* (accession No.: AT4G05320), whose promoter has been extensively used for driving transgene expressions [30–32], was used as the query to search for polyubiquitin genes in the reference genome of *Hevea brasiliensis* (http://hevea.catas.cn (accessed on 1 November 2019)) [33]. Three *Hevea* polyubiquitin genes having high sequence similarities with that of *AtUBQ10* were obtained and designated as *HbUBI10.1*, *HbUBI10.2*, and *HbUBI10.3*, respectively. Multiple sequence alignment was performed using DNAMAN 6.0 (Lynnon Biosoft, San Ramon, CA, USA). These polyubiquitin genes and their upstream fragments of approximately 1000–1500 bp in length were amplified by PCR using the primers listed in Table S1 with the *Hevea* genomic DNA as a template, and then were sequenced. The isolated promoter sequences upstream of three *HbUBI* genes were analyzed using the PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on 21 October 2020)) for the identification of putative *cis*-acting elements.

2.3. Analysis of the Expressions of Three HbUBI Genes in Hevea Plants

Expression profiles of *HbUBI10.1* and *HbUBI10.2* polyubiquitin genes in various tissues of multiple *Hevea* clones under different conditions were obtained from the HeveaDB website (http://hevea.catas.cn/tool/v1/toExpression (accessed on 1 June 2021)) [34]. The normalized expression levels for each gene were calculated as fragments per kilobase of transcript per million fragments mapped (FPKM). A heatmap was constructed using TBtools to display the expressions of these *HbUBI* genes [35].

Semi-quantitative RT-PCR was performed using the primers listed in Table S1. Three individual 1-year-old transgenic *Hevea* plants, T1, T2, and T3 were analyzed. First-strand cDNA was synthesized from one microgram of total RNA in a 20 μL reaction mixture using the EasyScript® First-Strand cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China). To achieve proper results, a pre-experiment was performed to determine the appropriate cycle number and the optimum annealing temperature range for each primer pair. The cycle number of 30 and annealing temperature of 57 °C were shown to be applicable for all primer pairs. The semi-quantitative RT-PCR was performed using 100 ng cDNA templates, 0.2 μM of each primer, and Premix Taq DNA Polymerase (TakaraBio, Shiga, Japan) in a reaction volume of 20 μL. The reactions were performed in triplicate for each sample using the following program: initial denaturation at 94 °C for 2 min, followed by 30 cycles consisting of denaturation at 98 °C for 10 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s. The final extension step was a 72 °C hold for 10 min. The resulting PCR products were then sequenced to confirm the specificity. The *Hevea* DEAD-box RNA helicase 8 gene (*HbRH8*, LOC110669478) was used as the internal control [36]. Agarose gels (1%, *w/v*) were used to analyze the PCR products, and the nucleic acids were stained with ethidium bromide. Gel images were taken and the band intensities were determined by Image Lab 6.0 software (Bio-Rad, Hercules, CA, USA). Relative expressions of three *HbUBI* genes and *GUS* gene were calculated by normalizing their band intensities to the band intensity of the *HbRH8* reference gene.

2.4. Vector Construction

The promoters of three *HbUBI* genes were amplified by PCR from the *Hevea* genomic DNA using the primers listed in Table S1. For the construction of the transient expression vector, each PCR fragment was constructed into the pJIT163hGFP vector [37] using the restriction enzymes *Sac*I (NEB, MA, USA) and *Nco*I (NEB, MA, USA) to replace the

2× CaMV35S promoter (Figure 1a). The resulting proHbUBI-pJIT163hGFP plasmids were used for transient transformation of *Hevea* protoplasts. Subsequently, a PCR reaction was carried out with proHbUBI-pJIT163hGFP plasmids as the template to create proHbUBI-hGFP fragments flanked by *Sac*I/*Pst*I restriction sites using the primers listed in Table S1. These PCR products were then introduced into the linearized pCAMBIA3300 vector (Cambia, Australia) to create the stable transformation vector proHbUBI-hGFP-pCAMBIA3300 (Figure 1b). The resulting plasmids were then transformed into *Agrobacterium tumefaciens* strain EHA105 by the freeze–thaw method [38] and used for the transformation of *Hevea* embryos.

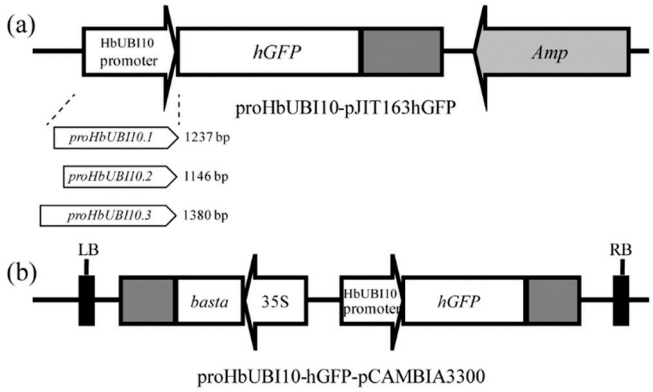

Figure 1. Schematic representation of the vectors used for *Hevea* transformation. (**a**) Transient expression vectors used for protoplasts transformation contain an *GFP* reporter gene driven by three *Hevea* polyubiquitin promoters, respectively. (**b**) T-DNA region of the binary vector used for *Agrobacterium*-mediated transformation of *Hevea* embryos.

2.5. Hevea Protoplasts Isolation and Polyethylene Glycol (PEG)-Mediated Transformation

The isolation and PEG-mediated transformation of *Hevea* mesophyll protoplasts were performed as previously described [6]. Briefly, about 2 g of 5–7-day-old etiolated leaves in the color-changing stage were collected for protoplasts' isolation. Two hundred microliters of protoplast solution (6×10^6 /mL) was transformed with 50 µL of transient expression vector DNA (1 µg/µL), and then incubated in the dark at 26 °C for 48 h. The pJIT163hGFP plasmid carrying a green fluorescent protein (GFP) gene driven by the 2× CaMV35S promoter was transformed as the control. Ten microscope fields were surveyed for each sample under a Leica AF6000 fluorescence microscope (Leica, Wetzlar, Germany). Three biological replicates were performed for statistical analysis to determine the transformation efficiency.

2.6. Agrobacterium-Mediated Transformation of Hevea Somatic Embryos

Hevea embryos about 1.5 cm in size were collected and transformed by *Agrobacterium tumefaciens* EHA105 bearing the proHbUBI-hGFP-pCAMBIA3300 construct, following the methods previously described [8]. After transformation, 0.75 mg/L basta was used for the selection of positive transformants. Basta-resistant embryos were then surveyed for *GFP* expression under a Leica AF6000 fluorescence microscope (Leica, Wetzlar, Germany).

3. Results

3.1. Isolation of Three Polyubiquitin Promoters in Hevea

In search for endogenous polyubiquitin promoters in *Hevea* that could potentially be used as alternatives to the CaMV35S promoter, we performed BLAST searches of the reference genome of *Hevea* using *AtUBQ10* as a query. Three polyubiquitin genes that share high sequence similarities to the query were identified and designated as *HbUBI10.1*, *HbUBI10.2* and *HbUBI10.3* (Figure S1). The *HbUBI10.1* and *HbUBI10.2* genes encode polyubiquitins consisting of six ubiquitin repeats in a head-to tail, spacerless arrangement, which is consistent with that of *AtUBQ10*. In contrast, the *HbUBI10.3* has a shorter coding sequence encoding five ubiquitin monomers. Additionally, the ubiquitin monomers encoded by these three

HbUBI genes have an amino acid sequence identical to that of *AtUBQ10*, except for a single amino acid difference in the first ubiquitin monomer in *HbUBI10.1* and *HbUBI10.2* (Figure S2). All of these *HbUBI* genes consist of a long leading intron, ranging from 760 to 1175 bp in length (Figure 2a), which is required in the polyubiquitin promoters to induce strong gene expression [32,39,40]. Therefore, in this study, DNA fragments of 1237, 1146 and 1380 bp in length upstream of the ATG start codon of the *HbUBI10.1*, *HbUBI10.2* and *HbUBI10.3* genes, including the leading introns, were respectively amplified as putative *HbUBI* promoters for subsequent experiments (Figure 2a). The sequences for *HbUBI10.1*, *HbUBI10.2* and *HbUBI10.3* promoters were deposited at the National Center for Biotechnology Information (NCBI) under the accession numbers ON110822, ON110823 and ON110824, respectively. PlantCARE analysis showed that these three *HbUBI* promoters consist of a large number of TATA-box and CAAT-box motifs at numerous positions, which are core elements required for transcription initiation (Figure 2b, Table S2). Two light responsive elements, G-box and the GATA-motif that were commonly present in other constitutive promoters were also found in these three *HbUBI* promoters. The promoters of *HbUBI10.1* and *HbUBI10.3* respectively harbor two and one G-box motifs. The GATA-motif appeared one and two times in the *HbUBI10.2* and *HbUBI10.3* promoter sequences, respectively. Additionally, multiple putative *cis*-acting elements that are associated with hormones and abiotic stresses response are present on these *HbUBI* promoters (Figure 2b, Table S2).

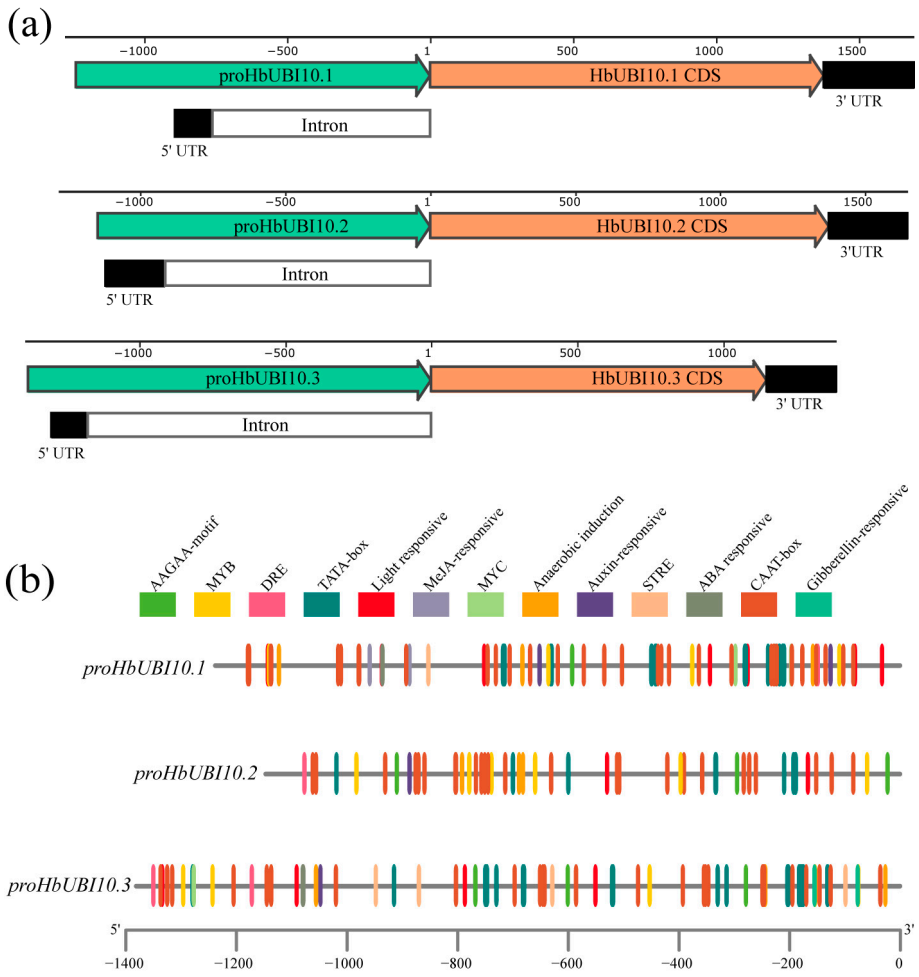

Figure 2. Analysis of three *HbUBI* genes and their promoters. (**a**) Schematic representation of the genomic structures of three polyubiquitin genes. (**b**) Putative *cis*-elements identified in these three *HbUBI* promoter regions. Abbreviations: CDS, coding sequence; UTR, untranslated region; DRE, dehydration-responsive element; MeJA, methyl jasmonic acid; STRE, stress response element; ABA, abscisic acid.

3.2. Expression Analysis of Three HbUBI Genes

We obtained the expression profiles of *HbUBI10.1* and *HbUBI10.2* in *Hevea* plants by mining the transcriptome data downloaded from HeveaDB [34]. The results show that these two genes are constitutively expressed in diverse *Hevea* tissues at different development stages (Figure 3a). Notably, *HbUBI10.1* is highly expressed in laticifers and latex, where natural rubber is biosynthesized. The expression of *HbUBI10.3* was not yet available in HeveaDB. Further study is required to investigate the expression profiles of *HbUBI10.3*.

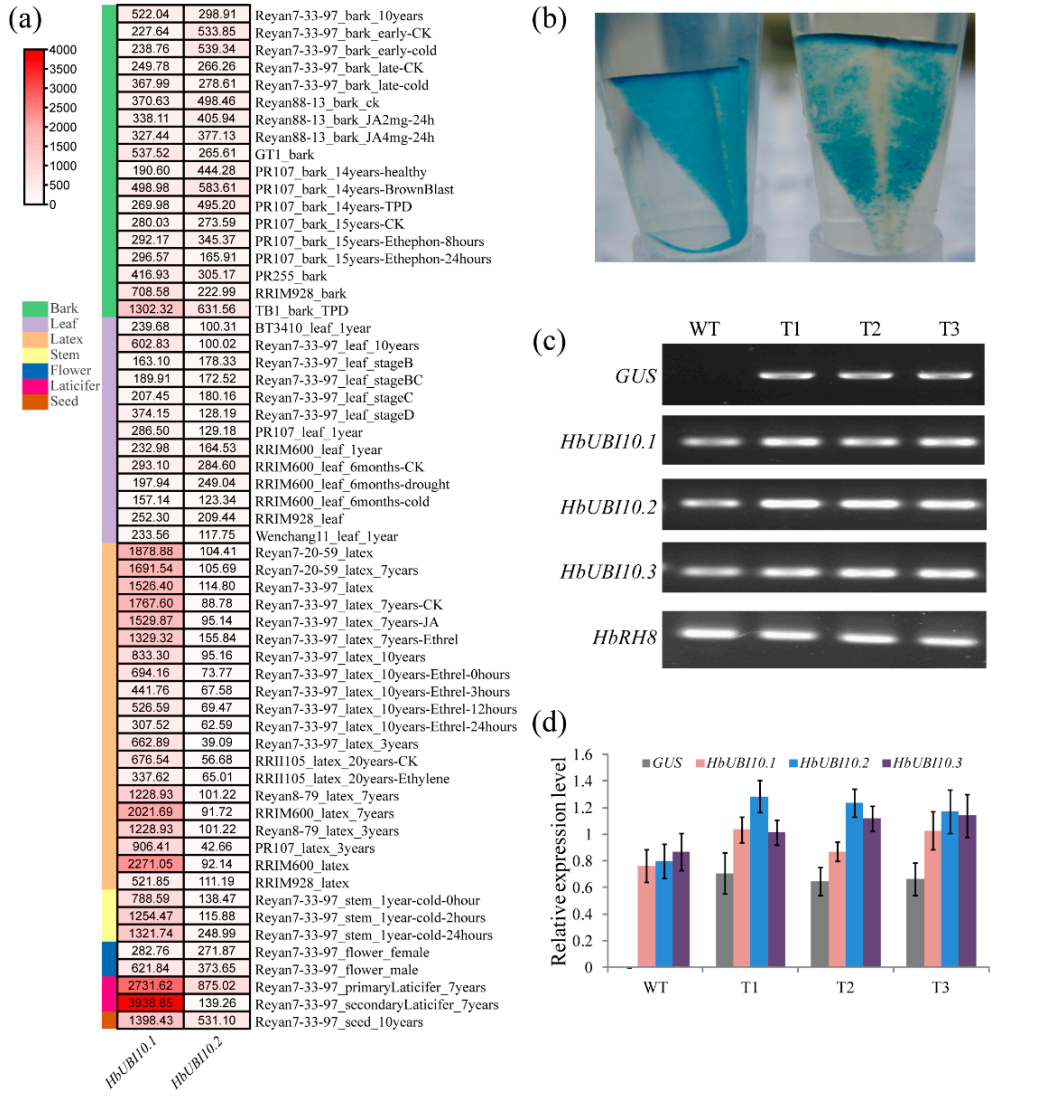

Figure 3. Expressions of three *HbUBI* genes in *Hevea brasiliensis*. (**a**) Expression profiles of *HbUBI10.1* and *HbUBI10.2* genes in various tissues of multiple *Hevea* clones at different developmental stages. Normalized transcript levels are displayed as FPKM values in the heatmap. Row names denote the *Hevea* clone, type of tissue, age and treatment. Abbreviations: JA, jasmonic acid; TPD, tapping panel dryness; stageB, bronze stage; stageBC, color-changing stage; stageC, light green stage; stageD, stable stage. (**b**) *GUS* staining of the leaves from 1-year-old transgenic *Hevea* plants harboring a *GUS* gene driven by the CaMV35S promoter. (**c**) Semi-quantitative RT-PCR analysis of the endogenous expression levels of three *HbUBI* genes in transgenic *Hevea* plants bearing a *GUS* gene driven by the CaMV35S promoter. WT represents the wild-type control, while T1, T2 and T3 represent three individual transgenic *Hevea* plants. (**d**) Relative expressions of three *HbUBI* genes and *GUS* in the leaves of 1-year-old transgenic *Hevea* plants. The mean relative expressions with standard deviation were calculated from three replicates of semi-quantitative RT-PCR reactions.

Semi-quantitative RT-PCR was performed to compare the endogenous expression levels of three *HbUBI* genes with that of the *GUS* gene driven by the CaMV35S promoter in the leaves of 1-year-old transgenic *Hevea* plants (Figure 3b–d). The results show that the *GUS* gene driven by CaMV35S promoter is comparably expressed among three transgenic *Hevea* plants, while these three *HbUBI* genes show slightly higher endogenous expression levels than that of the *GUS* gene (Figure 3c,d). These results suggest that the use of promoters from these *HbUBI* genes may give higher levels of transgene expression than that of the CaMV35S promoter in *Hevea* transformation.

3.3. Functional Evaluation of Three HbUBI Promoters in Hevea Tissues

To determine whether these *HbUBI* promoters could be used as alternatives to CaMV35S for *Hevea* transformation, both transient and stable transformation assays were performed to evaluate the capabilities of three *HbUBI* promoters in directing transgene expression in *Hevea* tissues.

These three *HbUBI* promoters were fused to the *GFP* reporter gene, and transformed into *Hevea* protoplasts. *GFP* fluorescence was observed in *Hevea* protoplasts transformed by plasmids containing each of these three *HbUBI* promoters (Figure 4), confirming their transcriptional activities. Transformation efficiencies were calculated according to the numbers of protoplasts with *GFP* fluorescence. The control plasmid incorporating the 2× CaMV35S promoter gave a transformation efficiency of 48%. However, when the *GFP* gene was associated with three *HbUBI* promoters, the transformation efficiencies were around 35% (Figure 4), which were lower than that of the 2× CaMV35S promoter control.

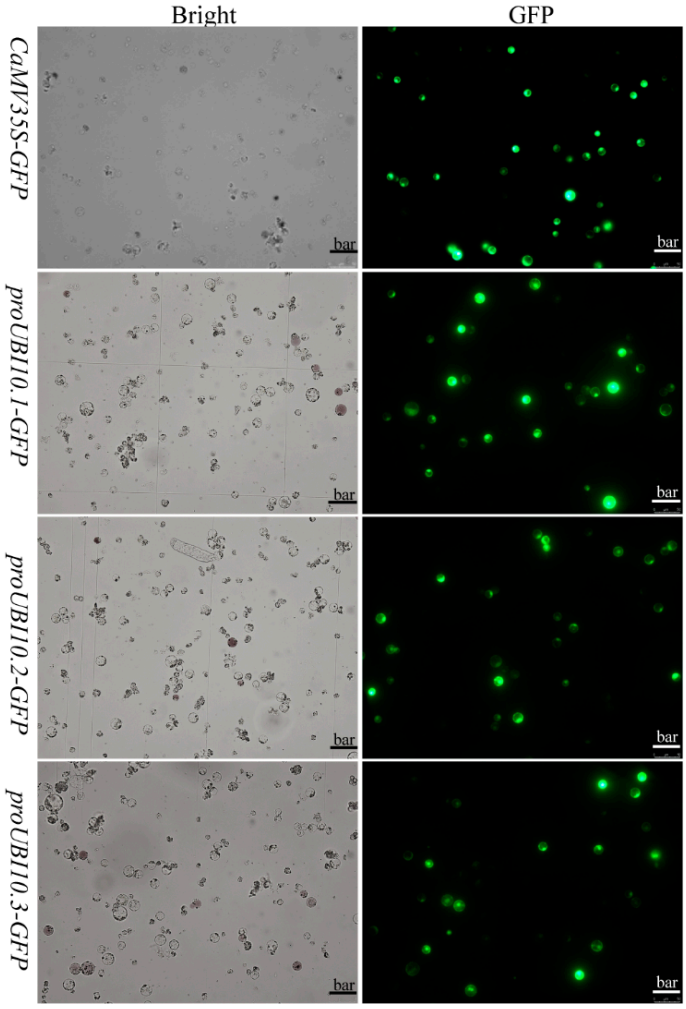

Figure 4. Transient expression assays in *Hevea* mesophyll protoplasts. Transformation efficiencies were calculated from three replicates. Size bars indicate 50 μm.

Agrobacterium-transformed *Hevea* embryos could be used as a preliminary indicator of the transcription activities of promoters in directing stable transgene expression. As shown in Figure 5, all three *HbUBI* promoters successfully promoted the expression of *GFP* in *Hevea* embryos. These results indicate that these three *HbUBI* promoters faithfully drive the expression of transgenes, and are therefore suitable alternatives to the CaMV35S promoter in *Hevea* transformation.

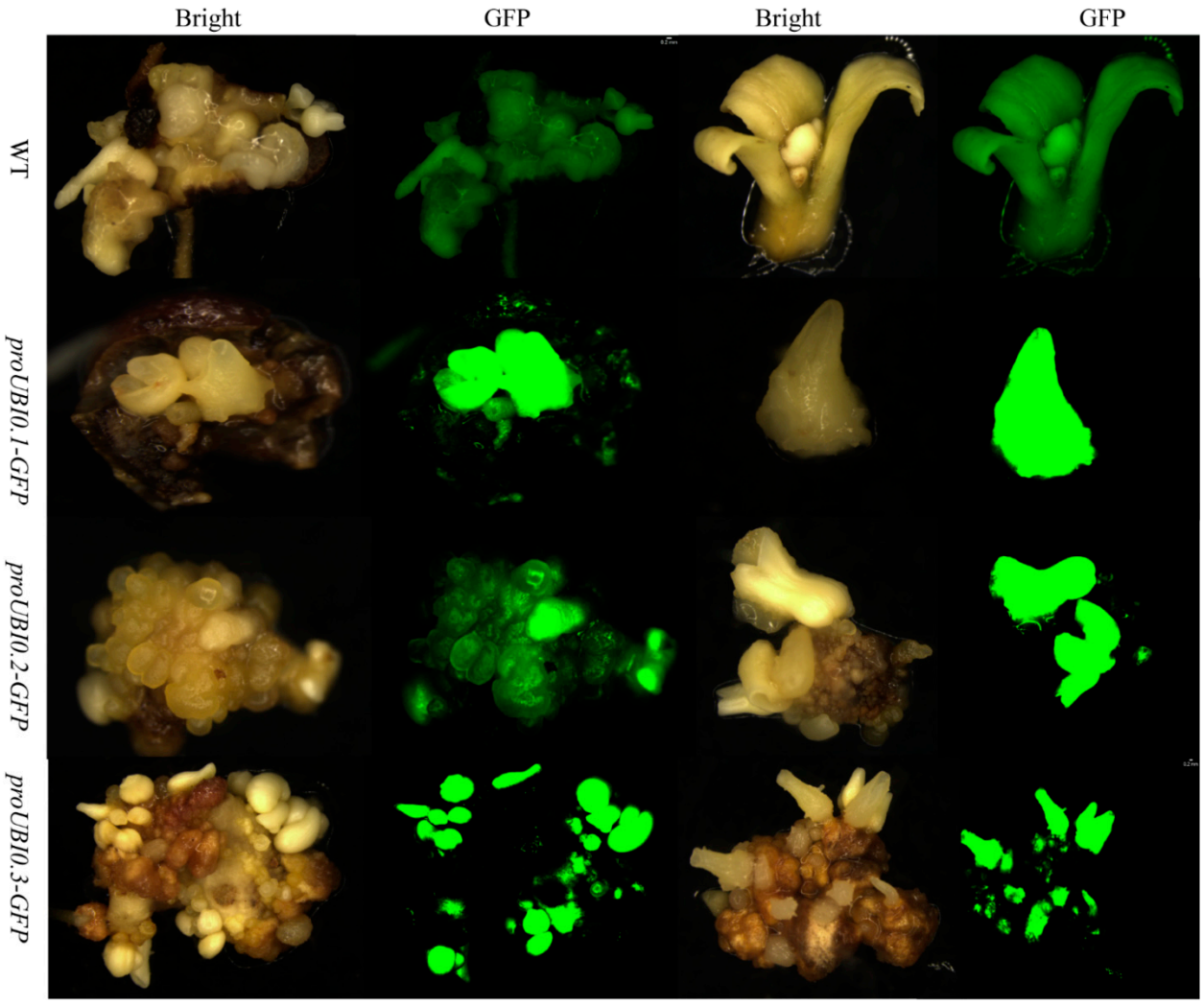

Figure 5. Transgenic *Hevea* embryos bearing a *GFP* reporter gene that was respectively driven by *HbUBI10.1*, *HbUBI10.2* and *HbUBI10.3* promoters. Non-transformed embryos were used as negative controls.

4. Discussion

In previously reported *Hevea* transformation systems, the CaMV35S promoter was extensively used to direct the constitutive expressions of selectable markers, reporter genes and genes of interest [3,5,7,8]. However, the use of the CaMV35S promoter for driving transgene expression in plant transformation is not ideal, either because of the incidence of promoter methylation-induced transgene repression during the development of transgenic plants [12,13], or the biosafety concerns raised by the pathogen—the-origin of the CaMV35S promoter. With the aim to provide alternative promoters to the CaMV35S for *Hevea* genetic transformation, we settled on the polyubiquitin promoters that confer a higher transformation efficiency, transgene expression levels and expression stability in various plant species when compared with those of the CaMV35S promoter [23,24,31].

The polyubiquitin genes encode the polyubiquitin precursor protein containing tandemly repeated ubiquitin monomers [18]. This study identified three polyubiquitin genes *HbUBI10.1*, *HbUBI10.2* and *HbUBI10.3* in *Hevea*, which respectively encode six, six and five repeats of ubiquitin monomers, having an identical amino acid sequence to *AtUBQ10* except for one amino acid difference in the first monomer of *HbUBI10.1* and *HbUBI10.2* (Figures S1 and S2). These findings support the previous reports that the ubiquitins are highly conserved among different plant species [18,21].

Previous studies showed that polyubiquitin promoters of around 1000 bp in length are generally used to drive transgene expression [20]. A longer sequence upstream of tomato *UBIQUITIN10* directs higher adjacent gene expression [32]. However, small promoters enable the elaboration of compact vectors, and therefore may produce higher transformation efficiencies [41]. All three *Hevea* polyubiquitin genes identified in the present study possess a long leading intron upstream of the ATG start codon (Figure 2a), which is a common feature of polyubiquitin genes. This intron is considered part of the promoter, and contributes to very high levels of adjacent gene expression in transiently and stably transformed plant tissues [27,30,32,39,40]. Removal of the intron from the promoter region either reduces the strength of the promoter or results in a complete loss of promoter activity [23,27]. Taking into consideration the above, the fragments upstream of three *HbUBI* genes including the signature leading intron, ranging from 1146–1380 bp in length, were used for further analysis.

The promoters of these three *HbUBI* genes were shown to contain numerous copies of TATA-box and CAAT-box motifs (Figure 2b, Table S2), which act as major determinants of promoter efficiency during gene transcription [42]. In addition to these core promoter elements, two light-responsive elements, the GATA-motif and G-box motif, were identified, which are well conserved motifs in other strong constitutive promoters [27,42]. The G-box motif, which has been proposed to contribute considerably to the strong expression mediated by polyubiquitin promoters [27,43], was present two and one times in the promoters of *HbUBI10.1* and *HbUBI10.3*, respectively. One and two copies of GATA-motifs were identified in the promoters of *HbUBI10.2* and *HbUBI10.3*, respectively. These results suggest that these three *HbUBI* promoters might be involved in effective transcription. Additionally, the presence of a large number of environment-responsive *cis*-acting elements in these *HbUBI* promoters may also contribute to the total transcription activities [27].

Transcriptomic data provide a better understanding of the global regulation of gene expression, and therefore facilitate the identification of endogenous promoters in economically important crops [26,41]. Several transcriptomes for a number of *Hevea* clones are now available [34,44,45]. According to the transcriptome database, *HbUBI10.1* and *HbUBI10.2* genes were highly expressed in various tissues and development stages (Figure 3a), indicating that their promoters should have constitutive activities. Although the activities of these three *HbUBI* promoters in directing transgenes in whole *Hevea* plants were not accomplished in this study, the higher endogenous expression levels of three *HbUBI* genes, as compared with the expression driven by the CaMV35S promoter in transgenic *Hevea* leaves, suggest that these *HbUBI* promoters may be capable of directing the strong expression of transgenes (Figure 3c,d).

Both the constitutive promoters of apple *MdUBQ10* and *Arabidopsis AtUBQ10* drove the expression of target genes in apple protoplast cells significantly better than the CaMV35S promoter [46]. However, in our present study, the vector incorporating the 2× CaMV35S promoter produced higher protoplast transformation efficiency than the *HbUBI* promoter-based transient vectors (Figure 4), which may be attributed to its smaller size (737 bp in length), independent of the transcriptional activities of those incorporated promoters. These results demonstrate the capabilities of *HbUBI* promoters in driving transient expression in *Hevea* protoplasts. However, further promoter truncation analysis is required to reduce the length of these *HbUBI* promoters while maintaining their transcriptional activities, thus reducing the construct size, and therefore facilitating genetic transformation [27].

Transient expression is not subject to chromatin-based gene regulation and, thus, the transient assay does not reflect the full functionality of promoters [27]. Given that the recovery of whole transgenic *Hevea* plants takes at least several months, *Agrobacterium*-transformed *Hevea* embryos were therefore used for the rapid validation of the transcription activities of these *HbUBI* promoters in directing stable transgene expression. In basta-resistant embryos, all three *HbUBI* promoters produced *GFP* fluorescence (Figure 5), demonstrating their capabilities in driving the stable expression of reporter genes or selection markers in *Hevea* transformation. Further long-term investigations are required to document the comprehensive expression profiles of these *HbUBI* promoters in whole transgenic *Hevea* plants at different development stages. The maize ubiquitin promoter ZmUbi1 produced significant higher maize transformation efficiency than those of two viral promoters, the CaMV35S promoter and sugarcane bacilliform virus promoter, when used in driving the expression of selectable marker genes [24]. Therefore, the future use of these *HbUBI* promoters as replacements of the CaMV35S promoter for expressing selectable markers may also produce higher transformation efficiencies in *Hevea*.

Additionally, polyubiquitin promoters show great potential in CRISPR-mediated genome editing systems. In allotetraploid tobacco plants, *UBIQUITIN10* promoters from several plant species directed the higher expression of selection markers than that of the CaMV35S promoter, and thereby significantly improved the efficiencies of *Agrobacterium*-mediated transformation and CRISPR/Cas9-mediated mutagenesis [32]. The use of a grape endogenous polyubiquitin promoter UBQ2 can significantly improve the editing efficiencies in grape cells and stable transgenic plants by increasing the expression of Cas9 [26]. In a multiplexed genome editing system in alfalfa, the replacement of the CaMV35S promoter by the *Arabidopsis* ubiquitin promoter to drive Cas9 expression led to a significant improvement in genome editing efficiency [25]. Targeted genome editing in *Hevea* was recently achieved using the CRISPR/Cas9 system, wherein the expression of Cas9 was driven by the CaMV35S promoter [47]. Future applications of these *HbUBI* promoters in *Hevea* CRISPR systems may also improve the efficiencies of targeted mutagenesis.

5. Conclusions

This study identified three *Hevea* polyubiquitin genes by BLASTing the *Hevea* genome. Expression analysis showed that *HbUBI10.1* and *HbUBI10.2* were constitutively expressed in *Hevea* plants, and all three *HbUBI* genes exhibited higher endogenous expression than the *GUS* gene driven by the CaMV35S promoter in transgenic *Hevea* leaves. We isolated the promoter fragments of these three *HbUBI* genes, and confirmed that these promoters could direct *GFP* expression in both transient and stable assays. These results suggest that these *HbUBI* promoters could be potentially used as alternatives to the CaMV35S promoter for driving the constitutive expression of transgenes in *Hevea* genetic modification programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13060952/s1, Figure S1: Sequence alignment of coding regions of three *Hevea* polyubiquitin genes with that of *AtUBQ10*; Figure S2: Alignment of the amino acid sequences of three *HbUBI* polyubiquitins and *AtUBQ10*; Table S1: Primers used in present study; Table S2: Putative *cis*-acting elements identified in three *HbUBI* promoters.

Author Contributions: Conceptualization, H.H. and T.H.; Data curation, S.X.; Funding acquisition, Y.H.; Investigation, S.X., J.U. and X.D.; Methodology, S.X.; Project administration, T.H.; Resources, X.D.; Supervision, H.H.; Validation, S.X., J.U. and Y.F.; Writing—original draft, S.X.; Writing—review & editing, S.X. S.X. and J.U. contributed equally to this study. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (grant number 31872708), the Hainan Provincial Natural Science Foundation of China (grant number 322MS137), and the National Key R&D Program of China (grant number 2019YFD1001102).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequences for the three *Hevea* polyubiquitin promoters have been deposited at National Center for Biotechnology Information (NCBI) under the following accession numbers: ON110822 (*HbUBI10.1*), ON110823 (*HbUBI10.2*) and ON110824 (*HbUBI10.3*). The materials used in this study are available from the authors upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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