Full Length Article

Strictosidine Synthase Coding Gene Expression towards Quinine Biosynthesis and Accumulation: Inconsistency in Cultured Cells and Fresh Tissues of *Cinchona ledgeriana*

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Abstract

Strictosidine synthase, encoded by the gene *STR*, facilitates the regeneration of strictosidine, a critical intermediate for the synthesis of many plant alkaloids. The gene has, however, never been studied in *Cinchona* spp. The plants produce quinine alkaloid used for malaria medication, SARS-CoV-2 treatment and other industrial purposes. Cultured cells can produce the alkaloid but only at a much lower yield than the natural tree. This study describes *STR* expression and quinine content in various plant materials. Bioinformatic analyses were conducted on nine species of Rubiaceae to obtain reference sequences to design conservative primers for *Cinchona ledgeriana STR* (*ClSTR*). *ClSTR* expression was analyzed using qRT-PCR and quinine content was determined using HPLC. A complete coding sequence (CDS) of *ClSTR* was deposited in NCBI GenBank under the accession number MK422544.1. *ClSTR* was expressed in cultured cells, young and mature leaves, and stem bark. The elicited cells have higher expression than the control and they performed since the fourth week. However, the quinine content was greater in older cells. The gene expression in young leaves was superior, but quinine was most abundant in the stem bark. Every cell of *C. ledgeriana*, in culture or in the plant, expressed *ClSTR* and was capable of synthesizing the alkaloid quinine. The alkaloid from the leaves of the plant might be translocated and accumulated in the bark. No efflux of alkaloids from the confined cultured cells might contribute in triggering feedback inhibition in the biosynthetic pathway. This study revealed a critical obstacle in cell culture as a means of secondary metabolites production that needs further development of metabolic engineering. © 2021 Friends Science Publishers

Introduction

Cinchona or quina plants produce quinoline alkaloids, such as quinine, one of the potential candidate molecules for SARS-CoV-2 treatment (Grosse *et al.* 2020; Lestari *et al.* 2020). Plants produce secondary metabolites (SMs) to serve a wide range of biological activities, including physiological adaptation to various environmental conditions. The type and concentration of SMs in plants depend on the species (Nair 2010), its physiology and developmental stage, and the environmental factors during their growth (Ramakrishna and Ravishankar 2011; Maldonado *et al.* 2017; Isah 2019). Changes in environmental factors can also lead to alteration of the composition and concentration of SMs. Naturally, SM production is very low because of their role in the host plant as signaling agents or of their direct functionality as chemical protection against predators or invasive microorganisms (Ramakrishna and Ravishankar 2011; Ncube and Staden 2015).

The biosynthetic pathway of SM production may occur in different organelles within a cell, in multiple tissues within an organ, or in various organs (Rischer *et al.* 2006; Zhou *et al.* 2010; Shitan 2016). Furthermore, SMs are transported from their source to a sink for storage, which may be unique in different plant species. Excessive amounts of SMs can toxify the producing cells (Sirikantaramas *et al.* 2008; Wink 2010; Shitan 2016); therefore, SMs are generally transported and stored in particular compartments, such as the vacuoles, endoplasmic reticulum and secretory vesicles, or are simply excreted into the apoplast (Yazaki 2005; Pratiwi *et al.* 2020). Transporting the SMs to some other organelles or organs away from where the biosynthesis occurs and accumulating them therein suggest a mechanism of self-tolerance (Sirikantaramas *et al.* 2008;

Ramakrishna and Ravishankar 2011; Shitan 2016). Previous research has reported high variation in quinine content in cell suspension cultures, making large-scale production difficult and very expensive. There is a large discrepancy between the quinine content in cultured cells and plant tissues of *Cinchona* spp., in the range of 0.009 to 0.66% in *C. ledgeriana* cell cultures (Ratnadewi and Sumaryono 2010; Pratiwi *et al.* 2018; Hasibuan *et al.* 2021) compared with 4.8 to 9% in the plant stem bark (McCalley 2002; Kurian and Sankar 2007). It is questionable whether the low percentage of quinine in cultured cells is due to low capacity of biosynthesis or some self-tolerance mechanism.

Strictosidine is the main product of tryptophan and secologanin catalyzed by strictosidine synthases (STRs). Strictosidine becomes a critical intermediate for the synthesis of various terpenoid indole alkaloids, such as ajmalicine, vinblastine, vincristine and quinoline (Ratnadewi 2017). Depending on the plant species, many enzymes take part in this biosynthetic pathway by which specific alkaloids are produced. We focused on the *STR* gene, as it has a clear correlation with quinine and general quinoline alkaloid biosynthesis. The expression of this gene drives STR enzyme synthesis that would further activate the pathway toward the end product of cinchona alkaloids. By comparing the *STR* gene transcription and quinine content in cultured cells and fresh plant tissues, we sought to determine the source of the observed discrepancy.

Since we did not find any reference for the *STR* gene of *Cinchona* species, it was likely that this gene had never been sequenced before. Therefore, we performed bioinformatic analysis to determine the conserved regions within the coding sequence (CDS) of the *STR* gene in the order Gentianales, especially in the family Rubiaceae or subfamily Cinchonoideae. The results were used as references to design conservative primers for *STR* in *C. ledgeriana*. Phylogenetic trees were also generated for accessions of the Gentianales clade and the Rubiaceae family.

Aerts *et al.* (1991) investigated the localization of cinchona alkaloid biosynthesis by studying the distribution and activity of STR enzymes in diverse plant parts of six-month-old *C. ledgeriana*. Based on the enzyme activity, they reported that young organs were the sites of biosynthesis and accumulation of cinchona alkaloids. Our research aimed to study the correlation between the relative expression of the *STR* gene (namely, *ClSTR*) and alkaloid content, represented by quinine, in various fresh plant tissues and cultured cells of *C. ledgeriana*. The results of this study may be used to overcome current obstacles to improving the quinine content of *C. ledgeriana* cell culture through genetic and/or metabolic engineering in the future.

Materials and Methods

Plant materials

The friable callus used in this experiment was obtained from

Pratiwi *et al.* (2018) and the cell suspension culture was performed following their procedure. Young (YL) and mature leaves (ML), as well as stem bark (SB), were collected from a mature *C. ledgeriana* tree belonging to the State Plantation of PTPN VIII, Gunung Mas, West Java, Indonesia.

Cell suspension culture and treatments

Only several cell treatments were taken from those conducted by Pratiwi *et al.* (2018), notably those which produced the highest quinine content, highest total quinine, and control cells. A3K refers to the treatment with ABA (3 mg·L^{-1}) and the standard amount of sucrose (30 g·L^{-1}), while A3S denotes the treatment with ABA (3 mg·L^{-1}), sorbitol (5.3 g·L^{-1}) and sucrose (20 g·L^{-1}). The molarity of 5.3 g·L^{-1} sorbitol is equivalent to that of 10 g·L^{-1} sucrose. "Control" means that the culture media consisted of only the basic composition, without any elicitor or sucrose substitution. Each treatment was replicated 10 times; the cell suspension cultures were maintained for 4 and 7 weeks. After harvest, the cells were prepared for extraction and subsequent HPLC analysis.

Extraction and quinine content determination

Extraction was performed according to the method described by Hasibuan *et al.* (2021). The dried extract was made up to 2 mL with 100% methanol (HPLC grade) and a 15 μL aliquot was injected into the HPLC column at 30°C. A Hypersil ODS-2 C18 column (250 × 4.6 mm, 5 μm particle size; Thermo Fischer Scientific, USA) was used for the HPLC (Shimadzu 10 A VP, Kyoto, Japan). The HPLC eluent consisted of sodium acetate (6 g·L^{-1}): acetic acid (glacial): methanol (100:1:100). The flow rate was 0.1 mL·min^{-1}. The eluate from the column was monitored through a UV-vis detector at 240 nm. Quinine sulfate CRS (EDQM-DEQM, Germany) was used as a standard. The experiment was performed in three replicates for each treatment.

Bioinformatic analysis of *ClSTR* and primer design

We performed bioinformatic analysis to determine the conserved regions of the CDS of the *STR* gene in the group Gentianales, especially the family Rubiaceae or subfamily Cinchonoideae. The CDS accessions of *STR* analyzed were of *Mitragyna speciosa* (HM543187.1), *Ophiorrhiza japonica* (EU670747.1), *O. pumila* (AB060341.1), *Gelsemium sempervirens* (MF401945.1), *Rauvolfia verticillata* (DQ87216), *R. serpentina* (X62334.1), *Tabernaemontana elegans* (JN644947.1), *Catharanthus roseus* (X53602.1) and *Vinca minor* (JN644948.1) (NCBI Database). We then designed conservative primers of these *STR* genes using the primers application in U-Gene (Okonechnikov *et al.* 2012) to be employed in *C. ledgeriana*. In addition to designing the

primers, we generated phylogenetic trees by incorporating accessions of the Gentianales clade, including X61932, Y00756, DQ872163, DQ017054, JX118639, HM543187, EU288197, JF412823, EU670747, AB060341, MF401945, FD661083, GT020720, GT664657, FD660801, FJ004233, AF329450, MK422544, KM524258, AY027510, AF084972, AF084971, FJ004235, FJ004234, AF283506, JN226128, JN644947, JN644948 and X53602 (NCBI Database). We also made a phylogenetic tree based on the family Rubiaceae accessions EU670747, GT020720, GT664657, MK422544, EU288197, HM543187, JF412823 and AB060341 to understand the evolutionary status of the *ClSTR*.

Isolation of total RNA

RNA isolation was performed using the RNeasy Plant Mini Kit (Qiagen, Germany). We used 100 mg of the cultured cells, fresh portions of young leaves (YL), ML and SB of *Cinchona ledgeriana*. The quantity of RNA was measured using GenQuant (Bio-Rad, California, USA) and quality was visualized using agarose gel electrophoresis following the procedure described by Satrio *et al.* (2019).

cDNA synthesis

cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (# K1622, Thermo Fisher Scientific, USA) with slight modification, as described by Satrio *et al.* (2019). Total RNA (5 *μ*g) were treated with DNase I and then mixed with 1 *μ*L oligo-dT primer (20 pmol). The mixture was then added to 4 *μ*L 5X reaction buffer, 1 *μ*L Ribolock RNase Inhibitor (20 U·μL^{-1}), 2 *μ*L 10 m*M* dNTP Mix and 1 *μ*L RevertAid M-MuLV reverse transcriptase (200 U·μL^{-1}). The total reaction volume was 20 *μ*L. All the reactions were mixed and centrifuged twice. The mixture was then incubated for 60 min at 42°C and the reaction was terminated by heating at 70°C for 5 min. The concentration and purity of cDNA obtained were measured using a NanoDrop. The cDNA concentration was diluted to 50 ng·μL^{-1} before storage.

ClSTR gene sequencing from *Cinchona ledgeriana* cell culture

DNA was isolated following the modified CTAB method (Wahyuningtyas *et al.* 2016; Fendiyanto *et al.* 2019a), where the *STR* genomic regions were amplified using four forward and reverse primers (Table 1). The primers were also used to amplify *STR* of the previously synthesized cDNA. The amplified *STR* gene was identified and sequenced with an ABI PRISM 310 Genetic Analyzer machine with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) to obtain full-length *STR* gene sequences, following the method described by Fendiyanto *et al.* (2019b). The sequence data were analyzed for the codon start area, CDS, and stop codon with the U-Gene program (Okonechnikov *et al.* 2012).

ClSTR expression analysis by qRT-PCR

Gene expression was analyzed following the procedure of Satrio *et al.* (2019) with a slight modification to the PCR program with respect to annealing temperature. The expression was analyzed using the primers *STR1-forward*: 5'-CCCCATTCTGGAACAGAAAA-3' and *STR1-reverse*: 5'-CCTCCTTCAGGTCCAACCAC-3', and normalized to the expression of the housekeeping gene *actin* (*Actin-forward*: 5'-CCTCTTAACCCGAAGGCTAA-3' and *Actin-reverse*: 5'-GAAGGTTGGAAAAGGACT-3'). The programs used included the following steps: 95°C predenaturation for 2 min, 95°C denaturation for 5 s, 50°C (for *STR1*) or 55°C (for *actin*) annealing for 10 s and 60°C extension for 10 s. The qRT-PCR reaction was performed using 2x SensiFAST SYBR® High-ROX Mix (Bioline, United Kingdom) on the StepOne™ Plus Real-Time PCR System (Applied Biosystems, USA). The composition of the reaction mixture was 5 *μ*L 1X SensiFAST SYBR® High-ROX Mix, 0.4 *μ*L forward primer (10 *μM*), 0.4 *μ*L reverse primer (10 *μM*), 1 *μ*L cDNA template (50 ng) and 10 *μ*L ddH_2O. The results were analyzed for relative expression, following the protocols of Livak and Schmittgen (2001) and Ratnasari *et al.* (2016).

Pearson correlation analysis

To determine the correlation between quinine content and the *STR* gene expression, we performed a Pearson correlation analysis, followed by a principal component analysis (PCA). The data were analyzed in R program version 3.51, using corrplot mixed packages version 0.80, as described by Lander (2014) and Fendiyanto *et al.* (2019b).

Results

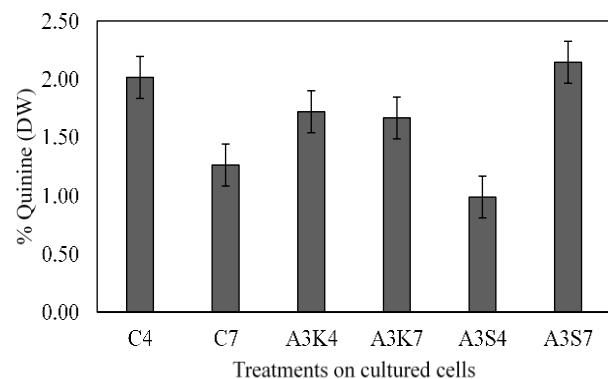**Quinine contents in cultured cells and fresh tissues**

The HPLC analysis demonstrated that both control and treated cells produced quinine (Fig. 1). The four-week-old cell culture yielded higher content of the alkaloid than the seven-week-old culture. A3S4 and A3S7 showed exceptional results; elicitation with ABA (3 mg·L^{-1}) and partial substitution of sucrose with sorbitol for seven weeks (A3S7) enhanced quinine production. The SB contained a substantial amount of quinine among the organ tissues of the plant, reaching 19.83%. The YL displayed twice the quinine content of the ML (Fig. 2).

Bioinformatic analysis of *ClSTR* and primer design

Using U-Gene program to analyze nine species of the family Rubiaceae, we used the conserved area of the *STR* gene to design primers. We successfully synthesized four primers and determined the annealing temperature, as presented in Table 1.

Table 1: Four primer designs resulted from bioinformatic analysis on the conserved regions of *STR* coding sequence in nine referent plant species

Primers	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	T Annealing (°C)
STR1	CCCCATTCTGGAACAGAAAA	CCTCCTTCAGGTCCAACCAC	50
STR2	GCCGGAGTTCTTCCAATTTA	GTTGTTCTCACAAAATGCTT	50
STR3	ATGGGTAGTTCAGAAGCCATGG	TCAGAAAGAAGAAAATTCCTTG	55
STR4	CACACCTAACATGAACACT	TAGAAACAAAATGTTCAAGT	55

Fig. 1: Quinine content in cultured cells of *Cinchona ledgeriana* treated with various elicitors

The data are mean $\pm$ SD of three analysis taken randomly from ten cultures per treatment. C4: 4 weeks-old control cells; C7: 7 weeks-old control cells; A3K4: cells treated with ABA 3 mgL^{-1} and standard sucrose, 4 weeks old; A3K7: cells treated with ABA 3 mgL^{-1} and standard sucrose, 7 weeks old; A3S7: cells treated with ABA 3 mgL^{-1}, partial sorbitol, 7 weeks old: cells treated with ABA 3 mgL^{-1}, partial sorbitol, 7 weeks old
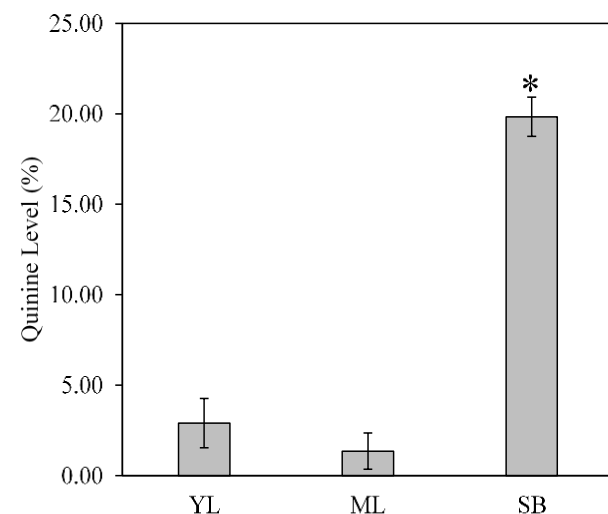
Fig. 2: Quinine contents in fresh tissues of *Cinchona ledgeriana*
The data of quinine are mean $\pm$ SD of three analysis. YL: young leaves; ML: mature leaves; SB: stem bark. The asterisk signs indicate statistically significant difference (Welch's t-test; *$P < 0.05$)

The cDNAs produced from the isolated total RNA of the cultured cells (concentrations ranged from 395 to 2115 ng·mL^{-1}) were used as templates to determine the coding region of *STR* in *C. ledgeriana*. These four primers were then employed for PCR. Some full-length sequences of *STR* were obtained through cDNA sequencing. One consistent
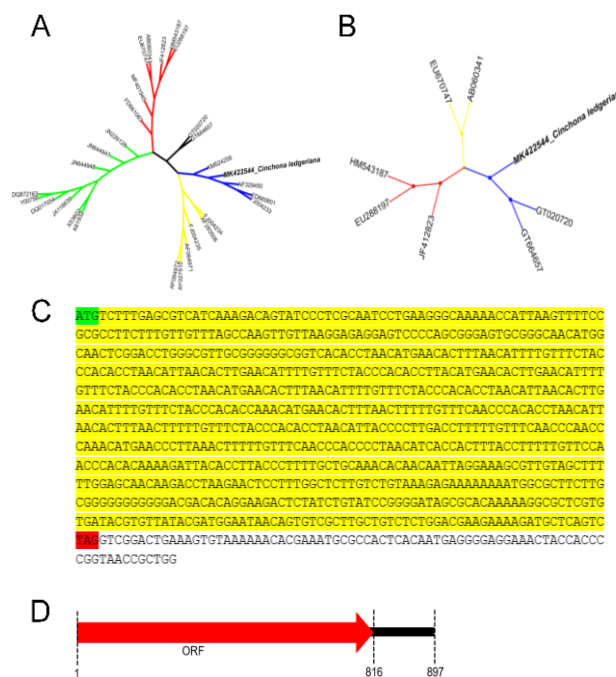
Fig. 3: Phylogenetic tree and sequence of the *ClSTR* in clade Genetiales and family Rubiaceae
(**A**) Phylogenetic tree of *Cinchona ledgeriana's Strictosidine synthase* (*ClSTR*) among the clade Genetiales
(**B**) Phylogenetic tree of *Cinchona ledgeriana's Strictosidine synthase* (*ClSTR*) among the family Rubiaceae
(**C**)The novel sequence of *Cinchona ledgeriana's Strictosidine synthase* (*ClSTR*), 897 bp consists of ORF (open reading frame); and 3'-UTR (3'-untranslated region)
(**D**) Schematic structure of *Cinchona ledgeriana's Strictosidine synthase* (*ClSTR*). ORF length: 1-816 bp; 3'-UTR length: 816-897 bp

result has been deposited as a complete CDS in NCBI GenBank with the accession No. MK422544.1 (https://www.ncbi.nlm.nih.gov/nuccore/MK422544.1). The gene size is 897 bp, in which the first 816-bp sequence is the open reading frame and the rest includes the 3'-unstranslated region (Fig. 3).

Two phylogenetic trees were generated: one of the Gentianales clade and the second based on the family Rubiaceae. In the Gentianales clade, homologous sequences were divided into five groups, where the *Cinchona STR* gene was classified into the same group as the accessions KM524258, AF329450, FD660801 and FJ004233 (Fig. 3A). In the family Rubiaceae, homologous sequences were classified into three groups, where the *STR* gene of *Cinchona* was in the same group with the accessions GT020720 and GT664657 (Fig. 3B).
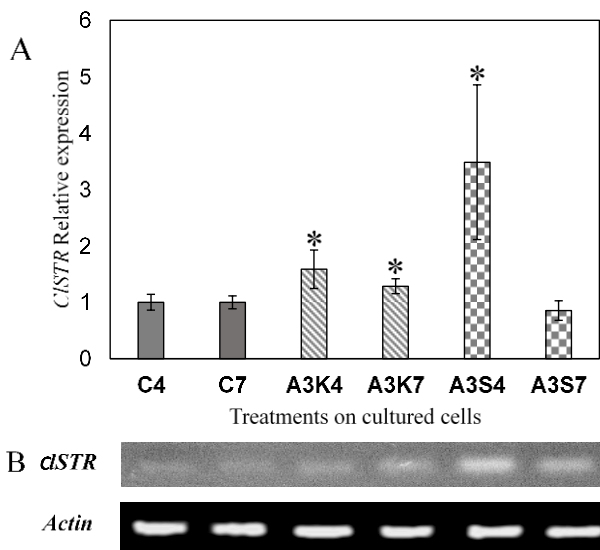
Fig. 4: The levels of *ClSTR* gene expression in *Cinchona ledgeriana* cell cultures at weeks 4 and 7 with various elicitor treatments

(**A**). The level of *ClSTR* gene expression was demonstrated by qRT-PCR method and was calculated using relative expressions following Livak and Schmittgen (2001). The data are mean ± standard error (S.E.) of the normalized *ClSTR* expression in the control, C4 and C7, respectively. C4: 4 weeks-old control cells; C7: 7 weeks-old control cells; A3K4: cells treated with ABA 3 mg/L^{-1} and standard sucrose, 4 weeks old; A3K7: cells treated with ABA 3 mg/L^{-1} and standard sucrose, 7 weeks old; A3S4: cells treated with ABA 3 mg/L^{-1}, partial sorbitol, 4 weeks old; A3S7: cells treated with ABA 3 mg/L, partial sorbitol, 7 weeks old The asterisk signs indicate statistically significant difference (Welch's t-test; *$P < 0.05$) to C4.
(**B**). RT-PCR gel images of the *ClSTR* as the gene of interest and *Actin* as a housekeeping gene in *C. ledgeriana* cell cultures. Each experiment was repeated at least three times (3 biological and 3 technical repeats)

ClSTR expression in cultured cells

The suspension cultures were harvested in the fourth and seventh weeks. By taking the control cells (no elicitation) at one harvesting time as the point of reference, relative expression of *ClSTR* in the cultured cells was determined (Fig. 4). The gene expression in the treated cultured cells was usually higher than that in the control cells, except in the A3S7 cultured cells. The highest expression was found in A3S4 cultured cells. With elicitation treatment, it was evident that the *ClSTR* gene was more active when the cell culture aged four weeks (A3K4 and A3S4) and its activity diminished by the seventh week (A3K7 and A3S7).

ClSTR expression in fresh tissues

The level of *ClSTR* expression in YL was higher compared with that in ML and SB (Fig. 5). The expression level of *ClSTR* in the YL and SB was 1.4- and 1.1-fold higher than that in the ML, respectively.

Pearson correlation analysis

The Pearson correlation analysis indicated a significant correlation between *ClSTR* expression and quinine content
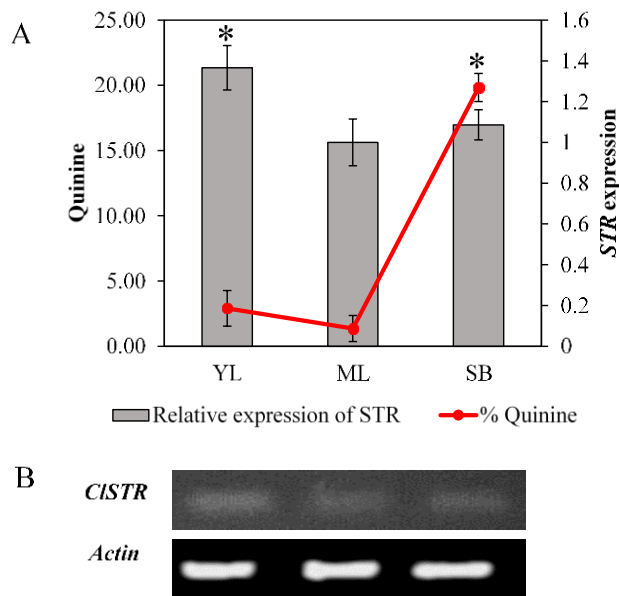
Fig. 5: The level of *ClSTR* gene expression is plotted with the quinine content in fresh tissues of mature plants of *Cinchona ledgeriana*

(**A**) The level of *ClSTR* gene expression in the fresh tissues was demonstrated by qRT-PCR method and was calculated using relative expressions following Livak and Schmittgen (2001). The quinine contents are plotted with the relative expression of *STR*. The data are mean ± standard error (S.E.) of the normalized *ClSTR* expression in ML. YL: young leaves; ML: mature leaves; SB: stem bark. The asterisk sign indicates statistically significant difference (Welch's t-test; *$P < 0.05$) to ML
(**B**) RT-PCR gel images of the *ClSTR* as the gene of interest and *Actin* as a housekeeping gene, particularly in fresh tissues. Each experiment was repeated at least three times (3 biological and 3 technical repeats)

in *Cinchona* cells and fresh organ tissues (Fig. 6). It was shown that the elicited cells tended to have closer correlation with YL and ML in terms of gene expression and quinine production. The highest content of quinine given by A3S7-treated cells had only 59% similarity with that of the SB. A PCA considers all the correlation data (Fig. 7). There are three different clusters taking both factors into consideration; one cluster contains A3S4, the second cluster consists of SB, and the rest of the treated cells (including the control cells) and the YL and ML tissues are confined in the third cluster. According to that biplot analysis, the *STR* and quinine parameters are relatively similar between the cultured cells and fresh organ tissues. It also indicates that *ClSTR* expression is tightly correlated to the quinine content.

Discussion

For years, we have tried to increase alkaloid content in cell culture of *C. ledgeriana* by applying various elicitors (Ratnadewi and Sumaryono 2010; Pratiwi *et al.* 2018; Hasibuan *et al.* 2021). The results were inconsistent from one trial to the next, despite the same treatments being applied. However, the one persistent finding was that control cells, without any elicitation, can produce alkaloids,
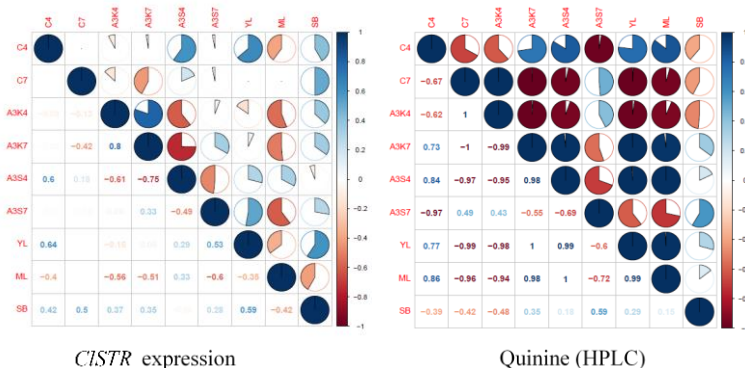
Fig. 6: Pearson correlation between the treated cells and fresh tissues, based on the *ClSTR* gene expression and the quinine content in *Cinchona ledgeriana*

C4: 4 weeks-old control cells; C7: 7 weeks-old control cells; A3K4: cells treated with ABA 3 mgL^{-1}, standard sucrose, 4 weeks old; A3K7: cells treated with ABA 3 mgL^{-1}, standard sucrose, 7 weeks old; A3S4: cells treated with ABA 3 mgL^{-1}, partial sorbitol, 4 weeks old; A3S7: cells treated with ABA 3 mgL^{-1}, partial sorbitol, 7 weeks old; YL: young leaves; ML: mature leaves; SB: stem bark. The data were analyzed with R program 3.51 version; packages corrplot mixed 0.80 version
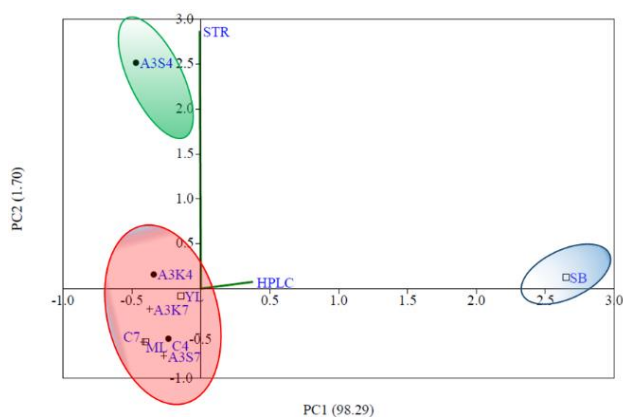
Fig. 7: Principal Component Analysis (PCA) and bi-plotting identify the cluster's performance based on the *ClSTR* gene expression and quinine content

C4: 4 weeks-old control cells; C7: 7 weeks-old control cells; A3K4: cells treated with ABA 3 mgL^{-1}, standard sucrose, 4 weeks old; A3K7: cells treated with ABA 3 mgL^{-1}, standard sucrose, 7 weeks old; A3S4: cells treated with ABA 3 mgL^{-1}, partial sorbitol, 4 weeks old; A3S7: cells treated with ABA 3 mgL^{-1}, partial sorbitol, 7 weeks old; YL: young leaves; ML: mature leaves; SB: stem bark; *STR*: Strictosidine synthase coding gene; HPLC, quinine content determined by high-performance liquid chromatography; PC1, principal component 1; PC2, principal component 2

but still a higher content found in the elicited cells. This was also reconfirmed in this research by A3S7 treatment (Fig. 1).

Strictosidine is a key intermediate and the general precursor in the biosynthesis of TIAs, including quinoline alkaloids. STR, by which strictosidine is generated from the condensation of tryptamine and secologanin, had been previously purified from cell cultures of *C. robusta* (Stevens *et al.* 1993). In addition, Aerts *et al.* (1991) had obtained crude extract of the enzyme from six-month-old plants of *C. ledgeriana*. For the first time here, the *STR* coding gene of *C. ledgeriana* has been sequenced.

The level of gene expression is indicative of the synthesis of the STR enzyme that leads to the alkaloid quinine biosynthesis. Quinine content increased from the fourth to the seventh week of culture when the cells were

treated with double elicitors, ABA and sorbitol (A3S4 and A3S7) (Fig. 1). In contrast, the four-week-old cells exhibited higher gene expression than the older cells (Fig. 4). The YL contained twice as much as quinine as the ML, but the SB contained 6.8 times higher quinine than the YL (Fig. 2), while the YL had higher gene expression than the ML and SB (Fig. 5). Thus, the patterns of quinine content were not in agreement with *ClSTR* gene expression in either type of plant material.

As C4 had 64% similarity with the YL, followed by 60% similarity with the A3S4 cells in terms of the *STR* expression (Fig. 6), it is suggested that, in the four-week-old cells, high *STR* transcription has occurred, similar to that in the YL. These findings indicate that *STR* transcription is active in all cells, regardless of the age or plant organ, but its activity in young cells and organs, *i.e.*, four-week-old cells and YL, are generally higher than in mature cells and leaves. From the cultured cells, we know that both *STR* expression and alkaloid production occur in the same cell. However, the alkaloids in the cultured cells are confined to those cells as they age. No alkaloids were detected when we performed HPLC determination on the culture media (data not shown). When gene expression was diminishing in the older cells, the alkaloids (*e.g.*, quinine) had already accumulated therein, represented by the higher quinine content, as in the case of A3S4 to A3S7.

At the plant level, YL demonstrated the highest expression of the *STR* gene and also exhibited moderate quinine content. On the other hand, the SB presented moderate gene expression but had very high quinine content (Fig. 5). From this, we concluded that all the organs (leaves and stem bark) express the *STR* gene and also produce the alkaloid. Quinine produced in the YL and ML, and most probably in the other organs too, is then translocated and stored in the SB. As a result, the SB is the richest site of the quinoline alkaloids. Similar results were found by Hotchandani *et al.* (2019), who worked on alkaloids in *Amaryllidaceae* and reported that the negative correlation

between the gene expression level and the amount of the corresponding compounds in an organ was due to the translocation of the biosynthetic enzymes and/or the products to other parts of the plant. Pratiwi *et al.* (2020) reported that the alkaloids of *C. ledgeriana* were found in the idioblasts, hypodermis, and palisade of the leaves, while in the stem, they were abundantly spread over the cortex, secondary phloem, and even in the intercellular space.

Inconsistency in the production of alkaloids in cell culture may result from many factors, such as the culture age, media composition, elicitor type and length of application, culture environmental conditions, and even skill of the operators in performing the culture and extraction, up to determination. Alternatively, despite the treatment applied, the low alkaloid content in the cultured cells is probably due to the absence of other enhancing factors. From the trees of *C. ledgeriana*, Maehara *et al.* (2010, 2019) isolated and characterized several endophytic fungi that might engage in beneficial symbiosis with the host plants. Those fungi may also partially contribute to the alkaloid production of trees, which may explain why the alkaloid content in trees is substantially higher than that in cultured cells, where the culture media and *in vitro* environment are totally free from microorganisms.

Some SM biosynthetic pathways have been reported to be active in several different organs within a plant (Zhou *et al.* 2010; Dewey and Xie 2013; Shitan 2016). However, the full pathway of alkaloid biosynthesis in Cinchona plants, including the availability of the substrates and enzymes, might be present within the same cells, even in every single cell, independent of cell differentiation.

At the physiological level, alkaloids are toxic to the cells producing them if the metabolites accumulate in those same cells to an excessive concentration (Yazaki 2005; Sirikantaramas *et al.* 2008; Shitan 2016). Therefore, naturally, the alkaloids will not accumulate in high concentration in a closed compartment system like a cell because of feedback inhibition. In the entire plant, self-toxicity is avoided by long-distance transportation such as efflux detoxification (Yazaki 2005) to less sensitive tissues or organs, namely, accumulation sites such as intercellular spaces, cuticle, resin duct (apoplasts) (Wink 2010; Shitan *et al.* 2015; Shitan 2016). Ideally, membrane permeability may be loosened to facilitate the excretion of SMs from the *in vitro* producing cells into the culture media. Some efforts have been made to increase the continuous production of SMs in cultured cells, for instance, by the application of certain chemicals (Boitel-Conti *et al.* 2000; Zhang and Franco 2005), or by improving the activity of certain efflux carriers such as plant ABC transporter (Wink 2010) or AtDTX1 in *Arabidopsis thaliana*, which belongs to the multidrug and toxic compound extrusion family (Li *et al.* 2002). At least four families of transporter proteins for SMs have been identified (Shitan 2016). For SMs that are entirely synthesized in the same cells, future research can be directed toward finding any technology suitable for this purpose.

Conclusion

The *STR* gene was expressed, and quinine biosynthesis occurred in all cells and tissues of *C. ledgeriana* under investigation. The low quinine content in the cultured cells might be due to the absence of intercellular transportation of the alkaloid that further blocks its continuous production. Cinchona alkaloid content may be increased by strengthening *STR* expression in conjunction with development of certain efflux carriers in the cultured cells through genetic means along with technical improvements.

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Author Contributions

D Ratnadewi conceptualized and designed the experiment; D Ratnadewi, MH Fendiyanto, RD Satrio, and AN Laily conducted the laboratory experiments; M Miftahudin, MH Fendiyanto, and RD Satrio assisted with the bioinformatic analysis; and D Ratnadewi and MH Fendiyanto analyzed the data and wrote the manuscript.

Conflict of Interest

Authors declare no conflict of interest.

Data Availability

The data and supplementary material are all available online.

Ethics Approval

The research does not involve the ethical approval.

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