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Enhancement of Phenylpropanoid Accumulation and Antioxidant Activities of *Agastache rugosa* Transgenic Hairy Root Cultures by Overexpressing the Maize *Lc* Transcription Factor

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Abstract: *Agastache rugosa* is also known as Korean mint, and it has numerous health benefits due to its rich source of phenolic compounds. The main objective of this study was to produce a *ZmLC*-overexpressing transgenic hairy root line via *Agrobacterium rhizogenes*-mediated transformation. The overexpressing transgenic lines were screened using qRT-PCR after exposure to light conditions. The best hairy root line was selected, and the expression levels of phenylpropanoid biosynthetic pathway genes and phenylpropanoid compound accumulation were analysed using qRT-PCR and HPLC, respectively. In addition, antioxidant activities (RPA, ABTS, and DPPH), total phenolic content, and total flavonoid content were analysed. The *ZmLC*-overexpressing transgenic line upregulated all the phenylpropanoid pathway genes, which led to the higher accumulation of phenylpropanoid compounds in the transgenic line than in the control line. In addition, the total phenolic and flavonoid content was significantly higher in the transgenic line. The antioxidant activity assay showed that the transgenic hairy root line had significantly higher activity than that of the control lines. Thus, *ZmLC* positively enhances the phenylpropanoid biosynthetic pathway and antioxidant activities in *A. rugosa*. The results show that *ZmLC* can be used to enhance phenylpropanoid compounds and antioxidant activities in transgenic *A. rugosa* hairy root lines via the genetic engineering approach.

Keywords: *Agastache rugosa*; Korean mint; transcription factor; *ZmLC*; phenolic compound; antioxidant activities

1. Introduction

Agastache rugosa is a precious medicinal plant from the Lamiaceae family that is grown in many Korean and East Asian areas. *Agastache rugosa* is also a famous plant in traditional Chinese medicine and is included in the list of fifty basic herbs [1]. It is a plant source containing both bioactive compounds [2] used to treat anxiety, cholera, infections, nausea, and gastrointestinal problems [3,4], and antimicrobial and antifungal agents [4]. It

also possesses HIV integrase inhibitory activity and is a potential drug candidate against COVID-19 [5].

Antioxidants are compounds that play an important role in delaying or preventing the oxidation of lipids or other molecules by preventing the induction or proliferation of oxidative chain reactions [6]. Phenylpropanoids are a large group of plant secondary metabolites that contain one or more C6-C3 fragments, playing a central role in phenolic compound biosynthesis in plants [7]. Phenylpropanoids and other phenolic compounds are synthesised from L-phenylalanine and, to a lesser extent, L-tyrosine through the phenylpropanoid pathway. Phenylpropanoids have been classified into five groups: flavonoids, phenolic acids, lignin, stilbenes, and coumarin [8]. In addition to important biological functions in defence, survival, and structural support related to plant growth [9,10], phenylpropanoids are also valuable metabolites. These compounds have protective effects on human health, especially related to their antioxidant activity [11], their anti-inflammatory, antibacterial, antiviral, anti-skin ageing, anti-cancer properties, and to osteoporosis, insulin sensitivity, obesity, and cardiovascular disease [12–14].

In plants, light is an important environmental factor that regulates growth and development and promotes photosynthesis [15]. At the same time, light exposure also affects the accumulation of secondary compounds in plants, including phenylpropanoids [16,17].

Transcription factors are proteins involved in the conversion or transcription of DNA into RNA. They play a role in initiating and regulating gene transcription [18]. Several studies have stated that MYB transcription factors play a significant role in plant phenylpropanoid production, and this has been proven in a number of plants, including *Arabidopsis thaliana* [19], *Fagopyrum esculentum* [20], *A. rugosa* [15], *Radish* callus [21], *Fagopyrum tataricum* [22], *Rose* [23], *Nicotiana tabacum* [24], *Solanum lycopersicum* [25], and *Saussure involucre* [26]. LC is a type of regulatory protein identified from *Zea mays* and this protein is mainly involved in anthocyanin biosynthesis. Several plant studies have reported that the ectopic expression of the *ZmLC* protein leads to anthocyanin accumulation and purple colouration in transgenic plants. In addition, it enhances the accumulation of phenylpropanoid compounds such as phenolic acids and flavonoids. For instance, exogenous expression of *ZmLC* protein in the leaves of apples [27] and flowers of tobacco [28] leads to enhanced accumulation of phenylpropanoid compounds via the regulation of flavonoid biosynthesis. In another study, it has been the overexpression of *ZmLC* in the hairy root of *S. baicalensis* that leads to an increased accumulation of flavone content such as baicalein, baicalin, and wogonin via triggering of the phenylpropanoid biosynthetic pathway [29]. In contrast, in several plants, *ZmLC* did not show a significant effect [30,31], whereas when *ZmLC* co-expressed with *Pl* (purple leaf), an eventual increase in anthocyanin accumulation occurred; however, expression of *ZmLC* did not show any noticeable effect in creeping bentgrass [32]. Several studies reported that light enhances the anthocyanin accumulation in *ZmLC*-transgenic alfalfa [33], petunia [34], cotton leaves [35], and maize seeds [36]. Similarly, light triggers *ZmLC*-overexpression and enhances phenylpropanoid accumulation in a few plants (*S. involucre*, *F. tataricum*, and *Scutellaria baicalensis*) [26,29,37]. In addition, it has been reported that *Lc* also enhances the ferric-reducing antioxidant power (FRAP) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay. However, to date, none of the studies have been conducted on the enhancement of phenylpropanoid production and antioxidant activities on *ZmLc* overexpression in *A. rugosa* transgenic hairy root cultures after exposure to light conditions.

The main aim of this study was to produce a *ZmLC*-overexpressing transgenic hairy root line via *Agrobacterium rhizogenic*-mediated transformation. The best hairy root line was selected, and expression levels of phenylpropanoid biosynthetic pathway genes and accumulation of phenylpropanoid compounds were analysed using qRT-PCR and high-performance liquid chromatography (HPLC). In addition, antioxidant activities reducing power assay (RPA), ABTS, and 2,2-Diphenyl-1-Picryl Hydrazyl (DPPH), total phenolic content (TPC), and total flavonoid content (TFC) were also analysed. These results show that *ZmLC* can be used to enhance phenylpropanoid compounds and antioxidant activities in transgenic *A. rugosa* hairy root lines via the genetic engineering approach.

2. Materials and Methods

2.1. Plant Materials

A. rugosa seeds were provided by Aram Company (Seoul, Republic of Korea) and were washed with soap under tap water. The seeds were then quickly shaken for 30 s in 70% (v/v) ethanol containing 1% Tween 20. The seeds were shaken for 12 min in 2% (v/v) sodium hypochlorite solution, rinsed with sterilised distilled water, and dried with sterilised papers. Seeds were sown on ½ MS medium containing 0.8% plant agar (pH 5.8) that had been autoclaved 4 days previously. For 3 weeks, they were cultured in a growth chamber using white, fluorescent bulbs with a flux rate of $30 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ under long-day conditions (light/dark = 16/8 h) at 25 °C and 60% humidity. The leaves were used for hairy root induction.

2.2. Hairy Root Induction

The hairy root induction method followed that presented by Do et al. [15] with some modifications. The target gene (pB7FWG2-*ZmLC*) and control vector (GUS-pB7FWG2) were constructed according to the previous protocol described by Park et al. [29]. Korean mint seeds were sown, and after three weeks the leaves were infected with the *A. rhizogenes* R1000 strain carrying the vector system containing the target gene and the control. The leaves were then cultured on solid medium (1/2 SH containing cefotaxime 500 mg/L) to induce hairy roots. After one week, the materials were transferred to screening medium (1/2 SH containing cefotaxime 500 mg/L and kanamycin 50 mg/L). After three weeks, we selected the most promising hairy root lines and continued culturing for another three weeks. A 5 g sample of stably grown hairy roots from the control and transgenic were nourished in 30 mL of 1/2 SH liquid and cultured with a 16-/8-h light/dark photoperiod and shaking at 100 rpm. After two weeks, we harvested and froze the samples in liquid nitrogen. A small number of samples were used to extract RNA, and the remaining samples were used to analyse phenylpropanoids.

2.3. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from 14 *ZmLC*-transgenic hairy root lines and the *A. rugosa* control using the CTAB method. RNA purification was carried out using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). RNA samples were assessed for quality using NanoVue Plus (GE Healthcare Life Sciences, Marlborough, MA, USA). The cDNA was synthesised using the ReverTra Ace-α Kit (Code No. FSK-101), Toyobo Co., Ltd. (Life Department, Osaka, Japan).

2.4. Gene Expression Analysis

The cDNA of each sample was synthesised, and we diluted it 20-fold to be used for gene expression analysis. The total volume for an RT-PCR reaction (10 µL) included the following ingredients: 2.5 µL cDNA, 1 µL primers (forward and reverse), 5 µL of 2X- Realtime Mix SFC green (BioFACT™, Daejeon, Republic of Korea), and 1.5 µL nuclease-free water. After vortexing, the reaction mix was centrifuged to remove air bubbles. The samples were run using the CFX Opus 96 Real-Time PCR System with the following thermal cycle: 95 °C for 10 min, 39 cycles of 95 °C for 15 s, 59 °C for 20 s, 72 °C for 20 s, and 72 °C for 10 min. In this study, the housekeeping gene β-actin was used as a reference gene to measure target gene expression. The qRT-PCR primer sequences are shown in Table S1 [1,38].

2.5. Analysis of Phenylpropanoid Content by HPLC

Korea mint hairy root samples were ground finely after being freeze-dried at −45 °C for 72 h. A 100 mg sample of each type was added to a centrifuge tube with 2 mL of 80% methanol, vortexed, and placed in an ultrasonic bath for 60 min. The tubes were maintained at 45 °C and vortexed every 20 min. Samples were then centrifuged at 12,000 rpm for 10 min, after which the supernatant was filtered through a 0.45 µm poly-filter membrane (Sartorius Stedim Biotech, Göttingen, Germany). HPLC analysis was performed according

to the procedure previously described by Sathasivam et al. [39]. The detailed protocol is described in Table S2.

2.6. Analysis of TPC and TFC

Total polyphenols and total flavonoids were extracted from the samples using methanol based on a previous description by Lim et al. [40]. Briefly, 2 mL of 70% methanol was added to 100 mg of sample, mixed gently, and sonicated for 1 h at room temperature. Following sonication, samples were centrifuged in the same manner as in the TA method, and the supernatant was filtered using a 0.45 µm PTFE hydrophilic syringe filter.

The TPC was quantified by determining the sample's Folin-Ciocalteu reduction capacity (FCRC) and was slightly modified by the previous methods from Lim et al. To estimate the FCRC, the diluted sample was prepared at a concentration of 5 mg/mL. A volume of 0.5 mL of 2 N Folin-Ciocalteu phenol reagent (Junsei, Yongin, Republic of Korea) was mixed with 0.1 mL of diluted sample extracts, and the mixture was incubated for 3 min. After adding 4 mL of 10% sodium carbonate, the mixtures were left in the dark for 90 min. Their absorbances were read at 760 nm using a UV-Vis spectrophotometer, and the TPC in the samples was determined as gallic acid equivalent (GAE) using a calibration curve of the standard: ranging from 31.25 to 1000 mg/L; $y = 0.0014x + 0.0162$, $R^2 = 0.9997$.

The TFC was determined based on the spectrophotometric method previously described by Lim et al. [40], with slight modifications. First, 0.5 mL of the diluted sample, which was prepared in the same manner as in the TPC method, was mixed with 2.0 mL of deionised water and 0.15 mL of 5% sodium nitrite and incubated for 5 min. We added 0.15 mL of 10% aluminium chloride to this mixture and measured the absorbance 15 min later at 415 nm using a UV-Vis spectrophotometer. The TFC in the samples was determined as rutin equivalent (RE) using a calibration curve of the standard ranging from 31.25 to 1000 mg/L; $y = 0.0016x + 0.009$, $R^2 = 0.9998$.

2.7. Determination of RPA from Extracts of *A. rugosa*

RPA, which was estimated as the transformation of Fe^{3+} to Fe^{2+} , was determined following the method described by Lim et al. [40]. A sample extract volume of 0.3 mL at 6 concentrations was mixed with 0.3 mL of 1% potassium hexacyanoferrate (III) and 0.3 mL of 0.2 M phosphate buffer (pH 6.6). After 20 min of incubation at 50 °C, 0.3 mL of trichloroacetic acid (10%) was added, and the mixture was centrifuged at 10,000 rpm for 10 min. Subsequently, 0.5 mL of deionised water and 0.1 mL of 0.1% iron trichloride were added to 0.5 mL of the supernatant mixture. The absorbance was measured at 700 nm using a UV-Vis spectrophotometer, and the increase in absorbance value indicated the strength of the reducing power.

2.8. In Vitro Antioxidant Activity

Agastache rugosa extracts were used to determine antioxidant activity in the same manner as in the TPC and TFC assays. The DPPH radical scavenging activity (RSA) was evaluated based on previous reports by Lim et al. [40], with slight modifications. Briefly, 0.1 mL of 0.2 mM DPPH, which was dissolved in 99.9% methanol, was added to a 96-well plate, and 0.1 mL of the extracts was added to each well at 6 concentrations: 31.25, 62.5, 125, 250, 500, and 1000 mg/L. After incubation in the dark for 30 min, the absorbance was measured at 517 nm using a UV-Vis spectrophotometer. For the control, 70% methanol, which was used for the extraction solvent, was added instead of the sample extracts.

The ABTS RSA was evaluated following the protocol previously reported by Lim et al. [40], with slight modifications. Briefly, 7 mM ABTS powder was thoroughly dissolved in potassium persulfate (2.5 mM) solution, which was prepared in deionised water, and the mixture was incubated in the dark for 16 h. After incubation, the absorbance of the ABTS buffer was adjusted to 0.7 ± 0.002 at 734 nm, and 0.15 mL of the ABTS buffer was transferred to a 96-well plate. Then, 0.05 mL of *A. rugosa* extract was added to each well, ranging from 31.25 to 1000 mg/L, and incubated in the dark for 3 min. The decrease in

absorbance was measured at 734 nm using a UV-Vis spectrophotometer, and 70% methanol was used in place of the sample extracts for the control. The sample's RSA was calculated based on a previous report by Lim et al., and the plotted curve was used to indicate the amount of antioxidants needed (mg/mL) to reduce the initial free radical concentration by 50% (IC₅₀).

2.9. Statistical Analysis

The results were stated as the mean values with a standard deviation (SD) from every result in triplicate, and the statistical analysis of this study was performed using analysis of variance (ANOVA) in SPSS 20 (SPSS Inc., Chicago, IL, USA) and Graph pad Prism 8. Duncan's multiple range test was used to establish significance at the $p < 0.05$ level. Asterisks denote statistical significance (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). A correlogram was drawn by using the MATLAB software (R2020b).

3. Results

3.1. Hairy Root Induction

We selected hairy root lines on a medium containing kanamycin, resulting in 14 transgenic root lines and 2 control lines capable of fast and stable growth (Figure 1). The gene expression results of the hairy root lines are presented in Figure 2. These 14 lines (*ZmLC*) showed higher expressions than the control sample (*GUS*), especially hairy root lines 1 and 10. Homogenized line 10, thereafter LC10, was used to conduct further evaluations.

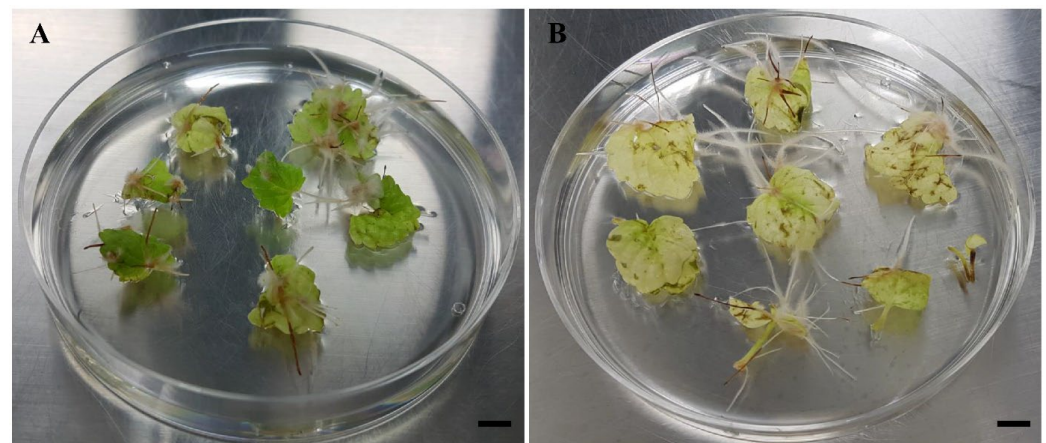


Figure 1. Hairy root induction of *A. rugosa* using *A. rhizogenes*. (A) Control hairy root harbouring *GUS* construct. (B) Transgenic hairy root harbouring *ZmLC* construct. The scale bar represents 1 cm.

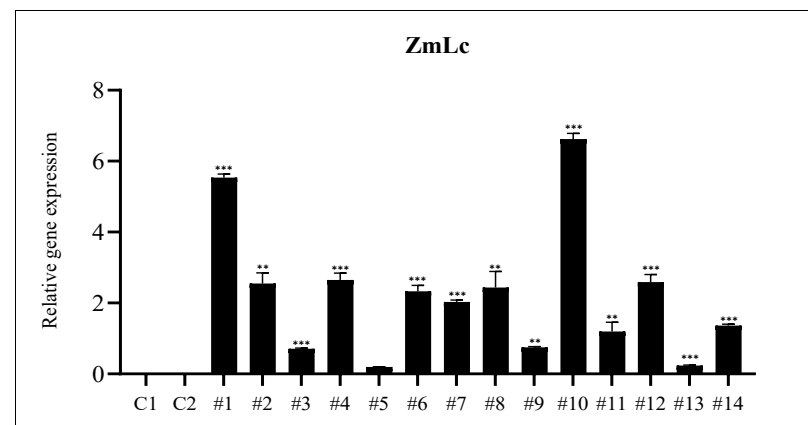


Figure 2. qRT-PCR analysis of control hairy root lines expressing *GUS* (C1 and C2) and transgenic hairy root lines expressing *ZmLC* (#1, #2, #3, #4, #5, #6, #7, #8, #9, #10, #11, #12, #13, and #14). Asterisks denote statistical significance (** $p < 0.01$, and *** $p < 0.001$).

3.2. Analysis of Phenylpropanoid Biosynthesis Pathway Genes Expression in *A. rugosa* Grown under Light Condition

The results of the qRT-PCR analysis are shown in Figure 3. The expression level of eight phenylpropanoid pathway genes was higher in the LC-10 sample than in the control sample. The highest expression was obtained for the *CHS* gene (2.7-fold higher), and the lowest expression was 1.28-fold for the *HPPR* gene compared to the control. There were two genes, *RAS* and *PAL*, with similar expression levels, and their expression was 2.48- and 2.44-fold higher, respectively, than that in the control. The expression of the *TAT*, *C4H*, and *4CL* genes was 1.69-, 1.40-, and 1.30-fold higher, respectively, than that in the controls.

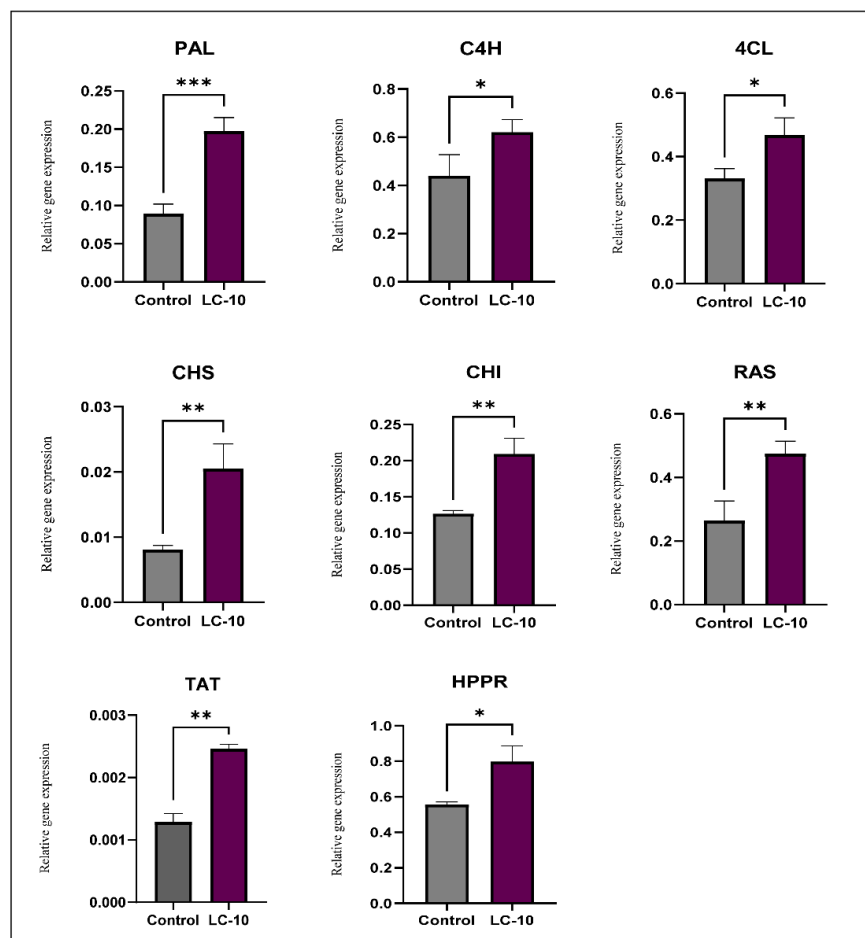


Figure 3. Effect of light treatment on phenylpropanoid biosynthetic pathway genes expression in *A. rugosa* transgenic hairy root lines. Control: *GUS* overexpressing hairy root line; LC-10: *ZmLC*-overexpressing hairy root line. Asterisks denote statistical significance (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; RAS, hydroxycinnamoyl-CoA: hydroxyphenyllactate hydroxycinnamoyl transferase; TAT, tyrosine aminotransferase; HPPR, hydroxyphenylpyruvate reductase.

3.3. Analysis of Phenylpropanoid from *A. rugosa* Extracts

We identified eight phenylpropanoid compounds in *A. rugosa* extracts, and the LC-10 extracts accumulated higher contents in every compound compared to the control. Rosmarinic acid showed the highest content in the control and LC-10 (24.4 ± 0.394 and 26.803 ± 0.635 mg/g DW, respectively) (Table 1). In the LC-10 extracts, the second highest content (mg/g DW) was caffeic acid (0.951 ± 0.026), followed by rutin (0.292 ± 0.003), benzoic acid (0.148 ± 0.003), acacetin (0.03 ± 0.003), *p*-coumaric acid (0.022 ± 0.001), trans-cinnamic acid (0.012 ± 0.001), and ferulic acid (0.011 ± 0.004). However, in the

control hairy root, *p*-coumaric acid, ferulic acid, trans-cinnamic acid, and acacetin showed a content (mg/g DW) of 0.01 mg or less (0.005 ± 0.001 , 0.01 ± 0.001 , 0.004 ± 0.001 , and 0.007 ± 0.001 , respectively).

Table 1. Effects of overexpressing the *ZmLC* transcription factor on the phenylpropanoid content (mg/g DW) in *A. rugosa* extracts.

No.	Compound	Control	LC-10	Fold Change
1	Caffeic acid	0.687 ± 0.015	0.951 ± 0.026	1.38
2	<i>p</i> -coumaric acid	0.005 ± 0.001	0.022 ± 0.001	4.40
3	Ferulic acid	0.01 ± 0.001	0.011 ± 0.004	1.10
4	Benzoic acid	0.141 ± 0.007	0.148 ± 0.003	1.05
5	Rutin	0.29 ± 0.001	0.292 ± 0.003	1.01
6	Trans-cinnamic acid	0.004 ± 0.001	0.012 ± 0.001	3.00
7	Rosmarinic acid	24.4 ± 0.394	26.803 ± 0.635 ***	1.10
8	Acacetin	0.007 ± 0.001	0.03 ± 0.003	4.29

All results were carried out in triplicate, and the values are shown as mean \pm SD. Asterisks denote statistical significance (***) $p < 0.001$. The column without asterisks denotes no significant differences between the control and LC-10.

3.4. Quantification of TPC and TFC

As shown in Table 2, the LC-10 sample, which overexpressed the *ZmLC* gene, showed a significantly higher TPC (28.07 ± 0.22 GAE mg/g DW) and TFC (42.35 ± 0.67 QE mg/g DW) (Figure 4), whereas the control showed a lower TPC (19.92 ± 1.66 GAE mg/g DW) and TFC (30.55 ± 0.15 QE mg/g DW). Based on these results, we can conclude that the sample overexpressing the *ZmLC* gene contains more TPC and TFC than the control.

Table 2. Relative TPC and TFC from two *A. rugosa* extracts.

	Control	LC-10
Total Polyphenol (GAE mg/g DW)	19.92 ± 1.66	28.07 ± 0.22 ***
Total Flavonoid (QE mg/g DW)	30.55 ± 0.15	42.35 ± 0.67 ***

All results were carried out in triplicate, and the values are shown as mean \pm SD. Asterisks denote statistical significance (***) $p < 0.001$. The column without asterisks denotes no significant differences between the control and LC-10.

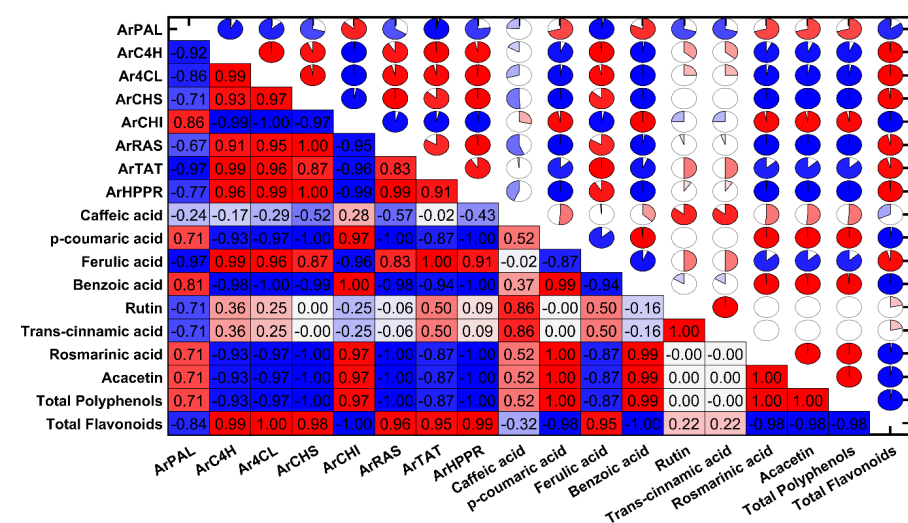


Figure 4. Correlation matrix using correlogram between the phenylpropanoid biosynthetic pathway genes and identified metabolites in *ZmLC*-overexpressing *A. rugosa* transgenic hairy root line. The correlation values are shown as a heatmap in the bottom left, using colour boxes to denote the strength of association, and the pie chart shown in the top right provides a quick overview.

3.5. Determination of RPA and Antioxidant Activities

The RSA, which was evaluated at 6 different concentrations (ranging from 31.25 to 1000 mg/L) of *A. rugosa*, showed a gradual increase with increased concentrations regardless of the samples. At all concentrations, the LC-10 sample showed more effective reducing power than the control, and ascorbic acid was used as the positive control (Figure 5a). The DPPH and ABTS RSA (%) results of different *A. rugosa* extracts were also determined at 6 different concentrations, and ascorbic acid was used as the positive control. At a concentration of 1000 mg/L, the LC-10 sample extracts showed about 90% scavenging activity for DPPH ($86.2 \pm 0.31\%$), whereas the control showed about 70% activity for DPPH ($68.6 \pm 5.29\%$) (Figure 5b). In addition, their values confirmed the significant difference between them. The IC_{50} for DPPH does not show any significance difference between the control and LC-10 lines. The highest efficiency was achieved in LC-10 (0.53 ± 0.0 mg/mL) (Table 3). ABTS RSA (%) followed the same tendency as the other assays. The LC-10 samples extracts, at 1000 mg/mL, attained $88.03 \pm 0.92\%$ activity in ABTS scavenging, followed by the activity of the control at $75.63 \pm 1.83\%$ (Figure 5c). The higher efficiency of the IC_{50} for ABTS, which indicated the required sample's concentration to inhibit their free radicals at 50% of the ABTS, was achieved in LC-10 (0.48 ± 0.01 mg/mL), whereas the control required a relatively high concentration of 0.63 ± 0.03 mg/mL to achieve 50% inhibition of the ABTS (Table 3). However, the IC_{50} for ABTS result showed that there is no difference between the LC-10 and control lines.

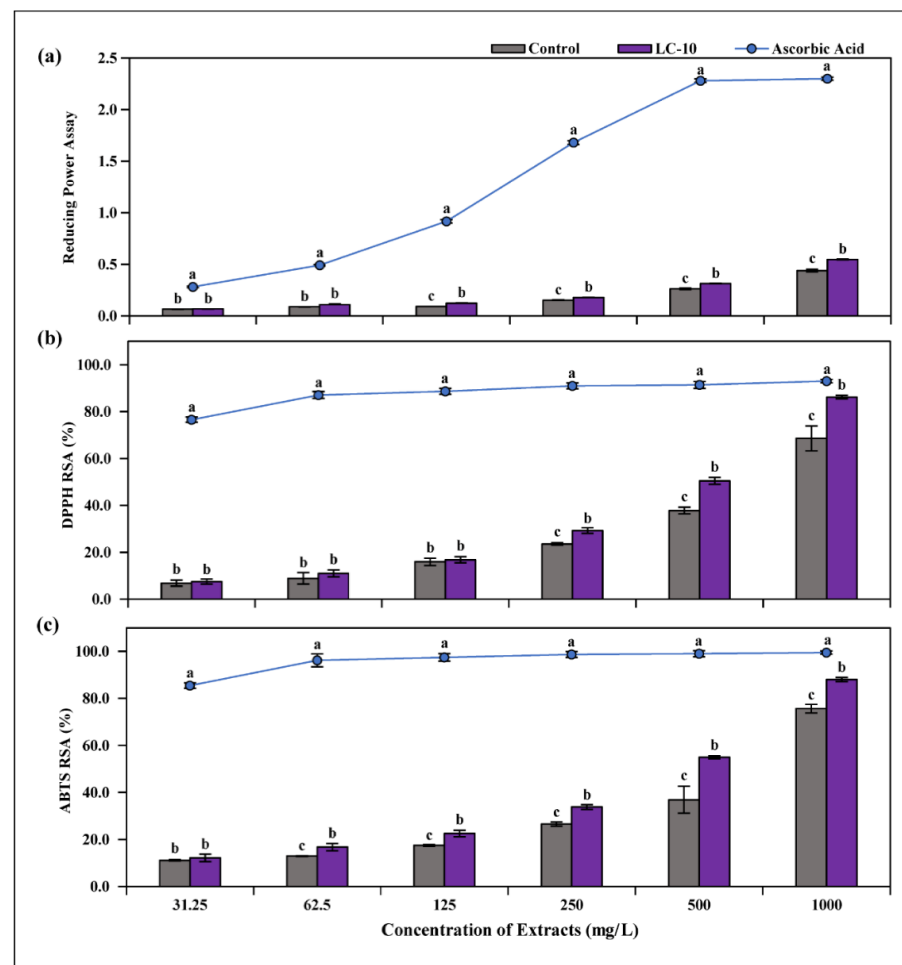


Figure 5. Reducing power and RSA (%) of six concentrations of *A. rugosa* extracts. (a) Reducing Power Assay; (b) DPPH RSA (%); (c) ABTS RSA (%). Values are shown as mean \pm SD, and different letters a–c are the statistically significant differences among the means using Duncan's multiple range test (ANOVA, $p < 0.05$).

Table 3. IC₅₀ values for DPPH and ABTS (mg/mL) from two *A. rugosa* extracts.

	IC ₅₀ of DPPH	IC ₅₀ of ABTS
Control	0.7 ± 0.03	0.63 ± 0.03
LC-10	0.53 ± 0.00	0.48 ± 0.01

All results were carried out in triplicate, and the values are shown as mean ± SD. The column without asterisks denotes no significant differences between the control and LC-10.

4. Discussion

In this study, the results showed that the following genes are responsible for the phenylpropanoid biosynthetic pathway: *PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *TAT*, *HPPR*, and *RAS*. These genes were expressed very clearly in the transformed hairy root lines compared to the control within two weeks of light exposure. Previously, several plant species maize *LC* genes have been used to manipulate the anthocyanin and flavonoid biosynthetic pathways. The introduction of the *LC* gene might work individually or co-ordinately with *C1* to increase flavonoid biosynthesis by triggering flavonoid biosynthetic pathway genes [29]. In dicot plants such as *N. tabacum* and *A. thaliana*, expression of the *LC* gene leads to enhanced accumulation of anthocyanin pigmentation [41]. In another study, in petunia, the overexpression of *ZmLC* genes leads to a higher accumulation of flavonoid content by triggering the flavonoid biosynthetic pathway genes such as *anthocyanidin synthase*, *dihydroflavonol 4-reductase (DFR)*, *flavonoid 3'5' hydroxylase (F3'5'H)*, *flavonoid 3'-hydroxylase (F3'H)*, and *UDP-glucose:flavonoid-3-O-glucosyltransferase*. In addition, it was found that there is a slight increase in the *CHI*, *CHS*, and *flavanone 3-hydroxylase* gene expression, whereas the *flavonol synthase (FLS)*, *PAL*, *C4H*, *rhamnosyltransferase*, and *UDP-rhamnose: anthocyanidin 3-glucoside* were not influenced by the *LC* overexpression [31]. Similar results were obtained in tobacco where overexpression of *LC* leads to activation of *CHS* and *DFR* gene expression [42]. Moreover, exogenous expression of the *LC* gene in *S. baicalensis* hairy root leads to increased expression of most of the flavonoid biosynthetic pathway genes [29]. In contrast, in chrysanthemum pigmentation, the expression of *LC* does not have a noticeable effect. Similarly, in plants such as *Pelargonium* and *Lisianthus*, ectopic expression of the *ZmLC* gene does not trigger the *CHI*, *CHS*, *FLS*, and *F3'5'H* genes [30]. However, in our study, we found that all the phenylpropanoid pathway genes were slightly overexpressed in the *LC* overexpressed transgenic lines, which leads to a higher accumulation of individual phenolic content, TPC, and TFC. From these results, it is shown that the effect of *ZmLC* overexpression on flavonoid and phenylpropanoid compounds might be dependent on the species of the plant.

In medicinal plants, the phenylpropanoid biosynthetic pathways genes such as *PAL*, *C4H*, and *4CL* play an important role in rosmarinic acid production. In *Salvia miltiorrhiza*, *PAL* is a main enzyme in the rosmarinic acid biosynthetic pathway, which indicates that alteration in the rosmarinic acid content is directly proportional to the *PAL* expression [43]. Another study reported that in *S. miltiorrhiza*, decreased expression of *PAL* has an impact on the *C4H* and *4CL* expression [44]. Similarly, in *Melissa officinalis*, it has been reported that the *PAL* and *4CL* expressions are associated with their corresponding enzymatic activities and with the rosmarinic acid content [45]. Park et al. [1] reported that in *A. rugosa*, the highest expression of *PAL*, *C4H*, and *4CL* genes leads to a significant accumulation of rosmarinic acid content. These results were consistent with this study's result that the expression of *PAL*, *C4H*, and *4CL* genes was significantly high in the *LC* overexpressed transgenic lines, which leads to a significant accumulation of rosmarinic acid content. From this result, it is shown that *PAL*, *C4H*, and *CL* genes are the most important genes responsible for the rosmarinic acid content in most of the plant species.

The hypocotyl elongation transcription factor (HY5) and two major photosensitizers, phytochrome (PHY)B and cryptochrome (CRY)2, are involved in light-dependent phenylpropanoid accumulation in *Arabidopsis* roots, and many phenylpropanoid genes are highly expressed [46]. Quantitative results using HPLC also demonstrated that *ZmLC* participated in the phenylpropanoid biosynthesis process in *A. rugosa* hairy roots, especially the out-

standing accumulation of three substances, namely trans-cinnamic acid, *p*-coumaric acid, and acacetin, which increased 3.46-, 4.35-, and 4.6-fold, respectively. The total polyphenol content increased 1.41-fold, and the flavonoid content increased 1.33-fold. These results are consistent with previous work showing light-induced phenolic biosynthesis and flavone accumulation in *A. rugosa* hairy roots [15], flavonoid accumulation in *Alnus glutinosa* roots [47], and phenylpropanoids, anthocyanins, and distinct proanthocyanidins in *Malus domestica* Borkh. [27]. Treating *Scutellaria lateriflora* with light and 15 mM of methyl- β -cyclodextrin increased phenolic compounds (baicalein, aglycones, and wogonin) [48], and this treatment increased rutin in *F. tataricum* Hokkai T10 hairy roots [37]. Furthermore, caffeic acid derivatives were significantly increased in *Echinacea purpurea* hairy roots [49].

The radical scavengers DPPH and ABTS are often used for the rapid assessment of the antioxidant activity of natural compounds [50]. Previous research has shown that all parts of the plant contain natural antioxidants [51], with flowers showing higher antioxidant capacity than stems and leaves in *A. rugosa*. However, flower harvesting depends on the weather and season; thus, it is difficult to proactively source raw materials for large-scale antioxidant production. Therefore, we evaluated the antioxidant activity of phenylpropanoids extracted from *A. rugosa* hairy roots using ABTS and DPPH. The results showed that LC-10 had a more effective reduction ability than the control. At a concentration of 1000 mg/L, the LC-10 sample extract showed 86.2% scavenging activity against DPPH, while that in the control sample was 68.6%. ABTS removal was 88.03% in LC-10 and 75.63% in the control. An increase in the free radical content in cells will lead to ageing and diseases, including atherosclerosis, a weakened immune system, reduced intelligence, diabetes, and cancer [52]. Therefore, the antioxidant activity of *A. rugosa* transgenic hairy roots in this report enrich the materials that can be used in the pharmaceutical industry to serve human health.

5. Conclusions

Knowledge about enhancing phenylpropanoid biosynthesis in *A. rugosa* is continuously being researched because it has excellent therapeutic properties for health, especially in eliminating free radicals that cause certain human diseases. Our results indicate that *ZmLC* gene overexpression and light treatment induce phenylpropanoid accumulation in *A. rugosa* hairy roots. This accumulation is related to the control of the following genes: *PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *TAT*, *HPPR*, and *RAS*. The DPPH and ABTS free radical scavenging capacity of phenylpropanoids tested at 6 concentrations (from 31.25 to 1000 mg/L) showed a gradual increase as the concentration increased, regardless of the sample, with an IC_{50} for DPPH of 0.53 mg/mL after 30 min of incubation in the dark and IC_{50} for ABTS of 0.48 mg/mL after 16 h of incubation in the dark. This is the first report on the enhancement of phenylpropanoid production and antioxidant activity in *A. rugosa* transgenic hairy root cultures by overexpressing the maize Lc transcription factor. Further research is needed on exploring the effects of other transcription factors or environmental stressors in *A. rugosa* hairy roots.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app14209617/s1>, Table S1: Primers used for qRT-PCR analysis. Table S2: HPLC conditions for phenylpropanoid compounds detected in this study.

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