LcERF19, an AP2/ERF transcription factor from Litsea cubeba, positively regulates geranial and neral biosynthesis

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A short running head: LcERF19 promotes geranial and neral biosynthesis

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Abstract

The APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factors (TFs) are involved in the regulation of specialized terpenoids biosynthesis. However, the AP2/ERF TFs in the Litsea cubeba have not been characterized, and their role in the biosynthesis of terpenoids is unknown. Here, 174 LcAP2/ERF TFs were identified in L. cubeba and categorized into four subfamilies: 27 AP2, 7 RAV, 1 Soloist, and 139 ERF (I to Xb-L). Transcriptomic and qRT-PCR assays both showed that the expression levels of LcERF19 were similar to that of terpene synthase LcTPS42 in pericarp, which is related to the synthesis of geranial and neral in L. cubeba. LcERF19 was further identified to encode a nuclear-localized protein and was strongly induced at the transcriptomic level by jasmonate. Furthermore, a yeast one-hybrid and dual-luciferase experiment showed that LcERF19 bonded to the GCC box elements of the LcTPS42 promoter and promoted its activity. Transient overexpression of LcERF19 in L. cubeba and overexpression of LcERF19 in tomato both resulted in a significant increase in geranial and neral. Our findings show that LcERF19 enhances geranial and neral biosynthesis mightily through activation of LcTPS42 expression, which provides strategies to improve the flavor of tomato fruit and other crops’ fruit.

Keywords: AP2/ERF family; flavour; geranial; neral, Litsea cubeba; plant secondary metabolism; terpenoids

Introduction

Litsea cubeba (Lour.) Person, an aromatic species in the Lauraceae, produces essential oil in the pericarp and is an important raw material for fragrance and medicine biosynthesis. The major components of L. cubeba essential oil (LCEO) are monoterpenes (94.4–98.4%) of about 41 kinds, including geranial and neral (78.7–87.4%), limonene, linalool, pinene, eucalyptus, etc.1) Common monoterpenoids appear as one or several main compounds in aromatic plants, which endow plants with the ability to cope with environmental stress, including biological and abiotic stresses, attracting pollinators, and seed disseminators to reproduce2. These terpenoids with an aromatic smell and medicinal value are also beneficial to humans. In particular, citral (geranial and neral) is widely utilized in fragrance, cosmetics, and pharmaceutics because of its unique and sensitive aroma, antibiotic, and oxidative characteristics3-5. To improve the production and quality of essential oils with significant commercial value, a better understanding of monoterpenoid metabolism and control mechanisms is necessary.

The monoterpenoid metabolic process has been widely researched, and it is known to be influenced by factors including pathway flux, enzyme biosynthetic step limitation6,7. Controlling the process through the biosynthesis pathway is linked with enzymatic activity, which would be influenced in effect by gene expression regulation8.
Transcription factors (TFs) can usually coordinate the transcription of multiple metabolic pathways and affect the transcription of genes in the same metabolic pathway\textsuperscript{9-11}.

The APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) genes play key roles in regulating the terpenoids biosynthesis and significantly improving the yield of target terpenoids in many plants, including *Catharanthus roseus*, *Artemisia annua*, maize (*Zea mays*), sweet orange (*Citrus sinensis*), etc\textsuperscript{12-16}. AP2/ERFs are classified into the 12 phylogenetic groups\textsuperscript{17}. In particular, group IX is implicated in the control of secondary metabolism and is stimulated by defense-related hormones, including jasmonate (JA)\textsuperscript{17}. Such as, CiERF71 activates the CiTPS16 promoter, which impacts E-geraniol biosynthesis in sweet orange\textsuperscript{18}. AaERF2 and AaORA induced ADS or CYP71AV1 promoter activities to positively regulate artemisinin biosynthesis\textsuperscript{13,19}. EREB58 induces sesquiterpene biosynthesis and improves maize disease resistance\textsuperscript{15}. These ERFs specifically recognize the cis-regulatory GCC box element to directly or indirectly regulate the genes of the secondary metabolic biosynthesis pathway and respond to the JA, indicating that the functions of AP2/ERF from different clusters are conserved and interchangeable\textsuperscript{20,21}. All these ERFs in response to JA signals belong to subgroup IXa, but not subgroup IXb, and we neglected the research on the regulation of plant secondary metabolism by subgroup IXb.

As described above, AP2/ERF TFs are very significant in terpenoid biosynthesis, but the AP2/ERF genes have not been studied in *L. cubeba*. The candidate AP2/ERF TFs are implicated in the biosynthesis of the main components, geranial, and neral, in *L. cubeba* and were investigated here. Our previous work reported that the expression of *LcTPS42* is favorably connected with the yield of LCEO (geranial and neral) and that *LcTPS42* could catalyze geranyl pyrophosphate to produce geraniol. Geraniol was the precursor for the geranial's direct biosynthesis. In the present study, the regulators responsible for the activation of the expression of *LcTPS42* were further studied. A total of 174 *LcAP2/ERF* TFs were identified based on the *L. cubeba* genome database and transcriptome data. *LcERF19*, a subgroup of IXb AP2/ERF TF, encodes a nuclear-localized protein and directly binds the promoter of *LcTPS42*. Transient expression of *LcERF19* in *L. cubeba* and transgenic tomato revealed that *LcERF19* could promote the biosynthesis of geranial and neral. Yeast one-hybrid and dual-luciferase experiments confirmed that *LcERF19* activates the *LcTPS42* promoter and potentially regulates *LcTPS42* gene expression. Our study provides a basis for the genetic improvement of geranial and neral *L. cubeba*, and also for the gene-engineering of these monoterpenoids products in aromatic plants.

**Results**

**Identification of the *LcAP2/ERF* TFs in *L. cubeba***

To confirm the potential regulation of AP2/ERF on main monoterpenoids biosynthesis (geranial and neral) in *L. cubeba*, we first identified the AP2/ERF TFs in *L. cubeba*. According to the HMMER and BLAST, 174 genes coding
proteins with the AP2/ERF domain were identified and named based on chromosome location and their phylogenetic classification (Table S2). Phylogenetic analysis indicated that the *L. cubeba* AP2/ERFs could be divided into 27 AP2, 7 RAV, 1 Soloist, and 139 ERF (I to Xb-L) based on the priority classification rule of *A. thaliana* AP2/ERF TFs (Fig. 1). Importantly, 16 AP2/ERF members were clustered into the ERF group IX (a + b), which affects the biosynthesis of plant secondary metabolism and is induced by JA\textsuperscript{17,20,23}.

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**Fig. 1** LcAP2/ERF family classification and phylogenetic analysis in *L. cubeba*. Different colors represent groups, and all LcAP2/ERF genes in *L. cubeba* were clustered into subclades based on the priority classification rule of *A. thaliana* AP2/ERF genes\textsuperscript{17}. 
**LcERF19 and LcTPS42 have a similar expression pattern and were induced by JA in the pericarp**

Considering monoterpenoids are mainly produced in the pericarp of *L. cubeba*, the LcAP2/ERF TFs highly expressed in the pericarp are probably involved in monoterpenoid biosynthesis. Previous studies have revealed that *LcTPS42* is highly expressed in the pericarp and catalyzed the biosynthesis of main components of monoterpenoid, geranial, and neral, in *L. cubeba*\(^2\). Hence, we included *LcTPS42* in the expression mode analysis. According to the results of the transcriptional sequencing (PRJNA763042) of *L. cubeba* pericarp at different stages of development, the connection among AP2/ERFs and *LcTPS42* gene expression was examined to screen the specific AP2/ERF. The results showed that the expression trends of several members (3 Ixa and 5 Ixb) in subgroup IX were consistent with that of *LcTPS42* in the pericarp of *L. cubeba* (Fig. 2a). In particular, the expression of LcERF19 was highly consistent with *LcTPS42* gene expression (Fig. 2a), suggesting that LcERF19 may be involved in the regulation of *LcTPS42*.

To further understand the relationship between *LcERF19* and *LcTPS42* gene expression, the expression trends of *LcERF19* and *LcTPS42* were investigated using qRT-PCR. As shown in Fig. 2b, *LcERF19* exhibited the highest expression in roots and the late development of the pericarp. Both *LcERF19* and *LcTPS42* were shown highly expressed in the later period of the pericarp. In addition, to examine whether the expression of *LcERF19* and *LcTPS42* were induced by JA, we selected the fruits whose contents of LCEO and JA increased rapidly in the pericarp at the development stage of 75DAF for JA treatment experiment\(^3\). We found that the transcript levels of JA responsive genes (*LcJAR1*, *LcCOI1*, *LcJAZ1*, *LcMYC2*), *LcERF19*, and *LcTPS42* were all increased with MeJA treatment (Fig. 2c).
Fig. 2 LcERF19 and LcTPS42 have a similar expression pattern and are induced by JA in the pericarp. a Heat map showing AP2/ERF genes and LcTPS42 expression in the pericarp based on FPKM values. Only the average FPKM > 2 in three biological replicates over at least one sample was used for the heat map. b The expression levels of LcERF19 and LcTPS42 in different tissues and developmental stages of the L. cubeba pericarp, respectively. Days after full bloom is termed DAF. c MeJA treatment increased the expression levels of JA responsive genes (LcJAR1, LcCOI1, LcJAZ1, LcMYC2), LcERF19, and LcTPS42 in L. cubeba pericarp at 75 DAF. The data contains the averages and standard deviations of three replicates. The L. cubeba Ubiquitin-conjugating enzyme (UBC) gene was served as the reference gene for internal control (Lin et al., 2013).
LcERF19 encodes a nuclear-localized protein

Transcription factors usually perform transcriptional regulatory functions in the nucleus. The LcERF19 coding sequence of fusion GFP was injected into tobacco leaves with A. tumefaciens. We observed that the GFP fluorescence of empty vector distributed throughout tobacco leaves cells (Fig. 3a), while the fluorescence of the nuclear marker (Fig. 3b) and LcERF19-GFP was only observed in the nucleus and showed yellow after fusion in the same cell. The results showed that LcERF19 belongs to a nuclear-localization protein (Fig. 3c).

![Fig. 3 Subcellular localization of LcERF19 in tobacco leaf cells. A. tumefaciens harboring (a) empty vector (35S: GFP), or (b) Nucleus marker (35S:h26-RFP), or (c) 35S: LcERF19-GFP and Nucleus marker infected four-week large tobacco leaves. Bar = 50 μm.](image)

LcERF19 bound and activated the LcTPS42 promoter

Our results showed that the expression trends of several members (3 IXa and 5 IXb) in subgroup IX were consistent with that of LcTPS42 in the pericarp of L. cubeba (Fig. 2a), indicating that these TFs may regulate the expression of LcTPS42. We further confirmed these TFs regulating LcTPS42 gene expression from the candidate unigenes by dual-LUC assay. These TFs were embedded into a pGreenII62-SK vector, and the LcTPS42 promotor was embedded into the pGREENII0800-LUC vector. The results showed that LcERF32, LcERF19, LcERF109, LcERF17, and LcERF110 could significantly activate the LcTPS42 promotor compared with the control (Fig. 4a). Among these, LcERF19, a subgroup of IXb AP2/ERF TF, was chosen for additional assays dependent on the high activation of the LcTPS42 promotor.

AP2/ERF subgroup IX TFs play a key role in regulating plant secondary metabolism through interacting with a GCC-box region in the promoters\textsuperscript{14, 15, 18, 21}. We noticed a GCC-box element on the LcTPS42 promotor and subsequently performed Y1H tests. Y1H tests revealed that interaction of the LcERF19 to three tandem duplicates of the GCC-box...
(rather than the mutated GCC-box) effectively triggered the AbA gene expression, allowing yeast to survive over an
ABA antibiotic, indicating that LcERF19 binds to GCC-box of the LcTPS42 promoter.

**Fig. 4** LcERF19 bound and activated the *LcTPS42* promoter. *a* dual-LUC assay further screened the candidate
TFs. *a* The AP2/ERFs were significantly activated compared with the control and highlighted in red. The data contains
the averages and standard deviations of three replicates (** **P < 0.001, **P < 0.01; *P < 0.05). *b* LcERF19 binds to the
GCC-box regions of the *LcTPS42* promoter, according to Y1H assays. Three repeats of GCC-box elements were used
as baits (pAbAi-3×mGCC-box). Yeast cells coexpressing pGADT7-LcERF19 and the pAbAi-3×mGCC-box from the
*LcTPS42* promoter were cultured for 5 days at 30°C in a selective medium SD/-Trp/-Ura containing 600 ng/mL AbAi.

**LcERF19** promoted geranial and neral biosynthesis in *L. cubeba*

We investigated whether LcERF19 may improve monoterpene yield in *L. cubeba* via activating the *LcTPS42*
promoter. Because stable transforming protocols in *L. cubeba* are hard to come by, an efficient and simple transient
expression test was used to investigate the function of LcERF19. After transient expression of LcERF19, we have
detected that the expression level of LcERF19 itself has increased by about 12.5-fold compared with control (Fig. 5a).
Transient expression test results revealed that the expression of monoterpene biosynthesis-related genes (*LcDXS,*
*LcDXR, LcHMGS, LcHMGR, LcCMS, LcMDS, LcHDS, LcGPPS.SSU1,* and *LcTPS42*) significantly increased
compared with control (Fig. 5a) and induced change in the monoterpene content (Fig. 5b-c). The primary
components of the LCEO, such as geranial and neral, were raised by about 242.02% and 266.72% compared with control (Fig. 5b-c), respectively. These findings suggested that LcERF19 enhances geranial and neral biosynthesis.

Fig. 5 transient overexpression of LcERF19 resulted in increased monoterpenoid biosynthesis and expression of related genes in L. cubeba. a Expression level of monoterpenoid biosynthesis pathway genes. b-c The content of monoterpenoids of LcERF19 transient overexpressed sterility seedling leaves were determined by GC-MS. Sterility seedling leaves of L. cubeba infected with A. tumefaciens containing LcERF19 driven by the 35S promoter. The data contains the averages and standard deviations of three replicates (*P < 0.05).

LcERF19 promote geranial and neral biosynthesis in tomato [Conserved regulation of terpenoids biosynthesis in plants]

To further confirm the conserved role of LcERF19 in monoterpenoid biosynthesis in L. cubeba fruit, we heterologously overexpressed LcERF19 in tomatoes, which is an ideal crop to produce terpenoids. A total of 13 transgenic tomato lines were obtained, and we selected tomato fruits from two independent transgenic lines with the highest expression...
levels of *LcERF19* (*LcERF19*-1, *LcERF19*-2) and two wild-type lines (WT-1, WT-2) for volatile analysis and qRT-PCR (Supplementary Fig. S1; Fig. 6a). The results indicated that overexpression of *LcERF19* resulted in the limonene, linalool, neral, and geranial content significantly increased in transgenic lines than in WT tomato fruit (Fig. 6b–c).

Compared with the control, the content of geranial in the fruit of the *LcERF19*-2 transgenic tomato line was increased from 0.0012 μg·g⁻¹ towards 0.0140 μg·g⁻¹ (11.7 fold), and the content of neral was increased from 0 μg·g⁻¹ towards 0.0046 μg·g⁻¹ (Fig. 6b–c). These findings suggested that *LcERF19* promotes geranial and neral biosynthesis in tomato plants.

![Fig. 6 Overexpression of *LcERF19* induces the accumulation of monoterpenes in tomato fruits, including geranial and neral.](https://academic.oup.com/hr/advance-article/doi/10.1093/hr/uhac093/6572270)

**Discussion**

*LcERF19* belongs to subgroup IXb AP2/ERF TF and directly binds and activates to *LcTPS42* promotor

The AP2/ERF family is involved in plant hormones signal defenses such as jasmonate, ethylene, and salicylic acid, as well as controlling the synthesis of specialized compounds. LCEO is mainly composed of specialized aromatic monoterpenoid metabolites, including geranial, neral, limonene, linalool, etc. The yield and content of these terpenoids
determine the economic value of LCEO\(^1\). In this work, we observed 174 AP2/ERF TFs in the \(L.\ cubeba\) and subgroup IX, including 9 IXa, 7 IXb, and 11 IXc member genes. The expression trend of several subgroup IX members (3 IXa and 5 IXb) is consistent with that of \(LcTPS42\), and these genes were highly expressed in the later phases of fruit peel growth (Fig. 2a). These findings indicated that these TFs may be important to \(L.\ cubeba\) terpenoid biosynthesis control.

The subgroups IXa and IXb contain the same basic AP2/ERF domain, recognize specific cis-regulatory elements (GCC-box element), and also share a CMIX2 motif. The CMIX2 motif is a hypothetical acidic region and may play a role as a transcriptional activation domain\(^17\). In particular, the subgroup IXa has emerged as a broad-spectrum JA-responsive transcriptional regulator in plant specialized metabolite pathways\(^20,21\). Such as, the ORCA2-ORCA6 regulates monoterpenoid indole alkaloids in \(C.\ roseus\)\(^20,22\), GAME9 regulates steroidal glycoalkaloids in tomato and potato\(^16\), ERF189 regulates nicotine in tobacco\(^14\), and AaERF2 and AaORA both regulate artemisinin in \(A.\ annua\)\(^13,19\). However, we lacked research on the regulation of plant-specific metabolites by subgroup IXb. In this study, we noticed that the expression trends of 5 IXb and 3 IXa TFs were consistent with those of the \(LcTPS42\) gene (Fig. 2a).

Interestingly, the activation effect of 5 IXb TFs (LeERF32, LeERF19, LeERF109, LeERF17, LeERF110) on the \(LcTPS42\) promoter was better than that of IXa, especially LeERF19 leading to a 7.6-fold improvement in \(LcTPS42\) promoter activity compared to the control treatment (Fig. 4a). These IXb TFs are similar to the \(C.\ roseus\) ORCA TFs, which activate terpenoid biosynthesis pathway genes in a redundant manner\(^20,23\). Our results showed that the subgroup IXb LeERF19 directly binds and activates the \(LcTPS42\) promoter, and it will be crucial to investigate the control of other ERF family members on specialized metabolite biosynthesis in the future. Considering that IXa takes a crucial role in the control of secondary metabolites in plants, although we have not found that IXa TFs regulate the \(LcTPS42\) gene, IXa TFs may also be implicated in the control of other \(L.\ cubeba\) terpenoid genes. In addition, LeERF19 recognizes and binds the GCC-box element on the \(LcTPS42\) promoter. The binding elements are typical binding sites of subgroup IX or even the ERF TF subfamily, suggesting that other ERF subfamily members may also regulate terpenoid biosynthesis by binding to this site.

**LeERF19 induces the expression of terpenoid metabolic genes and functions as a positive regulator for terpenoid biosynthesis engineering**

In plants, the subgroup IX TFs induce the expression of terpenoid biosynthesis genes and improve terpenoid yield and quality\(^20,23\). Overexpression of AaORA induces the expression of multiple terpenoid biosynthesis genes and increases artemisinin content\(^19,29\). AaERF1 and AaERF2 bind and activate \(A.\ annua\) ADS and CYP71AV1 promoters to increase their expression and increase artemisinin content\(^13\). Similarly, in this study, we found that transient overexpression of LeERF19 boosted the expression of genes involved in the geranial and neral biosynthesis pathway genes. Consistently,
the contents of geranial and neral were increased in *LcERF19* overexpressed tomato fruits. Our results demonstrate that *LcERF19*, a subgroup of IXb, favorably controls geranial and neral biosynthesis.

Unexpectedly, although *LcERF19* overexpression increased geranial and neral contents, the contents of pinene and camphene decreased. This may be because the metabolic flow of plants in this monoterpenic biosynthesis pathway is relatively stable. *LcERF19* greatly improves the expression of the *LcTPS42* gene and requires more substrates to synthesize geranial and neral, resulting in a reduction in the content of other terpenoids. Similarly, *A. annua AaTCP15* increased the conversion efficiency of dihydroartemisinic acid to artemisinin by activating the activity of the ALDH11 promoter, resulting in the increase of artemisinin content and a decrease of dihydroartemisinic acid content in *AaTCP15* transgenic plants. However, this is favorable for the genetic improvement of citral (geranial and citral) with fewer other terpenoids. In addition, overexpression of *LcERF19* in tomato fruit contributed to a significant improvement in citral content, suggesting that *LcERF19*'s role in promoting citral accumulation is conservative. These findings demonstrate that *LcERF19* is an essential key regulator that mightily is exploited to increase citral production and quality in *L. cubeba*.

**Conclusion**

In brief, we functionally identified that *LcERF19*, a member of the IXb AP2/ERF TFs, controls geranial and neral biosynthesis. Overexpression of *LcERF19* in *L. cubeba* and tomato significantly increased the contents of geranial and neral. Furthermore, the expression profile of *LcERF19* in the pericarp was similar to that of *LcTPS42*, and both were induced by JA. *LcERF19* could also directly bind and activate the *LcTPS42* promoter. Our results indicated that *LcERF19* promotes geranial and neral biosynthesis, likely through the activation of *LcTPS42* expression. In the future, more research will be needed to provide knowledge of the AP2/ERF TF interaction members and their involvement in regulating terpenoids metabolism. Besides, we are constructing the genetic transformation system of *L. cubeba* and trying to transform these TFs.

**Materials and methods**

**Identification of AP2/ERF gene family in *L. cubeba* and phylogenetic analysis**

The AP2/ERF domain was acquired from the PFAM database. TBtools was used to extract LcAP2/ERF genes from the *L. cubeba* genome. In parallel, the homologs of *Arabidopsis* AtAP2/ERF transcription factor protein arrangements were downloaded from the PlantTFDB 5.0. The AtAP2/ERF TFs were then shown as a query in TBtools to scan the *L. cubeba* protein dataset, using an e-value limit of 1e-5 and a 45 percent filtering. In addition, the putative LcAP2/ERF proteins were obtained by analyzing HMMER and BLAST results and manually deleting duplicated sequences. Following that, batches from the NCBICDD, SMART, and PFAM databases were used to double-check the predicted
LcAP2/ERF genes\textsuperscript{32}. Finally, 174 LcAP2/ERF TFs were successfully determined in the \textit{L. cubeba} genome. The LcAP2/ERFs sequences were multiply aligned by MAFFT, and the phylogenetic tree was manufactured by PhyML 3.0 with the default parameters\textsuperscript{33}.

**Dual-luciferase (dual-LUC) assay**

Dual-LUC analysis experiments were used to identify LcERF19 activated \textit{LcTPS42} promoters. TFs were embedded into a pGreenII62-SK plasmid (effectors), and \textit{LcTPS42} promoters were embedded into a pGreenII0800-LUC plasmid (reporters). The empty pGreenII62-SK plasmid was used as control for the effector. The effectors and reporters were individually transformed into \textit{Agrobacterium} strain GV3101. The overnight \textit{Agrobacterium} cultures were collected and suspended in infection solution (10 mM MES, 10 mM MgCl\textsubscript{2}, 200 mM acetosyringone, pH 5.7) to an OD\textsubscript{600} = 1.6 and incubated for 4 h at 23-26°C. \textit{Agrobacterium} suspension containing effectors and reporters was mixed (1:1), and infiltrated into the leaves of 30 days old tobacco plants by needleless syringes. Plants were grown at 26°C for 40-72 h with a 16 h light/8 h dark photoperiod. LUC and REN enzymatic activity were analyzed by luciferase assay reagents kit (Promega, USA).

**Subcellular localization**

An \textit{Agrobacterium} GV3101 strain containing the recombinant overexpression plasmid LcERF19 (\textit{pCambia1300S-LcERF19}) and \textit{pCambia1300S} empty vector was used in the subcellular localization experiments. The \textit{Agrobacterium} was suspended through infection solution (10 mM MES, 10 mM MgCl\textsubscript{2}, 200 mM acetosyringone, pH 5.7) to adapt the OD\textsubscript{600} to 0.8 and then incubated for 4 h at 23-26°C, and then infiltrated 30-days-old tobacco leaves. Fluorescence signals were analyzed 40–72 h after infiltration by laser scanning microscope (ZEISS LSM 880, Germany). Positive control was established using OsRed nuclear localization protein with red fluorescence.

**Yeast one-hybrid (Y1H) assay**

Y1H assays were performed as described by Wang et al. (2022)\textsuperscript{24}. The LcERF19 gene was integrated into the pGADT7 plasmid to obtain pGADT7-prey, while three tandem repeats of GCC-box were integrated into the pAbAi plasmid to obtain pAbAi-bait. The bait was cleaved with the reagent BstBI (NEB, USA) and cotransporter into yeast with prey. Select positive colonies and grow on medium (SD/-Trp/-Ura) with 600 ng/mL AbA.
RNA extraction and quantitative Real-Time PCR (qRT-PCR)

Different tissues and different development stages of fruits of L. cubeba were gathered from the farms in Zhengjiang, China (30°27′94″N, 119°58′43″E), quickly frozen and saved at -80°C for qRT-PCR analysis. RNA extraction and reverse transcription methods are described by Zhao et al. (2020)\(^\text{34}\). qRT-PCR was conducted by using the TB Green® Premix Ex Taq™ II kit and ABI PRISM 7500 instrument. All primers have been provided in Table S1.

Transient overexpression of LcERF19 in L. cubeba

An Agrobacterium LBA4404 strain containing the recombinant overexpression plasmid LcERF19 (pCAMBIA1300S-LcERF19) and pCambia1300S empty vector were used for the transient overexpression assays. Transient overexpression methods are described by Wang et al. (2022)\(^\text{24}\). Sterility seedling leaves of L. cubeba infected with A. tumefaciens were placed on MS medium, and cultured at 25-27°C with a photoperiod of 16 h light/8 h dark for 40–72 h. The leaves were gathered as well as saved at -80°C for volatile and qRT-PCR analysis. The volatiles were analyzed using GC-MS\(^\text{34}\).

Transgenic overexpression of LcERF19 in tomato

The recombinant vector containing LcERF19 (pCAMBIA1300S-LcERF19) was transformed into Agrobacterium LBA4404 strains for tomato genetic transformation. The tomato transformation was carried out as reported by Chetty et al. (2013)\(^\text{35}\). Cotyledon explants 7-day-old plants infected with Agrobacterium were planted on KCMS medium and cultured for 40–48 h in the dark. The cotyledon explants were then subcultured in callus induction 2Z medium with 4 mg/L Hygromycin B, and 180 mg/L Timentin to induce callus growth. After 30–45 d, the calluses were transferred to 0.2Z media to induce shoots, and the shoots were placed in MS medium to induce root. Following the formation of its roots, the seedlings are placed into the soil and cultivated in the greenhouse. Genomic PCR using specific primers for both the Hygromycin gene and the LcERF19 gene was performed to verify positive transformations. Samples containing two independent tomato lines with high expression of LcERF19 were gathered and saved at -80°C for volatile and qRT-PCR analysis.

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Author contributions

CYC, WYD, GM: Conceptualization, Funding acquisition, Supervision, Writing-review & editing. WMY, ZYX, YHF, WLW: Writing-original draft, Investigation, Methodology, Writing-review & editing. YJH, WSQ, XSF, YY, WJ: Writing- Proofreading of initial manuscript format and spelling, Investigation.

Data availability

All data are presented inside the manuscript and its supplementary data.

Competing interests

The authors declare no competing interests.

Supplementary information

Table S1 Specific primers used in this manuscript.
Table S2 List of the 174 LcAP2/ERF genes identified in this manuscript.
Fig. S1 Detection of DNA and RNA expression levels of LcERF19 gene in tomato fruit.

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