



LaeA Controls Citric Acid Production through Regulation of the Citrate Exporter-Encoding *cexA* Gene in *Aspergillus luchuensis* mut. *kawachii*

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ABSTRACT The putative methyltransferase LaeA is a global regulator of metabolic and development processes in filamentous fungi. We characterized the homologous *laeA* genes of the white koji fungus *Aspergillus luchuensis* mut. *kawachii* (*A. kawachii*) to determine their role in citric acid hyperproduction. The $\Delta laeA$ strain exhibited a significant reduction in citric acid production. Cap analysis gene expression (CAGE) revealed that *laeA* is required for the expression of a putative citrate exporter-encoding *cexA* gene, which is critical for citric acid production. Deficient citric acid production by a $\Delta laeA$ strain was rescued by the overexpression of *cexA* to a level comparable with that of a *cexA*-overexpressing $\Delta cexA$ strain. In addition, chromatin immunoprecipitation coupled with quantitative PCR (ChIP-qPCR) analysis indicated that LaeA regulates the expression of *cexA* via methylation levels of the histones H3K4 and H3K9. These results indicate that LaeA is involved in citric acid production through epigenetic regulation of *cexA* in *A. kawachii*.

IMPORTANCE *A. kawachii* has been traditionally used for production of the distilled spirit shochu in Japan. Citric acid produced by *A. kawachii* plays an important role in preventing microbial contamination during the shochu fermentation process. This study characterized homologous *laeA* genes; using CAGE, complementation tests, and ChIP-qPCR, it was found that *laeA* is required for citric acid production through the regulation of *cexA* in *A. kawachii*. The epigenetic regulation of citric acid production elucidated in this study will be useful for controlling the fermentation processes of shochu.

KEYWORDS *Aspergillus luchuensis* mut. *kawachii*, citric acid, putative methyltransferase, *laeA*, citrate exporter, *cexA*

Shochu is a traditional Japanese distilled spirit (1). The black koji fungus *Aspergillus luchuensis* and its albino mutant, white koji fungus *Aspergillus luchuensis* mut. *kawachii* (*A. kawachii*), are primarily used for the production of shochu. *A. luchuensis* and *A. kawachii* produce enzymes that decompose the starch contained in ingredients such as rice, barley, buckwheat, and sweet potato (2). In addition, they also produce a large amount of citric acid during the fermentation process, which prevents microbial contamination.

A. luchuensis is phylogenetically related to *Aspergillus niger*, which has been used for industrial citric acid fermentation (3–5). Studies investigating citric acid production have been performed for *A. niger* with respect to various aspects (6–8). In previous studies, nonacidifying mutant strains of *A. niger* were analyzed and *laeA* was found to

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play a significant role in the production of citric acid and secondary metabolites (9). In addition, *laeA* disruption also caused a significant decrease in citric acid production by *Aspergillus carbonarius*, a species closely related to *A. niger* (10). *LaeA* was initially identified as a regulator of secondary metabolism in *Aspergillus* spp. (11). Subsequently, *LaeA* has been primarily studied as a regulator of secondary metabolic and development processes in filamentous fungi (12, 13). A transcriptomic study also supported the idea that *laeA* overexpression and disruption caused the production of secondary metabolites to dramatically change in *A. niger* (14). However, why *LaeA* is required for citric acid production in *A. niger* and *A. carbonarius* remains to be determined.

In this study, we characterized three *laeA* homologous genes, namely, *laeA*, *laeA2*, and *laeA3*, to determine the regulatory mechanism underlying citric acid production in *A. kawachii*. Study of gene disruption indicated that *laeA* significantly reduced citric acid production; therefore, we further analyzed *LaeA*-dependent gene expression by cap analysis gene expression (CAGE) and found that *laeA* is required for expression of a putative citrate exporter-encoding *cexA* gene, which plays a crucial role in citric acid production (15, 16). Further analysis via complementation test and chromatin immunoprecipitation coupled with quantitative PCR (ChIP-qPCR) indicated that *LaeA* is required for citric acid production through epigenetic regulation of *cexA* in *A. kawachii*.

RESULTS

LaeA-like putative methyltransferases in *A. kawachii*. Protein BLAST analysis (<https://www.ncbi.nlm.nih.gov/>) using the amino acid sequences of *Aspergillus nidulans* *LaeA* (AN0807) and *A. niger* *LaeA* (An01g12690) as search queries identified three *LaeA*-like putative methyltransferases in *A. kawachii* using criteria of >50% identity and E values of <1.00E–100: AKAW_04823, AKAW_11001, and AKAW_06012. AKAW_04823 is extremely similar to *A. niger* *LaeA*, with 94% identity over 375 amino acid residues, likely owing to the close phylogenetic relationship between *A. kawachii* and *A. niger*. In addition, all three *A. kawachii* proteins showed a reciprocal best BLAST hit to *A. nidulans* *LaeA*, and alignments of these proteins demonstrated that structural homology with the S-adenosylmethionine (SAM) binding motif (11) is highly conserved (Fig. 1A). Thus, AKAW_04823, AKAW_11001, and AKAW_06012 as *LaeA*-like putative methyltransferases were termed *LaeA*, *LaeA2*, and *LaeA3*, respectively.

To further clarify the relationship between *LaeA*, *LaeA2*, and *LaeA3*, we performed a phylogenetic analysis of *LaeA*-like methyltransferases conserved in *Aspergillus* spp. (Fig. 1B). The data set for analysis was obtained by protein BLAST of the *Aspergillus* Genome Database (AspGD; <http://www.aspgd.org/>) using the amino acid sequences of *A. kawachii* *LaeA*, *LaeA2*, and *LaeA3* as search queries. *A. kawachii* *LaeA* is classified into the putative *LaeA* ortholog group. Conversely, *LaeA2* and *LaeA3* were assigned to different positions; however, they remain closer to *LaeA* than to *A. nidulans* *Llm* (*LaeA*-like-methyltransferase) proteins (17). This result supports the hypothesis that *LaeA2* and *LaeA3* are also *LaeA*-like methyltransferases.

Colony formation and citric acid production by control, $\Delta sC \Delta laeA$, $\Delta sC \Delta laeA2$, and $\Delta sC \Delta laeA3$ strains. To investigate the role of *LaeA*-like methyltransferases in citric acid production by *A. kawachii*, we constructed strains with each gene disruption and observed their colony formation (Fig. 2A). For comparison with the respective disruption, the *A. kawachii* control strain was defined to show the same auxotrophic background.

The $\Delta sC \Delta laeA$ strain exhibited colony morphology similar to that of the control strain, but its color was found to be paler than that of control, $\Delta sC \Delta laeA2$, and $\Delta sC \Delta laeA3$ strains upon observation of the bottom of the minimal (M) medium agar plate (Fig. 2A). This result indicates that *LaeA* positively regulates the production of the hyphal yellow pigment in *A. kawachii* in addition to being involved in secondary metabolism in *A. niger* (9, 14). However, a difference was observed in the *A. kawachii* $\Delta sC \Delta laeA$ strain in that the *A. niger* $\Delta laeA$ strain showed yellowish mycelia compared with those of the *A. niger* control strain on the minimal medium agar plate (9).

A

AnidLaeA	134	PHPTNGRFLDLGCGTGIWAIEVANKYFDADFVAGVDLAPIQPPNHFKNCFYAPFDFAEPWAMGEDSWDLIHLQMGCGSSVM	213
AnigLaeA	132	PHPPNGRFLDLGCGTGIWAIDVAHKYPNAFVAGVDLAPIQPPNHEDNCFYAPFDFAEPWTLGENSWDLIHLQMGCGSVL	211
AkawLaeA	132	PHPPNGRFLDLGCGTGIWAIDVAHKYPNAFVAGVDLAPIQPPNHEDNCFYAPFDFAEPWTLGENSWDLIHLQMGCGSVL	211
AkawLaeA2	101	PHPRNGRFLDVGCGTGIWAIDVAHEYENAFVVGVDLAPIQPPNHFNCCQFYAPFDFAEPWTLGEDSWDLIHLQMGCGSSV	180
AkawLaeA3	152	PHPENGRFLDLGCGTGIWAIDVIAQFPCAFVVGDIPIQPPSNKPEKNCSEFRAPFDLESPTWTLGENEWDVLYLRMGCGYIS	131
*** ** *			
AnidLaeA	214	GWPPLYRRIFAHLRPGAWFEQVEIDDFEPRCDDRSLDGTLALRHWDCLKQATAEATMRPIAHSSRDITIKDLQDAGEFTE	289
AnigLaeA	212	GWQNLKRLRLRHLQPGAWFEQVEIDDFEPRCDDRSLDGLALREWYQYLKQATQDTMRPIAHSSRDITIRHLEEAGFTQ	287
AkawLaeA	212	GWPPLYRRIFAHLRPGAWFEQVEIDDFEPRCDDRSLDGLALREWYQYLKQATQDTMRPIAHSSRDITIRHLEEAGFTQ	287
AkawLaeA2	181	AWPNLYRRIILRHLQPGAWFEQVEIDDFEPRCDDCSLDGLALDQWYQCLKQATQATMRPIAHNSRDITIRALEEAGFTQ	256
AkawLaeA3	132	DWQGLYRKVYAHLCGGWLEQIEIDFTPRSRVHRTKGCAMQFWYQSLTQATEKAVRPLAHCPOWTMOKLQAGAVE	207

B

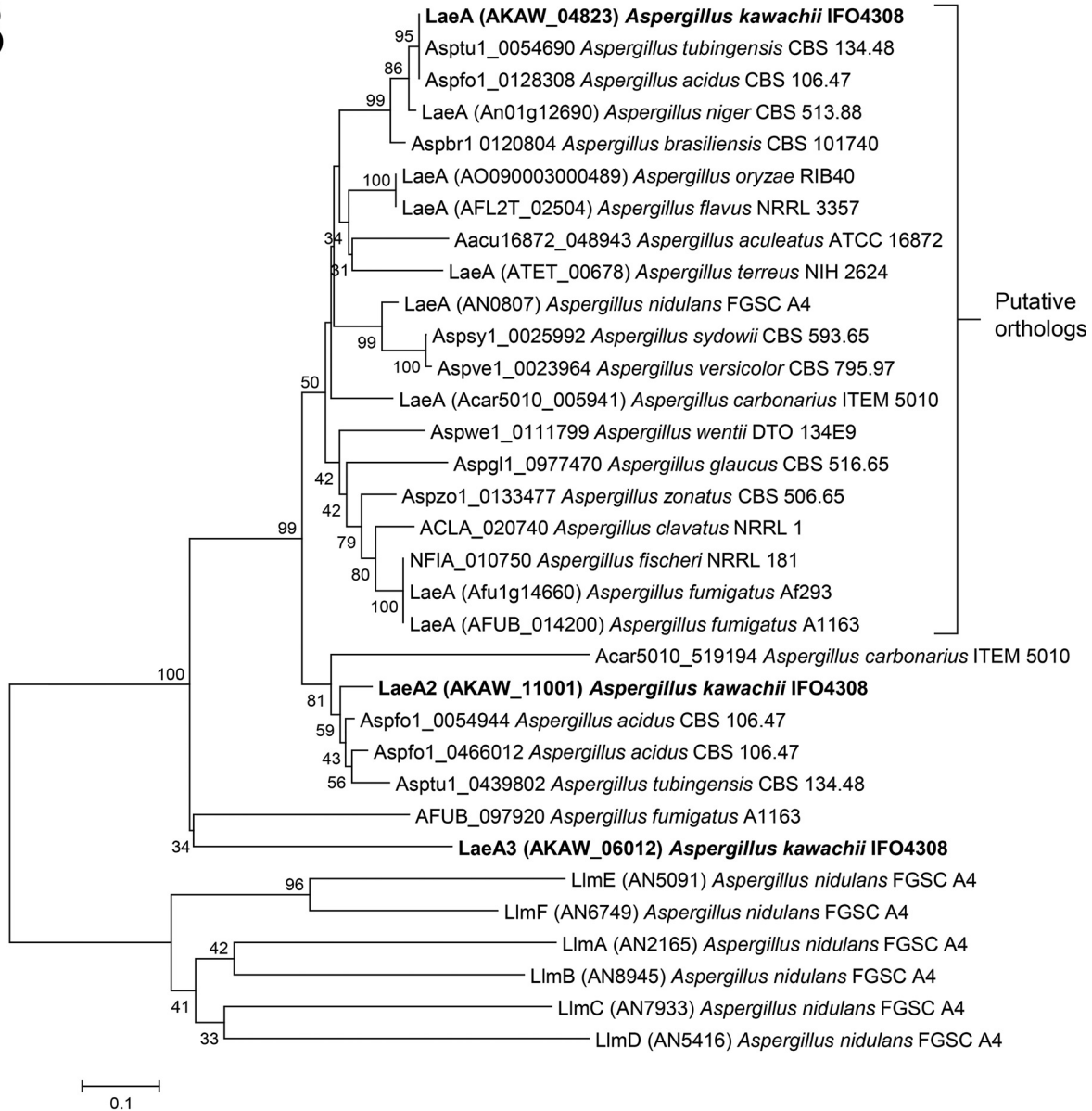


FIG 1 (A) Putative methyltransferase domain sequences of *A. nidulans* (Anid) LaeA, *A. niger* (Anig) LaeA, and predicted *A. kawachii* (Akaw) LaeA-like proteins. The domains were identified by Pfam (<https://pfam.xfam.org/>). Sequence alignment was performed using ClustalW program in the BioEdit Sequence Alignment Editor (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Putative intron sequences of *A. kawachii* *laeA* and *laeA2* were confirmed by transcriptome sequencing (RNA-seq; data not shown) and translated to the amino acid sequences. Asterisks indicate putative SAM binding sites (11). (B) Phylogenetic tree of amino acid sequences of LaeA-like methyltransferases identified from the *Aspergillus* genome database (<http://www.aspgd.org/>). The tree was constructed via the neighbor-joining method with complete gap deletion on MEGA version 6.0 (53). Bootstrap values (1,000 replicates) are indicated at the branches. Low bootstrap values (<25) were removed.

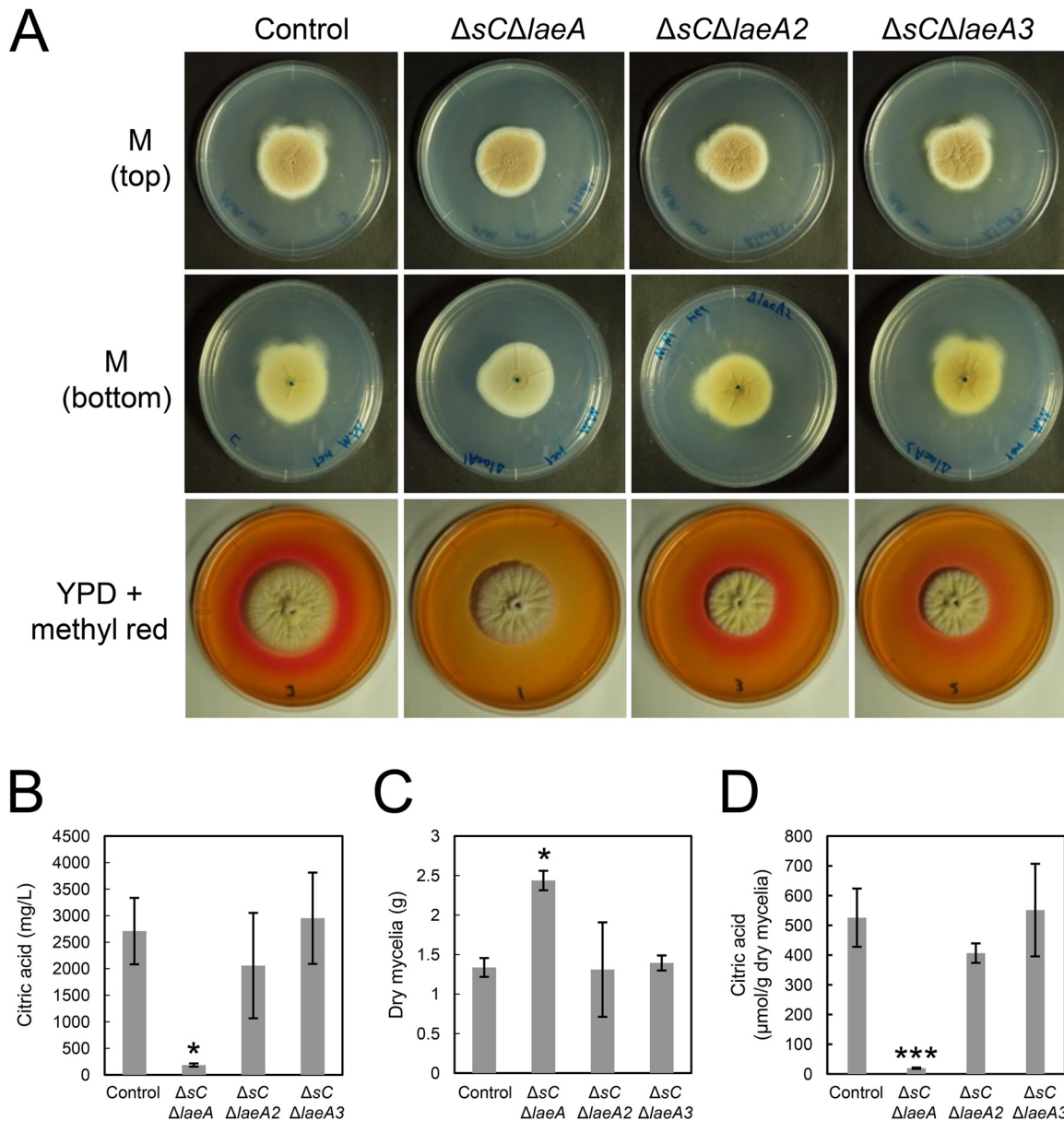


FIG 2 (A) Colony formation of *A. kawachii* control, $\Delta sC \Delta laeA$, $\Delta sC \Delta laeA2$, and $\Delta sC \Delta laeA3$ strains. Strains were cultured at 30°C for 5 days on M medium or YPD medium containing methyl red. Agar medium was inoculated with 1×10^4 conidiospores. Methyl red was added as a pH indicator; it turned red at pH values of ≤ 4.4 . (B to D) Citric acid level in culture supernatant (B), mycelial biomass production (C), and extracellular citric acid production per mycelial weight (D) by *A. kawachii* strains. The control, $\Delta sC \Delta laeA$, $\Delta sC \Delta laeA2$, and $\Delta sC \Delta laeA3$ strains were precultured in M medium for 36 h and then transferred to CAP medium for 48 h. Means and SDs were determined from the results of 3 independent cultivations. Asterisks indicate significant difference (*, $P < 0.05$; ***, $P < 0.001$; Student's *t* test) from the results for the control strain.

In addition, red color surrounding the colonies disappeared only with the $\Delta sC \Delta laeA$ strain in yeast extract-peptone-dextrose (YPD) medium containing methyl red as a pH indicator, indicating that the $\Delta sC \Delta laeA$ strain shows a nonacidifying phenotype (Fig. 2A). Therefore, we compared the organic acid productivities of control, $\Delta sC \Delta laeA$, $\Delta sC \Delta laeA2$, and $\Delta sC \Delta laeA3$ strains to further investigate the role of LaeA-like methyltransferases in citric acid production. The strains were precultured in M medium at 30°C for 36 h and then transferred to citric acid production (CAP) medium and further cultured at 30°C for 48 h. Next, the measurement of citric acid levels in culture supernatant (Fig. 2B) and mycelial biomass (Fig. 2C) allowed for the determination of extracellular citric acid production per mycelial weight (Fig. 2D). CAP medium was used for evaluating

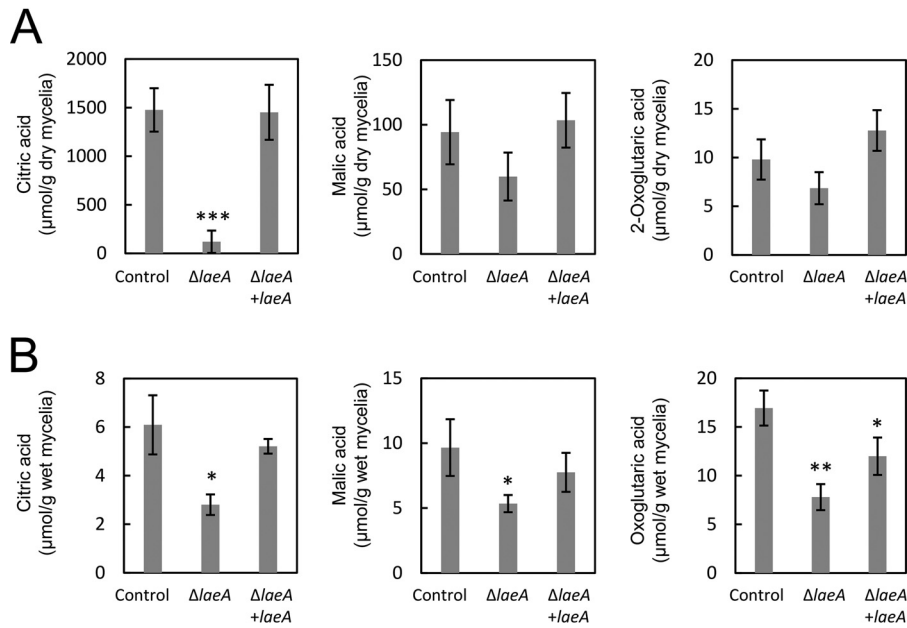


FIG 3 Extracellular (A) and intracellular (B) organic acid production by *A. kawachii* strains. The control, $\Delta laeA$, and $\Delta laeA$ plus *laeA* strains were precultured in M medium for 36 h and then transferred to CAP medium for 48 h. Means and SDs were determined from the results of 3 independent cultivations. Asterisks indicate significant difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's *t* test) from the results for the control strain.

organic acid production because it contains a high concentration of a carbon source (10% [wt/vol] glucose) as well as appropriate trace elements (6–8).

The citric acid level in culture supernatant for the $\Delta sC \Delta laeA$ strain was 14.3-fold lower than that for the control strain (Fig. 2B). In contrast, the production of mycelial biomass by the $\Delta sC \Delta laeA$ strain was 1.82-fold higher than that by the control strain (Fig. 2C). Based on citric acid level in culture supernatant and amount of mycelial biomass produced, the $\Delta sC \Delta laeA$ strain exhibited a 25-fold-lower citric acid production than the control strain, whereas the $\Delta sC \Delta laeA2$ and $\Delta sC \Delta laeA3$ strains exhibited similar citric acid levels (Fig. 2D). This result indicates that LaeA plays a significant role in citric acid production and hyphal growth of *A. kawachii*.

Test for complementation of the $\Delta laeA$ strain with wild-type *laeA*. Because *laeA* disruption caused a significant decline in citric acid production by *A. kawachii*, we examined the complementation of wild-type *laeA* in the $\Delta sC \Delta laeA$ strain using an *sC* marker. Control, $\Delta laeA$, and $\Delta laeA$ plus *laeA* strains were cultured as described above and culture supernatant and mycelia were separated as the extracellular and intracellular fractions, respectively. In the extracellular fraction, the $\Delta laeA$ strain exhibited 12.5-fold-lower citric acid levels than the control strain (Fig. 3A). Conversely, decreases in the production of malic acid and oxoglutaric acid (1.6-fold and 1.4-fold, respectively) were not statistically significant. Decreases in the production of citric acid, malic acid, and oxoglutaric acid were rescued by the complementation of wild-type *laeA*.

Additionally, in the intracellular fraction, the $\Delta laeA$ strain exhibited productivities of 2.2-fold-lower citric acid, 1.8-fold-lower malic acid, and 2.2-fold-lower oxoglutaric acid (Fig. 3B). These decreases in organic acid production were rescued by the complementation of wild-type *laeA*, although the $\Delta laeA$ plus *laeA* strain still exhibited 1.4-fold-lower oxoglutaric acid production than that of the control strain.

These results indicate that LaeA plays a significant role in both extracellular and intracellular organic acid production in *A. kawachii*. In addition, extracellular citric acid accumulation was most significantly affected by *laeA* disruption.

Colony formation of control, $\Delta laeA$, and $\Delta laeA$ plus *laeA* strains. To explore the physiologic roles of LaeA, we characterized the colony morphology of *A. kawachii*

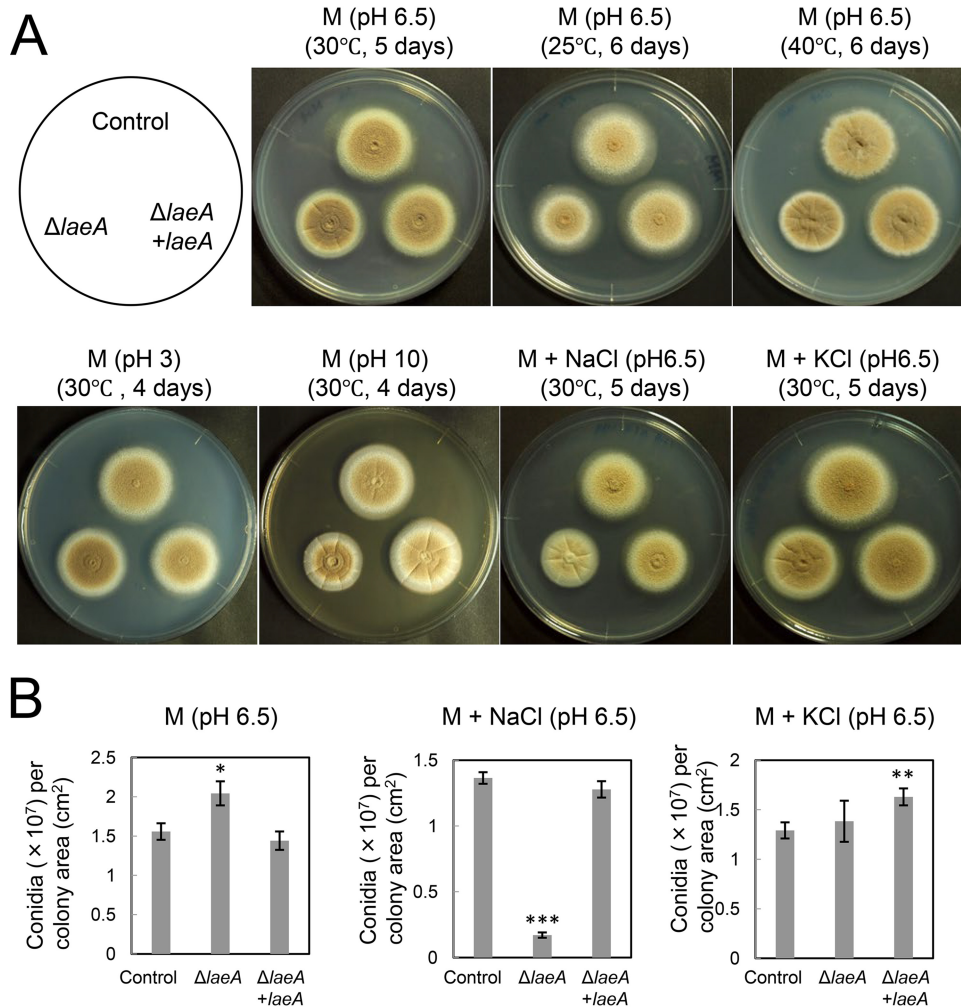


FIG 4 Strains were cultured on M medium with or without stress conditions, and agar medium was inoculated with 1×10^4 conidia. (A) Colony morphology of *A. kawachii* control, $\Delta laeA$, and $\Delta laeA$ plus *laeA* strains. (B) Conidiation of *A. kawachii* control, $\Delta laeA$, and $\Delta laeA$ plus *laeA* strains. All results are expressed as means and SDs. Asterisks indicate significant difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's *t* test) from the results for the control strain.

control, $\Delta laeA$, and $\Delta laeA$ plus *laeA* strains (Fig. 4A). The $\Delta laeA$ strain exhibited a slightly smaller average colony diameter than those of the control and $\Delta laeA$ plus *laeA* strains on M medium at all tested temperatures at pH 6.5. This growth deficiency was restored at pH 3, whereas it worsened at pH 10.

In addition, colonies of the $\Delta laeA$ strain were paler than those of the control and $\Delta laeA$ plus *laeA* strains in M medium with 0.8 M sodium chloride but not in that with 0.6 M potassium chloride (Fig. 4A). Thus, we next assessed conidium formation (Fig. 4B). Strains were cultured on M medium with or without 0.8 M sodium chloride or 0.6 M potassium chloride at 30°C for 5 days, at which time the number of conidia formed was determined. The number of conidia per square centimeter of the $\Delta laeA$ strain was significantly reduced, to approximately 13% of the number formed by the control strain, in M medium containing 0.8 M sodium chloride. Alternatively, the number of conidia per square centimeter for the $\Delta laeA$ strain was 1.32-fold higher and similar to that of the control strain in M medium and M medium with 0.6 M potassium chloride, respectively. Complementation of *laeA* rescued the deficient conidium formation of the $\Delta laeA$ strain, although the $\Delta laeA$ plus *laeA* strain exhibited 1.26-fold-higher conidium formation in M medium containing 0.6 M potassium chloride.

These results indicate that *LaeA* is required for asexual development, particularly in the presence of sodium-specific stress versus high osmotic stress. The deficiency in

conidium formation was consistent with previous reports that the *laeA* disruption caused a significant defect in conidium formation in *A. oryzae* (18) and *Penicillium chrysogenum* (19). Although LaeA plays a significant role in the sexual development of *A. nidulans* (20), sexual development has not been observed in *A. kawachii*.

Gene expression related to citric acid production. To identify LaeA-regulated genes related to citric acid production, the expression profiles of *A. kawachii* control and $\Delta laeA$ strains during citric acid production were compared. These strains were precultured in M medium at 30°C for 36 h and then transferred to CAP medium and further cultured at 30°C for 12 h, at which time the *A. kawachii* control strain vigorously produced citric acid. Next, gene expression profiles were compared using CAGE.

Gene expression change of a total of 9,647 genes was evaluated by CAGE (see Data Set S1 in the supplemental material). The change in gene expression was considered to be significant if the false-discovery rate (FDR) was <0.05 and the \log_2 fold change was less than -0.5 or greater than 0.5 . Using these criteria, a total of 1,248 differentially expressed genes were identified, including 590 upregulated and 658 downregulated genes. Gene ontology (GO) term enrichment analysis of these gene data sets revealed the enrichment of GO terms related to transport and metabolic processes (data not shown); however, it was difficult to interpret the results to understand the reason as to why *laeA* disruption reduced citric acid production.

Next, we mapped the differentially expressed genes, 15 of which were mapped to metabolic pathways related to citric acid production (Fig. 5; Table 1). For example, genes related to glycerol synthesis (reactions 1 [AKAW_07170] and 12 [AKAW_08691] in Fig. 5) and pentose phosphate (reaction 11 [AKAW_00489]) pathways were upregulated by *laeA* disruption, whereas the Embden-Meyerhof-Parnas (EMP) pathway (reactions 2 [AKAW_04737] and 3 [AKAW_03026]) and citrate synthase in the tricarboxylic acid (TCA) and glyoxylate cycles (reactions 4 [AKAW_00170] and 5 [AKAW_06223]) were downregulated by *laeA* disruption. The citrate synthase-encoding gene (AKAW_06223) showed most significant reduction in gene expression ($-11.35 \log_2$ fold); however, it was not the most highly expressed citrate synthase gene, indicating that the reduced expression level of AKAW_06223 might not explain the deficient citric acid production.

Next, we focused on the citric acid export process, as it is considered to play a significant role in the high citric acid production by *A. niger* (21–23) (Table 2). Our group previously reported that the two mitochondrial citrate transporters CtpA (*ctpA* [AKAW_03754]) and YhmA (*yhmA* [AKAW_06280]) (24) are involved in the transport of citric acid from mitochondria to cytosol; however, their gene expression levels did not significantly change. Conversely, a significant reduction in gene expression ($-6.09 \log_2$ fold) of the putative citrate exporter-encoding *cexA* (AKAW_07989). CexA was identified as the main citrate exporter of the plasma membrane in *A. niger* (15, 16). AKAW_07989 showed the best reciprocal BLAST hit to *A. niger* CexA, with 96% identity over 502 amino acid residues, indicating that AKAW_07989 represents CexA in *A. kawachii*.

Citric acid was the most significantly decreased organic acid in the extracellular fraction resulting from the disruption of *laeA* (Fig. 3A); however, citric acid, malic acid, and oxoglutaric acid productions were reduced at similar levels within the intracellular fraction of the $\Delta laeA$ strain (Fig. 3B). Thus, we focused on the significantly reduced expression level of *cexA*, which could explain the reduced citric acid accumulation in the extracellular fraction (Table 2).

Test of complementation of *laeA* disruption by *cexA* overexpression. To examine whether the downregulation of *cexA* was crucial for the decrease in citric acid production caused by *laeA* disruption, we compared citric acid production by control and $\Delta laeA$ strains with that by $\Delta cexA$, $\Delta cexA$ plus OE*cexA* (*cexA* overexpression with deletion of *cexA*), and $\Delta laeA$ plus OE*cexA* (*cexA* overexpression with deletion of *laeA*) strains (Fig. 6A). *gpdA* encodes glyceraldehyde-3-phosphate dehydrogenase (25, 26) and was used for overexpression of *cexA* because there was no significant change in *gpdA* expression between control and $\Delta laeA$ strains in the CAGE data set (Data Set S1), indicating that the *gpdA* promoter is not controlled by LaeA.

TABLE 1 Citric acid production-related genes among up- and downregulated genes following *laeA* disruption

Reaction no. in Fig. 5	Locus tag	Putative function	Log ₂ fold change	FDR
1	AKAW_07170	Fructose-bisphosphate aldolase	4.01	0.0011
2	AKAW_04737	Glyceraldehyde 3-phosphate dehydrogenase	-1.05	0.0005
3	AKAW_03026	Phosphoglycerate mutase	-0.78	0.0335
4	AKAW_00170	Citrate synthase	-0.92	0.0014
5	AKAW_06223	Citrate synthase	-11.35	1.10E-98
6	AKAW_07594	Succinate-CoA ligase (GDP-forming) alpha chain	1.27	0.0052
7	AKAW_07352	Succinate dehydrogenase	-0.71	0.0352
8	AKAW_00119	Isocitrate lyase	-1.40	1.063E-05
9	AKAW_05189	Alpha, alpha-trehalosephosphate synthase (UDP forming) 1 (TpsA, TpsB)	-0.76	0.0108
10	AKAW_03597	Alpha, alpha-trehalosephosphate synthase (UDP forming) 1 (TpsA, TpsB)	0.87	0.0024
11	AKAW_00489	Ribose-5-phosphate isomerase (B)	1.03	0.0035
12	AKAW_08691	Alcohol oxidase	1.65	3.18E-09
13	AKAW_02095	Phosphoenolpyruvate carboxykinase (ATP)	0.80	0.0139
14	AKAW_00804	Pyruvate carboxylase (cytosol)	-1.15	0.0168
15	AKAW_06947	Pyruvate decarboxylase	0.83	0.0205

A. kawachii strains were precultured with M medium at 30°C for 36 h and were then transferred to CAP medium and further cultured at 30°C for 72 h. Then we measured the citric acid (Fig. 6A) and glucose (Fig. 6B) levels in culture supernatant. In addition, we measured the amount of mycelial biomass produced after precultivation in M medium for 36 h (defined as 0 h), which is the time point just before strain transfer to CAP medium, and after further cultivation in CAP medium for 72 h (Fig. 6C).

The $\Delta cexA$ strain exhibited lower extracellular citric acid production than the control strain, similar to that observed for the $\Delta laeA$ strain, whereas the $\Delta cexA$ plus OE*cexA* and $\Delta laeA$ plus OE*cexA* strains exhibited higher extracellular citric acid production than the control strain throughout the cultivation period of 72 h (Fig. 6A). There was no significant difference in citric acid production between the $\Delta cexA$ plus OE*cexA* and $\Delta laeA$ plus OE*cexA* strains, indicating that the overexpression of *cexA* alone was sufficient to rescue deficient citric acid production in the $\Delta laeA$ strain.

The amount of mycelial biomass produced by the $\Delta laeA$ strain was not considerably different from that produced by the control strain at 0 h; however, this amount became 1.42-fold higher than that produced by the control strain after further cultivation in CAP medium for 72 h (Fig. 6C). This result indicates that the hyphal growth of the $\Delta laeA$ strain was enhanced in CAP medium compared with that of the control strain. In addition, the amounts of mycelial biomass produced by the $\Delta laeA$ plus OE*cexA* and $\Delta cexA$ plus OE*cexA* strains were 3.8-fold and 2.9-fold lower than those produced by the control strain, respectively, at 0 h (Fig. 6C). This result indicates that the overexpression of *cexA* negatively affects the hyphal growth of *A. kawachii* in M medium.

The glucose level in culture supernatant was 1.3-fold lower in the $\Delta laeA$ strain than that of the control strain at 24 h (Fig. 6B). Therefore, the $\Delta laeA$ strain exhibited a higher glucose consumption rate than the control strain in CAP medium. Because the initial amounts of mycelial biomass for the control and $\Delta laeA$ strains were at similar levels at 0 h, the higher glucose consumption rate led to a higher mycelial biomass yield by the $\Delta laeA$ strain at 72 h (Fig. 6C). In contrast, glucose levels were 1.1-fold higher for the $\Delta cexA$ plus OE*cexA* strain than for the control strain at 12 h (Fig. 6B). In addition, glucose levels were 1.1-fold, 1.2-fold, and 1.2-fold higher for the $\Delta laeA$ plus OE*cexA* strain than for the control strain at 12, 48, and 72 h, respectively. This may be due to the smaller

TABLE 2 Gene expression change of citric acid excretion-related genes following *laeA* disruption

Gene	Locus tag	Putative function	Log ₂ fold change	FDR
<i>ctpA</i>	AKAW_03754	Mitochondrial citrate transporter	0.08	0.9432
<i>yhmA</i>	AKAW_06280	Mitochondrial citrate transporter	0.09	0.9313
<i>cexA</i>	AKAW_07989	Plasma membrane localized citrate exporter	-6.09	3.72E-35

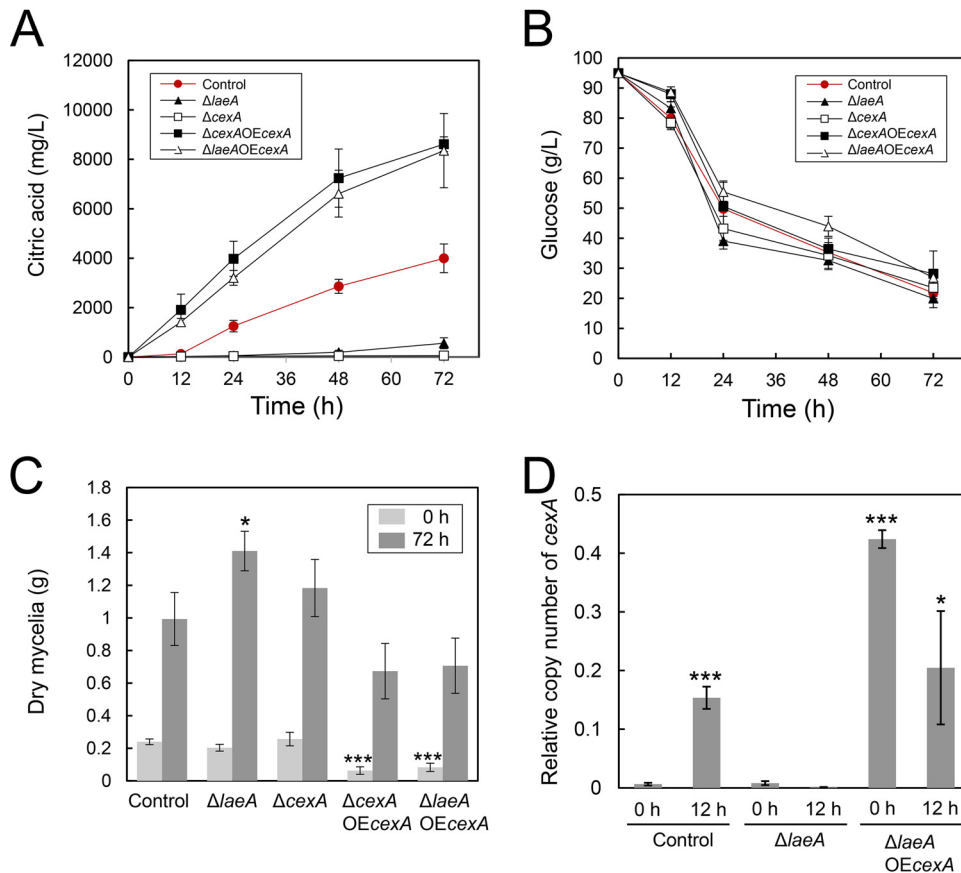


FIG 6 Citric acid level (A) and glucose level (B) in culture supernatant and mycelial biomass production (C) by *A. kawachii* strains. Control, $\Delta laeA$, $\Delta cexA$, $\Delta cexA$ plus OE*cexA*, and $\Delta laeA$ plus OE*cexA* strains were precultured in M medium for 36 h, then transferred to CAP medium, and further cultured for 0, 12, 24, 48, and 72 h. Means and SDs were determined from the results of 3 independent cultivations. Asterisks indicate significant difference (*, $P < 0.05$; ***, $P < 0.001$; Student's *t* test) from the results for the control strain. (D) Transcriptional levels of *cexA*. The control, $\Delta laeA$, and $\Delta laeA$ plus OE*cexA* strains were precultured in M medium for 36 h, then transferred to CAP medium, and further cultured for 0 or 12 h. Means and SDs were determined from the results of 3 independent cultivations. Asterisks indicate significant difference (*, $P < 0.05$; ***, $P < 0.001$; Student's *t* test) from the results for the control strain cultured for 0 h.

initial amounts of mycelial biomass for the $\Delta cexA$ plus OE*cexA* and $\Delta laeA$ plus OE*cexA* strains than that for the control strain at 0 h (Fig. 6C).

Transcriptional levels of *cexA* in the control, $\Delta laeA$, and $\Delta laeA$ plus OE*cexA* strains. Because the $\Delta laeA$ plus OE*cexA* strain exhibited higher citric acid production than the control strain (Fig. 6A), we determined gene expression levels of *cexA* in the control, $\Delta laeA$, and $\Delta laeA$ plus OE*cexA* strains. Strains were precultured in M medium at 30°C for 36 h and then transferred to CAP medium. The time point of 36 h of precultivation just before transfer was defined as 0 h (i.e., starting time). We compared the gene expression levels of *cexA* at 0 and 12 h of cultivation in CAP medium via quantitative reverse transcription-PCR (RT-PCR) analysis (Fig. 6D). The control strain showed a 24-fold-greater expression level of *cexA* at 12 h than that at 0 h, whereas the $\Delta laeA$ strain showed similar expression levels of *cexA* at 0 and 12 h. These results indicate that *cexA* expression is induced under the condition of citric acid production via *LaeA*.

Control and $\Delta laeA$ plus OE*cexA* strains showed similar expression levels of *cexA* at 12 h; however, the $\Delta laeA$ plus OE*cexA* strain exhibited a 66-fold-higher expression level of *cexA* than the control strain at 0 h, likely because the *gpdA* promoter is active in M as well as CAP medium. Thus, the higher citric acid production by the $\Delta laeA$ plus OE*cexA* strain (Fig. 6A) could be attributable to a higher gene expression level of *cexA* when beginning culture in CAP medium.

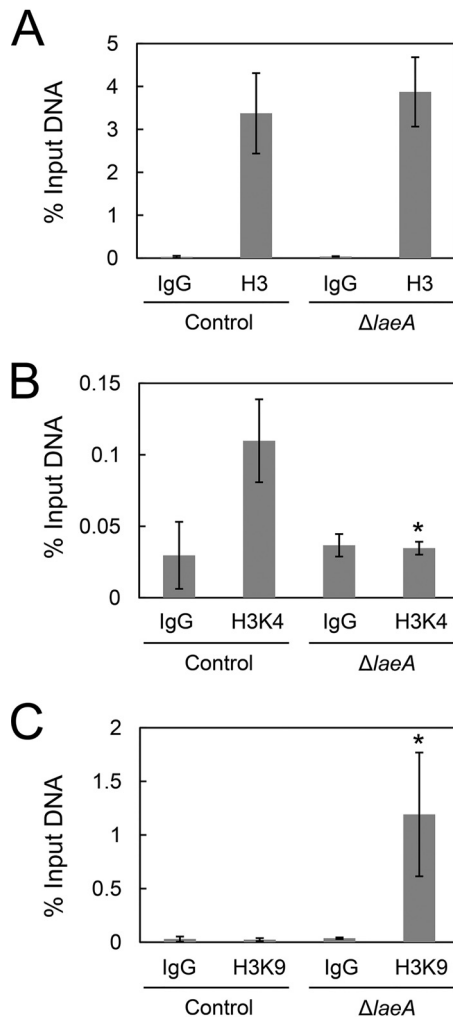


FIG 7 ChIP-qPCR of *cexA* promoter region. Control and $\Delta laeA$ strains were cultured as described in the text. Histone H3 (A), histone H3K4me3 (B), and histone H3K9me3 (C) occupancies of the *cexA* promoter region were investigated. The means and SDs were determined from the results of 3 independent cultivations. Asterisks indicate significant difference (*, $P < 0.05$; Student's *t* test) from the results for the control strain.

Histone trimethylation level in the *cexA* promoter region. LaeA is believed to widely regulate gene expression via controlling the methylation level of histones (11–13). To determine the mechanism underlying LaeA-dependent *cexA* expression through histone methylation, we performed ChIP-qPCR analysis of histone H3, histone H3 trimethyl K4 (H3K4me3), and histone H3 trimethyl K9 (H3K9me3) in control and $\Delta laeA$ strains. H3K4me3 and H3K9me3 are known to be euchromatin and heterochromatin markers, respectively.

The histone H3 occupancy at the *cexA* promoter did not change between the control and $\Delta laeA$ strains (Fig. 7A). However, the euchromatin marker H3K4me3 at the *cexA* promoter was decreased to a level similar to that of the negative control (i.e., normal anti-mouse IgG) in the $\Delta laeA$ strain (Fig. 7B). In addition, the heterochromatin marker H3K9me3 was greatly enriched in the $\Delta laeA$ strain compared with that in the control strain (Fig. 7C). These results indicated that LaeA controls *cexA* expression via modulation of euchromatin/heterochromatin ratios at the *cexA* promoter region in *A. kawachii*.

DISCUSSION

In this study, we characterized three LaeA-like methyltransferases, LaeA, LaeA2, and LaeA3, in *A. kawachii* to reveal their role in citric acid production. LaeA2 and LaeA3 are

not conserved in all *Aspergillus* spp. (Fig. 1B), implying that these two LaeA-like methyltransferases are paralogs. For example, LaeA-like methyltransferases, similar to *A. kawachii* LaeA2, were only conserved in the *Aspergillus* section *Nigri* (*A. carbonarius* ITEM 5010, *Aspergillus acidus* CBS 106.47, and *Aspergillus tubingensis* CBS 134.48), with the exception of *A. niger* CBS 513.88. In addition, LaeA-like methyltransferases similar to *A. kawachii* LaeA3 were identified only in *A. fumigatus* A1163.

Among the three LaeA-like methyltransferase genes, only *laeA* disruption caused significant reduction in citric acid production by the *A. kawachii* strain (Fig. 2D). This result is consistent with the results of previous studies showing that LaeA is required for citric acid accumulation in culture supernatants of *A. niger* and *A. carbonarius* (9, 10).

As LaeA is known to control secondary metabolism and development in filamentous fungi (11–13), the $\Delta laeA$ strain showed deficient production of yellow mycelium pigment (Fig. 2A) and formation of conidia (Fig. 4B). The $\Delta laeA$ strain exhibited deficient formation of conidia in the presence of sodium chloride but not in the presence of potassium chloride (Fig. 4B), thereby indicating that LaeA is required for the formation of conidia, particularly in the presence of sodium-specific stress. Sodium is known to demonstrate toxic effects above a certain Na^+/K^+ ratio and has to be excreted from the cytosol to prevent the toxic effects caused by sodium transporters (27–29). However, there is no significant change in the expression of genes encoding putative sodium transporters *Nha1* (AKAW_09133 and AKAW_03679) and *Nhx1* (AKAW_07982) in the CAGE data set (Data Set S1). In addition, CAGE could not link the transcription start sites with predicted coding sequences of *Ena1* (AKAW_08368 and AKAW_08719), which are also the homologs of sodium transporters.

CAGE identified 1,248 genes differentially expressed between the control and $\Delta laeA$ strains (Data Set S1). These genes included approximately equal numbers of upregulated and downregulated genes (590 and 658, respectively). LaeA should regulate these genes not only directly but also indirectly because they include at least 9 transcription factor gene homologs. For example, the upregulation of *IreB* (AKAW_00032) (30), *tamA* (AKAW_02564) (31), and *dbaA* (AKAW_01762) (32) and the downregulation of *amyR* (AKAW_09854) (33), *zipA* (AKAW_05495) (34), *oefC* (AKAW_03045) (35), *farB* (AKAW_07498) (36), *cpcA* (AKAW_00007) (37), and *devR* (AKAW_08419) (38) were identified in this process.

CAGE identified considerable reduction in the gene expression of the citrate exporter-encoding *cexA* gene by *laeA* disruption (Table 2). A further study showed that *cexA* overexpression remedied deficient citric acid production by $\Delta laeA$ and $\Delta cexA$ strains at similar levels (Fig. 6A). These results indicated that the deficient gene expression of *cexA* led to the deficient citric acid production by the $\Delta laeA$ strain. For the evaluation of citric acid production, *A. kawachii* strains were cultivated in CAP medium after precultivation in M medium. CAP medium was used for enhancing citric acid production concomitant with reduced hyphal growth (24). Fungal pellet morphology is known to affect the production of organic acids, proteins, and secondary metabolites (39), and elevated citric acid production occurs with shorter hyphae or hyperbranched phenotypes in *A. niger* (40, 41). The production of mycelial biomass by the $\Delta laeA$ strain did not considerably change during precultivation in M medium compared with that by the control strain (Fig. 6C); however, it considerably increased after cultivation in CAP medium (Fig. 2C, Fig. 6C), thereby indicating that the hyphal growth of the $\Delta laeA$ strain was enhanced compared with that of the control strain under the condition of citric acid production. On the other hand, the production of mycelial biomass by the $\Delta cexA$ plus OE*cexA* and $\Delta laeA$ plus OE*cexA* strains reduced during precultivation in M medium compared with that by the control strain (Fig. 6C). In addition, we observed that there were no considerable changes in the pellet sizes of the control, $\Delta laeA$, $\Delta cexA$, $\Delta cexA$ plus OE*cexA*, and $\Delta laeA$ plus OE*cexA* strains after cultivation in CAP medium; however, the surface roughness of the pellets of the $\Delta cexA$ plus OE*cexA* and $\Delta laeA$ plus OE*cexA* strains was smoother than those of the control strains (data not shown). Unlike in the control strain, these growth phenotypes and morphological changes due to *laeA* deletion and *cexA* overexpression might be related to citric acid production (Fig. 6A).

Subcultivation of the control strain from M medium to CAP medium led to elevated

TABLE 3 *Aspergillus kawachii* strains used in this study

Strain name or description	Genotype	Reference
SO2 ($\Delta sC \Delta argB$)	<i>ligD</i> ⁻ <i>argB::hph sC</i> ⁻	44
ΔsC	<i>ligD</i> ⁻ <i>argB::argB sC</i> ⁻	This study
$\Delta sC \Delta laeA$	<i>ligD</i> ⁻ <i>argB::hph sC</i> ⁻ <i>laeA::argB</i>	This study
$\Delta sC \Delta laeA2$	<i>ligD</i> ⁻ <i>argB::hph sC</i> ⁻ <i>laeA2::argB</i>	This study
$\Delta sC \Delta laeA3$	<i>ligD</i> ⁻ <i>argB::hph sC</i> ⁻ <i>laeA3::argB</i>	This study
CK2	<i>ligD</i> ⁻ <i>argB::argB sC::sC</i>	24
$\Delta laeA$	<i>ligD</i> ⁻ <i>argB::hph sC::sC laeA::argB</i>	This study
$\Delta laeA$ plus <i>laeA</i>	<i>ligD</i> ⁻ <i>argB::hph sC</i> ⁻ <i>laeA::laeA-sC-argB</i>	This study
$\Delta cexA$	<i>ligD</i> ⁻ <i>argB::hph sC::sC cexA::argB</i>	This study
$\Delta cexA$ plus OE <i>cexA</i>	<i>ligD</i> ⁻ <i>argB::hph sC</i> ⁻ <i>cexA::argB</i> pGS-PgpdA- <i>cexA</i>	This study
$\Delta laeA$ plus OE <i>cexA</i>	<i>ligD</i> ⁻ <i>argB::hph sC</i> ⁻ <i>laeA::argB</i> pGS-PgpdA- <i>laeA</i>	This study

transcript levels of *cexA*, whereas the $\Delta laeA$ strain lost its transcriptional response, thereby indicating that LaeA is involved in the transcriptional regulation of *cexA* (Fig. 6D). Although details concerning the molecular function of LaeA remain unclear (11–13), LaeA is known to counteract the heterochromatinization of the promoter region of secondary metabolite gene clusters by histone H3K9 methylation via heterochromatin protein 1 (HepA) and a H3K9 methyltransferase (ClrD) in *A. nidulans* (42). Our findings indicated that the euchromatin structure of the *cexA* promoter nearly disappeared with *laeA* disruption (Fig. 7B), and therefore, the heterochromatin level of the *cexA* promoter might be enriched by HepA (AKAW_02119) and ClrD (AKAW_07568) orthologs in *A. kawachii* (Fig. 7C). Gene expression levels of AKAW_02119 and AKAW_07568 were not significantly altered in the $\Delta laeA$ strain (Data Set S1), but loss of LaeA might affect histone modification balance and euchromatin/heterochromatin ratios. The molecular mechanism of LaeA-dependent histone modification should be confirmed through additional experiments because the methyl-accepting substrate of LaeA still remains elusive (43). In addition, whether gene expression of *cexA* requires a specific DNA-binding transcriptional factor remains unclear and should be further studied.

In conclusion, LaeA plays a significant role in citric acid production in *A. kawachii* by controlling *cexA* expression via histone modification at the *cexA* promoter region. Because *A. kawachii* is widely used in the production of shochu and elsewhere in the fermentation industry, our findings are expected to enhance the understanding of citric acid production mechanism(s) and facilitate optimization of strategies for controlling *A. kawachii* activity.

MATERIALS AND METHODS

Strains and growth conditions. The *Aspergillus kawachii* strains used in this study are listed in Table 3, and strain SO2 (44) was used as the parental strain.

For construction and characterization, the strains were grown in minimal medium (1% [wt/vol] glucose, 0.6% [wt/vol] NaNO₃, 0.052% [wt/vol] KCl, 0.052% [wt/vol] MgSO₄·7H₂O, and 0.152% [wt/vol] KH₂PO₄, plus Hutner's trace elements [pH 6.5]). The medium was adjusted to the required pH using HCl or NaOH. For the cultivation of *sC*⁻ and *argB*⁻ strains, 0.02% (wt/vol) methionine and/or 0.211% (wt/vol) arginine was added to M medium, respectively.

To evaluate acidification occurring on agar medium, strains were grown in YPD with methyl red (2% [wt/vol] glucose, 1% [wt/vol] yeast extract, 2% [wt/vol] peptone, and 2% methyl red solution) prepared as follows: 100 mg of methyl red (Nakalai Tesque, Kyoto, Japan) was dissolved in 100 ml of ethanol and titrated by 0.1% (wt/vol) NaOH solution until observation of an obvious color change from red to yellow; the solution was then sterile filtered (0.2- μ m pore size; Toyo Roshi Kaisha, Japan).

To investigate citric acid production, *A. kawachii* strains were also grown in CAP medium (10% [wt/vol] glucose, 0.3% [wt/vol] (NH₄)₂SO₄, 0.001% [wt/vol] KH₂PO₄, 0.05% [wt/vol] MgSO₄·7H₂O, 0.00005% [wt/vol] FeSO₄·7H₂O, 0.00025% [wt/vol] ZnSO₄·5H₂O, 0.00006% [wt/vol] CuSO₄·5H₂O [pH 4.0]). CAP medium was adjusted to the required pH with HCl.

Construction of putative methyltransferase gene disruption strain. *laeA*, *laeA2*, and *laeA3* were disrupted in *A. kawachii* SO2 (44) by insertion of *argB*. A gene replacement cassette encompassing the homology arm at the 5' end of the putative methyltransferase genes, an *argB* selection marker, and the homology arm at the 3' end of the putative methyltransferase genes was constructed by recombinant PCR using the primer pairs AKlaeX-FC/AKlaeX-R1, *argB*-F2/*argB*-R2, and AKlaeX-F3/AKlaeX-RC, respectively (where "X" indicates A, A2, or A3) (Table 4). For amplification of the *argB* gene, the pDC1 plasmid was used as the template DNA (45). For amplification of other DNA fragments, *A. kawachii* IFO 4308

TABLE 4 Primers used in this study

Primer name	Sequence (5'–3')	Reference
AKlaeA-FC	TGATGCTCGGAAGGCACCAG	This study
AKlaeA-F1	AGCCAGCGCATTTCACCAC	This study
AKlaeA-R1	GCATGCAAGCTTTCGCGAGCCGTCGCCATGGTATTGGTGGGTGC	This study
AKlaeA-F3	CCGGGTACCGAGCTCGAATTCGCGGGGGCGGCCGTCCTTTG	This study
AKlaeA-R3	CGGCGCGGATGAGGATATTG	This study
AKlaeA-RC	CCCGACGTTCTCACAATCG	This study
AKlaeA2-FC	CTGATGAGGATGACTCAGC	This study
AKlaeA2-F1	GCAGTTGAAGACGTCAAAGG	This study
AKlaeA2-R1	GCATGCAAGCTTTCGCGAGCCGTCGGCTCCTTTCATCACACGG	This study
AKlaeA2-F3	CCGGGTACCGAGCTCGAATTCGCGGGCACGTATACCAAGCACGG	This study
AKlaeA2-R3	CAGCCTGGTCATTGACCATG	This study
AKlaeA2-RC	GGACTGGGATACCGACGAG	This study
AKlaeA3-FC	CTCAACGTGATTGCGGCAC	This study
AKlaeA3-F1	CCACTTGGAGTGTGACGAG	This study
AKlaeA3-R1	GCATGCAAGCTTTCGCGAGCCGTCGCGTCAATCTGCGTCTTGG	This study
AKlaeA3-F3	CCGGGTACCGAGCTCGAATTCGCGGGCGCTGCCCCATGTAG	This study
AKlaeA3-R3	TGCAGCCGAAACAGGCAC	This study
AKlaeA3-RC	CCAGCCTCAATATAGCGCGC	This study
argB-F2	CGACGGCTCGGAAAGCTTGCATGC	This study
argB-R2	CCGCGAATTCGAGCTCGGTACCCGG	This study
sC-comp-F	CAATCAGCAAGCCGAGCTG	24
sC-comp-R	CTCACCGATGTAGGTCATG	24
AKlaeAcomp-R1	CTGGGACACCATGACAACGGCAGCATTTTCAAAGGGACGGGG	This study
AKlaeAcomp-F2	GCCCGTCCCTTTGAAAATGCTGCCGTTGTATGGTGTCCCG	This study
sC-argB-F2	CCGTTGTCATGGTGTCCAGCAGCA	24
sC-argB-R2	AATTCGAGCTCGGTACCCGG	24
pGS-PgpdA-cexA-inf-F	CCGCCGAACAGTGAACACATCTACACAATGTCTTCAACCACATCTTCATC	This study
pGS-PgpdA-cexA-inf-R	CTCCCATATGGTTCGACctcctaATTTCCGTTGGC	This study
cexA-ChIP-F	GTCTTCAACCACATCTTCATCAAG	This study
cexA-ChIP-R	GAGACATCATCCAGGGCAGG	This study
cexA-RT-F	GGTCCCTGTACCACAGGTCA	This study
cexA-RT-R	GTGGCTTCTCGGACGACTGA	This study
actA-RT-F	GGTATGGGTCAGAAGGACTC	51
actA-RT-R	CTCCATGTCATCCAGTTCG	51

wild-type genomic DNA was used as the template DNA. The resultant DNA fragment amplified with primers AKlaeX-F1 and AKlaeX-R3 was used to transform *A. kawachii* SO2, yielding $\Delta sC \Delta laeA$, $\Delta sC \Delta laeA2$, and $\Delta sC \Delta laeA3$ strains. M agar plates lacking arginine were used for the selection of transformants. Introduction of *argB* into each methyltransferase gene locus was confirmed by PCR using the primer pair AKlaeX-FC and AKlaeX-RC (Fig. S1).

The SO2 strain was transformed using the *argB* cassette to employ the same auxotrophic genetic background strains for comparative study. This *argB* gene cassette was generated with PCR using *A. kawachii* genomic DNA and pDC1 as the template DNA and was used to transform the SO2 strain, yielding the ΔsC strain (Table 3). Transformants were selected on M agar medium lacking arginine.

Complementation of the *laeA* disruption strain. For complementation analysis of *laeA* disruption using wild-type *laeA*, a gene replacement cassette encompassing a homology arm at the 5' end of *laeA*, wild-type *laeA*, an sC selection marker, and a homology arm at the *argB* locus was constructed with recombinant PCR using the primer pairs AKlaeA-FC/AKlaeAcomp-R1 and AKlaeAcomp-F2/argB-R2 (Table 4). For amplification of DNA fragments, *A. kawachii* IFO 4308 wild-type genomic DNA and a plasmid carrying tandemly connected sC and *argB* were used as the template DNA. The resultant DNA fragment amplified with primers AKlaeA-F1/argB-R2 was used to transform *laeA* disruption, yielding the $\Delta laeA$ plus *laeA* strain. Transformants were selected on M agar medium lacking methionine. Introduction of *laeA* and sC into the target locus was confirmed by PCR using primers AKlaeA-FC and argB-R2 (Fig. S2).

The $\Delta sC \Delta laeA$ strain was transformed using the sC cassette for use of the same auxotrophic genetic background strains for the comparative study. The sC cassette was generated by PCR using *A. kawachii* genomic DNA as the template and primers sC-comp-F and sC-comp-R (Table 4) and was used to transform the $\Delta sC \Delta laeA$ strain and yield the $\Delta laeA$ strain (Table 3). Transformants were selected on M agar medium lacking methionine.

Construction of putative citrate exporter gene disruption strain. *cexA* was disrupted in *A. kawachii* SO2 (44) by insertion of *argB*. A gene replacement cassette encompassing the homology arm at the 5' end of the *cexA*, *argB* selection marker, and homology arm at the 3' end of *cexA* was constructed using recombinant PCR with the primer pairs AKcexA-FC/AKcexA-R1, AKcexA-F2/AKcexA-R2, and AKcexA-F3/AKcexA-RC, respectively (Table 4). For amplification of *argB*, plasmid pDC1 was used as the template DNA (45). For amplification of the other DNA fragment, *A. kawachii* IFO 4308 wild-type genomic DNA was used as a template. The resultant DNA fragment was amplified with primers AKcexA-F1 and AKcexA-R3 and was used to transform *A. kawachii* SO2 and yield the $\Delta sC \Delta cexA$ strain. M agar plates lacking arginine

were used for selection of transformants. Introduction of *argB* into the *cexA* locus was confirmed by PCR using the primer pair AKcexA-FC and AKcexA-RC (Fig. S3). After confirmation of gene disruption, the Δ cC Δ cexA strain was transformed with an sC cassette to use the same auxotrophic genetic background strains for comparative study. This cassette was synthesized by PCR using primers sC-comp-F and sC-comp-R and *A. kawachii* genomic DNA as the template (Table 4). Transformants were selected on M agar medium lacking methionine.

Construction of the putative citrate exporter overexpression strain. Plasmid pGS-PgpdA (46), which carries *A. kawachii* sC (28), was used for overexpression of *cexA*. The *cexA* gene was amplified by PCR with primers pGSG-cexA-inf-F/pGSG-cexA-inf-R using *A. kawachii* genomic DNA as the template (Table 4). The amplicon was inserted into the Sall site of pGS-PgpdA, thereby yielding pGS-PgpdA-cexA, which was used to transform the Δ sC Δ cexA and Δ sC Δ laeA strains, yielding the Δ cexA plus OE*cexA* and Δ laeA plus OE*cexA* strains, respectively (Table 3). Transformants were selected on M agar medium lacking methionine. Single-copy integration of pGS-PgpdA-cexA was confirmed by real-time RT-PCR (data not shown). In addition, the transformants were transferred to YPD with methyl red; then the appearance of a red border around the colonies was confirmed.

Measurement of extracellular and intracellular organic acids and extracellular glucose. Levels of extracellular and intracellular organic acids were measured as described previously (24). Briefly, 2×10^7 conidial cells of *A. kawachii* were inoculated into 100 ml of M medium, precultured with shaking (180 rpm) at 30°C for 36 h, and then transferred into 50 ml of CAP medium and further cultured with shaking (163 rpm) at 30°C for 12, 24, 48, or 72 h. Culture supernatant was harvested as the extracellular fraction. Mycelia were used for preparation of the intracellular fraction using a hot-water extraction method (47) with modifications. The freeze-dried and wet mycelial weights were measured as extracellular and intracellular fractions, respectively. Wet mycelia were ground into a powder using mortar and pestle in the presence of liquid nitrogen and then dissolved in 10 ml of hot water (80°C) per 1 g of mycelial powder, vortexed, and centrifuged at $18,800 \times g$ at 4°C for 30 min. The supernatant was taken as the intracellular fraction.

To measure organic acid level, extracellular and intracellular fractions were filtered through a polytetrafluoroethylene (PTFE) filter (0.2- μ m pore size; Toyo Roshi Kaisha) and analyzed with HPLC on a Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a CDD-10AVP conductivity detector (Shimadzu). The organic acids were separated using two tandem Shimadzu Shim-pack SCR-102H columns (internal diameter, 8 by 300 mm; Shimadzu) at 50°C using 4 mM *p*-toluenesulfonic acid monohydrate as the mobile phase at a flow rate of 0.8 ml/min. The flow rate of the postcolumn reaction solution (4 mM *p*-toluenesulfonic acid monohydrate, 16 mM bis-Tris, and 80 μ M EDTA) was 0.8 ml/min.

To measure the glucose level, the filtrated extracellular fraction was analyzed with the Prominence HPLC system equipped with a RID-10A refractive index detector (Shimadzu). Glucose was separated using a COSMOSIL Sugar-D column (internal diameter, 4.6 by 250 mm; Nacalai Tesque, Kyoto, Japan) at 40°C using acetonitrile and water (3:1) as the mobile phase at a flow rate of 1.0 ml/min.

CAGE analysis. Total RNA was extracted from mycelia. Briefly, 2×10^7 conidial cells of the *A. kawachii* strains were inoculated into 100 ml of M medium, precultured with shaking (180 rpm) at 30°C for 36 h, and then transferred to 50 ml of CAP medium and further cultured with shaking (163 rpm) at 30°C for 12 h. The mycelia were ground to a powder as described above. Then, RNA was extracted using RNAiso Plus reagent (TaKaRa Bio, Shiga, Japan). RNA samples were treated with the SV total RNA isolation system (Promega, Madison, WI) according to the manufacturer's protocol.

Library preparation, sequencing, and data analysis for CAGE (48) were performed by Kabushiki Kaisha DNAFORM (Kanagawa, Japan). All CAGE experiments were performed three times with RNA samples obtained from independently prepared mycelia. First-strand cDNAs were transcribed to the 5' end of capped RNAs and attached to CAGE barcode tags, and these tags were sequenced using the NextSeq 500 system (Illumina, San Diego, CA) and mapped to the *A. kawachii* IFO 4308 genome (49) using BWA software (v0.5.9) after discarding ribosomal or non-A/C/G/T-base-containing RNAs. For tag clustering, CAGE tag 5' coordinates were input for RECLU clustering (50). The criteria for linking transcriptional start sites and predicted coding sequences were within 600 bp upstream or downstream of the predicted start codon. Triplicate data were analyzed and the expression ratio was also calculated as the log (base 2) ratio through the RECLU pipeline.

Transcriptional analysis. For RNA extraction from mycelia, conidia (2×10^7 cells) of the *A. kawachii* control, Δ laeA, and Δ laeA plus OE*cexA* strains were inoculated into 100 ml of M medium and cultured with shaking (180 rpm) for 36 h at 30°C. After incubation, mycelia were collected and divided into two equal portions, transferred individually to CAP medium, and cultured with shaking (163 rpm) for 12 h at 30°C. Mycelia were ground as described above and RNA was extracted using RNAiso Plus (TaKaRa Bio), and then cDNA was synthesized from total RNA using a PrimeScript Perfect real-time reagent kit (TaKaRa Bio) according to manufacturer's protocols. Real-time RT-PCR was performed using a Thermal Cycler Dice real-time system MRQ (TaKaRa Bio) with SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Bio). The following primer sets were used: AKcexA-RT-F and AKcexA-RT-R for *cexA* and AKactA-RT-F and AKactA-RT-R for *actA* (Table 4) (51).

ChIP-qPCR. *A. kawachii* conidia were cultured as described above. ChIP was performed as previously described (52) using normal anti-mouse IgG as a negative control (Cosmo Bio, Tokyo, Japan), as well as anti-histone H3 (Medical and Biological Laboratories, Nagoya, Japan), anti-H3K4me3 (Medical and Biological Laboratories), and anti-H3K9me3 (Medical and Biological Laboratories) antibodies. Two micrograms of antibody was used with 200 mg of total protein in each ChIP experiment. DNA quantification was performed with real-time qPCR using SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Bio) and the primer set *cexA*-ChIP-F and *cexA*-ChIP-R (Table 4). Positions of the primers relative to the ATG site of *cexA*

were +2 to +26 for cexA-ChIP-F and +238 to +257 for cexA-ChIP-R. Relative amounts of DNA (i.e., percent input DNA) were calculated by dividing immunoprecipitated DNA by input DNA.

Data availability. CAGE data were deposited in the Gene Expression Omnibus under accession number GSE135849 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135849>).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.6 MB.

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