Functional analysis of the putative citrate transporter CitT responsible for the ability to produce citric acid in koji fungi

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Research aims

The white koji fungus, *Aspergillus luchuensis* mut. *kawachii*, has been used for the production of shochu, a Japanese traditional distilled spirit. Koji fungi play an important role in producing amylolytic enzymes for the production of fermented foods. In addition, the white koji fungus produces a large amount of citric acid, which decreases the pH of "*moromi*" (mash) and prevents microbial contamination during the fermentation process. This ability is a characteristic feature of the white and black koji fungus (*Aspergillus luchuensis*) among the koji fungi because the yellow koji fungus, *Aspergillus oryzae,* and the shoyu koji fungus, *Aspergillus sojae,* do not produce such large amounts of citric acid. In this study, we characterized the citrate exporter CexA (referred as CitT in the Research Subject) in *A. kawachii* and *A. oryzae* to elucidate the differences in the citric acid productivity of koji fungi. We also investigated the epigenetic regulation of *cexA* in *A. kawachii*.

Methods

The *cexA* homolog of white koji fungus was designated as *AkcexA* (AKAW_07989). In addition, *cexA* homologs of yellow koji fungus were designated as *AocexA* (AO090009000645) and *AocexB* (AO090023000271).

We constructed an *AkcexA* disruptant strain and an *AkcexA* overexpression strain (using the *tefA* promoter) in *A. kawachii*. We also constructed *A. oryzae* strains overexpressing *AkcexA*, *AocexA*, and *AocexB* using the *amyB* promoter. We then made rice koji using these strains, and measured the resulting citric acid concentrations. Citric acid productivity was evaluated per estimated mycelial weight in rice koji based on the nucleic acid content. In addition, the intracellular metabolites of the *A. oryzae* control and *AkcexA* overexpression strains were comprehensively analyzed using CE-MS.

We found that the transcriptional level of *AkcexA* was significantly reduced by the disruption of a putative methyltransferase encoding *laeA* in *A. kawachii*. Thus, we constructed an *AkcexA* overexpression strain in the *A. kawachii laeA* disruptant strain using the *gpdA* promoter, which is not controlled by LaeA. We also performed chromatin

immunoprecipitation coupled with quantitative PCR (ChIP-qPCR) to analyze the euchromatin/heterochromatin ratios of the *AkcexA* promoter region in *A. kawachii*.

Results

Recently the citrate exporter CexA was identified in *Aspergillus niger* and excretion of intracellular citric acid was demonstrated as a significantly important step for the high citric acid productivity[1,2]. Thus, we analyzed the relationship between the citric acid productivity of koji fungi and the expression level of *cexA*. The *A. kawachii AkcexA* disruptant strain showed significantly lower (2.3%) citric acid productivity compared to the control strain[3]. In contrast, the *A. kawachii AkcexA* overexpression strain showed significantly higher (2.6-fold) citric acid productivity compared to the control strain. These results suggest that AkCexA is a major citrate transporter and that the citrate export process is a rate-limiting step in *A. kawachii*. Next, we examined whether *cexA* overexpression could enhance the citric acid production in *A. oryzae*. Interestingly, the *A. oryzae AkcexA* overexpression strain showed citric acid productivity similar to that of *A. kawachii*. In addition, *A. oryzae AocexA* and *AocexB* overexpression strains also showed significantly higher citric acid production compared to the control strain, indicating that AoCexA and AoCexB are citrate transporters in *A. oryzae*. Moreover, the intracellular citrate concentration was decreased in the *A. kawachii AkcexA* disruptant strain, whereas it was increased in the *A. oryzae AkcexA*, *AocexA*, and *AocexB* overexpression strains. This result indicates that CexA enhances both extracellular and intracellular citric acid accumulation in A. *kawachii* and *A. oryzae*. Taken together, these results suggest that CexA is a significantly important factor in determining the citrate production ability of koji fungi.

Next, we analyzed the regulatory mechanism of *AkcexA* gene expression in *A. kawachii*. Disruption of a putative methyltransferase encoding *laeA* gene wqas reported to cause a defect in the citric acid production by *A. niger*[4]. Disruption of *laeA* also markedly reduced citrate production in *A. kawachii*[5]. Because LaeA is known as an epigenetic regulator, we analyzed the gene expression changes caused by *laeA* disruption using the Cap Analysis Gene Expression (CAGE)-sequence. We found that *AkcexA* expression was significantly reduced in the *laeA* disruptant strain and that the forced expression of *AkcexA* in the *laeA* disruptant strain remedied the citric acid productivity. This suggests that LaeA is involved in citric acid production through gene regulation of *AkcexA*. LaeA is believed to regulate gene expression by controlling the methylation level of histones. To determine the mechanism underlying LaeA-dependent *AkcexA* expression, we performed ChIP-qPCR analysis of histone H3, histone H3K4me3, and histone H3K9me3 in *A. kawachii* control and *laeA* disruptant strains. H3K4me3 and H3K9me3 are known as euchromatin and heterochromatin markers, respectively. The histone H3 occupancy at the *AkcexA* promoter did not change

between the control and *AklaeA* disruptant strains. However, the euchromatin marker H3K4me3 at the *AkcexA* promoter was decreased to a level similar to that of the negative control in the *laeA* disruptant strain. Further, the heterochromatin marker H3K9me3 was significantly enriched in the *laeA* disruptant strain compared with that in the control strain. These results indicate that LaeA controls *AkcexA* expression by modulating euchromatin/heterochromatin ratios at the *AkcexA* promoter region in *A. kawachii*.

Conclusion

Overexpression of the *A. kawachii AkcexA* in *A. oryzae* significantly enhanced citric acid accumulation to a level comparable with that in *A. kawachii*[3]. This suggests that CexA is a significantly important factor determining the citric acid productivity of koji fungi. In addition, we revealed that *AkcexA* expression was regulated by LaeA in *A. kawachii*[5]. The epigenetic regulation of citric acid production elucidated in this study will be useful for controlling the citric acid production of koji fungi.

References

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