# Elucidation of the mechanism of excessive heat generation during koji making

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**Research objective**

During koji fermentation, the koji molds, *Aspergillus oryzae* and *Aspergillus luchuensis*, degrade the starch in steamed rice by producing large amounts of amylolytic enzymes, and these molds use the degraded products as an energy source. It is generally known that koji molds emit a large amount of fermentation heat during the rice degradation process. Although temperature control of koji is important for koji mold growth and enzyme production, prolonged high temperatures are disadvantageous for koji mold growth. If the fermentation heat of koji molds is not controlled, excessive temperatures may be reached at which koji molds cannot grow. Despite this evidence, the mechanism of excessive koji mold heat generation is as yet unknown. In this study, to identify the gene responsible for triggering excessive fermentation heat during koji making, we focused on an alternative oxidase (Aox), reported to be involved in the generation of fever in plants such as *Symplocarpus foetidus* (Fig. 1A), and conducted an investigation to clarify the relationship between Aox in koji molds and fermentation heat production.

**Methods**

Using the known *aoxA* in *Aspergillus niger* as a template, we searched the homologous genes in the genomes of *A. oryzae* and *A. luchuensis*. We named the genes *AoaoxA* and *AlaoxA*, respectively. These *AoaoxA* and *AlaoxA* disruptants were constructed using the *Ao*Δ*ligD*[1)] and *Al*Δ*ligD*[2)] strains as hosts. We also constructed an *AoaoxA*-overexpressing strain. To reveal the relationship between Aox and fermentation heat, we made koji using these disruptants and the overexpressing strain. The fermentation heat generated during koji making was determined using a non-airflow box[3)], which produced uniform conditions in the culture substrate with high reproducibility and enabled the control of favorable conditions without agitation of the substrate during culture.

**Results**

1. Construction of *AoaoxA* and *AlaoxA* disruptants and observation of these phenotypes

To investigate whether the alternative oxidase, Aox, in *A. oryzae* and *A. luchuensis* is

involved in the production of fermentation heat, we constructed *AoaoxA* and *AlaoxA* disruptants and an *AoaoxA*-overexpressing strain. Thereafter, we observed the growth response of the phenotypes of the *AoaoxA* and *AlaoxA* disruptants to inhibitors of electron transport chain complex proteins, such as cyanide. The *AoaoxA* and *AlaoxA* disruptants were sensitive to sodium azide, which is an inhibitor of complex protein IV (Fig. 1B). This result suggests that *the AoaoxA* and *AlaoxA* genes encode an alternative oxidase. In contrast, no difference was observed in the growth of the *AoaoxA* disruptant compared to the parent strain under ROS stress and temperature stress.

2. The examination of fermentation heat using *AoaoxA* and *AlaoxA* disruptants

To investigate whether AoxA in *A*. *oryzae* and *A*. *luchuensis* is involved in the production of fermentation heat, we made koji using *AoaoxA* and *AlaoxA* disruptants and an *AoaoxA*-overexpressing strain. The *AoaoxA* disruptant and the overexpressing strain showed a similar fermentation temperature course compared to that of the parent strain. The amount of fermentation heat produced by the *AlaoxA* disruptant was initially slightly higher than that produced by the parent strain; however, after 48 h of incubation, the fermentation heat produced by the *AlaoxA* disruptant and the parent strain was similar (Fig. 1C). In addition, there was no difference in the activity of enzymes such as alpha-amylase and alpha-glucosidase in the koji produced by each of the constructed strains compared to that in the parent strain koji.

**Conclusion**

At present, we have not obtained any data that indicate that the *aoxA* genes of *A*. *oryzae* and *A*. *luchuensis* are involved in the production of fermentation heat. These findings suggest that factors other than AoxA in *A*. *oryzae* and *A*. *luchuensis* may be involved in fermentation heat generation, although AoxA is involved in cyanide-resistant respiration. We will conduct further research to identify the genes involved in fermentation heat generation in *A*. *oryzae* and *A*. *luchuensis*.

**References**

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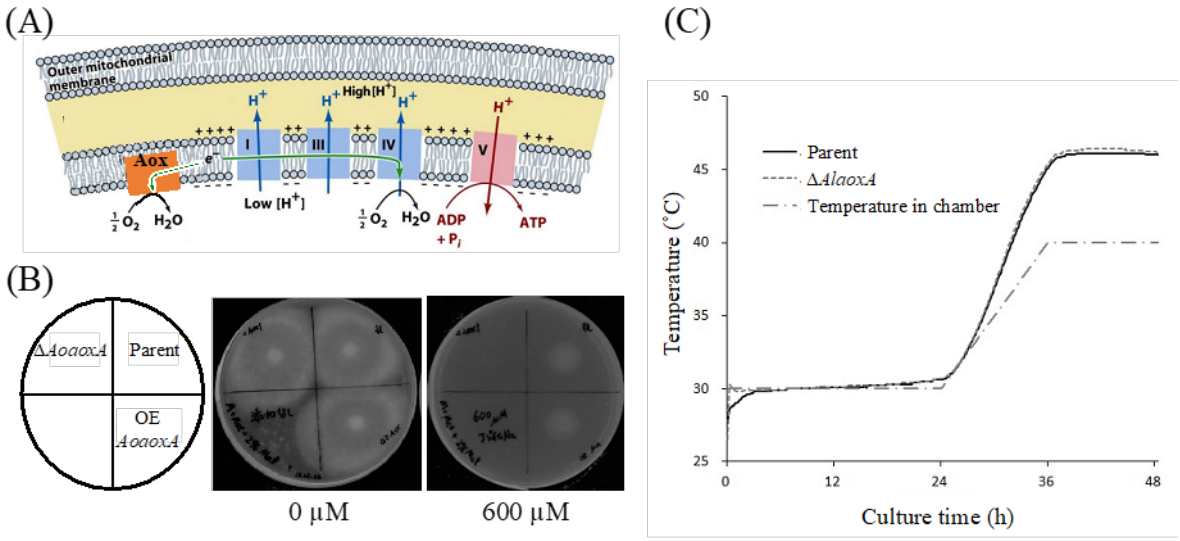


Fig. 1 (A) Schematic representation of the localization and role of alternative oxidase (Aox) in mitochondria. (B) The phenotype of the *AoaoxA* disruptant (Δ*AoaoxA*) and the *AoaoxA*-overexpressing strain (OE*AoaoxA*) under sodium azide treatment. (C) Fermentation heat generation using the *AlaoxA* disruptant (Δ*AlaoxA*) during koji making.