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Full Length Research Paper

Secretory expression of *Rhizopus oryzae* α-amylase in *Kluyveromyces lactis*

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***Kluyveromyces lactis* is a non-conventional yeast species extensively used in the expression of heterologous genes. In this study, a genetically modified *K. lactis* with high-level expression of α-amylase from *Rhizopus oryzae* was obtained, which could successfully hydrolyze and use starch for growth very well. Shake flask fermentation indicated that, the recombinant yeast was able to produce the α-amylase at considerable secretion levels using a variety of carbon sources. The highest level of amylase expression was 22.4 U/ml when cultivated at 30°C and pH 7.0 in the presence of galactose. Moreover, it was shown that the recombinant yeast, which could efficiently degrade starch, yielded a final biomass of 12.25 g/l with enzyme activity of 11 U/ml in the culture medium using 20 g soluble starch/l as the sole carbon source.**

Key words: *Kluyveromyces lactis,* α-amylase, secretion, starch, fermentation.

INTRODUCTION

Yeasts are eukaryotic micro-organisms that have been used for centuries in traditional fermented food (Romanos et al., 1992). They have recently become attractive host organisms for the production of foreign proteins because of developments in recombinant DNA technology (Romanos et al., 1992; Dominguez et al., 1998). Compared with the extensively used *Escherichia coli* expression system, the yeast expression system has important advantages in the expression of proteins from eukaryotic micro-organisms; these advantages stem from its effective post-translational modification system and ability to produce foreign proteins in soluble and correctly folded form (van Ooyen et al., 2006).

Kluyveromyces lactis is a Crabtree-negative, non-conventional yeast that is able to grow solely on lactose as the carbon source (Dominguez et al., 1998). For decades, *K. lactis* has been used in the production of low-lactose milk because of its lactase-producing ability, which is necessary for lactose degradation (Freitas et al., 2008). Its GRAS (generally regarded as safe) status, efficient secretion capacity and low catabolite repression makes it an attractive alternative expression system to traditional baker's yeast in the production of certain proteins (Dominguez et al., 1998; Schaffrath and Breunig, 2000). As an expression host, *K. lactis* is best known for its use in the production of bovine chymosin on an industrial scale, widely recognised as a major biotechnological achievement (van Ooyen et al., 2006). The yeast was successfully used in the production of β-galactosidase in the food industry (Dagbagli and Goksungur, 2008) and was able to achieve high-level expression of human serum albumin (Lodi et al., 2005) and human interleukin-1β (Fleer et al., 1991) in pharmaceutical research. In addition, a large variety of proteins from different sources such as bacteria, fungi, plants and mammals (van Ooyen et al., 2006), have been produced by *K. lactis*, including α-amylase from *Bacillus amyloliquefaciens* (Bartkeviciute and Sasnauskas, 2003), mouse (Tokunaga et al., 1997) and wheat (Russell et al., 1993).

The aim of this study was to enhance the expression and secretion of the *Rhizopus oryzae* α-amylase using *K. lactis*. The amylase gene was integrated into the *K. lactis* genome and expressed under the control of the *K. lactis* lactase (*LAC4*; β-galactosidase) promoter and *Saccharomyces cerevisiae* α-mating factor pre-pro sequence. Different cultivation conditions, including tem-

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perature, pH and carbon sources were investigated to study their effects on the production of the *R. oryzae* α-amylase in *K. lactis*. Specifically, the starch fermentation characteristics of the recombinant *K. lactis* strain were also observed.

MATERIALS AND METHODS

Strains, plasmids and culture media

E. coli JM109 was used for the propagation of plasmids. The host strain *K. lactis* and the expression plasmids pKLAC1 were purchased from New England BioLabs Company. Recombinant plasmid pMD-*RoAmy* containing the *R. oryzae* α-amylase gene coding sequence was constructed in previous work (Li et al., 2011) LB medium (5 g yeast extract, 10 g peptone, 10 g NaCl per litre) was used for cultivation of *E. coli*. YPD medium (10 g yeast extract, 20 g peptone, 20 g dextrose per litre) and YCBA agar plate (10 g yeast carbon base, 15 g agar powder per litre, 5 mM acetamide) were prepared for the pre-cultivation of *K. lactis* and selection of yeast recombinants, respectively. YPDS agar plate (YPD medium supplemented with 5 g soluble starch, 15 g agar powder per litre) was used for halo assays of α-amylase activity. YPX medium (10 g yeast extract, 20 g peptone, 20 g carbon source per litre) was used for the expression of α-amylase.

Construction and transformation of expression plasmid

The mature RoAmy coding sequence (*RA*) was amplified by the polymerase chain reaction (PCR) from pMD-*RoAmy* with primers: 5'-ATTGTCGACGTGCCTGTCATCAA-3' and 5'-GTAGCGGCCGCGATAAGCTTGCACAAACGAAC-3'. The amplification yielded a copy of the gene flanked by *Sal*I and *Not*I sites provided by the two primers (underlined), respectively. The PCR product was gel-purified, digested with the two indicated enzymes and inserted at the corresponding site of pKLAC1 vector; this procedure yielded a recombinant expression plasmid pKLRA, in which the α-amylase gene was under the control of the *K. lactis* lactase (*LAC4*; β-galactosidase) promoter and the *S. cerevisiae* α-mating factor pre-pro sequence.

The secretion cassette pKLRA was linearized with *Sac*II and introduced into *K. lactis* cells by electroporation transformation method (Delorme, 1989). The electroporated cells were spread on YCBA agar plates. After incubation, transformants appeared on the plates at 30°C for 3 to 4 days and were selected for further study.

α-Amylase halo assay

For halo assay, the transformed yeast cells were patched onto YPDS agar plates and incubated for 48 h at 30°C. The halo-forming ability of transformants was detected by staining with weak iodine solution (20 g KI and 2 g I_2 per litre).

Expression of α-amylase

The selected *K. lactis* transformants with different halo-forming abilities were cultured in shake flasks to screen the RoAmy expression levels. The recombinant strains were inoculated into 10 ml YPD medium and grown in a shaking incubator with 200 rpm at 30°C overnight; 0.2 ml of the overnight culture was transferred into 50 ml fresh medium and cultivated to an OD_{600}=6.0 to 10.0. Then, the pre-cultures were inoculated into 50 ml YPX medium to a final optical density (600 nm) of 0.2. In all the shake flask cultivation processes, 250 ml baffled flasks were used.

Analytical techniques

The α-amylase activity was determined as follows: 1 ml 1% (w/v) soluble starch was mixed with 0.25 ml citric acid–Na_2HPO_4 buffer (0.2 M, pH 5.0) and incubated at 55°C for 5 min, followed by addition of 0.1 ml enzyme solution. It was further incubated for 5 min. The reducing sugar formed was determined according to the method described previously (Miller, 1959), using maltose as a standard. One unit of α-amylase activity was defined as the amount of enzyme that released 1 mg reducing sugar per minute under the mentioned conditions.

The yeast growth was measured in terms of dry weights, which were determined by drying 3 ml samples to constant weight at 65°C. The starch concentration was quantified according to the method described by Xiao et al. (2006).

RESULTS

Expression of α-amylase in *K. lactis*

More than one hundred transformants appeared on YCBA agar plates. Eleven colonies were randomly selected and replicated to YCBA agar plates for further purification. Then, the purified clones were patched onto YPDS agar plates and grown at 30°C for 3 to 4 days. After staining with weak iodine solution, clear zones were formed around clones no. 1, 2, 5 and 11, as shown in Figure 1. It was seen that clones no. 1 and 11 shown larger zones on the plate assay, but the α-amylase activities observed in shake flask fermentation were almost the same (data not shown).

Effects of culture conditions on α-amylase expression in *K. lactis*

To investigate the effects of initial pH on α-amylase expression, this study tested the YPD media with initial pH values ranging from 4.0 to 8.0. The highest obtained level of amylase expression, calculated from the triplicate fermentations, ranged from 1.4 to 11.2 U/ml, as shown in Figure 2a. To study the effects of temperature on α-amylase expression, the temperature of the shake flask incubator was set from 20 to 35°C and the initial pH value of the medium was adjusted to 7.0. Among the cultivation temperatures observed, the highest amylase expression level (11.8 U/ml) was achieved at 30°C (Figure 2b).

α-Amylase expression with different carbon sources

The carbon source for amylase expression in the shake flask culture was screened and the initial amount of different carbon source was kept at 20 g/l. Figure 3 shows that, amylase was successfully secreted in all the

Figure 1. Test of the amylase activity of selected clones of the *K. lactis* transformats. The clones' labelled no. 3, 4, 6 to 10 and the colony labelled "C" of non-transformed control host *K. lactis* cells show no clearance zones indicating the absence of amylase activity.

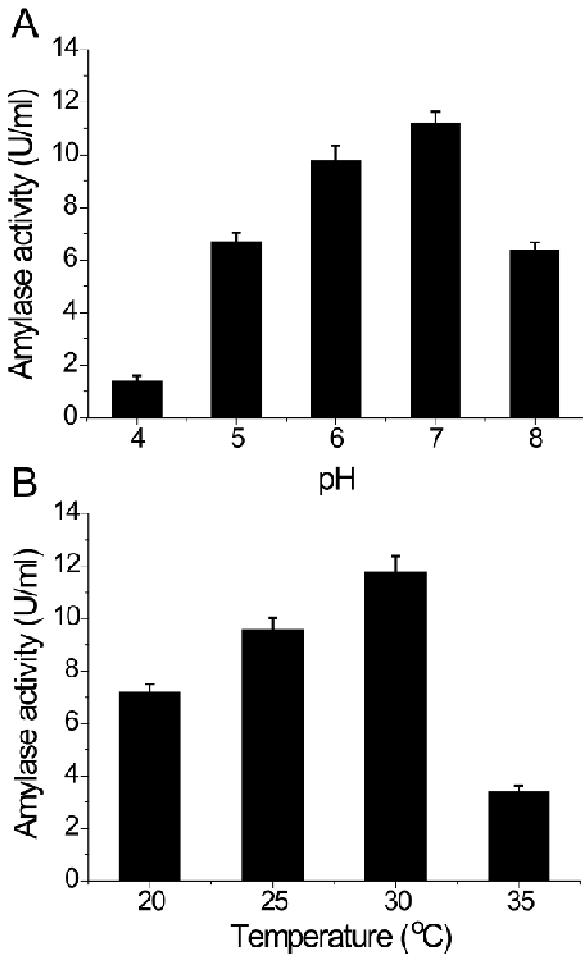

Figure 2. (A) Effects of pH and (B) temperatures on α-amylase production in YPD culture medium. Samples were harvested from 48 h fermentation cultures. All data are mean values of three independent experiments; error bars indicate standard deviation.

tested culture media, of which the galactose-containing culture provided the highest level of amylase secretion at 22.4 U/ml- approximately two times higher than that obtained in other carbon source-containing cultures. However, the highest biomass (15.75 g/l) was achieved using glycerol as the carbon source. Interestingly, at the end of the shake flask fermentation, the starch- and maltose-containing cultures yielded similar biomass, which was slightly higher than that observed in the glucose-containing culture. Samples from the galactose-containing culture media of recombinant *K. lactis* were analyzed by SDS-PAGE for the presence of the α-amylase, as shown in Figure 4, both glycosylated and non-glycosylated α-amylases were secreted into the culture medium.

Starch fermentation characteristics of the recombinant *K. lactis* strain

The characterisation of the growth of the recombinant *K. lactis* strain and the *K. lactis* host strain as well as their starch hydrolysis and fermentation, were observed using soluble starch as the sole carbon source. Figure 5a shows that, the native *K. lactis* strain could not attain continuous growth in starch, whereas the recombinant strain, which has the α-amylase secretion cassette, displayed a high growth rate in starch. The effects of initial starch concentrations ranging from 10 to 40 g/l were examined to study the growth of recombinant yeast. Because of the α-amylase secreted by the recombinant yeast, the starch in the cultures was efficiently and completely degraded during fermentation from 12 to 24 h (Figure 5b). The maximum cell density increased with rising initial starch concentrations from 10 to 30 g/l; although, the 40 g starch/l in the medium was completely degraded in the first 24 h of fermentation, the maximum cell density observed at 84 h was slightly lower than that obtained at 20 and 30 g starch/l concentrations. In addition, the maximum yeast growth rate during the first 12 h was achieved with 10 g starch/l and the yeast exhibited better growth with 20 g starch/l during the 24 to 60 h cultivation time. Further details are given in Table 1.

DISCUSSION

It is difficult to accurately compare the expression levels of various α-amylases in *K. lactis*, because of variations in the starch origin and α-amylase activity definition, as well as in the details of the enzyme assays. However, the

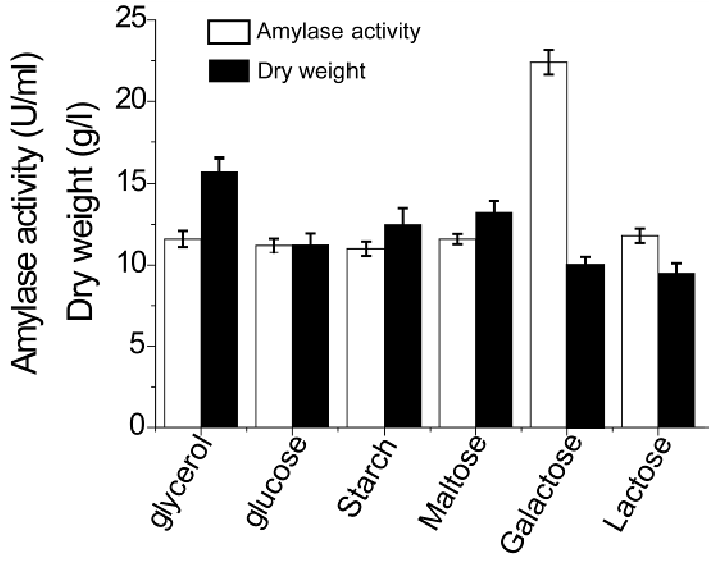

Figure 3. Effects of carbon sources on α-amylase secretion and biomass production. Samples were harvested from 48 h fermentation cultures. All data are mean values of three independent experiments; error bars indicate standard deviation.

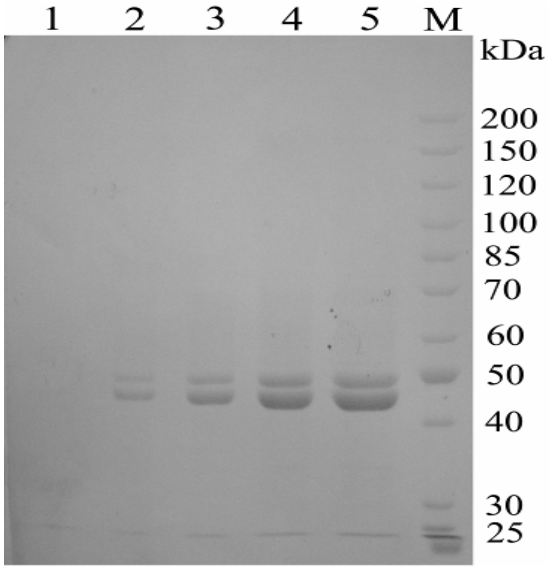

Figure 4. SDS-PAGE analyses of α-amylase produced in galactose-containing culture medium by recombinant *K. lactis*. Proteins were concentrated 5-fold by 10% (w/v) trichloroacetic acid precipitation, before loading (15 µl). Protein bands were stained with Coomassie brilliant blue R-250. Lane 1 to 5, samples from 0, 6, 12, 24 and 48 h fermentation cultures, respectively; lane M, molecular mass marker.

highest yield of the *R. oryzae* α-amylase obtained in the galactose-containing culture medium by shake-flask fermentation was 22.4 U/ml (approximately 20 mg/l), which was much higher than the expression level of mouse α-amylase (0.527 U/ml) (Tokunaga et al., 1997) or *Schwanniomyces occidentalis* α-amylase (30 mU/ml) (Strasser et al., 1989) obtained in *K. lactis*. To the best of this study's knowledge, among various α-amylases expressed in *K. lactis*, the expression level of the *R. oryzae* α-amylase obtained in this study is one of the highest reported? In the previous work of these authors, the *R. oryzae* α-amylase expression levels observed in *E. coli* and *S. cerevisiae* were only approximately 0.11 and 1.3 U/ml, respectively. In addition, the α-amylase expression level obtained by recombinant *Pichia pastoris* was up to 45 U/ml in shake-flask fermentation, however, the application of recombinant α-amylase in food industry could be limited due to the characteristics of the host stain (not a GRAS status strain) and slight residual methanol in the final fermentation broth. Thus, it was conclude that *K. lactis* could be more promising for the expression of the *R. oryzae* α-amylase at food status.

Among physical parameters, cultivation temperature and pH of growth media are important for organism growth and enzyme secretion (Gupta et al., 2003). Similar to the temperatures used in many studies (Rocha et al., 1996; Mincheva et al., 2002), 28 to 30°C yielded the highest α-amylase activity and biomass. Attempts to express amylase at an initial pH of 4.0 resulted in an absence of enzyme activity and the amylase activity produced at pH 8.0 was only half of the highest amylase expression level at pH 7.0 (Figure 2). This result indicates that pH control is crucial for *R. oryzae* α-amylase expression in *K. lactis*.

The expression and secretion of *R. oryzae* α-amylase

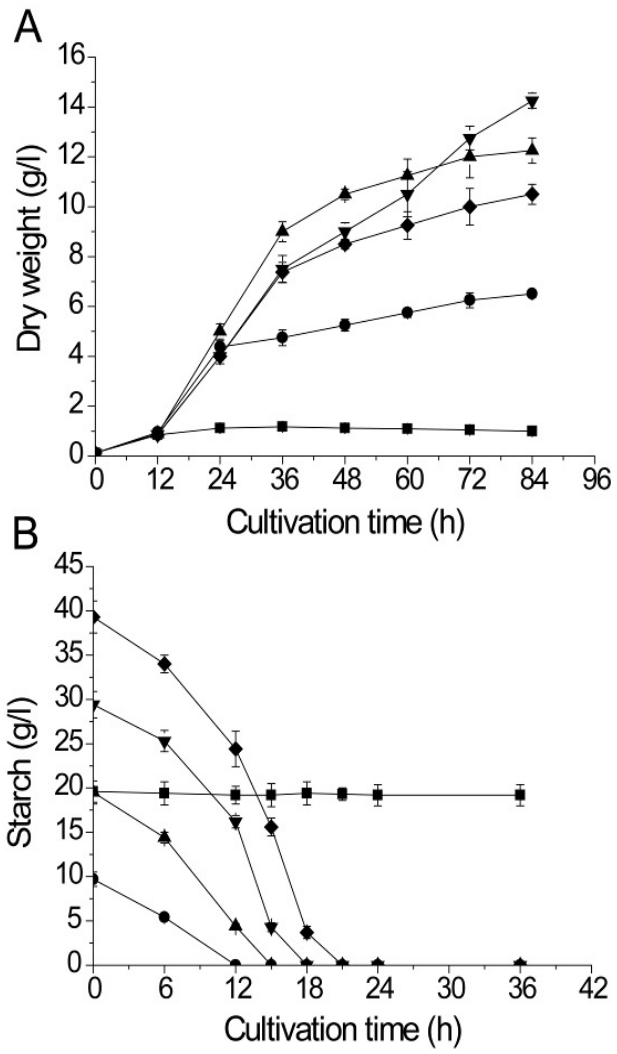

Figure 5. Time courses in the fermentation of starch using recombinant *K. lactis* and host strain. Biomass produced (A) and starch hydrolyzed (B) by recombinant *K. lactis* with initial starch concentrations of 10 g/l (●), 20 g/l (▲), 30 g/l (▼) and 40 g/l (□) and host strain with 20 g starch/l (■). All data are mean values of three independent experiments; error bars indicate standard deviation.

in *K. lactis* was under the control of the *LAC4* promoter, which is one of the most frequently used promoters for high-level heterologous gene expression in *K. lactis* and induced by the presence of lactose or galactose in the growth medium, but not fully repressed in the absence of the inducer (van Ooyen et al., 2006). A number of carbon sources were tested for the growth and α-amylase expression of recombinant *K. lactis*. Galactose generated the highest α-amylase expression probably because the *LAC4* promoter was induced by galactose present in the culture medium (Rubio-Texeira, 2006). By contrast, α-amylase expressed in lactose-containing cultures were almost the same as that observed in other carbon source cultures, indicating that lactose failed to induce the *LAC4* promoter for the enhancement of α-amylase expression. From another point of view, the *LAC4* promoter was not repressed by the presence of a variety of studied carbon sources. However, considering the price of galactose, other carbon sources, such as glucose, glycerol, lactose and starch, are more suitable for α-amylase production.

Most of the wild-type yeast strains cannot directly utilise starches because of their inability to produce starch-decomposing enzymes (Jamai et al., 2007). Numerous studies have demonstrated that, both α-amylase and glucoamylase are required for efficient starch hydrolysis in industrial ethanol production using *S. cerevisiae* strains (Knox et al., 2003; Kosugi et al., 2009). In previous study, *R. oryzae* α-amylase was secreted through an *S. cerevisiae* strain. However, the recombinant strain in the starch-containing culture medium showed extremely low α-amylase activity (approximately 0.06 U/ml), a level insufficient for efficient starch degradation without exogenous glucoamylase. In this study, the host *K. lactis* strain used could not grow in the medium with starch as the sole carbon source, whereas the recombinant *K. lactis* strain obtained was able to hydrolyse soluble starch efficiently because of its ability to express α-amylase at high concentrations in the starch-containing culture (11.2 U/ml), as shown in Figure 5 and Table 1.

The maltose uptake system of *S. cerevisiae* has been extensively studied and is a highly specific strain-dependent. For example, Weusthuis et al. (1993) observed a linear decrease in cell yield with increasing amounts of maltose in the medium feed for *S. cerevisiae* CBS 8066 strain, whereas Batistote et al. (2006) reported that, *S. cerevisiae* VIN7 wine strain yielded a higher final biomass in the presence of maltose rather than glucose under certain conditions. Similar results were found for both *S. cerevisiae* 70 and 254 strains, as described by Zastrow et al. (2000). These findings are similar with those observed in the recombinant *K. lactis* strain obtained in this study. Specifically, the recombinant *K. lactis* produced higher final cell density in the presence of soluble starch rather than glucose, probably because of its ability to produce *R. oryzae* α-amylase. Unlike bacterial α-amylase, fungal α-amylase can be used to produce high-maltose syrup (Doyle et al., 1989), in which the maltose concentration is up to 50% (w/w) and the glucose concentration is usually around 10% (w/w). In addition, considerable amounts of maltotriose, which also yielded higher final biomass than in the presence of glucose (Zastrow et al., 2000), can be released during starch hydrolysis (Doyle et al., 1989). In previous study, soluble starch hydrolysed with *R. oryzae* α-amylase yielded a final concentration of glucose and maltose at 12 and 67% (w/w), respectively and a large amount of maltotriose was still released at the initial hydrolysis stage of the gelatinised starch (data not shown). Overall, it can be conclude that the ability of *R. oryzae* α-amylase to produce high levels of maltose and maltotriose accounts for the higher cell density obtained by the recombinant *K. lactis* in the presence of soluble

Table 1. Soluble starch fermentation characteristics of recombinant *K. lactis* strain.

Parameter	Starch concentration (g/l)				
	Time (h)	10	20	30	40
Biomass (g l^{-1} h^{-1}) [a]	0-12	0.064	0.064	0.063	0.601
	12-24	0.289	0.343	0.261	0.262
	24-36	0.031	0.333	0.292	0.281
	36-84	0.146	0.271	0.563	0.261
Starch (g l^{-1} h^{-1}) [b]	0-6	0.717	0.852	0.683	0.883
	6-12	- [c]	1.667	1.517	1.602
	12-15		-	4.001	2.933
	15-18			-	3.967
	18-21				-

[a] Biomass production rate, gram of dry weight per litre per hour; [b] starch degradation rate, gram of soluble starch per litre per hour; [c] the calculation of starch degradation rate in its running out time is not accurate and the data are not shown. All data are mean values of three independent experiments.

starch.

Conclusion

In conclusion, because of the high-level expression and secretion of *R. oryzae* α-amylase in the culture medium, the recombinant *K. lactis* was able to efficiently grow using soluble starch as the sole carbon source and attained a relatively high cell density probably because of the considerable amounts of maltose and maltotriose released by *R. oryzae* α-amylase. These findings indicate that, the production of heterologous proteins or other specific products from *K. lactis* using starchy materials as the sole carbon source may be achieved through the co-expression of the genes of interest and *R. oryzae* α-amylase gene.

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