

Hyperproduction of β -Glucanase Exg1 Promotes the Bioconversion of Mogrosides in *Saccharomyces cerevisiae* Mutants Defective in Mannoprotein Deposition

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Supporting Information

ABSTRACT: Bacteria and fungi can secrete extracellular enzymes to convert macromolecules into smaller units. Hyperproduction of extracellular enzymes is often associated with alterations in cell wall structure in fungi. Recently, we identified that *Saccharomyces cerevisiae* *kre6* Δ mutants can efficiently convert mogroside V into mogroside III E, which has antidiabetic properties. However, the underlying efficient bioconversion mechanism is unclear. In the present study, the mogroside (MG) bioconversion properties of several cell wall structure defective mutants were analyzed. We also compared the cell walls of these mutants by transmission electron microscopy, a zymolyase sensitivity test, and a mannoprotein release assay. We found zymolyase-sensitive mutants (including *kre1* Δ , *las21* Δ , *gas1* Δ , and *kre6* Δ), with defects in mannoprotein deposition, exhibit efficient MG conversion and excessive leakage of Exg1; such defects were not observed in wild-type cells, or mutants with abnormal levels of glucans in the cell wall. Thus, yeast mutants defective in mannoprotein deposition may be employed to convert glycosylated bioactive compounds.

KEYWORDS: *Saccharomyces cerevisiae*, *KRE6*, mogrosides, bioconversion, *EXG1*, mannoprotein

INTRODUCTION

Mogrosides (MGs) from the extracts of *Siraitia grosvenorii* Swingle fruits, also called monk fruit or Luo Han Guo (LHK), are a mixture of curcubitane-type triterpene saponins.¹ Several studies have reported the various biological functions of MG extracts, which include anti-inflammatory and antioxidation activities, insulin secretion stimulating effects, and antihepatitis virus activity.^{2–5} Suzuki et al. also demonstrated that mogroside III E (MG III E), a minor form of natural MGs with three glucose molecules attached to the C-3 and C-24 positions, exhibits the highest rat intestinal maltase inhibition activity among various MGs, including mogroside V (MG V), mogroside IV (MG IV), and siamenoside I (S I).⁶ However, further studies of MG III E have been hindered by the lack of efficient techniques for obtaining sufficiently large quantities of pure compounds, which is a considerable problem as several types of glycosylated forms of MGs exist in natural extracts. We previously identified that a budding yeast (*Saccharomyces cerevisiae*) mutant with a single *KRE6* gene deletion (*kre6* Δ) efficiently produces MG III E from MG V when monk fruit extract is added to the culture medium (Figure 1).⁷ However, the underlying efficient bioconversion mechanism is unclear.

KRE6 (killer toxin resistant) encodes a β -glucan synthase required for β -(1,6)-glucan biosynthesis in yeast.⁸ *Kre6* is classified as a type II membrane protein with its C-terminus exposed to the extracellular or luminal space.⁹ Disruption of

KRE6 results in slow growth with a 50% reduction of β -(1,6)-glucan synthesis, as well as an increase of amorphous regions in the cell wall.⁹ Thus, *Kre6* has been shown to affect cell wall integrity in yeast.⁹ Accordingly, it was previously hypothesized that the *kre6* Δ strain may possess increased levels of extracellular enzymes to hydrolyze MGs, or other genes involved in bioconversion may be upregulated to enhance MG III E production from MG V.⁷ It was also hypothesized that specific cell wall abnormalities of the *kre6* Δ mutant may relate to the facilitative bioconversion phenotype.⁷ In addition, earlier studies identified that yeast Exg1, an *exo*-(1,3)- β -glucanase located in the periplasmic space of the cell wall which diffuses into extracellular spaces,^{10,11} is responsible for MG and flavonoid glucoside bioconversion.^{7,12}

On the basis of the above observations and hypotheses, we decided to investigate how MG bioconversion is promoted in *kre6* Δ mutants, specifically whether it is mediated through regulation of Exg1 or other mechanisms.

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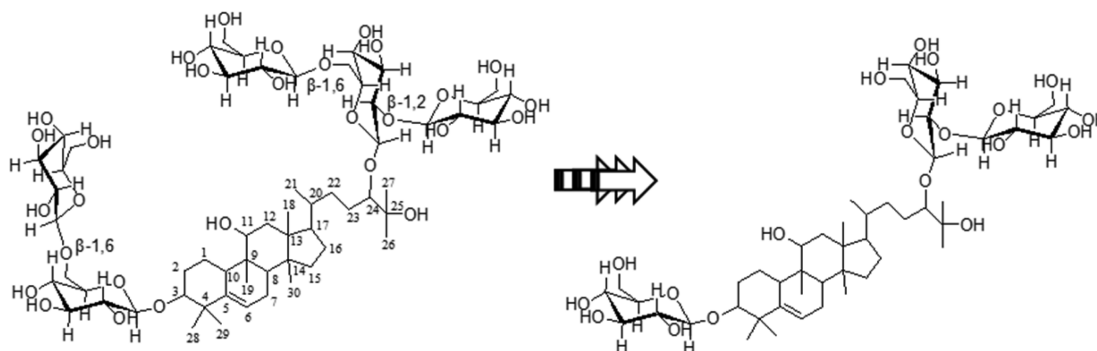


Figure 1. Chemical structures of mogroside V and mogroside III E. The cucurbitane-type triterpene saponin mogroside V (MG V; left panel) is converted into mogroside III E (MG III E; right panel) by the removal of two glucose residues from the C-3 and C-24 positions of MG V during yeast fermentation.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions. *S. cerevisiae* strains BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), W303 (*ade2-1 trp1-1 leu2-3,112 his3-11, 15 ura3-1 can1-100 [psi⁺]*), and S288C (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) were used as background strains in our studies. Cell wall defective mutants were obtained from yeast deletion sets (Table S1). Double-deletion (*kre6Δexg1Δ*) mutants were obtained by replacing *KRE6* with a nourseothricin-resistant sequence (*NAT*) and *EXG1* with *KanMX* (both were accomplished using the polymerase chain reaction (PCR)-based gene-targeting homologous recombination method). The resulting cells with the *kre6Δ::Nat, exg1Δ::KanMX4* genotype were resistant to both nourseothricin and kanamycin antibiotics. MG medium was made by adding 1% of a commercially available powder of monk fruit extracts containing 25% MGs (Changsha Huir Biological-tech Co. Ltd.) to yeast extract–peptone–dextrose (YPD) medium.

Mogroside HPLC–ESI-MS/MS Analysis. The MGs in the culture media after whole cell fermentation or enzymatic conversion in vitro were analyzed as follows. An aliquot of fermentation media was purified using solid-phase extraction cartridges (C-18, 500 mg/3 mL, Chrome Expert, Sacramento, CA). The C-18 packing material in the extraction cartridge was first activated by adding methanol. Water was then added to equilibrate the extraction system before sample loading. Methanol (45–100%) was used as the mobile phase, and fractions were collected for HPLC–ESI-MS/MS analysis as described by Chiu with minor modifications.⁷ A 10 μ L volume of sample was injected for analysis. ESI parameters were as follows: -4.8 kV of spray voltage, 400 °C capillary temperature, 25 arbitrary units of sheath gas, 8 arbitrary units of auxiliary gas, 20% collision energy, 2.0 Da isolation width. The mass scan range was 50–2000 m/z . Xcalibur 2.0.7 software was used for data analysis (San Jose, CA). The methods for isolating standard compounds and determining the purities of MG V, MG IV, S I, and MG III E were described previously.⁷ The dominant molecular ions in the ESI/MSⁿ spectra for MGs are $[M + Na]^+$; sequential glucose loss (-162) was observed in the fragmentation pattern in the collision-induced dissociation mode. MG V, S I, MG IV, and MG III E were identified on the basis of the presence of ions with m/z 1309, 1147, 1147, and 985 for $[M + Na]^+$, respectively.

Glucosidase Activity and Protein Assay. Cells were inoculated into YPD medium and cultured for 24 h to reach the stationary phase. Culture supernatants were collected and concentrated using a 50 kDa MWCO (molecular weight cutoff) ultrafiltration membrane (Vivaspin 20, GE Co., Taipei, Taiwan). Total protein recovery of each sample ranged from 90% to 100%. The extracellular proteins were collected from 20 mL of supernatant and then concentrated into a final volume of 400 μ L. The protein concentrates were subsequently desalted and stored at -80 °C. A 10 μ L volume of the final extracellular protein concentrates was added to 0.5 mL of 1% LHK media and incubated for 24 h at 30 °C. After 24 h of incubation, the reaction was stopped by the addition of 0.5 mL of methanol. Glucosidase activity was determined by measuring the levels of *p*-nitrophenol (*p*NP) released

from *p*-nitrophenyl β -D-glucoside (*p*NPG; 2.5 mg/mL) after the glucose was hydrolyzed by glucosidase in citrate–phosphate buffer (pH 5.0) at 40 °C for 30 min. The released *p*NP was measured at a wavelength of 405 nm.¹² One unit of glucosidase activity resulted in the formation of 1 μ M *p*NP per minute under the experimental conditions. The volumetric activity of each concentrated supernatant was calculated as previously described;¹³ the volumetric activity reflects the original glucosidase activity in the supernatant before it was concentrated. The protein concentration was determined by the Bradford method.¹⁴ Extracellular proteins were also analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with silver staining to visualize protein band patterns.

Extracellular Protein Release Kinetic Analysis. Fermentation cultures were first centrifuged at 5000g for 5 min to obtain cell-free supernatants. Extracellular proteins were collected through concentration of supernatants followed by desalting, both of which were accomplished by using Amicon centrifugal ultrafiltration devices with a 50 kDa pore size (Amicon Ultra 4, Millipore Co., Taipei, Taiwan). Total protein recovery rates for each sample were all between 90–100%, as calculated according to the manufacturer's user guides. Extracellular protein concentrations with the same concentration folds were determined using the Pierce bicinchoninic acid (BCA) protein assay reagent kit from Bio-Rad, using bovine serum albumin (BSA) as the standard.

Epitope Tagging. The protein tagging procedure was described previously by Schneider.¹⁵ To tag *Exg1* in the W303 strain background, we designed a pair of primers (5'-AACCATTGAC-TGACAGAAAGTATCCAAACCAATGTGGCACAATTTCTAAC-AGGGAACAAAAGCTGG-3' and 5'-AAAATAACATTAGAA-AATTCAGCTAAAATGAGCGGACTGAGGGCGACTTACTATAGGGCGAATTGG-3') using *pMPY3xHA* as the template. A 1.5 kb PCR product was synthesized for yeast transformation, and transformants were selected using URA dropout plates. Colonies that appeared on the URA dropout plates were further confirmed using confirmation primers (5'-AAAATGAAGAATGACTACTTGGCAC-3' and 5'-TAGTGCAATGAGGTAAGAAAGGAAAC-3'). Colonies shown to have correct amplification bands were introduced with pop-out assays using 5-fluoroorotic acid (5-FOA) selection plates. These pop-out colonies were also confirmed using confirmation primers. Correct expression of tagged *Exg1*-3HA was identified by Western blotting using anti-HA (hemagglutinin) antibody (α -HA 3F10) as the primary antibody and goat antirat as the secondary antibody (Sigma Co., Taipei, Taiwan).

Gene Expression Analysis. RNA was extracted from W303 strain background yeast using a traditional hot-phenol method. RNA was reverse transcribed to cDNA with random primers using the Superscript II kit (Invitrogen Co., Taipei, Taiwan). The sequences of primers for *EXG1* quantitation were 5'-TTCGCTTAAACGTT-ACTGTGTACG-3' and 5'-GGCATCTAGTTGTGCCTCCA-3', and a housekeeping gene, *ACT1*, was amplified using the following primers: 5'-TCACGCCATTTTGAGAATCG-3' and 5'-TTCAG-CAGTGGTGGAGAAAGAG-3'. Primer efficiencies were tested before

Table 1. Specific Activities, Volumetric Activities, and Mogroside Conversion Activities of Extracellular Protein Extracts from Wild-Type and Mutant Cells^a

strain	specific activity ^b (U/mg)	volumetric activity (U/mL)	relative ratio ^c (%)			
			MG V	S I	MG IV	MG III E
nonfermented LHK water extract			66.16 ± 2.91 c	10.78 ± 0.16 a	14.28 ± 1.61 a	4.51 ± 0.48 a
wild type	77.20 ± 6.10 b	35.84 ± 2.54 b	12.26 ± 2.07 b	18.77 ± 1.15 c	30.22 ± 0.37 c	38.75 ± 1.05 b
<i>kre6Δ</i>	220.67 ± 0.57 c	112.19 ± 0.50 c	5.18 ± 1.16 a	14.61 ± 3.46 b	25.48 ± 2.47 b	54.74 ± 2.14 c
<i>exg1Δ</i>	3.56 ± 1.23 a	2.24 ± 0.76 a	67.26 ± 2.24 c	10.24 ± 1.06 a	13.52 ± 1.42 a	3.26 ± 1.62 a
<i>kre6Δexg1Δ</i>	2.69 ± 0.89 a	1.86 ± 1.11 a	66.35 ± 1.08 c	9.98 ± 1.24 a	13.35 ± 0.83 a	4.65 ± 1.06 a

^aAbbreviations: MG V, mogroside V; S I, siamenoside I; MG IV, mogroside IV; MG III E, mogroside III E. Within each column, values that share the same online letter (a, b, or c) are not significantly different; significance was set at $p < 0.05$. The data are shown as the mean ± SD from three independent experiments, and were analyzed by one-way ANOVA followed by Duncan's multiple test. ^bSpecific activity = volumetric activity/protein concentration. ^cRelative MG ratios were calculated from HPLC–MS quantitation results, on the basis of respective peak areas.

the experiments. Real-time PCR analysis was performed with the SYBR Green Fast qPCR kit from KAPA in a Roche Light Cyclers system (Roche Applied Science Co., Taipei, Taiwan). The expression levels of *EXG1* were normalized to those of *ACT1*.

Electron Microscopy (EM). Cells from the exponential and stationary growth stages were analyzed by EM as described, with minor modifications.⁹ Cell pellets were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 70 min. Next, the cells were washed three times with 0.1 M phosphate buffer for 15 min each. Postfixation steps were performed with 1% OsO₄ for 1 h at rt (room temperature). The cells were washed three times with 0.1 M phosphate buffer for 15 min, and dehydrated by incubation with increasing concentrations of acetone (30%, 50%, 70%, 90%, and 100%) for 30 min each. After dehydration, the cells were infused with gradually increasing concentrations of Spurr's resin (25%, 50%, 75%, and 100%) for three consecutive days, and then embedded at 70 °C for 48 h. Gold sections (90 nm) were mounted on 0.25% Formvar-coated grids, and sections were stained with 2% uranyl acetate; this was followed by six washes with water (20 min per wash). The sections were viewed on a Hitachi H-7650 electron microscope at an operating voltage of 80 kV.

Zymolyase Sensitivity Test. Overnight stationary phase growing cells were first refreshed in rich YPD media, and then cultured from OD 0.1 to OD 0.6. Cell cultures were then harvested to obtain exponential-phase cells, which were washed three times with deionized water. The cells were subsequently suspended in Tris–HCl (10 mM, pH 7.5) buffer to OD 0.6/1 mL in a 1.5 mL Eppendorf tube. The mixture was thoroughly mixed with Zymolyase (Zymo Research, Irvine, CA) at a final concentration of 2 μg/mL, and 200 μL of the cell suspension were transferred to a 96-well flat-bottom plate. The light absorbance at an OD of 660 nm was immediately measured at 25 °C, and the optical density was recorded at 5 min intervals until the values became constant. To calculate the LT (lag time) and maximal lysis rate (MLR) of each lysis curve, the values were first normalized by dividing the original optical density values of each time point (OD) by the initial optical density (OD_{init}). Replicate values of the ratio OD/OD_{init} were averaged, and standard deviations (SDs) were calculated. The error bars for the first point in each curve are the SD of OD_{init} divided by the mean OD_{init}. After normalizing, the values were logged relative to the reaction time (min). Log values for error bars were calculated from $\log(SD_x) = 0.5[\log(X + SD_x) - \log(X - SD_x)]$, where X is the mean OD/OD_{init}. The MLR was the absolute value for the slope of the least-squares fit for five consecutive points from the steepest part of the lysis curve. $LT = y_{int}/MLR$, where y_{int} is the y intercept of the MLR regression line.¹⁶

Mannoprotein Analysis. Extracellular protein samples were all diluted to the same concentration. Portions (5 μg) of the proteins were resolved by 6% SDS–PAGE. The proteins were transferred to a poly(vinylene fluoride) (PVDF) membrane, and mannoproteins were detected with peroxidase-conjugated concanavalin A (Sigma Co., Taipei, Taiwan), as described by Klis et al.¹⁷ Mannoprotein quantities in the medium can be expressed as the total polysaccharide quantity.

Most yeast carbohydrates dispersed in the medium are linked to mannoproteins composed mainly of mannose, and can be quantified by phenol–sulfuric methods.¹⁸

Coimmunoprecipitation Assay. Cells (50 OD) were disrupted in 400 μL of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, and 1× proteinase inhibitor) with acid-washed glass beads by six rounds of heavy vortexing at 4 °C for 30 s, with a 1 min pause between each round. Cell debris was removed by centrifugation at 1000g for 5 min, and the cleared lysate protein concentration was then measured. Proteins were diluted with lysis buffer to 5 mg/mL, and 950 μL of the diluted protein cell lysate was then mixed with prebinding beads (Dynabeads anti-HA) or activated immunoglobulin G (IgG) beads (anti-TAP (tandem affinity purification)) and incubated at 4 °C overnight. A 50 μL volume of nonbinding lysate was used as the input control. The beads were washed three times with lysis buffer, and then suspended in SDS sample loading buffer and incubated at 65 °C for 15 min to obtain the SDS–PAGE samples.

RESULTS

Deletion of *KRE6* Enhances Extracellular Protein Production and MG Conversion. Our previous study showed that *kre6Δ* mutants can efficiently convert MG V to MG III E. However, the underlying mechanism is still unclear. Here, we first examined the specific enzymatic conversion of MGs in cell-free supernatants separated from the culture medium of wild-type cells and *kre6Δ* mutants. We observed that extracellular proteins from the culture medium of *kre6Δ* mutants had higher enzymatic activities than samples from all other tested strains (Table 1; within each column of Table 1, values that share the same online letter (a, b, c, or d) are not significantly different). In particular, the specific enzyme activities and volumetric enzyme activities were significantly increased in culture medium of *kre6Δ* mutants (220 units/mg and 112 units/mL, respectively), as compared to those of wild-type cells (77 units/mg and 35 units/mL, respectively) (Table 1). After 24 h of incubation in MG media, MG III E made up 55% of the MGs produced by *kre6Δ* mutants, as compared to 39% of MGs in wild-type cells (Table 1). On the other hand, *exg1Δ* mutants do not convert MGs due to their low enzyme activities in the culture media. Furthermore, the requirement for Exg1 in the observed increased enzyme activity in the medium of *kre6Δ* mutants was demonstrated by the finding that mutants lacking both the *KRE6* and *EXG1* genes (*kre6Δexg1Δ* mutants) had levels of enzymatic activities similar to those of *exg1Δ* mutants (Table 1). We further analyzed the extracellular protein release kinetics and profiles of wild-type cells and *kre6Δ* mutants during fermentation, observing that *kre6Δ* mutants grew slower than wild-type cells. However, the

release of extracellular proteins (pg/cell) was 2-fold higher for *kre6Δ* cells than wild-type cells (Figure 2A). The cumulative

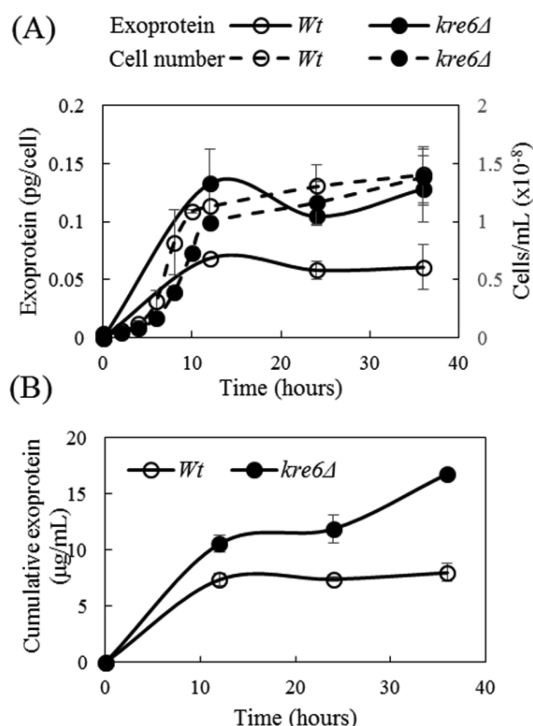


Figure 2. Kinetics of extracellular protein production by wild-type and *kre6Δ* cells during growth. (A) Release of extracellular proteins by individual cells and cell growth numbers during fermentation. (B) Cumulative extracellular protein contents in wild-type cells and *kre6Δ* mutants at the indicated time points. Dotted and solid lines represent growth curves and extracellular protein secretion levels, respectively. Open and closed circles represent wild-type and *kre6Δ* cells, respectively.

release of extracellular proteins over 36 h was also much greater for *kre6Δ* mutant cells (Figure 2B). Thus, these data suggest that deletion of *KRE6* results in high production of extracellular proteins and extensive MG conversion, and that *EXG1* may encode the bioconversion enzyme involved.

Effects of *KRE6* Disruption on Exg1 Secretion. To further examine the possible mechanisms underlying the elevated levels of extracellular proteins and the enhancement of MG III E production in the culture media of *kre6Δ* mutants, we compared Exg1 protein levels (Figure 3A,B) and gene expression levels (Figure 3C) in wild-type cells and *kre6Δ* mutants. Both intra- and extracellular proteins were extracted from wild-type cells and *kre6Δ* mutants, both of which produced either native Exg1 or Exg1 with a C-terminal 3HA epitope (Exg1-3HA). Anti-HA antibodies were used to probe the 3HA-tagged Exg1 proteins. Silver staining revealed that greater amounts of extracellular protein were released by *kre6Δ* mutants with either native Exg1 or Exg1-3HA than by wild-type cells (Figure 3A). Our Western blotting results further confirmed that intracellular levels of Exg1-3HA were lower in *kre6Δ* mutants than in the wild type (*kre6Δ* mutants/wild type, 0.69/1) (Figure 3B). In contrast, the amounts of extracellular Exg1-3HA were greater for *kre6Δ* mutants (*kre6Δ* mutants/wild type, 1.67/1) (Figure 3B). The amounts of extracellular Exg1-3HA relative to intracellular Exg1-3HA were on average 2.3-fold higher in the *kre6Δ* strain than in wild-type cells ($p <$

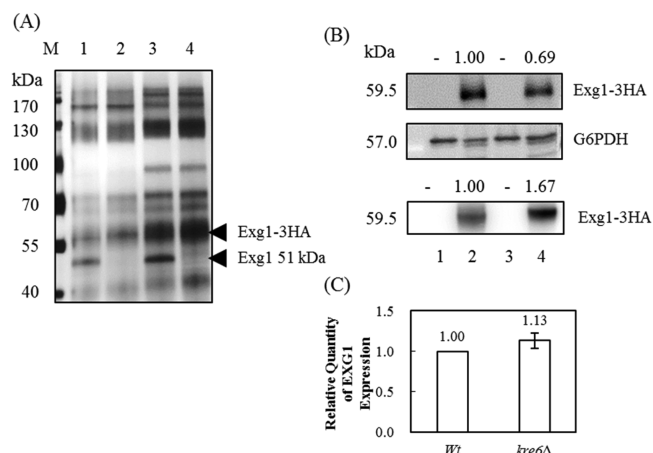


Figure 3. Comparison of intracellular/extracellular Exg1 levels and *EXG1* expression between wild-type and *kre6Δ* cells. (A) Extracellular protein silver staining. Arrowheads indicate bands predicted to be tagged and nontagged Exg1. (B) Intracellular/extracellular Exg1-3HA levels, as detected by Western blots. Lanes 1–4 contained intracellular or extracellular proteins from wild-type cells, wild-type cells with Exg1-3HA, *kre6Δ* mutants, and *kre6Δ* mutants with Exg1-3HA, respectively. Similar Western blotting trends were obtained from all samples ($n = 3$), and a representative figure is shown. (C) Comparison of *EXG1* expression levels of wild-type cells and *kre6Δ* cells. The average *EXG1* gene expression is shown ($n = 5$). There was no significant difference at $p < 0.05$ between wild-type cells and *kre6Δ* cells, as determined by Student's *t* test.

0.05). In addition, extracellular Exg1-3HA enzymes of the *kre6Δ* mutant still exhibited higher MG bioconversion ability than the same Exg1-3HA construct from wild-type cells for the same reaction (Figure S1). We also found that the *EXG1* gene expression levels were not significantly different between *kre6Δ* mutants and wild-type cells (Figure 3C). These data collectively imply that disruption of *KRE6* in yeast may result in enhanced Exg1 leakage and higher bioconversion activity in the culture medium. Interestingly, we observed that the extracellular protein profiles of wild-type and *kre6Δ* cells were vastly dissimilar, as revealed by SDS–PAGE with silver staining (Figures 3A and 4A). However, the differences in protein bands could not be attributed to the nearly 2-fold differences in extracellular protein concentration between *kre6Δ* mutants and wild-type cells (Figure 2) or the sensitivity of silver staining. We confirmed that the differences in protein band patterns were consistent through testing various protein loading concentrations of wild-type samples (data not shown). In addition, we established that the band profiles shown in Figures 3A and 4A were different due to the strain backgrounds used (W303 and BY4741, respectively). We also consistently found that cells with defective *EXG1* secreted fewer extracellular proteins. This may be because mannoprotein secretion was affected by the absence of Exg1. Consistent with this hypothesis, an earlier study suggested that the activities of β -glucanases, including Exg1, are likely to influence the release of mannoproteins from the cell wall of yeasts.¹⁹

Each protein band was given a specific number to represent its identity on the basis of its relative protein mobility value. Band number 10 may be Exg1, as this band is of the predicted size (about 51 kDa), and it was absent in both *exg1Δ* and *kre6Δexg1Δ* mutants, but greatly increased in *kre6Δ* mutants. On the other hand, the extracellular protein profiles of *kre6Δ* mutants and *kre6Δexg1Δ* mutants were distinct from those of

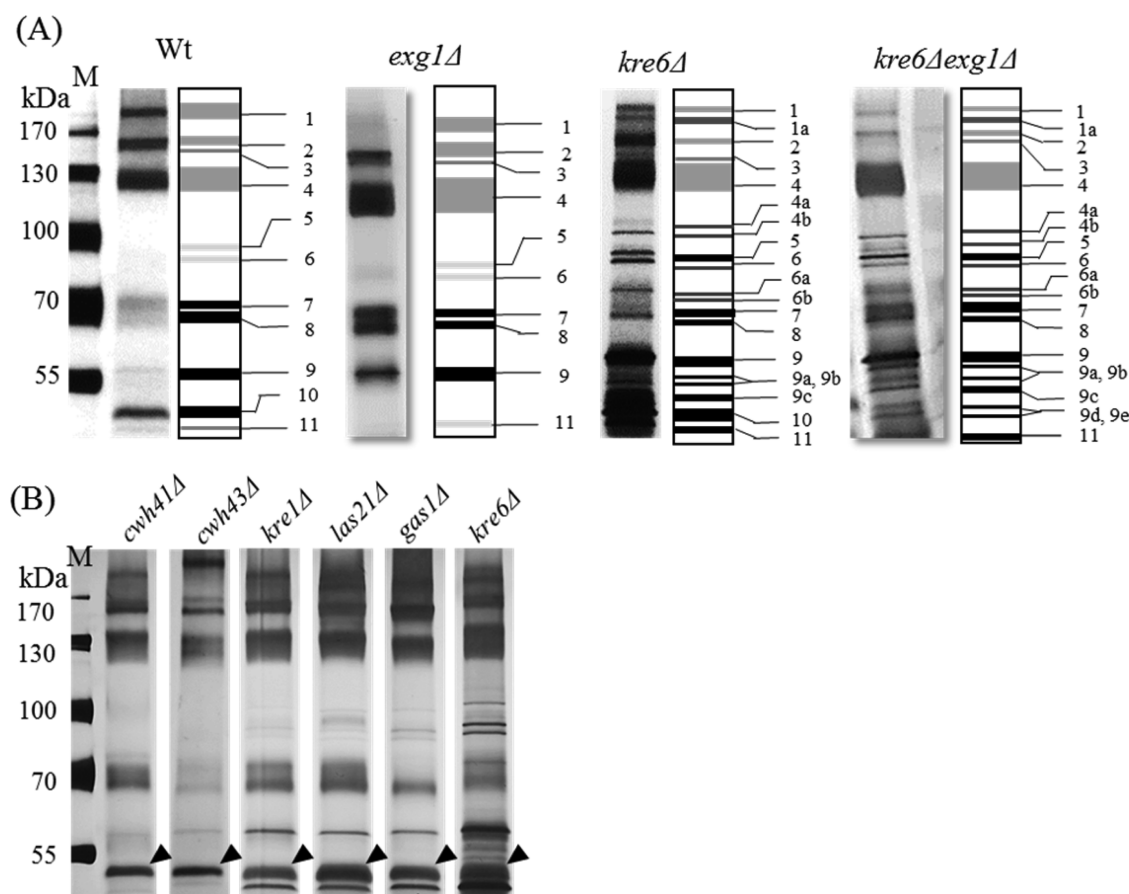


Figure 4. Extracellular protein banding patterns and extracellular Exg1 levels in mutants. (A) Extracellular protein banding patterns. Concentrated and dialyzed extracellular protein samples taken from wild-type, *exg1Δ*, *kre6Δ*, and *kre6Δexg1Δ* cells cultured for 24 h were analyzed by 8% SDS–PAGE and silver staining. A schematic drawing of the gel is shown next to each photograph. (B) Extracellular Exg1 levels in mutants. The same volumes of supernatants from *cwh41Δ*, *cwh43Δ*, *gas1Δ*, *gas1Δ*, *kre1Δ*, and *kre6Δ* cells cultured for 24 h were brought down to the same volume and analyzed by 8% SDS–PAGE and silver staining. Arrowheads indicate Exg1 (51 kDa).

wild-type cells (Figure 4A), with higher levels and more types of extracellular proteins being released from these two mutant strains as compared to the wild type. However, *kre6Δexg1Δ* mutants do not observe changes in any enzymatic activities related to either MGs or pNPG (Table 1). These results indicate that deletion of *KRE6* not only increases Exg1 secretion, but also changes the extracellular protein profiles of the culture medium. On the basis of our collective data, we further propose that deletion of *KRE6* leads to high production of extracellular proteins, and that cell wall defects in this mutant probably cause the observed increase in MG conversion.

Specific Cell Wall Defects Are Associated with Hypersecretion of Extracellular Enzymes and Facilitative Mogroside Conversion. An earlier study showed that hyperproduction of extracellular enzymes is associated with alterations in cell wall structure in *Neurospora crassa*.²⁰ Thus, we examined the cell wall structure of *kre6Δ* (*cwh48Δ*) and other yeast deletion mutants, including *cwh41Δ* and *cwh43Δ*, on the basis of their mutual calcofluor white hypersensitivity (CWH) phenotype, which can indicate cell wall defects (data not shown). It is known that *CWH41* encodes an α -glucosidase involved in the modification of cell wall β -(1,6)-glucan synthase glycosylation, and *CWH43* encodes a transporter protein involved in cell wall biogenesis.^{21,22} Cells bearing defective *CWH41* or *CWH43* are known to have cell wall defects.^{21,22} Here, we examined cell wall structure differences among wild-

type cells and these mutants by TEM; wild-type cells were found to have a darkly stained and smoothly delineated outer layer, which was absent in the *kre6Δ* mutant (Figure 5A). In addition, the cell walls of *cwh41Δ* and *cwh43Δ* mutants were surrounded by thickened, electron-lucent structures, with a rather vague and darkly stained outer layer. However, despite having cell wall structures that differed from those of wild-type cells, neither *cwh41Δ* nor *cwh43Δ* mutants could convert MGs as efficiently as *kre6Δ* mutants (Figure 6E,F). These results indicate that only the specific cell wall defects of the *kre6Δ* mutation are associated with hypersecretion of extracellular enzymes and facilitative MG conversion.

Mutants with a Facilitative Mogroside Conversion Phenotype Are Sensitive to Zymolyase Treatment and Exhibit Increased Mannoprotein Release. According to an earlier study, mannoproteins are mainly present on the cell wall surface.^{23,24} The amount of mannoproteins anchored to the cell wall, as indicated by dark staining of the outer layer, determines the cell wall porosity, which acts as a filter to control both the exit of secretory proteins and the entrance of macromolecules from the environment.^{25–28} Therefore, we hypothesized that the extent of mannoprotein deposition may impact extracellular enzyme release in *kre6Δ* mutants. Previous studies showed that the extent of mannoprotein anchorage to the cell wall can be evaluated directly using a zymolyase sensitivity test and indirectly by measuring the concentrations of polysaccharides

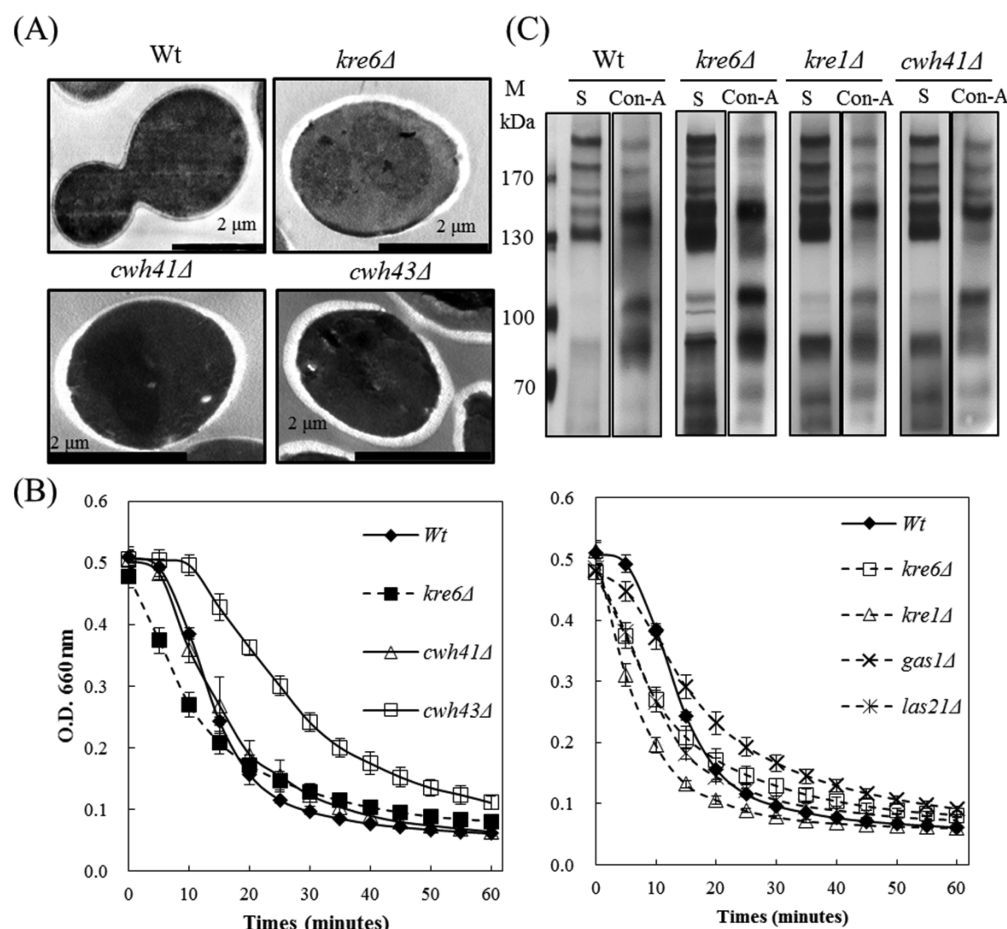


Figure 5. Cell wall structure analysis and extracellular mannoprotein detection. (A) Transmission electron micrographs of wild-type cells and mutant cells. The scale bar represents 2 μm. (B) Cell wall defective mutants showed zymolyase sensitivity phenotypes that differed from those of wild-type cells. Results were obtained from at least three independent experiments. Mean values were plotted with the standard deviation (SD). (C) SDS-PAGE analysis of extracellular mannoproteins by hybridization to peroxidase-conjugated concanavalin A (Con A). S indicates the silver staining results. Representative data are shown from at least three independent results.

in the medium.^{18,25} Mannoproteins can also be detected by Western blots with concanavalin A (Con A) antibody.²⁴ Here, we observed that the *kre6Δ* mutants exhibited increased sensitivity to zymolyase treatment, as compared to wild-type, *cwh41Δ*, and *cwh43Δ* cells (Figure 5B, left panel). The Con A antibodies bound to mannoproteins of various sizes (Figure 5C), and the concentrations of total mannoproteins and polysaccharides released into the media were greater for *kre6Δ* mutants than for wild-type cells and *cwh41Δ* mutants (Table 3). Together, these data support the hypothesis that defects in *KRE6* enhance both the release of mannoproteins and sensitivity to zymolyase treatment.

To further extend our hypothesis that sensitivity to zymolyase treatment and higher mannoprotein release in mutants may contribute to facilitative MG conversion, we analyzed the MG bioconversion efficiency in additional cell wall mutants, particularly those with defective mannoprotein anchorage, such as *kre1Δ*, *las21Δ*, and *gas1Δ* mutants. We report that the *kre1Δ*, *las21Δ*, *gas1Δ*, and *kre6Δ* mutants were more sensitive to zymolyase treatment than wild-type cells, as demonstrated by their relatively short lag time (LT) in the corresponding lysis curve (Figure 5B, right panel, and Table 2). In contrast, the LTs of the *cwh41Δ* and *cwh43Δ* mutants were much longer (Table 2). The shorter LTs of the *kre6Δ*, *kre1Δ*, *gas1Δ*, and *las21Δ* mutants may reflect a lack of integrity of the

outer mannoprotein layer. Indeed, both *kre1Δ* and *kre6Δ* mutants released significant amounts of mannoproteins into the culture medium, as compared to wild-type cells and *cwh41Δ* mutants (Table 3). The silver staining analysis also revealed that cells with defective *KRE1*, *GAS1*, or *LAS21* genes produced even higher levels of extracellular proteins, and in particular Exg1, as compared to *cwh41Δ* and *cwh43Δ* mutants (Figure 4B). We proceeded to examine the MG bioconversion abilities of these defective mannoprotein anchorage mutants. Remarkably, the LC-MS chromatograms of MGs (Figure 6) indicated that the *kre6Δ*, *gas1Δ*, and *las21Δ* mutants efficiently convert MG V to MG III E. Although the *kre1Δ* mutant was slightly less efficient than the other three mutants (51% MG III E in *kre1Δ* mutants and 61% MG III E in *kre6Δ*, *gas1Δ*, and *las21Δ* mutants), its conversion activity was still more efficient than that of wild-type cells (40% MG III E) and *cwh41Δ* (39%) and *cwh43Δ* (40%) mutants. Thus, our data are consistent with the hypothesis that defective mannoprotein anchorage at the outer layer of the cell wall is associated with hypersecretion of extracellular enzymes and enhanced MG conversion.

DISCUSSION

We previously identified that Exg1 is a major enzyme involved in the initiation of MG bioconversion, and that deletion of *KRE6* facilitates the production of MG III E from LHK extracts

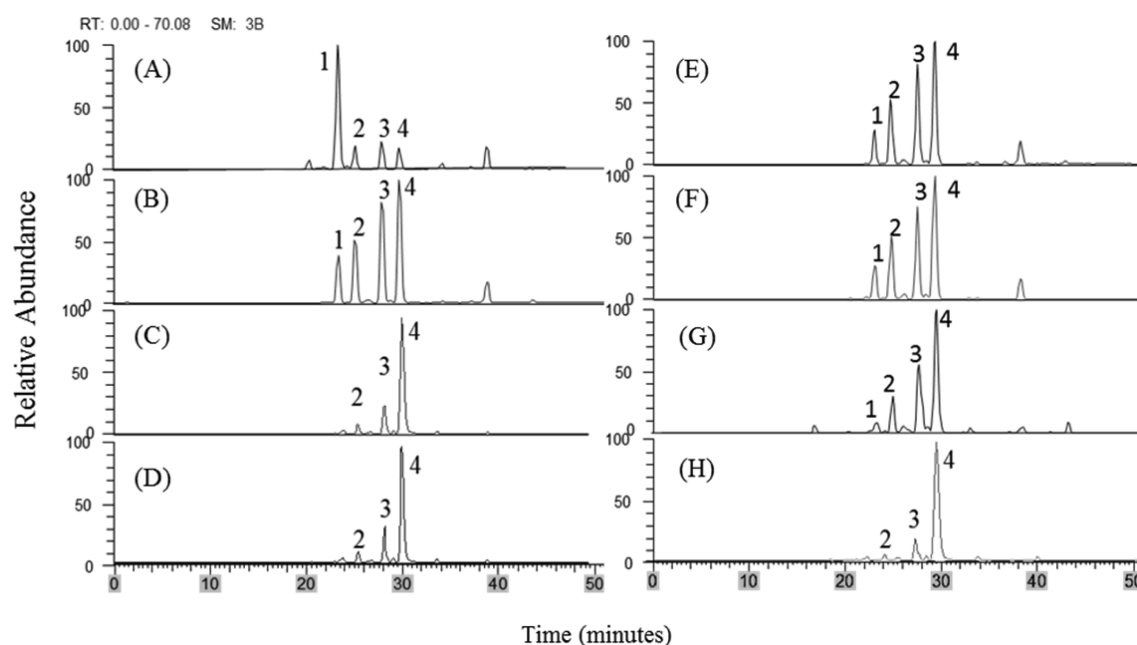


Figure 6. LC–MS chromatograms of mogrosides after 24 h of whole cell mogroside bioconversion: (A) nonfermentation and (B) wild-type, (C) *kre6Δ*, (D) *las21Δ*, (E) *cwh41Δ*, (F) *cwh43Δ*, (G) *kre1Δ*, and (H) *gas1Δ* cells, with nonfermentation LHK water extract used as the control. Peaks: 1, mogroside V (MG V); 2, siamenoside I (S I); 3, mogroside IV (MG IV); 4, mogroside III E (MG III E).

Table 2. Zymolyase Cell Wall Digestion Analysis of Cell Wall Mutants^a

strain	LT (min)	MLR (100 min ⁻¹)
wild type	6.99 ± 0.72 d	3.29 ± 0.26 d
<i>cwh41Δ</i>	6.24 ± 1.58 d	2.66 ± 0.23 c
<i>cwh43Δ</i>	13.98 ± 2.18 f	1.72 ± 0.16 a
<i>uth1Δ</i>	12.64 ± 1.36 e	1.59 ± 0.16 a
<i>kre6Δ</i>	0.29 ± 0.13 a	2.32 ± 0.01 b
<i>kre1Δ</i>	not detected	4.14 ± 0.26 e
<i>gas1Δ</i>	4.07 ± 0.55 c	2.07 ± 0.20 b
<i>las21Δ</i>	3.46 ± 1.21 b	2.63 ± 0.14 c

^aThe data are from at least three independent experiments, and are shown as the mean ± SD. Within each column, values that share the same online letter (a, b, c, d, e, or f) are not significantly different; significance was set at $p < 0.05$, as analyzed by one-way ANOVA followed by Duncan's multiple test.

Table 3. Concentrations of Total Mannoproteins and Polysaccharides Released by Wild-Type Cells and Mutants^a

strain	polysaccharide concn (mg/L)	polysaccharide concn/OD ₆₀₀ (mg/L/OD ₆₀₀)
wild type	96.96 ± 8.98 a	23.59 ± 1.67 a
<i>kre6Δ</i>	164.71 ± 4.82 b	42.97 ± 1.46 c
<i>cwh41Δ</i>	101.96 ± 4.18 a	21.63 ± 1.65 a
<i>kre1Δ</i>	156.81 ± 19.98 b	33.21 ± 2.39 b

^aPolysaccharide concentration and polysaccharide concentration/OD₆₀₀ were compared independently. Data are the mean ± SD from three independent experiments. Within each column, values that share the same online letter (a, b, or c) are not significantly different; significance was set at $p < 0.05$, as analyzed by one-way ANOVA followed by Duncan's multiple test.

in yeast.⁷ In the present study, we have delineated the underlying mechanisms by which *kre6Δ* mutants facilitate MG III E conversion. On the basis of our findings, we conclude that lack of cell wall integrity and loss of mannoproteins anchored

to the cell surface result in increased release of Exg1 into the culture medium, which promotes MG bioconversion.

Kre6 is a membrane protein required for β -(1,6)-D-glucan biosynthesis and cell wall synthesis in yeast.⁸ Kre6 in cells is localized to the endoplasmic reticulum (ER) and the plasma membrane.²⁹ It was previously reported that Kre6 is required for polarized growth and vesicle secretion during cell growth.²⁹ On the other hand, Exg1 is the major *exo*-(1,3)- β -glucanase of the cell wall, and it is involved in cell wall β -D-glucan assembly.^{10,11} As a secretory enzyme, Exg1 synthesized in the ER needs to be transported through the plasma membrane.³⁰ Thus, we first examined if there were any interactions between Kre6 and Exg1, particularly at the plasma membrane, which may hamper the mass secretion of Exg1. If such an interaction exists, deletion of *KRE6* would be expected to give rise to increased levels of Exg1 in extracellular regions. However, we ruled out this possibility as we were unable to detect any interactions between Kre6 and Exg1 in our pulldown assay (Figure S2).

Outer layer mannoproteins in yeast not only protect cells from external chemical damage, but also act as a molecular sieve with a distinctive pore size that affects the exit of secretory proteins.^{11,28} Cell wall porosity can be evaluated using the zymolyase resistance test.²⁵ Commercial zymolyase consists of Z-glucanase and minor amounts of Z-protease, which are responsible for cell wall glucan and mannoprotein digestion, respectively. Therefore, cells with less mannoprotein in the outer layer are more susceptible to direct digestion by zymolyase and are more easily lysed. Conversely, cells with normal mannoprotein content in the outer layer of the cell wall or with thicker cell walls are more resistant to zymolyase.²⁵ Notably, our results demonstrated that only certain zymolyase-sensitive (as evidenced by shortened LT values (Table 2) and increased cell wall porosity) mutants, including *kre1Δ*, *las21Δ*, *gas1Δ*, and *kre6Δ*, which possessed compromised mannoproteins, exhibited leakage of Exg1 enzymes and efficient MG conversion (Figures 5 and 6). Similarly, the integrity of the

mannoprotein structure has been reported to influence protein leakage, on the basis of the observation that chemical destruction of mannoproteins on the yeast outer cell wall facilitates the release of periplasmic proteins, such as invertase, into the medium (invertase is mainly expressed under glucose repression culture conditions).²⁶

We considered the suitability of total polysaccharide quantification methods for detecting mannoproteins in the culture medium, as it is possible that the mannoprotein content may be overestimated if β -glucans are present. However, a recent study conducted by Quiros et al. reported a strong correlation between extracellular mannose content and total polysaccharides ($p < 0.01$), but no significant correlation between glucose contents and total polysaccharides.¹⁸ They also showed that the mannose/glucose ratio in the macromolecular fraction of the supernatant of various yeast strains correlated with the supernatant mannose content, indicating that increased release of cellular mannoproteins does not reflect an increased release of other cell wall polysaccharides, such as β -glucans.¹⁹ Therefore, the differences in extracellular total polysaccharide contents between yeast mutants in our study may reflect differences in extracellular mannoprotein contents, and thus, our data suggest that defects in the *KRE6* gene enhance the release of mannoproteins and increase the sensitivity of zymolyase treatment.

Many studies have indicated that bacteria and fungi are able to secrete extracellular enzymes to convert macromolecules into smaller units for growth and adaptation.^{31–34} Indeed, alterations in cell wall structures were previously shown to be associated with hyperproduction of extracellular enzymes in fungi.^{20,35} However, we demonstrated here that specific cell wall structure abnormalities contribute to extra enzyme and protein release. Such abnormalities include those caused by deletion of *LAS21* (*GPI7*), a gene encoding an enzyme for glycosylphosphatidylinositol (GPI) anchor structure synthesis, which decreases GPI-anchored mannoproteins on the cell wall surface.³⁶ Deletion of *GAS1* affects protein anchorage, resulting in the release of more proteins and glucans into the culture medium during fermentation.^{37,38} *Kre6* and *Kre1* are responsible for inner chain β -(1,6)-glucan synthesis and β -(1,6)-glucan outer chain extension synthesis, respectively; both are the primary factors affecting the anchorage of mannoproteins.^{9,23} Moreover, Bowen and Wheals showed that GPI-anchored mannoproteins, such as Sed1p presented on the cell wall, are released into the medium upon *KRE6* deletion.³⁹ However, Jiang et al. suggested that *cwh41Δ* mutants release different cell wall mannoproteins, such as Cwp1, into the medium only when *KRE1* is also deleted.²¹ Together, all of these lines of evidence are consistent with our finding that *cwh41Δ* and wild-type cells exhibit normal mannoprotein anchorage and have regular MG III E production rates (Table 2, Figures 5C and 6).

Several studies have indicated that overproduction of Exg1 in wine yeast leads to an increase in the aroma of wine, as it yields higher levels of alcohol, terpene, and free volatile compounds.⁴⁰ Previous studies also suggested that increased mannoproteins can further maintain the stability of colors in wine.^{19,37,41,42} Thus, the use of certain yeast strains, such as the *kre6Δ*, *kre1Δ*, *gas1Δ*, and *las21Δ* mutants identified in our current study, may provide an alternative approach for producing high amounts of Exg1 proteins for increasing wine aroma.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b03909.

Table S1 listing the strains used in the study and their genotypes, Figure S1 showing the LC–MS chromatographic analysis of mogrosides after 24 h of extracellular protein and mogroside reaction, and Figure S2 showing the protein–protein interaction between Exg1 and *Kre6* proteins (PDF)

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Notes

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