

Deletion of *GBG1*/*AYR1* Alters Cell Wall Biogenesis in *Saccharomyces cerevisiae*

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We identified a gene for β -1,3-glucan synthase (*GBG1*), a nonessential gene whose disruption alters cell wall synthesis enzyme activities and cell wall composition. This gene was cloned by functional complementation of defects in β -1,3-glucan synthase activity of the the previously isolated *Saccharomyces cerevisiae* mutant LP0353, which displays a number of cell wall defects at restrictive temperature. Disruption of the *GBG1* gene did not affect cell viability or growth rate, but did cause alterations in cell wall synthesis enzyme activities: reduction of β -1,3-glucan synthase and chitin synthase III activities as well as increased chitin synthase I and II activities. *GBG1* disruption also showed altered cell wall composition as well as susceptibility toward cell wall inhibitors such as Zymolyase, Calcofluor white, and Nikkomycin Z. These results indicate that *GBG1* plays a role in cell wall biogenesis in *S. cerevisiae*.

KEYWORDS : β -1,3-glucan synthase, Cell wall composition, Chitin synthase, *GBG1*/*AYR1*, *Saccharomyces cerevisiae*

The cell wall of *Saccharomyces cerevisiae* supposedly plays a role in determining cell morphology and maintaining osmotic stability [1]. The major components of *S. cerevisiae* are mannoproteins, β -glucans, and chitin [2]. Yeast cell wall biogenesis is an example of cellular morphogenesis, since the wall expands during cell growth and a septum is synthesized at a specific time and location during the cell division cycle [3, 4]. Recently, there has been evidence to support connections between cell wall synthesis and other areas such as signal transduction, control of bud placement and emergence, and the roles of intermediate filaments [1, 5, 6]. From a practical standpoint, *S. cerevisiae* also provides a model for cell wall synthesis in pathogenic fungi. Therefore, investigating cell wall biogenesis, which is critical to maintaining cell wall integrity, can help elucidate which steps of the process are susceptible to antifungal agents [7]. To date, a variety of strategies have been used to identify cell wall synthesis mutants as well as genes involved in cell wall assembly [1]. Thus, thorough understanding of how a yeast cell synthesizes and assembles its wall components has been developed. However, identification of new cell wall biogenesis genes as well as the genetic interactions between them is still required to disclose the full mechanism of cell wall biogenesis.

We previously identified the two genes responsible for β -1,3-glucan biosynthesis in *S. cerevisiae* using a conditional osmo-sensitive mutant with lower β -1,3-glucan content and lower β -1,3-glucan synthase activity at non-

permissive temperature (37°C). We also cloned *SOO1*/*RET1*, which is essential and responsible for the defective β -1,3-glucan synthase activity as well as the temperature-dependent osmo-sensitive (Tos⁻) phenotype of mutant strain LP0353 by functional complementation [8]. We identified a gene for β -1,3-glucan synthase (*GBG1*), a non-essential gene that partially complements defective β -1,3-glucan synthase activity but not the Tos⁻ phenotype. Disruption of *GBG1* altered not only the activities of cell wall synthesis enzymes but also cell wall susceptibility toward various agents in *S. cerevisiae*. These results suggest that *GBG1* plays a role in cell wall biosynthesis in *S. cerevisiae*.

Materials and Methods

Strains and growth media. *S. cerevisiae* LP0353 (*MATa ura3-52 lys2-801 soo1-1 Bgs⁻*) and F808 (*MATa ura3-52 leu2-3,112 his4-519 ade1-100*) were used for cloning and disruption experiments. *S. cerevisiae* X2180-1A (*MATa SUC2 mal mel gal2 cup1*) cells were used to prepare synchronous culture. Rich medium containing glucose (yeast-extract peptone, dextrose [YPD]) and synthetic minimal medium (synthetic dextrose) for yeast culture were prepared as described elsewhere [9]. *Escherichia coli* DH5 α was used for the propagation of all plasmids.

DNA manipulation and yeast genetic techniques. Transformation of *E. coli*, plasmid preparation, restriction mapping, DNA ligation, and other DNA manipulations were performed by standard techniques [10]. Transforma-

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tion of yeast cells was carried out by the lithium acetate procedure [11]. The yeast plasmids YEplac195, YCplac33, and YIplac128 were used for cloning and gene disruption experiments.

Construction of *GBG1* null mutant. For gene disruption, ca 600 bp internal *BalI-SpeI* fragment of *GBG1* cloned in YEplac195 was replaced by the PCR-amplified *LEU2* gene in YIplac128 using primers (LEU2-*SpeI*-R: 5'-AAC TAG TTA GGC GTA TCA CGA GGC C-3', underlined is *SpeI* site, LEU2-*BalI*-L: 5'-TTG GCC AAG ACG AAA GGG CCT ACC C-3', underlined is *BalI* site). The *LEU2* gene in the resulting plasmid flanked on either side by the *GBG1* fragment was amplified by PCR using primers (B2-LEU2-L: 5'-GTG ATG ACG GTG AAA ACC TCC AAC TCT GAC CGG CAT TG-3', B2LEU2-R: 5'-CCT CGT GAT ACG CCT ATT TTC CCT TGC CTG AAA CCT CA-3') followed by introduction into the haploid F808 strain.

Induction of synchrony and Northern blot analysis. To synchronize yeast culture with α -factor, the X2180-1A strain was grown to early log phase at 30°C in YPD medium. α -factor purchased [12] from Sigma Chemical Co. (St Louis, MO, USA) was added to a final concentration of 5 μ g/mL. The cells were further incubated at 30°C for 1 hr, collected by centrifugation, washed with distilled water and then transferred to fresh medium. X2180-1A cells synchronized with α -factor were then washed and resuspended in fresh YPD medium. Aliquots of the cell suspension were injected into five separate flasks containing fresh media. After shaking at 30°C for 100 min, cultures were harvested at 10 min intervals for 40 min. Total RNA was extracted by hot acid phenol method [12]. For Northern blotting, 30 to 40 μ g of RNA was separated on a 1% agarose gel containing 6% formamide, followed by transfer to a Hybond-N+ membrane (Amersham, Buckinghamshire, UK) through capillary blotting and UV cross-linking. Northern hybridization was performed with modified church buffer using [α -³²P] dCTP-labeled probe and a random priming kit (Stratagene, La Jolla, CA, USA). The probe used was a 600 bp portion of the *EcoRI-BalI* fragment from *GBG1*. Following hybridization, the blot was exposed to X-ray film.

Preparation of holoenzyme from yeast and measurement of cell wall biosynthesis enzyme activities. Membrane fractions of cells were prepared using glass beads [8, 13]. Glucan synthase activity was measured following a previously described method [8, 14]. However, chitin synthase activity was measured by the method of Choi and Cabib [13]. All reactions were performed in duplicate and terminated by the addition of 10% trichloroacetic acid. The radioactivity of insoluble materials was counted

by a liquid scintillator after filtration through glass fiber filters (Whatman Inc., Clifton, NJ, USA). Specific activities of β -1,3-glucan synthase and chitin synthase were expressed as nanomoles of Glu and GlcNAc incorporated per hour per milligram of protein, respectively. The concentration of used protein was measured by the method of Lowry *et al.* [15] using bovine serum albumin as a standard.

Fractionation and analysis of cell wall preparations. Total cell wall fractionation was prepared by following a previously described method [8, 16]. Glucan content of the fractions was determined by the orcinol-sulphuric acid method [17]. For determination of chitin content, the amount of GlcNAc liberated after treatment of the acid-insoluble fraction with chitinase was measured by a previous method [18].

Susceptibility to cell wall damaging agents. Zymolyase resistance test was performed following a modified method [19]. Briefly, yeast cells were grown in YPD medium containing 1.2 M sorbitol to mid-log phase and then harvested, after which the cell pellet was washed and resuspended in distilled water to OD₆₀₀ = 0.4. Zymolyase 100T in 10 mM Tris-HCl (pH 8.0) was added to a final concentration of 2 mg/mL followed by incubation at 30°C. Every 90 min, aliquots were removed and read optically. Determination of minimum inhibitory concentration (MIC) was performed according to the method of Kurtz *et al.* [20].

Results and Discussion

Characterization of the *GBG1* gene. In a previous study, we isolated a clone that complements the defective glucan synthase activity of LP0353 [21]. Sequence analysis of the selected 8.5 kb DNA fragment revealed two open reading frames (ORFs), Yil124w and Yil125w. ORF Yil125w was identified as *KGD1*, which encodes α -keto-glutarate dehydrogenase [22]. Although both ORFs can complement defective glucan synthase activity (data not shown), the function of ORF Yil124w was not clear at the beginning of this study. We therefore performed functional analysis of the unknown gene, Yil124w, which is an ORF consisting of 891 bp that encode a polypeptide of 297 amino acids and with a calculated mass of 32.8 kDa. Based on previous results, this gene was named as *GBG1*.

Even during our own study, the function of ORF Yil124w has been reported by other groups [23, 24]. The ORF Yil124w, named as *AYR1*, encodes a NADPH dependent 1-acyl dihydroxyacetone phosphate reductase found in lipid particles, the endoplasmic reticulum, and mitochondrial outer membrane [23, 24]. Thus *AYR1* is involved in phosphatidic acid and phospholipid biosynthesis, and is also

Table 1. Assay of β -1,3-glucan synthase activity and quantitative analysis of cell wall composition in LP0353 strain expressing *GBG1*

Strain	β -1,3-glucan synthase ^a (%)	β -glucan ^b			
		Total glucan ^c	β -1,3 glucan ^d	β -1,6 glucan	β -1,3 glucan/Total glucan ^e
Wild type	103.6 (100)	8314.8	7590.0	724.8	91.3 (100)
LP0353	27.2 (26.3)	2976.0	1993.0	982.8	67.0 (73.4)
LP0353 + YCp	24.0 (23.2)	3954.0	2781.6	1172.4	70.3 (77.0)
LP0353 + YEp	24.8 (23.9)	ND	ND	ND	ND
LP0353 + YCpGBG1	39.8 (38.4)	3193.2	2482.8	710.4	77.8 (85.2)
LP0353 + YEpGBG1	35.3 (34.1)	ND	ND	ND	ND

GBG, gene for β -1,3-glucan synthesis; ND, not detected.

^aEnzyme activity was measured in membrane fractions and was expressed as mg (UDPG)/h·mg (protein).

^bThe units of β -glucan were expressed as mg/mg of cell wall dry-weight.

^cThe amount of total β -glucan was determined based on the carbohydrate content of the Zymolyase-insoluble pellet and solubilized supernatant before dialysis.

^dThe amount of β -1,3-glucan was the difference in between total carbohydrate content and β -1,6-glucan.

^eThe amount of β -1,3-glucan in GS-1-36 was taken as 100.

required for spore germination but not vegetative growth [24]. However, there are no reports on the function of *Gbg1*/*Ayr1* in cell wall biogenesis.

In order to explore the effect of copy number of the *GBG1* gene on β -1,3-glucan synthase activity, 1.6 kb *EcoRI* fragment containing the whole ORF of *GBG1* was cloned into a high-copy number plasmid (YEplac195) and low-copy number plasmid (Ycplac33). Each of the resulting recombinant plasmids were introduced into mutant LP0353. The levels of β -1,3-glucan synthase activity were almost identical, but both were lower than that of wild type (Table 1). Additional evidence that *GBG1* plays a role in β -1,3-glucan synthase activity was that the LP0353 strain expressing *GBG1* produces an increased amount of β -1,3-glucan, based on quantitative analysis of cell wall composition. Although further experiments including an allele test

should be performed, these results indicate that *GBG1* may account for the defect in β -1,3-glucan synthase activity [21].

***GBG1* disruption reduces cell wall polysaccharide synthesis.** To predict the *in vivo* function of the *GBG1* gene with regards to cell wall biogenesis, a *GBG1* disruption mutant was constructed and confirmed by Southern blot (Fig. 1). No difference in growth rate between the *GBG1* null mutant and isogenic wild type was observed (data not shown). However, as expected, the *GBG1* null mutant showed 36% reduction in β -1,3-glucan synthase activity (Table 2). On the other hand, *GBG1* disruption increased chitin synthase I and II activities by 23% and 51%, respectively, and decreased chitin synthase III activity by 12% (Fig. 2). The reduction in β -1,3-glucan and chitin III

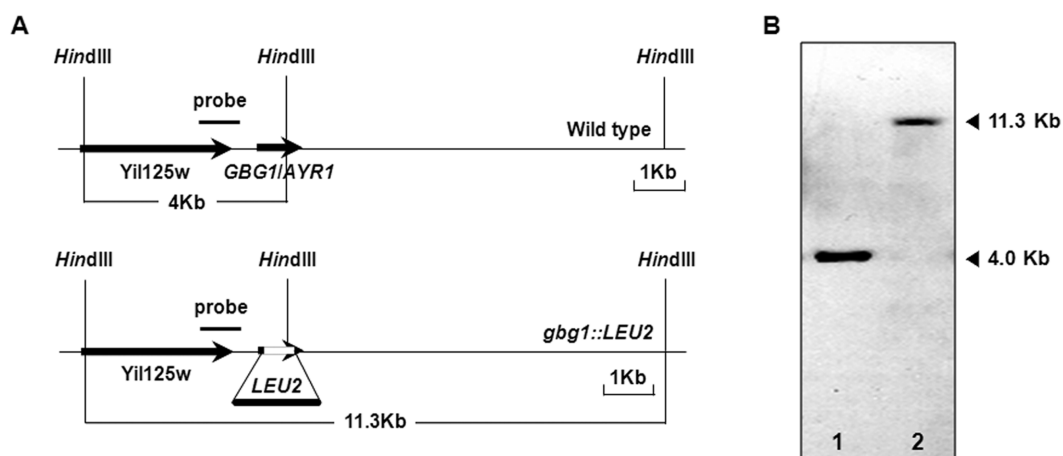


Fig. 1. Gene disruption of gene for β -1,3-glucan synthesis (*GBG1*) in *Saccharomyces cerevisiae*. A, A schematic representation of the gene disruption of *GBG1*. Black arrows stand for open reading frames. The predicted sizes of fragments generated by *HindIII*-digestion are depicted. A 4 kb fragment indicates *GBG1* wild type allele while the 11 kb fragment indicates the *gbg1::LEU2* allele disrupted by inserted *LEU2*; B, The disruption of *GBG1* was detected by Southern blot using the indicated probe. Analysis of wild type (lane 1) and Δ *gbg1* (lane 2) is shown.

Table 2. Assay of β -1,3-glucan synthase activity and quantitative analysis of cell wall composition in *Δgbg1* mutant

Strain	β -1,3-glucan synthase ^a (%)	β -glucan ^b			
		Total glucan ^c	β -1,3 glucan ^d	β -1,6 glucan	β -1,3 glucan/Total glucan ^e
Wild type	101.4 (100)	5268.0	4550.4	717.6	86.4 (100)
<i>Δgbg1</i>	68.3 (67.4)	4010.4	3141.6	868.8	78.3 (90.6)

gbg, gene for β -1,3-glucan synthesis.

^aEnzyme activity was measured in membrane fractions and was expressed as mg (UDPG)/h·mg (protein).

^bThe units of β -glucan were expressed as μ g/mg cell wall dry-weight.

^cThe amount of total β -glucan was determined based on the carbohydrate content of the Zymolyase-insoluble pellet and solubilized supernatant before dialysis.

^dThe amount of β -1,3-glucan was the difference between total carbohydrate content total and β -1,6-glucan.

^eThe amount of β -1,3-glucan in F808 was taken as 100.

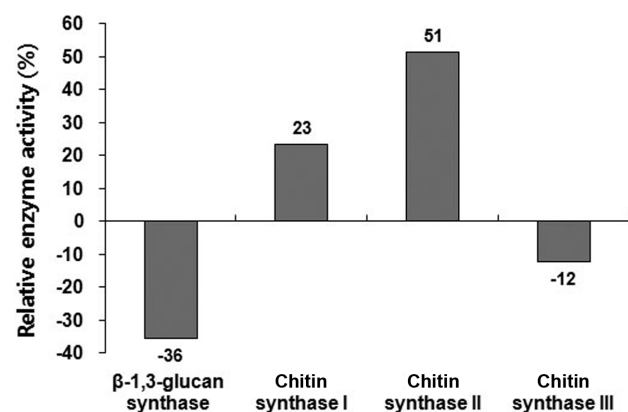


Fig. 2. Assays of enzymes related to cell wall biogenesis in *Δgbg1*. The values are presented as relative enzyme activities normalized to that of wild type. The experiment was performed three times. *gbg*, gene for β -1,3-glucan synthesis.

synthase activity by the disruption of a single is not well understood. However, the increase in chitin synthase I and II activities can be explained by the fact that gene(s) for cell wall biogenesis are activated by defect(s) in the cell wall caused by mutation. This compensation mechanism is known to increase chitin synthesis in a *FKS1* deletion mutant [25]. In this study, *GBG1* disruption caused defects in β -1,3-glucan synthase, which then may have altered chitin synthase activity.

Although the null mutant showed no significant change in the amount of cell wall carbohydrates in the alkali-soluble fraction, there was dramatic reduction in the acid-insoluble fraction containing β -1,3-glucan and chitin. Consistent with the result of the chemical extraction, the amount of GlcNAc liberated by chitinase treatment of the acid-insoluble fraction was also dramatically reduced (Table 2). According to a recent model of yeast cell wall architecture, chitin is linked to the cell wall through both β -1,3-glucan and β -1,6-glucan. Thus, a decrease in β -1,3-glucan and/or β -1,6-glucan synthesis in combination with defective chitin synthesis could dramatically reduce chitin content in the cell wall.

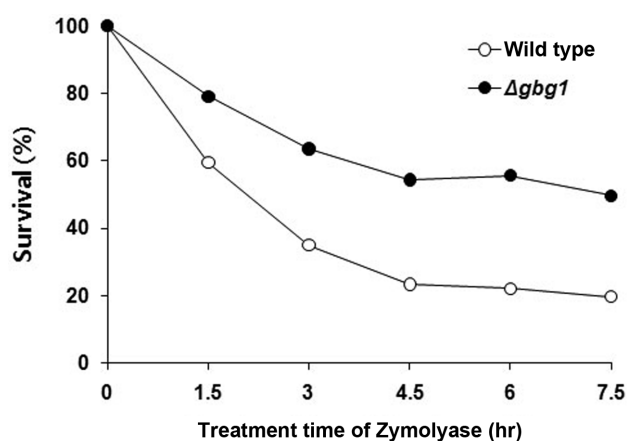


Fig. 3. The viability of *Δgbg1* cells after treatment with Zymolyase. Cells were grown to mid-exponential phase followed by treatment with Zymolyase 100T (final concentration is 2 μ g/mL). Cell survival was measured as OD₆₀₀ every 90 min for 7.5 hr. Data are the mean of three independent experiments. *gbg*, gene for β -1,3-glucan synthesis.

***GBG1* disruption alters susceptibility toward various agents affecting cell wall biogenesis.** In order to investigate other cell wall properties that are altered by *GBG1* disruption, the susceptibility of the strains toward various agents affecting cell wall biogenesis was tested. Usually, a reduction in cell wall components will increase resistance toward cell wall lytic enzyme. Upon Zymolyase treatment, the *GBG1* null mutant revealed more than two-fold increase in resistance, implying that β -1,3-glucan content in cell wall of the null mutant was reduced (Fig. 3). This is consistent with the result from the cell wall analysis that revealed a dramatic decrease in β -1,3-glucan. Many cell wall defects affect sensitivity to Calcofluor white, a chitin specific dye [26]. When measuring the MIC (minimum inhibitory concentration) of the strains, *GBG1* null mutant revealed an increased MIC value toward Calcofluor white and Nikkomycin Z (Table 3). Calcofluor white is known to interfere with the normal synthesis and assembly of cell wall polysaccharides by binding to the growing chitin

Table 3. Sensitivity of $\Delta gbg1$ mutant to cell wall damaging agents

	MIC ($\mu\text{g/mL}$)	
	Wild type	$\Delta gbg1$
Calcofluor white	4	20
L-688,786	4	4
L-733,560	0.8	0.8
Nikkomycin Z	4	> 100
Polyoxin D	> 100	> 100

gbg, gene for β -1,3-glucan synthesis; MIC, minimum inhibitory concentration.

polymer [26, 27]. Thus, decreased chitin content increases cell resistance toward this dye. On the other hand, Nikkomycin Z, an inhibitor of chitin synthesis, is a specific inhibitor of chitin synthase III in *S. cerevisiae* [28]. Therefore, the results from the MIC determination are consistent with those from the cell wall analysis and chitin synthase activity assays that showed reduction in chitin content and chitin synthase III activity. However, sensitivities to Polyoxin D, which inhibits chitin synthesis, as well as L-688,786 and L-733,560, which inhibit glucan synthesis, were not different between null mutant and wild type [28].

***GBG1* is expressed in a cell cycle-dependent manner.**

In fungi various cell types cause change in cell wall struc-

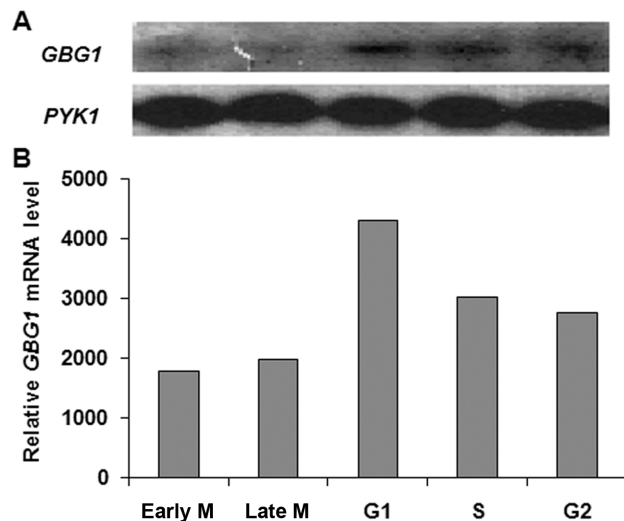


Fig. 4. Analysis of gene for β -1,3-glucan synthesis (*GBG1*) transcription during vegetative cell cycle. Total RNA was isolated from the X2180-1A strain synchronized with α -factor. A, Northern blotting of *GBG1* and pyruvate kinase (*PYK1*) was performed during the synchronized cell cycle. *PYK1* was used as a loading control; B, Level of *GBG1* mRNA was determined by densitometry of the Northern blot in A. The values were normalized to *PYK1* mRNA.

ture and composition [3]. Morphological differentiation in *S. cerevisiae* was observed during the cell cycle and mating process as well as in response to nutrient limitation [4]. It was also reported that *FKS1*, a β -1,3-glucan synthase gene, is mainly expressed for vegetative growth, whereas *FKS2* is induced for sporulation under starvation and in response to mating pheromones [6, 29]. To test the expression of *GBG1* during vegetative growth, RNA preparation from vegetative cells was subjected to Northern blot analysis. As shown in Fig. 4, *GBG1* was expressed during vegetative growth with a maximum level of transcription at G1 phase, after which it was decreased during the remainder of the cell cycle. A bud was observed in G1 phase, and maturation was observed in S phase. Increased *GBG1* transcription during G1 phase may be able to explain abnormally elongated bud formation in $\Delta ayr1$ mutant [30]. These results also suggest that *GBG1* may play a role during G1 phase when the bud starts to emerge.

In this study, we showed that disruption of *GBG1* reduced the activities of β -1,3-glucan synthase and chitin synthase III while increasing chitin synthase I and II activities. *GBG1* disruption also altered cell wall composition and thus increased cell wall resistance to inhibitors such as Zymolyase, Calcofluor white, and Nikkomycin Z. Since *GBG1/AYR1* codes for NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase, a key enzyme of the phosphatidic acid biosynthesis pathway [23], and not for a cell wall synthesis enzyme, the effect of *GBG1* deletion on cell wall biogenesis might be indirect and therefore explainable by the following. Phosphatidic acid plays a central role in the metabolism of phospholipids [31], which in turn determine the total amounts of membrane lipids [32]. The nature of lipids in the membrane affects membrane fluidity, protein-mediated transport, and membrane-associated enzyme activities [32, 33]. Although further experiments are required for the confirmation, alterations of yeast cell wall biogenesis caused by the *GBG1* deletion reported here can be supported by the low level of phospholipids and phosphatidic acid in the *gbg1/ayr1* deletion mutant [23, 31]. G1-specific expression of *GBG1* is also explainable by a previous report that found *GBG1* is required for spore germination, which is a G1-specific event in the yeast cell cycle [23, 30, 31]. The results presented here indicate that *GBG1* is not essential for the viability and growth of cells but instead is involved in cell wall biogenesis by affecting the activities of cell wall synthesis enzymes.

Acknowledgments

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