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Biochemistry

Chitin synthase 3 from yeast has zymogenic properties that depend on both the *CAL1* and the *CAL3* genes

(chitin/cell wall)

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ABSTRACT **In previous studies, chitin synthase 3 (Chs3), the enzyme responsible for synthesis of most of the chitin present in the yeast cell, was found to be inactivated by incubation with trypsin, in contrast to other yeast chitin synthases (Chs1 and Chs2), which are stimulated by this treatment (chitin synthase; UDP-*N*-acetyl-D-glucosamine:chitin 4-β-*N*-acetylglucosaminyltransferase, EC 2.4.1.16). It has now been found that the substrate UDPGlcNAc protects Chs3 against proteolytic inactivation. Treatment of Chs3-containing membranes with detergents drastically reduced the enzymatic activity. Activity could, however, be restored by subsequent incubation with trypsin or other proteases in the presence of UDPGlcNAc. Under such conditions, protease treatment stimulated activity as much as 10-fold. A change in divalent cation specificity after trypsin treatment suggests that the protease directly affects the enzyme molecule. Experiments with mutants in the three genes involved in Chs3 activity—*CAL1*, *CAL2*, and *CAL3*—showed that only *CAL1* and *CAL3* are required for the protease-elicited (zymogenic) activity. It is concluded that Chs3 is a zymogen and that the *CAL2* product functions as its activator. The differences and possible similarities between Chs3 and the other chitin synthases are discussed.**

In the yeast *Saccharomyces cerevisiae*, three chitin synthase enzymes (UDP-*N*-acetyl-D-glucosamine:chitin 4-β-*N*-acetylglucosaminyltransferase, EC 2.4.1.16) catalyze synthesis of the chitin that constitutes the primary septum formed at cytokinesis and is also interspersed in the structure of the cell wall. Each of these enzymes has a specific function: chitin synthase 3 (Chs3) is involved in formation of the ring of chitin found at the base of an emerging bud and of the cell wall chitin, whereas Chs2 participates in construction of the chitin disc that completes the primary septum at cytokinesis (1). Chs1 appears to have a repair function, counterbalancing the activity of a chitinase that facilitates cell separation (2, 3). Because of their different functions, each chitin synthase must be regulated by a specific mechanism acting when its function is executed. Recent work indicates that at least some of those regulatory mechanisms are posttranslational, presumably involving activation of the enzymes (W.-J.C., B. Santos, A. Durán, and E.C., unpublished results). In accordance with this notion, Chs1 and Chs2, and indeed many other fungal chitin synthases, are present in the cell as zymogenic forms that can be activated by partial proteolysis (4, 5). In contrast, Chs3 was originally described as existing in a functionally active state that was inactivated, rather than stimulated, by incubation with trypsin (6–8). Based on these findings, in a recent review (9) mutants defective in Chs3 were treated separately from the "zymogen-deficient" mutants that lack Chs1 or Chs2 activity.

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The availability of mutants that contain Chs3 as the only chitin synthase (1, 6) prompted us to reinvestigate the properties of this enzyme by taking advantage of the absence of interfering activities. We report here conditions under which a large stimulation of Chs3 by proteases can be shown—i.e., Chs3 behaves as a zymogen. This result indicates that Chs3 may be much more similar to Chs1 and Chs2 than was previously thought.

Three genes—*CAL1/CSD2/DIT101*, *CAL2/CSD4*, and *CAL3*—are required for Chs3 activity, as measured without protease treatment (7, 10, 11). We found that only *CAL1* and *CAL3* are necessary for the protease-dependent activity, which suggests that the *CAL2* gene product may act as an activator of the Chs3 zymogen.

MATERIALS AND METHODS

Strains and Culture Conditions. The strains used in this study are listed in Table 1. *S. cerevisiae* strains were grown in minimal medium (2% glucose/0.7% Difco yeast nitrogen base without amino acids) plus nutritional requirements and with the addition of succinate as a buffer to compensate for the Chs1 defect (2).

Membrane Preparation and Chitin Synthase Assay. Total cell membranes were prepared as described (7, 8). Briefly, cells suspended in 50 mM Tris·HCl (pH 7.5) containing 5 mM magnesium acetate were broken by Vortex mixing with glass beads. Cell walls were sedimented at 3000 × *g* for 5 min and the supernatant fluid was centrifuged for 30 min at 120,000 × *g*. The membrane pellet was suspended in the same buffer used in the breakage, to a final vol of 1.6 ml per g (wet weight) of cells used.

Detergent-treated membranes were prepared as follows: membranes from 1 g of cells were homogenized with 2 ml of 50 mM Tris·HCl (pH 7.5) containing 5 mM magnesium acetate, 2% Tergitol Nonidet P-40 (TNP40), or 0.4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Homogenized membranes were pelleted by ultracentrifugation at 120,000 × *g* for 40 min. The pellet was suspended in 1.6 ml of 50 mM Tris·HCl (pH 7.5) containing 5 mM magnesium acetate and 33% (vol/vol) glycerol, and chitin synthase activity was measured by a slight modification of a described procedure (5). For the proteolytic activation step, reaction mixtures contained 32 mM Tris·HCl (pH 7.5), 4.3 mM magnesium acetate, 1.1 mM UDP[U-^{14}C]GlcNAc (400,000 cpm/μmol; American Radiolabeled Chemicals, St. Louis), 2 μl of trypsin at the optimal concentration for activation (usually 1–3 mg/ml), and 20 μl of membranes in a total vol of 46 μl. When Co^{2+} was used in place of Mg^{2+}, the concentration of cobalt acetate was 2.7 mM and the pH of the Tris buffer was 8.0. Mixtures were

Abbreviations: Chs1, Chs2, and Chs3, chitin synthases 1, 2, and 3, respectively; TNP40, Tergitol Nonidet P-40; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
ECY36-3C	*MATa chs1-23 chs2::LEU2 trp1-1 ura3-52 leu2-2*	This laboratory
ECY36-3D	*MATa chs1-23 cal1 trp1-1 ura3-52 leu2-2*	This laboratory
CR4-2	*MATα chs1::URA3 cal2 ura3-52 his4*	C. Roncero*
CR4-3	*MATa chs1::URA3 cal2 ade1 ura3-52*	C. Roncero
HV23-3	*MATa chs1::URA3 cal3 ura3-52 his⁻*	H. Valdivieso*
5A	*chs1-23 cal3 ura3-52 his4 trp1 leu2*	H. Valdivieso

In strains ECY36-3C and ECY36-3D the *leu2* allele is *leu2-2*, not *leu2-3,112* as erroneously designated (1, 7).
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incubated for 15 min at 30°C. Proteolysis was stopped by adding 2 μl of a soybean trypsin inhibitor solution at a concentration 1.5 times that of the trypsin solution used, and tubes were placed on ice. GlcNAc was added to a final concentration of 32 mM and incubation at 30°C was carried out for 90 min. The chitin formed was assayed after filtration through glass fiber filters (12, 13). Protein was measured by the method of Lowry *et al.* (14). Specific activity is expressed as nmol of GlcNAc incorporated per 90 min per mg of protein.

RESULTS

As previously reported (6, 7), trypsin incubation of membrane preparations from strains lacking Chs1 and Chs2 in the presence of Mg^{2+} led to a decrease in the activity of the remaining synthase, Chs3 (Fig. 1). However, it has now been found that inclusion of the substrate UDPGlcNAc during incubation with the protease prevents the loss of activity and in fact leads to some increase (Fig. 1). With Co^{2+} the results were more variable, showing sometimes a decrease (7) and sometimes a small increase (Fig. 1) in the absence of substrate. Here too, however, the presence of the nucleotide sugar during trypsin incubation invariably enhanced the activity.

The relative increase by trypsin treatment became quite dramatic when the membranes were extracted with detergents before trypsin treatment. Extraction lowered the synthase activity, but incubation of the extracted membranes with trypsin in the presence of substrate resulted in activities considerably higher than the original ones (Fig. 2). CHAPS was more effective than TNP40 in lowering the activity. After treatment with CHAPS, a 10-fold increase in activity by trypsin was observed in the presence of Mg^{2+} (Fig. 2*A*).

The activation effect is not limited to trypsin. Among the proteases assayed, chymotrypsin and proteinase K were stimulatory, whereas *Staphylococcus* V8 protease and Pronase were without significant effect (Table 2). It is to be noted that after proteinase K treatment the activity in the presence of Co^{2+} was slightly greater than that with Mg^{2+}, whereas after incubation with trypsin or chymotrypsin the Mg^{2+}-stimulated activity was much higher (Table 2).

An indication that trypsin treatment modifies the synthase molecule rather than the membrane structure was provided by the marked change in metal specificity observed after protease incubation (Fig. 3). To eliminate any interference of Mg^{2+} with the effect of Co^{2+}, these experiments were carried out with membranes prepared in the absence of divalent cations (usually Mg^{2+} is present; see *Materials and Methods*); furthermore, the activity was assayed at pH 8 with both cations. Under such conditions, Co^{2+} was a better stimulator than Mg^{2+} with the intact preparation, but after detergent and trypsin treatment the situation was reversed. The ratio between activity with Mg^{2+} and that with Co^{2+} increased 5-fold, from 0.42 to 2.2 (Fig. 3). Furthermore, the shape of the curve

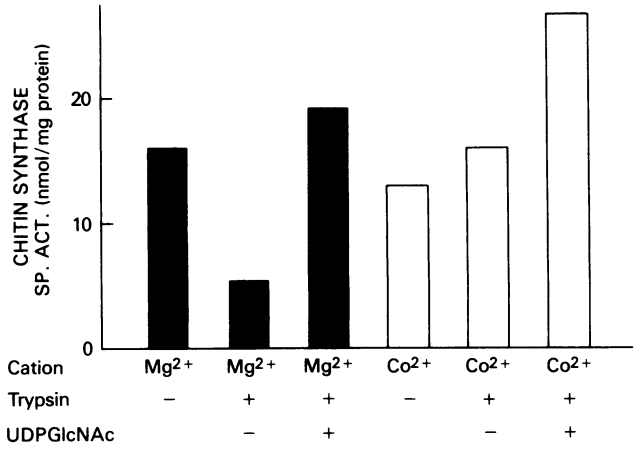

FIG. 1. Protection of Chs3 by substrate during trypsin treatment. Where indicated, the membrane preparation, from strain ECY36-3C, was incubated for 15 min with trypsin (20 μg per mg of protein) in the absence or presence of 1.1 mM UDPGlcNAc. Mg^{2+} (4.3 mM) or Co^{2+} (2.7 mM) was also present. The missing components of the reaction mixture were then added and the chitin synthase activity was assayed.

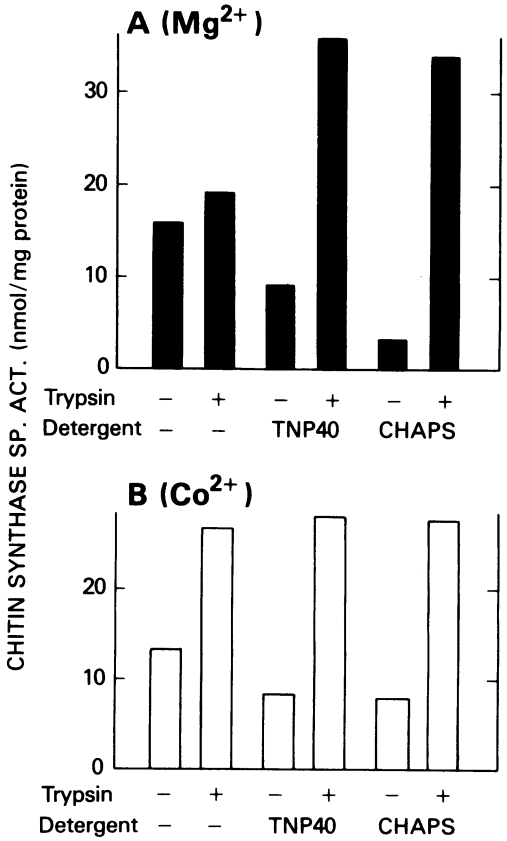

FIG. 2. Effect of trypsin treatment on Chs3 activity before or after extraction of the membranes with detergents. The conditions were the same as in Fig. 1, except that substrate was always present during trypsin treatment.

Table 2. Effect of different proteases on Chs3 activity

Protease used	Chitin synthase activity, nmol per mg of protein	
	With Mg^{2+}	With Co^{2+}
None	2.8	5.7
Trypsin	35	15
Chymotrypsin	28	13
Staphylococcus V8 protease	2.3	4.6
Pronase	6.4	7.9
Proteinase K	12	15.5

Membranes were prepared from ECY36-3C cells, treated with CHAPS, and incubated with trypsin or with the other proteases as described. The amounts of protease used were as follows: for trypsin and chymotrypsin, 20 μg per mg of membrane protein; for *Staphylococcus* V8 protease, Pronase, or proteinase K, 6 μg per mg of membrane protein. For trypsin, chymotrypsin, and proteinase K, the amounts used were the optimal amounts. Increasing *Staphylococcus* V8 protease or Pronase did not result in enhanced synthase activity. After a 15-min incubation with the respective protease, the reaction was stopped with an excess of soybean trypsin inhibitor for trypsin; with 1 mM phenylmethylsulfonyl fluoride for chymotrypsin, *Staphylococcus* V8 protease, and Pronase; and with 0.1 mM EGTA for proteinase K. Assay of chitin synthase activity was then performed as specified.

relating activity to Mg^{2+} concentration changed drastically, whereas the effect of Co^{2+} was only slightly modified (Fig. 4).

To confirm that the activity measured after detergent and trypsin treatment corresponded to a bona fide chitin synthase, purified chitinase (15) was added to the assay mixtures of several preparations, similar to those of Fig. 2. This addition drastically reduced the amount of trichloroacetic acid-insoluble reaction product, by ≈80% on the average (data not shown), confirming the nature of the product as chitin.

At least three genes—*CAL1*, *CAL2*, and *CAL3*—are required for activity of Chs3, as measured without trypsin treatment (7, 10, 11). Since mutants in all three genes are available (10, 16), it was desirable to test them for trypsin-elicited activity. These mutants contain, of necessity, Chs2,

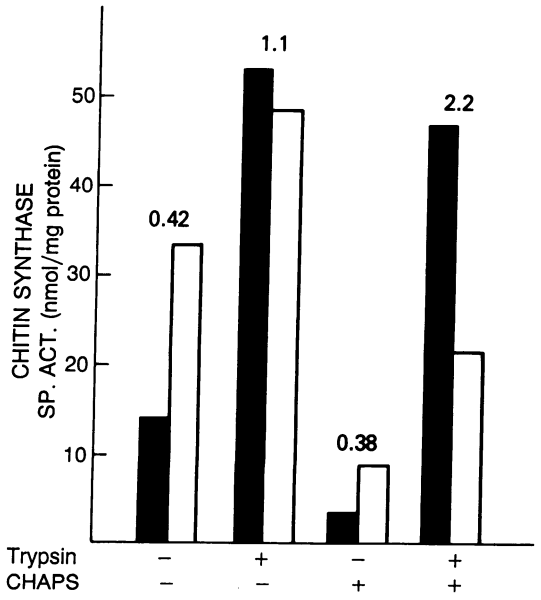

FIG. 3. Change in metal specificity of Chs3 after detergent extraction and trypsin treatment. In this experiment, membranes were prepared from strain ECY36-3C in the absence of added Mg^{2+} and the assay was carried out at pH 8 for both divalent cations. Solid bars, Mg^{2+} in assay; open bars, Co^{2+} in assay. Numbers above bars represent the ratio of activity with Mg^{2+} vs. that with Co^{2+} in the assay.

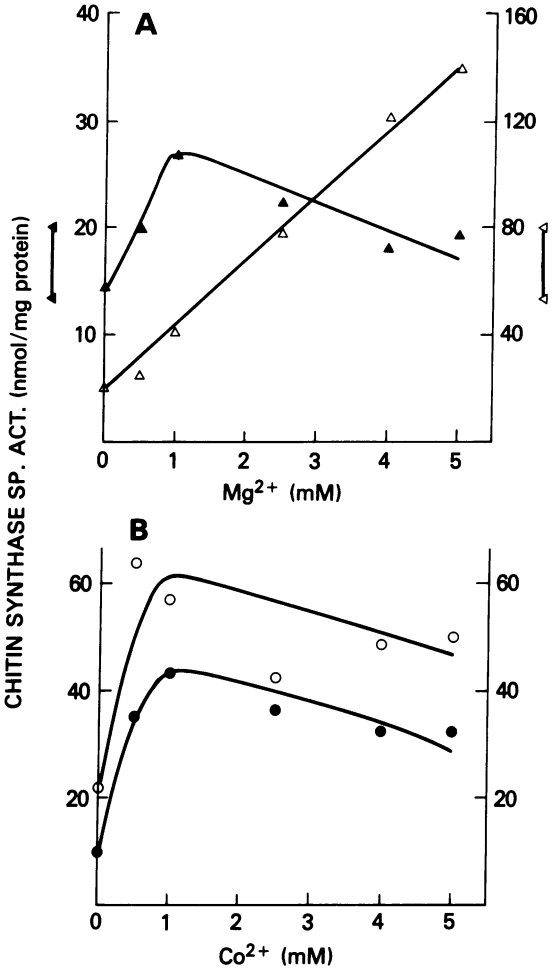

FIG. 4. Change in Mg^{2+} dependence of Chs3 activity after extraction of membranes with detergent and treatment with trypsin. Conditions were the same as in Fig. 3. (*A*) Mg^{2+} dependence of untreated (▲) and detergent- and trypsin-treated (△) preparations. Notice the different scale on the left and right ordinate. (*B*) Co^{2+} dependence of untreated (●) and detergent- and trypsin-treated (○) preparations.

because cells lacking both Chs3 and Chs2 are nonviable (1). Since Chs2 is also activated by trypsin, it interferes with the Chs3 determinations. We found, however, that Ni^{2+} ions almost completely inhibit Chs2 without affecting Chs3 when Co^{2+} is used as the stimulatory cation (W.-J.C. and E.C., unpublished data). Thus, when both Chs2 and Chs3 are present, only the latter is measured in the presence of Ni^{2+}. Under these conditions, only the *cal2* mutant exhibited trypsin-elicited activity, whereas both the *cal1* and *cal3* strains were devoid of activity with or without trypsin (Fig. 5). Similar results were obtained with two other *cal2* and *cal3* mutants, strains CR4-3 and 5A, respectively (data not shown). Although no detergent was used in these experiments, trypsin caused a 3-fold increase in the Chs3 activity of the *cal2* strain, about the same observed for the ECY36-3C enzyme when measured in the presence of Co^{2+} after CHAPS extraction (Fig. 2*B*). The Chs3 activity measured in strain CR4-2 is smaller than that of ECY36-3C; however, both that activity and the Chs2/Chs3 ratio are about the same as for wild-type cells (results not shown). ECY36-3C has a high chitin content (1), which correlates with a high Chs3 activity, although the level of this activity has been rather variable in our hands.

DISCUSSION

In this discussion, chitin synthase activity measured without protease treatment will be called "direct activity" to distin-

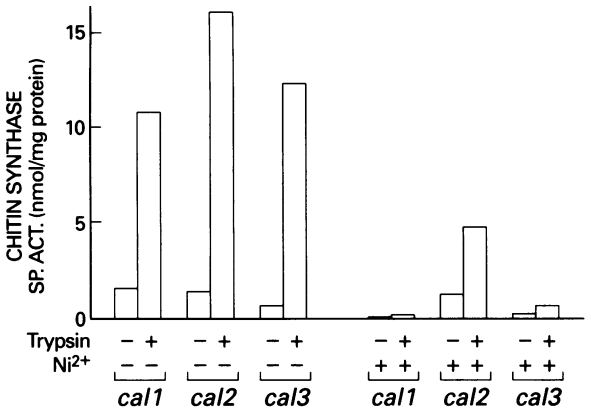

FIG. 5. Zymogenic activity of Chs3 in *cal* mutants. *cal1*, *cal2*, and *cal3* strains are, respectively, ECY36-3D, CR4-2, and HV23-3. Measurements in the absence of Ni^{2+} represent the sum of Chs2 and Chs3, and those in the presence of Ni^{2+} represent Chs3 activity alone. Where Ni^{2+} was present, the concentration was 5 mM. Co^{2+} was present at 5 mM throughout.

guish it from protease-elicited or trypsin-elicited activity. Whereas in previous work (6–8) the direct activity of Chs3 was decreased by trypsin treatment, inclusion of the substrate UDPGlcNAc during the protease incubation led to an increase in activity, presumably by affording protection to the enzyme. The relative increase in synthase activity was much larger when most of the direct activity was removed by prior treatment of the membranes with detergent. The change in metal specificity of the enzyme suggests that protease digestion has a direct effect on the catalytic component(s) of Chs3 rather than an indirect effect on membrane structure.

The ability of Ni^{2+} to inhibit Chs2 allowed us to test for trypsin-elicited activity in strains bearing mutations in each of the three genes that are required for Chs3 direct activity—*CAL1*, *CAL2*, and *CAL3*. Only the *cal2* mutant, carrying intact *CAL1* and *CAL3* genes, yielded trypsin-elicited activity. Neither direct nor trypsin-elicited activity was detected in the *cal1* or *cal3* mutant. The increase in activity found after trypsin treatment of the *cal2* membranes was about the same as that observed in detergent-extracted membranes of ECY36-3C, an indication that the detergent results were not artifactual.

The simplest interpretation of the above findings is that the *CAL1* and *CAL3* products are required for the Chs3 zymogen form, which can then be activated either proteolytically or by interaction with the *CAL2* gene product. Detergent presumably removes the latter product and results in a decrease of the Chs3 direct activity. Because of its reversibility by detergent treatment, it is likely that the direct activity results from association with an activating protein rather than from proteolysis or another covalent modification of the enzyme. The notion that the *CAL2* gene product acts as an activator of Chs3 is in agreement with the unpublished results cited by Bulawa (9), which showed an increase in Chs3 direct activity upon overexpression of *CAL2/CSD4*.

Despite several attempts, conditions have not yet been found for successful reconstitution of direct Chs3 activity with membrane preparations and detergent extracts from different strains (unpublished experiments). All three *CAL* genes have now been cloned, although the cloning of *CAL2* (mentioned in ref. 9) and that of *CAL3* (B. Santos and A. Durán, personal communication) have not yet been published. In the future, simultaneous overexpression of more than one of those genes may help in the reconstitution studies. Overexpression of *CAL1* alone does not result in an increase in either the direct (7) or the protease-elicited (W.-J.C. and E.C., unpublished results) Chs3 activity.

In view of the results reported here, a reassessment of the similarities and differences between Chs3 and Chs1 or Chs2 appears to be in order. *CAL1* was previously categorized as a structural gene for Chs3 on the basis of its homology with the *CHS1* and *CHS2* genes (7). That notion is in accordance with the results reported here, since an intact *CAL1* gene is required for both direct and protease-elicited activity of Chs3. The same requirement has been found for *CAL3*, which may code for another subunit of Chs3. In contrast, only one structural gene was supposed to exist for Chs1 or Chs2, because expression of *CHS1* or *CHS2* in *Schizosaccharomyces pombe* was sufficient to give rise to the corresponding synthase in zymogenic form (17, 18). At that time, *Sc. pombe* was supposed to be devoid of both chitin and the corresponding synthases. However, some chitin or chitin-like polysaccharide and also chitin synthase activity have been detected recently in *Sc. pombe* (19) as well as a genomic sequence with homology to *CHS* genes (20). It is not impossible, therefore, that both *Sc. pombe* and *S. cerevisiae* contain analogs of *CAL3* that are necessary for a functional Chs1 or Chs2. The reason why three genes were identified in the case of Chs3, and only one was identified for Chs1 or Chs2, may be of methodological origin. The two screens used for genes involved in Chs3 activity, resistance to Calcofluor (16) or incorporation of acetylglucosamine into chitin in intact cells (10), required synthase function *in vivo*. In contrast, *CHS1* and *CHS2* were cloned by detecting overproduction of protease-activatable enzyme in permeabilized cells (17, 18). Although these considerations are speculative, they have heuristic value inasmuch as they suggest that a search for genes interacting with either *CHS1* or *CHS2* may uncover additional information about the functioning of the corresponding synthases.

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