GATA-type Transcriptional Factor SpGAT1 Interacts with SpMIG1 and Promotes Lipid Accumulation in the Oleaginous Yeast Saitozyma podzolica zwy-2-3

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Research Article

GATA-type Transcriptional Factor SpGAT1 Interacts with SpMIG1 and Promotes Lipid Accumulation in the Oleaginous Yeast *Saitozyma podzolica* zwy-2-3

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Abstract

Background: In oleaginous yeast, nitrogen limitation is a critical parameter for lipid synthesis. GATA-family transcriptional factor GAT1, a member of the target of rapamycin (TOR) pathway and nitrogen catabolite repression (NCR), regulates nitrogen uptake and utilization. Therefore, it is significant to study the SpGAT1 regulatory mechanism of lipid metabolism for conversion of biomass to microbial oil in *Saitozyma podzolica* zwy-2-3.

Results: Compared with WT, $\Delta gat1$, and OE::$gat1$, the lipid yield of OE::$gat1$ increased markedly in the low carbon and nitrogen ratio (C/N ratio) mediums, while the lipid yield and residual sugar of $\Delta gat1$ decreased in the high C/N ratio medium. According to yeast two-hybrid assays, SpGAT1 interacted with SpMIG1, and its deletion drastically lowered $SpMIG1$ expression on the high C/N ratio medium. $MIG1$ deletion has been found in earlier research to affect glucose metabolic capacity, resulting in a prolonged lag period. Therefore, we speculated that SpGAT1 influenced glucose consumption rate across SpMIG1. Based on yeast one-hybrid assays and qRT-PCR analyses, SpGAT1 regulated the glyoxylate cycle genes $ICL1$, $ICL2$, and pyruvate bypass pathway gene ACS, irrespective of the C/N ratio. SpGAT1 also could bind to the $ACAT2$ promoter in the low C/N medium and induce sterol ester (SE) accumulation.

Conclusion: Our findings indicated that SpGAT1 positively regulated lipid metabolism in *S.podzolica* zwy-2-3, but that its regulatory patterns varied depending on the C/N ratio. When the C/N ratio was high, SpGAT1 interacted with SpMIG1 to affect carbon absorption and utilization. SpGAT1 also stimulated lipid accumulation by regulating essential lipid anabolism genes. Our insights might spur more research into how nitrogen and carbon metabolism interact to regulate lipid metabolism.

Keywords: *Saitozyma podzolica* zwy-2-3; C/N ratio; SpGAT1; SpMIG1; Lipid metabolism

Background

Because of the shortage of fossil fuels and the rising use of first-generation biofuels, renewable and sustainable alternatives [1], like microbial lipid, the third generation of biodiesel generated by oleaginous yeasts, need to be researched [2]. Conventional oleaginous yeasts (like *Lipomyces starkeyi*, *Rhodotorula toruloides*, *Yarrowia lipolytica*, *etc.*) have been applied extensively, but they still have drawbacks, for instance, restricted feedstock accessibility

and yields that fall short of theoretical lipid yields[3, 4]. When compared to existing conventional species, nonconventional oleaginous yeasts have become appealing research materials due to their benefits of species diversity, high biomass and lipid production, good tolerance to hydrolysate inhibitors, excellent biodiesel characteristics, and economically lucrative bioprocesses [3, 5].

In recent years, *Saitozyma podzolica*, a nonconventional oleaginous yeast of basidiomycetous species, has been discovered [6, 7, 8]. *S. podzolica* DSM 27192 could produce lipid and sugar acids from glucose or xylose, and its lipid accumulation is influenced by fermentation factors such as pH, temperature, and carbon sources concentration [7, 9]. To increase yields in

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S. podzolica DSM 27192, many lipid extraction techniques also have been developed [10, 11, 12]. However, lipid metabolism mechanisms in *S. podzolica* still have yet to be investigated. In conventional oleaginous yeasts, the lipid mechanism is usually considered to be that nitrogen limitation inhibits isocitrate dehydrogenase activity, raising cytoplasmic citric acid levels and boosting lipid formation in the high C/N ratio medium [13]. While even among closely related species and strains, there is enormous metabolic diversity like marked variances in xylose utilization, especially among non-conventional oleaginous yeasts [1]. Therefore, the focus of this study is on the mechanisms of carbon and nitrogen sources regulating lipid synthesis.

GATA-family transcriptional factor GAT1 plays a critical role in nitrogen sources response and utilization pathways, nitrogen catabolite repression (NCR) and target of rapamycin (TOR) pathway [14]. TOR pathway has been shown to regulate lipid metabolism in several studies [15]. For example, the mammalian TOR complex1 (mTORC1) modulates lipid homeostasis via regulating S6 kinase beta-1 (S6K) and transcription factor TEF3 [16]. Inhibition of the target of rapamycin complex 1 (TORC1) triggers lipid accumulation in fungi, and rapamycin (TORC1 inhibitor) therapy increases TAG accumulation in *S. cerevisiae* and oleaginous microorganisms (like *Chlamydomonas reinhardtii*, *Cyanidioschyzon merolae* , and *Trichosporon oleaginosus*) [17, 18, 19, 20]. Both TORC1 inhibition and deletion of *GAT*1, *GLN*3, and *SIT*4 in the TOR pathway affect lipid droplet replenishment in *S. cereviase* [17, 21]. In nitrogen-limited or non-preferred nitrogen source conditions, inhibited TORC1 interacts with Tap42–Sit4 complexes to dephosphorylate GAT1, and dephosphorylated GAT1 enters the nucleus to regulate NCR-related gene expression and improve nitrogen sources absorption [22]. Whereas, there is limited evidence that GAT1, a key nitrogen response protein, activates lipid metabolism-related genes directly.

Cell growth is linked to carbon and nitrogen metabolism. Carbon catabolite suppression (CCR) in yeast impacts carbon source uptake and utilization via the SNF1-MIG1 pathway [22]. The cross-talk between the TORC1 and SNF1-MIG1 pathways modulates cellular metabolic states [23]. During glucose replete growth, TORC1 is highly active to increase the anabolic process, while serine/threonine kinase AMP-activated protein kinase (AMPK, homolog protein of sucrose non-fermenting 1 (SNF1)) is relatively inactive. Upon glucose starvation, AMPK is activated to increase the catabolic process and inhibit mTORC1

[24, 25]. Deletion of the transcriptional repressor *MIG*1 also causes TOR pathway nutrient-sensing dysfunction in *Cryptococcus neoformans* [26]. Currently, little research has focused on how the TOR and SNF1-MIG1 pathways interact to modulate lipid metabolism, however, several genes in two pathways impact oleaginous yeast lipid synthesis. For instance, *SNF*1 and *MIG*1 deletion both improve *Y. lipolytica* lipid production [27, 28]. Active SNF1/AMPK could repress fatty acid and cholesterol synthesis by inactivating acetyl-coenzyme A carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) activities [29]. The C/N ratio of the fermentation medium is the most important parameter for lipid formation [30]. Therefore, unraveling the relationship between carbon and nitrogen source absorption will help us better comprehend lipid accumulation physiology.

In the current study, we explored how the transcription factor SpGAT1 with SpMIG1 regulated lipid metabolism in the promising non-conventional oleaginous yeast strain *S. podzolica* zwy-2-3. We tested biomass, lipid yield, and residual sugar in *S. podzolica* zwy-2-3 WT, $\Delta gat1$, and OE::*gat*1 strains which were cultivated at varying C/N ratio mediums. Then, with yeast one-hybrid and two-hybrid assays, we investigated whether lipid metabolism was influenced by SpGAT1 and the cross-talk between nitrogen and carbon metabolism.
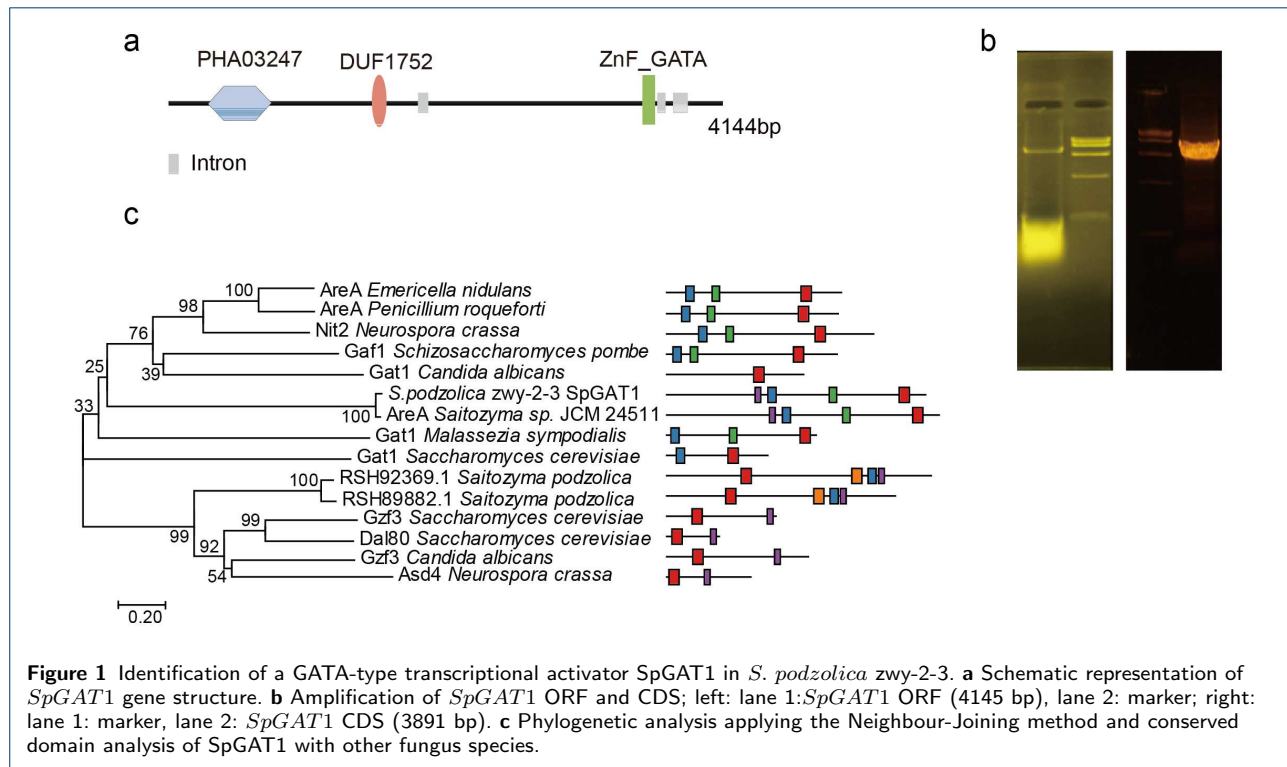
Results

Cloning, identification, and phylogenetic tree analysis of SpGAT1

SpGAT1 (GenBank accession number: RSH87488.1) was analyzed from the reference genome of *S. podzolica* DSM27192 strain to identify SpGAT1 in *S. podzolica* zwy-2-3. As shown in Fig. 1, SpGAT1 had two conserved domains, one of which was ZnF_GATA, a zinc-finger DNA binding domain (pfam: 00320), and the other of which was DUF1752 (pfam: 08550). SpGAT1 which cloned from *S. podzolica* zwy-2-3 was 4145 bp length and contained three introns and four exons. SpGAT1's CDS was 3891 bp (Fig. 1a,b), encoding 1297 amino acids with a predicted protein molecular weight of 135.23 kDa and an isoelectric point of 8.14. In addition, SpGAT1 was found to be most similar to AreA in *S. podzolica* JCM 24511 after phylogenetic analysis (Fig. 2c).

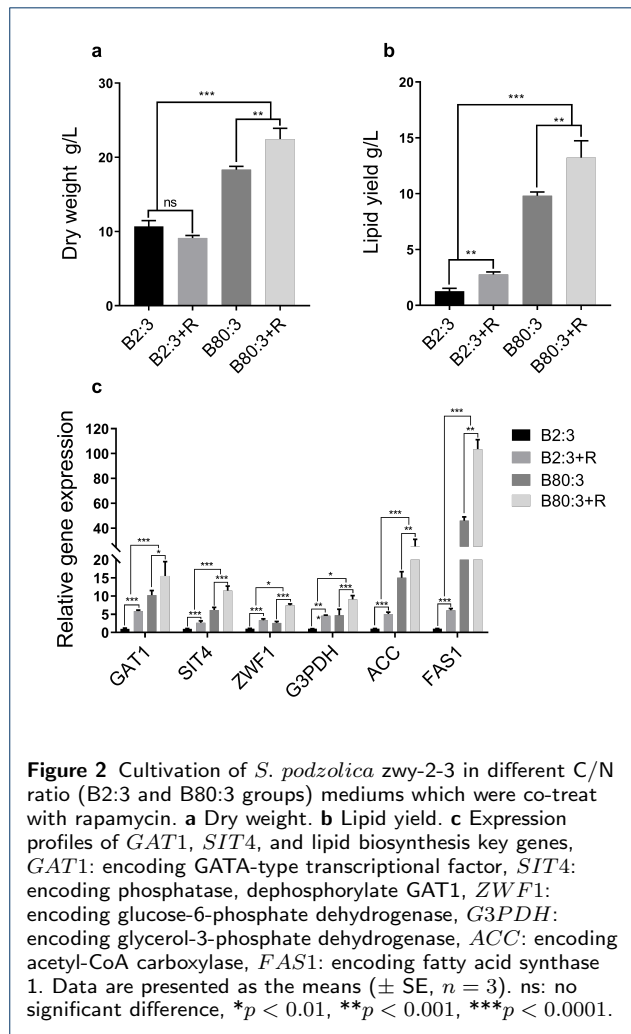### In *S. podzolica* zwy-2-3, the TOR pathway and NCR enhance lipid accumulation through SpGAT1

To determine if the TOR pathway and NCR enhance *S. podzolica* zwy-2-3 lipid production, we measured

Figure 1 Identification of a GATA-type transcriptional activator SpGAT1 in *S. podzolica* zwy-2-3. **a** Schematic representation of *SpGAT*1 gene structure. **b** Amplification of *SpGAT*1 ORF and CDS; left: lane 1:*SpGAT*1 ORF (4145 bp), lane 2: marker; right: lane 1: marker, lane 2: *SpGAT*1 CDS (3891 bp). **c** Phylogenetic analysis applying the Neighbour–Joining method and conserved domain analysis of SpGAT1 with other fungus species.

biomass and lipid yield in various C/N ratio mediums co-treated with rapamycin. Rapamycin treatment media (B2:3+R and B80:3+R) groups accumulated higher lipid than non-rapamycin treatment media (B2:3 and B80:3) groups. Low C/N media (B2:3 and B2:3+R) groups generated less lipid than high C/N ratio media (B80:3 and B80:3+R) groups. More specifically, after rapamycin treatment, the biomass of the B2:3 group decreased slightly from 10.69 g/L to 9.13 g/L, while the lipid yield increased significantly from 1.27 g/L to 2.76 g/L; the biomass of the B80:3 group increased from 18.33 g/L to 24.45 g/L, since the lipid yield increased significantly from 9.82 g/L to 13.22 g/L (Fig. 2a, b). The results of single cell lipid droplets stained with Nile red matched with those extracted by the Folch method (Fig. S1a). Fatty acid profiles in different groups did not differ significantly (Fig. S1b). In conclusion, our results indicated that the TOR pathway and NCR contributed to lipid accumulation.

To further confirm SpGAT1's role in the TOR pathway and NCR regulating lipid metabolism, we used qRT-PCR to analyze the transcription levels of *SpGAT*1, *SIT*4 (encoding PP2A-like protein phosphatase), and four genes encoding critical enzymes involved in lipid biosynthesis. *SpGAT*1 transcription level was strongly induced by rapamycin and nitrogen-limit treatment, and the relative expression levels of *SIT*4, *G3PDH* (encoding glycerol-3-phosphate dehydrogenase), *ZWF*1 (encoding glucose-6-phosphate dehydrogenase), *ACC* (encoding acetyl-CoA carboxylase), and *FAS*1 (encoding fatty acid synthase1) followed a similar expression trend with *SpGAT*1's (Fig. 2c). Specifically, when rapamycin was treated, all gene expression levels in the B2:3+R group generally increased in comparison to the B2:3 group. For example, *SpGAT*1 and *SIT*4 relative expression levels increased by 6 and 2.7 times, respectively; *FAS*1 and *ACC* increased by 6 and 5 times, respectively; *ZWF*1, which provides NADPH for lipid synthesis, increased by 3 times. In contrast with the B80:3 group, *SpGAT*1, *SIT*4, *FAS*1, *ACC*1 and *ZWF*1 relative expression levels in the B80:3+R group increased by 1.5, 2, 2.2, 1.7, and 7.4 times, respectively. In nitrogen-limit treatment groups, *SpGAT*1, *SIT*4 and *ZWF*1 relative expression levels were 10, 6, and 2.6 times higher than those in the B2:3 group, respectively. Strikingly, the relative expression levels of *FAS*1 and *ACC*1 in the B80:3 group were 46 and 15 times higher than in the B2:3 group, respectively. These results suggested that SpGAT1 might be activated by the TOR pathway and NCR to regulate lipid metabolism of *S. podzolica* zwy-2-3.

Figure 2 Cultivation of *S. podzolica* zwy-2-3 in different C/N ratio (B2:3 and B80:3 groups) mediums which were co-treat with rapamycin. **a** Dry weight. **b** Lipid yield. **c** Expression profiles of $GAT1$, $SIT4$, and lipid biosynthesis key genes, $GAT1$: encoding GATA-type transcriptional factor, $SIT4$: encoding phosphatase, dephosphorylate GAT1, $ZWF1$: encoding glucose-6-phosphate dehydrogenase, $G3PDH$: encoding glycerol-3-phosphate dehydrogenase, ACC: encoding acetyl-CoA carboxylase, $FAS1$: encoding fatty acid synthase 1. Data are presented as the means ($\pm$ SE, $n = 3$). ns: no significant difference, $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$.

SpGAT1's effects on lipid accumulation and the uptake and utilization of various carbon and nitrogen sources

To perform function analysis of SpGAT1 in *S. podzolica* zwy-2-3, a SpGAT1-knockout strain ($\Delta gat1$) and an overexpression strain (OE::$gat1$) were constructed. In the $\Delta gat1$ strain, $SpGAT1$ cannot be amplified by PCR and hygromycin had been inserted in the genome (Fig. S2a,b). qRT-PCR validated the OE::$gat1$ strain, and its $SpGAT1$ transcription level was enhanced 6-folds on the low C/N ratio medium compared to WT (Fig. S2c,d).

Then, in different C/N ratio mediums, we analyzed the biomass, lipid yield, and residual sugar of WT, $\Delta gat1$, and OE::$gat1$ to explore whether SpGAT1 had impacts on *S. podzolica* zwy-2-3 lipid metabolism (Fig. 3). In the B2:3 group, overexpression of $SpGAT1$ reinforced *S. podzolica* zwy-2-3 lipid accumulation. Because the biomass and glucose consumption rate of *S. podzolica* zwy-2-3 were dramatically decreased when $SpGAT1$ was knocked out, the B80:3 group's

lipid yield was drastically reduced. The biomass of WT, OE::$gat1$, and $\Delta gat1$ in the B2:3 group did not vary significantly, as shown in Fig. 3a. The biomass of $\Delta gat1$ declined from 20.6 g/L to 12.1 g/L in the B80:3 group as compared to WT, while OE::$gat1$ did not alter appreciably. SpGAT1 had distinct effects on lipid yield in media with high and low C/N ratios (Fig. 3b, c). The lipid yields of WT and $\Delta gat1$ in the B2:3 group were 1.02 g/L and 1.22 g/L, respectively, with no significant difference; however, the lipid yield of OE::$gat1$ soared to 3.24 g/L, and the lipid content increased from 9.9 % to 31.5 %. Unlike the B2:3 group, the lipid yields of WT, OE::$gat1$, and $\Delta gat1$ respectively were 11.05 g/L, 10.84 g/L, and 5.08 g/L, lipid content of $\Delta gat1$ declined obviously from 53.6 % to 41.4 %, while overexpression $SpGAT1$ had no significant impact on lipid accumulation in B80:3 group. Interestingly, the glucose consumption rates of the three strains were clearly dissimilar (Fig. 3d). The three stains all most exhausted glucose in the low C/N ratio medium; while in the high C/N ratio medium, residual glucose of WT was approximately 7.5g/L and OE::$gat1$ was about 2.98 g/L left, and glucose consumption rate of $\Delta gat1$, which had residual sugar of around 35.22 g/L, was much lower than WT and OE::$gat1$. In addition, like rapamycin and nitrogen-limit treatment, fatty acid profiles of $SpGAT1$ mutants revealed no significant differences with WT (Fig. S3a).

To further clarify how SpGAT1 impacts lipid metabolism, we analyzed relative expression levels of $FAS1$, $ZWF1$, $G3PDH$, and ACC in different C/N ratio mediums. SpGAT1 had a wide affect on several crucial lipid synthesis genes, as shown in Fig. 3e,f. In the B2:3 group, compared to WT, the transcription levels of $FAS1$, $G3PDH$, $ZWF1$, and ACC of OE::$gat1$ were significantly up-regulated 3.3, 2.1, 2.1, and 2.1 times, respectively; while the expression levels of these genes of $\Delta gat1$ versus WT were decreased by 0.14, 0.26, 0.59 and 0.22-folds, respectively (Fig. 3e). Except for transcription levels of $FAS1$ and ACC which were decreased by 0.57 and 0.31-folds, respectively. Lipid synthesis gene expression levels of $\Delta gat1$ in the B80:3 group exhibited only a slight change (Fig. 3f). qRT-PCR results were following the phenotypes of lipid yeild. Therefore, these results revealed that SpGAT1, as a transcription factor, could regulate lipid metabolism and the glucose consumption rate of *S. podzolica* zwy-2-3.

The capacities of the WT, $\Delta gat1$, and OE::$gat1$ growing on various nitrogen and carbon sources were assessed to further analyze the function of SpGAT1 in nitrogen and carbon absorption, as shown in Fig. S3. On the one hand, the effect of SpGAT1 on nitrogen absorption and uptake was related to the nitrogen

sources. Three strains grew well in the presence of preferred amino acids such as glutamine, glutamic acid, or inorganic nitrogen. However, the $\Delta gat1$ strain grew worse on non-preferred amino acids including serine, proline, methionine, tryptophan, isoleucine, and histidine (Fig. S3b). Despite this, the $\Delta gat1$ strain grew more quickly on arginine than the other two strains. On the other hand, there was no evident difference in the consumption rate of various carbon sources between OE::$gat1$ and WT. Whereas the $\Delta gat1$ strain used glucose, sucrose, mannose, xylose, and xylan at the slowest rate among the three strains (Fig. S3c). These results reflected that SpGAT1 affected the abilities of nitrogen and carbon uptake and $SpGAT1$ deletion might disrupt metabolism.

SpGAT1 promotes lipid accumulation by positively regulating key genes in the pyruvate bypass pathway and glyoxylate shunt pathway

SpGAT1-GFP subcellular localization was showed to be cytoplasmic, nuclear-cytoplasmic, or nuclear in Fig. S4. This discovery confirmed that SpGAT1 could distribute in the nucleus and bound to promoters to affect genes transcription and expression. Then we wondered whether SpGAT1 might directly activate transcript-level of genes associated with lipid biosynthesis, so we applied R package TFBSTools to screen the whole genome for genes with one or more binding sites on their promoters. After GO rich and conserved domains analyses, we ultimately selected six genes (ACS, encoding acetyl-CoA synthetase; $ICL1$ and $ICL2$, encoding isocitrate lyase1 and 2; $ACAT1$, encoding acetyl-CoA acyltransferase 1; $ACAT2$, encoding acetyl-CoA acyltransferase 2; PDC, encoding pyruvate decarboxylase) which had impacts on lipid metabolism.

We found SpGAT1 had distinct regulation patterns on these genes under different C/N ratio mediums (Fig. 4). SpGAT1 positively regulated ACS in the pyruvate bypass pathway and $ICL1/ICL2$ in the glyoxylate shunt pathway, irrespective of the nitrogen concentration. Besides, SpGAT1 could positively regulate $ACAT2$ expression only in a low C/N ratio medium (Fig. 4). In particular, yeast one-hybrid assays showed that SpGAT1 directly bound to promoters of $ACAT2$, PDC, ACS, $ICL1$, and $ICL2$, except $ACAT1$ (Fig. 4c). In the low C/N ratio medium, transcription levels of $ICL1$, $ICL2$, $ACAT1$, ACS, and $ACAT2$ of OE::$gat1$ increased (approximately 1.8, 3.1, 3.9, 1.8, and 2.7 times, respectively); whilst transcription levels of $ICL2$, $ACAT1$, ACS, and $ACAT2$ of $\Delta gat1$ reduced 0.4, 0.4, 0.5, and 0.2-folds versus WT, respectively. In the high C/N ratio medium, $ICL1$, $ICL2$, and ACS expression levels in OE::$gat1$

were significantly up-regulated 21, 2.8, and 4-folds, respectively, but expression levels of $ICL1$, $ICL2$, and $ACAT2$ of $\Delta gat1$ did not change significantly; ACS and $ACAT1$ expression levels both down-regulated about 0.5-folds. In the OE::$gat1$ and $\Delta gat1$ strains, PDC exhibited a special transcription pattern and its transcription level dropped 0.4 and 0.3-folds in the low C/N ratio medium and up-regulated by 8-folds and 83-folds in the high C/N ratio medium, respectively (Fig. 4a,b). Therefore, it was unclear how SpGAT1 regulated PDC.

SpGAT1 interacts with SpMIG1 to affect carbon source utilization rate to promote lipid metabolism

There was no overall substantial decrease in the expression levels of lipid biosynthesis key genes on the high C/N ratio medium (Fig. 3f and Fig. 4b), but a significant decrease in sugar consumption rate and biomass resulted in a major loss in lipid yield in the $\Delta gat1$ strain compared to WT (Fig. 3b). These findings demonstrated that transcripts of genes involved in lipid biosynthesis did not always correlate with lipid accumulation, suggesting that additional factors other than SpGAT1's direct activation might be engaged in SpGAT1's influence on lipid biosynthesis.

We questioned if SpGAT1 impacted the expression of genes involved in carbon uptake. Using *S. cerevisiae* as reference species, we predicted possible interactions between GAT1 and MIG1 in the String database (Fig. S5). Yeast two-hybrid assays revealed that SpGAT1 interacted with SpMIG1 (Fig. 5b). To learn more about how SpGAT1 regulates carbon metabolism, we examined $SpMIG1$ expression in the WT, $\Delta gat1$, and OE::$gat1$ strains in different C/N ratio mediums. Compared to WT, $SpMIG1$ expression in OE::$gat1$ was up-regulated to 2.7 and 2.0-folds, and $SpMIG1$ expression of $\Delta gat1$ was down-regulated to 0.1 and 0.5-folds in the B2:3 and B80:3 groups, respectively (Fig. 5a). To conclude, knockout $SpGAT1$ significantly reduced lipid accumulation by negatively regulating the expression of $SpMIG1$, which might impair glucose consumption rate in *S. podzolica* zwy-2- 3.

Discussion

In this study, the effects of SpGAT1, a GATA-type transcriptional activator, on carbon absorption and lipid biosynthesis in *S. podzolica* zwy-2-3 were investigated. As shown in Fig. 6, SpGAT1 could regulate carbon metabolism by interacting with SpMIG1; then, SpGAT1 could bind to promoters of key genes in the pyruvate bypass and glyoxylate shunt pathways to regulate cytoplasmic acetyl-CoA levels, and finally, SpGAT1 had different regulation patterns on
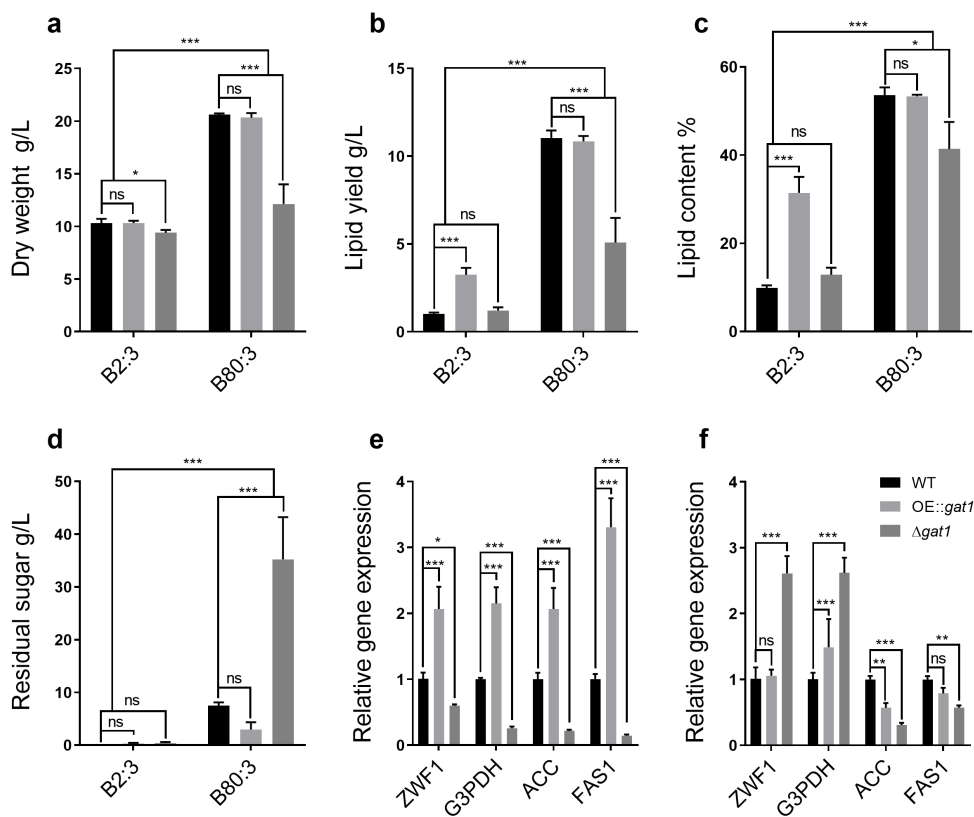
Figure 3 Effects of $SpGAT1$ mutants on cell growth and lipid biosynthesis in shake flasks. **a** Dry weight of WT, OE::$gat1$, and $\Delta gat1$ in the B2:3 and B80:3 groups. **b** Lipid yield of WT, OE::$gat1$, and $\Delta gat1$ in the B2:3 and B80:3 groups. **c** Lipid content of WT, OE::$gat1$, and $\Delta gat1$ in the B2:3 and B80:3 groups. **d** Residual glucose concentration in liquid supernatant of WT, OE::$gat1$, and $\Delta gat1$ in the B2:3 and B80:3 groups. **e** Expression profiles of $ZWF1$, $G3PDH$, ACC, and $FAS1$ of WT, OE::$gat1$, and $\Delta gat1$ in the B2:3 group. **f** Expression profiles of $ZWF1$, $G3PDH$, ACC, and $FAS1$ of WT, OE::$gat1$, and $\Delta gat1$ in the B80:3 group. $ZWF1$: encoding glucose-6-phosphate dehydrogenase, $G3PDH$: encoding glycerol-3-phosphate dehydrogenase, ACC: encoding acetyl-CoA carboxylase, $FAS1$: encoding fatty acid synthase 1. Data are presented as the means ($\pm$ SE, $n = 3$). ns: no significant difference, *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$.

S. podzolica zwy-2-3 carbon and lipid metabolism in low and high C/N ratio conditions.

SpGAT1 was found to regulate *S. podzolica* zwy-2-3 carbon source uptake and utilization to promote lipid synthesis, potentially by interacting with Sp-MIG1, a CCR transcription repressor(Fig. 6a). The $\Delta gat1$ strain had significantly lower biomass and glucose consumption ability, as well as worse growth on mannose, sucrose, xylose, and xylan carbon sources than WT and OE::$gat1$ (Fig. 3b,d and Fig. S3b). In *Fusarium graminearum*, *Pleurotus ostreatus*, and *Aureobasidium pullulans*, knockout of either $GAT1$ or $AreA$ also resulted in a profound decrease in growth rate [31, 32, 33]. Previous research has reported that SNF1-MIG1 pathway is a crucial regulatory pathway for glucose utilization, and deletion of $MIG1$ might hinder glucose metabolism capacity, resulting in a pro-

longed lag period [28, 34], while carbon absorption rate is strongly related to lipid synthesis [35]. Our results found that SpGAT1 interacted with SpMIG1 and deletion of $SpGAT1$ drastically lowered $SpMIG1$ expression (Fig. 5). Hence we hypothesized that Sp-GAT1 could regulate sugar utilization capacity and lipid accumulation by interacting with SpMIG1. Under low glucose concentrations, MIG1 is phosphorylated by activated SNF1 and localizes to the cytoplasm; conversely, under high glucose concentrations, SNF1 kinase is inhibited, leading MIG1 to be dephosphorylated and relocated to the nucleus, inhibiting downstream gene expression [36]. Reduced expression level of $SpMIG1$ caused by knockout $SpGAT1$ had an extremely pronounced effect on glucose utilization related genes in the high C/N ratio (high glucose concentration) medium, eventually leading to a decrease
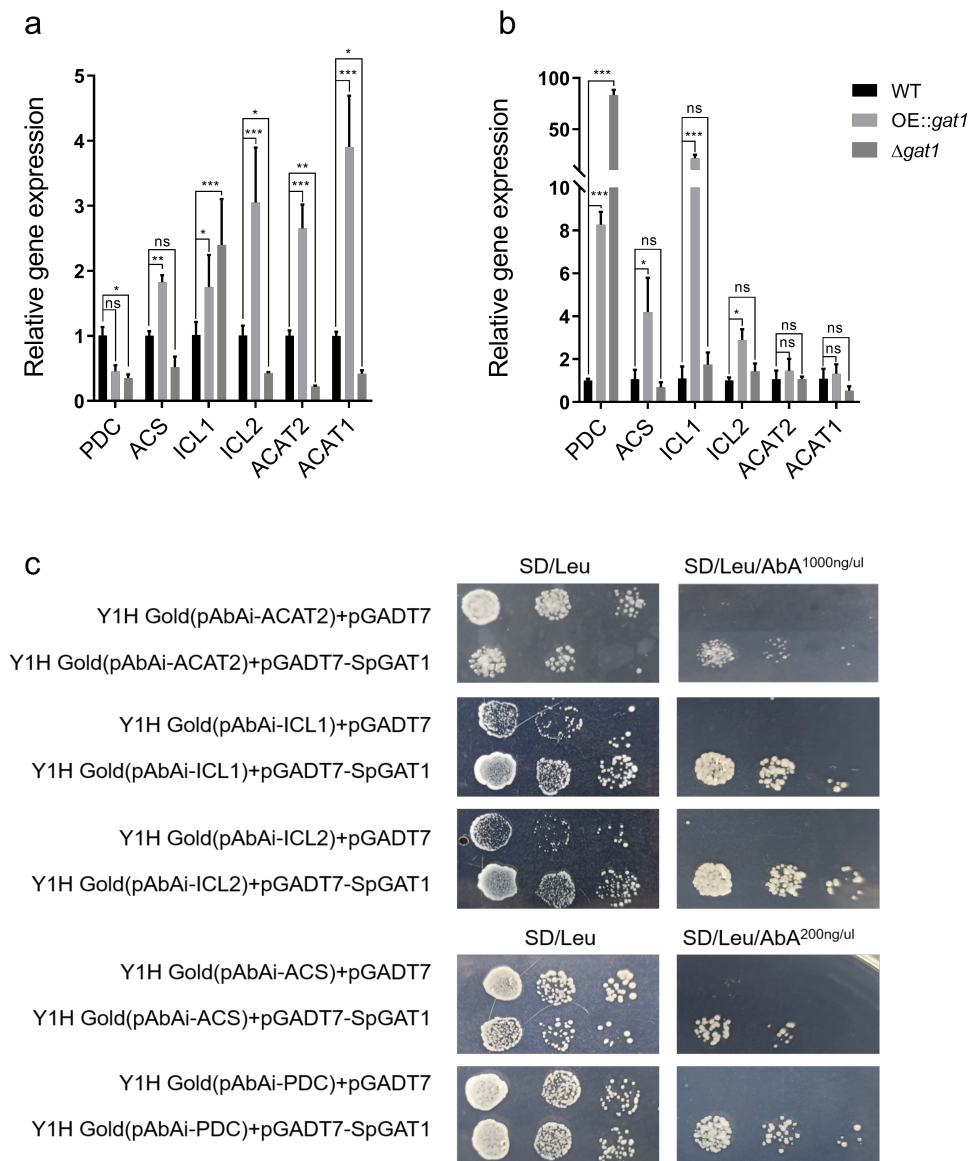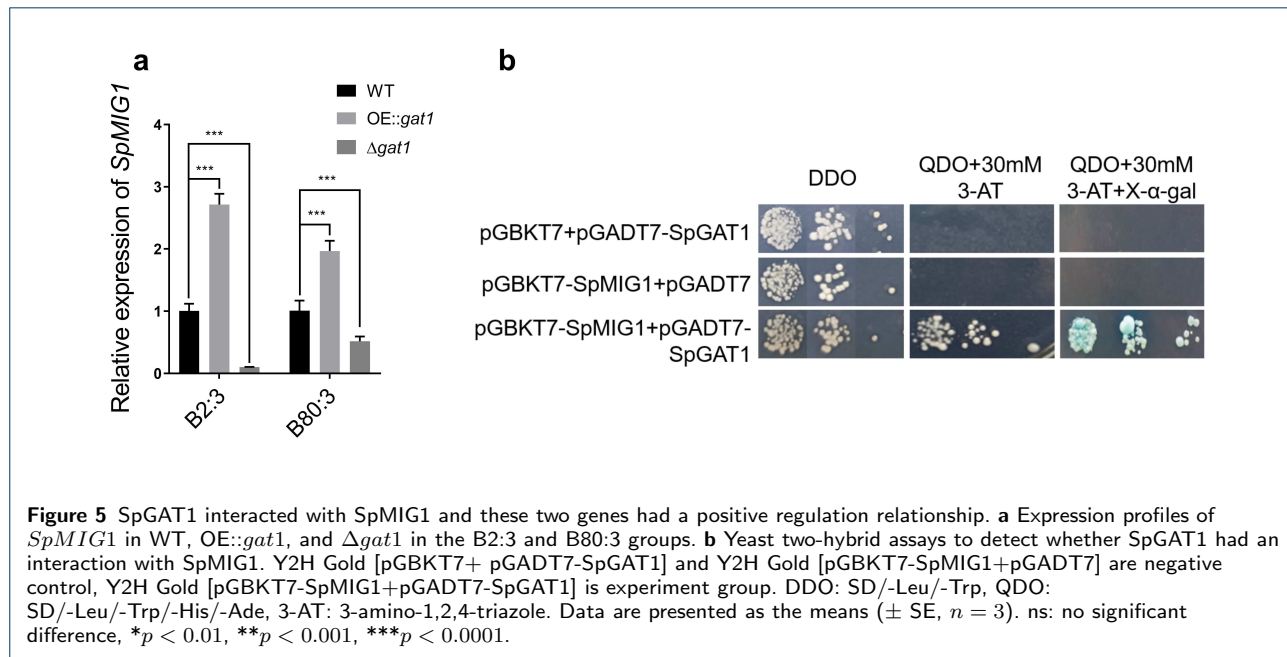
Figure 4 SpGAT1 directly bound to promoters of key genes in the pyruvate bypass pathway and the glyoxylate shunt pathway to promote lipid accumulation. **a** Expression profiles of PDC, ACS, $ICL1$, $ICL2$, $ACAT2$, and $ACAT1$ of WT, OE::$gat1$, and $\Delta gat1$ in the B2:3 group. **b** Expression profiles of PDC, ACS, $ICL1$, $ICL2$, $ACAT2$, and $ACAT1$ of WT, OE::$gat1$, and $\Delta gat1$ in the B80:3 group. PDC, encoding pyruvate decarboxylase; ACS, encoding acetyl-CoA synthetase; $ICL1$ and $ICL2$, encoding isocitrate lyase 1 and 2; $ACAT1$, encoding acetyl-CoA acyltransferase 1; $ACAT2$, encoding acetyl-CoA acyltransferase 2. **c** Yeast one-hybrid assays to detect whether SpGAT1 bound to promoters of PDC, ACS, $ICL1$, $ICL2$, $ACAT2$, and $ACAT1$. Y1H Gold [pAbAi-gene promoter + pGADT7] is the negative control, and Y1H Gold [pAbAi-gene promoter + pGADT7-SpGAT1] is the experiment group. Data are presented as the means ($\pm$ SE, $n = 3$). ns: no significant difference, *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$.

in total biomass and lipid yield (Fig. 3a,b); however, this phenomenon did not appear in the low C/N ratio (low glucose concentration) medium, probably because SpMIG1 was localized in the cytoplasm by SNF1 phosphorylation under low C/N ratio conditions. Therefore, SpGAT1 and SpMIG1 interaction revealed a tight

link between nitrogen source, carbon source, and yeast lipid metabolism.

SpGAT1 had different regulation patterns for lipid metabolism in low and high C/N ratio conditions. Our findings suggested that $SpGAT1$ transcription level was substantially positively related to lipid syn-

Figure 5 SpGAT1 interacted with SpMIG1 and these two genes had a positive regulation relationship. **a** Expression profiles of $SpMIG1$ in WT, OE::$gat1$, and $\Delta gat1$ in the B2:3 and B80:3 groups. **b** Yeast two-hybrid assays to detect whether SpGAT1 had an interaction with SpMIG1. Y2H Gold [pGBKT7+ pGADT7-SpGAT1] and Y2H Gold [pGBKT7-SpMIG1+pGADT7] are negative control, Y2H Gold [pGBKT7-SpMIG1+pGADT7-SpGAT1] is experiment group. DDO: SD/-Leu/-Trp, QDO: SD/-Leu/-Trp/-His/-Ade, 3-AT: 3-amino-1,2,4-triazole. Data are presented as the means ($\pm$ SE, $n = 3$). ns: no significant difference, *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$.

thesis genes expression levels, and lipid accumulation in both nitrogen limitation and rapamycin treatment indicated that SpGAT1 was likely to regulate lipid metabolism by NCR and TOR pathway (Fig. 2). But although, there were diverse regulation patterns, in a nitrogen-rich medium, overexpression of the $SpGAT1$ promoted lipid formation (Fig. 3b), which may raise the nuclear-located SpGAT1 levels and stimulate transcriptional activation of downstream genes. In particular, under nitrogen-rich conditions, lipid biosynthesis genes in the OE::$gat1$ strain were typically up-regulated as $SpGAT1$ expression levels increased. In contrast, same genes in the $\Delta gat1$ strain were generally down-regulated (Fig. 3e). As a result, the lipid yield of the OE::$gat1$ strain raised from 10 % to 30 % compared to WT (Fig. 3c). Under nitrogen-limited conditions, deletion of $SpGAT1$ affected nuclear localization of SpGAT1, which reduced transcriptional activation of downstream genes and lipid accumulation (Fig. 3b); however, only $FAS1$ and ACC expression were severely down-regulated in the $\Delta gat1$ strain (Fig. 3f), most likely owing to the compensatory effect of other nitrogen source response factors such as GLN3; the decrease in lipid yield of $\Delta gat1$ strain mostly due to a loss in biomass (Fig. 3a,b,c). Besides, because of $SpGAT1$'s high background expression level in the OE::$gat1$ strain, lipid synthesis genes were marginally up-regulated and lipid yield did not alter significantly in the high C/N ratio medium (Fig. 3b,f).

SpGAT1 bound to promoters of ACS in the pyruvate bypass pathway, $ICL1/ICL2$ in the glyoxylate shunt pathway, and $ACAT2$ in different patterns that were also dependent on the C/N ratios to modulate cytoplasmic acetyl-CoA levels. Among those genes, SpGAT1 positively regulated $ICL1$, $ICL2$, and ACS to increase lipid accumulation, irrespective of nitrogen concentrations (Fig. 4a,b). Increasing cytoplasmic acetyl-CoA levels, a crucial precursor of TAG production, is a smart way to boost lipid yield. Overexpression of ACS in *Chromochloris zofingiensis*, *C. reinhardtii* and *Schizochytrium sp.* TIO1101 greatly increased the intracellular acetyl-CoA levels [37, 38, 39]. The glyoxylate cycle pathway can recycle acetyl-CoA generated by fatty acid oxidation, with ICL catalyzing isocitrate acid to produce succinate, which speeded up the TCA cycle and accelerated citric acid storage in the cytoplasm, which could return to raise cytoplasmic acetyl-CoA levels [40, 41, 42]. Furthermore, SpGAT1 may influence the expression of $ACAT2$, which encoded a key enzyme for HMG-CoA production, to promote sterol ester (SE) synthesis only when the C/N ratio was low (Fig. 4a,c). Other research discovered that knocking out $ACAT2$ impeded the development of sheep precursor adipocytes, and knockout cells had less intracellular lipid droplets than $ACAT2$ overexpression cells and WT cells [43]. $RKACAT2$ overexpression increased carotenoid synthesis in *Rhodosporidium kratochvilovae*. Expressing a truncated $HMGR$ and $ERG10$ (homologous gene of $ACAT2$) in *S. cerevisiae* enhanced the mevalonate pathway and α-bisabolol titer by 2.9-folds [44]. The expression levels of $ACAT1$, $ACAT2$, $ICL1$, $ICL2$, ACS, and lipid synthesis-related genes in the $\Delta gat1$ strain were too low in the low C/N ratio medium (Fig.

3e and Fig. 4a), implying that the acetyl-CoA level used for lipid synthesis was already extremely low, and there was no more acetyl-CoA to consume to maintain *S. podzolica* zwy-2-3 metabolic homeostasis. In contrast, the levels of intracellular acetyl-CoA increased in the OE::*gat*1 strain because of increased expressions of *ACAT*2, *ACAT*2, *ICL*1, *ICL*2, *ACS*, and genes associated with lipid synthesis (Fig. 3e and Fig. 4a). Lipid accumulation requires a constant supply of carbon sources [45, 46], but the carbon source in the medium was virtually depleted at the late fermentation stage. Therefore, ACAT1 could promote fatty acid degradation and increased levels of β-oxidation, resulting in the release of acetyl-CoA. Finally, with positively regulating *ACAT*2, SpGAT1 returned acetyl-CoA to SE synthesis. However, the expression of *ACAT*2 did not change significantly in the high C/N ratio media (Fig. 4b), most likely due to the preponderance of TAG accumulation in the nitrogen-limit medium.

SpGAT1 not only regulated lipid and carbon metabolism but also influenced nitrogen uptake and utilization. On non-preferred nitrogen sources, the Δgat1 strain grew worse than WT and OE::*gat*1 (Fig. S3b). In *Candida albicans*, knockout *GLN*3 grew poorly on proline [47], while in *Aspergillus nidulans*, AreA (homologous protein of GAT1) nuclear localization signal sequence mutation dramatically influenced its growth under various nitrogen sources [48]. Therefore, it is probable that the deleting *SpGAT1* weakened the metabolic capacity to regulate nitrogen sources, making microorganisms impossible to properly utilize non-preferential nitrogen sources.

SpGAT1 enhanced lipid accumulation under high C/N ratio conditions by interacting with SpMIG1 and positively regulating *ICL*1, *ICL*2, and *ACS* (Fig. 6a). Under low C/N ratio conditions, SpGAT1 increased lipid yield by positively promoting expression levels of *ACAT*2, *ICL*1, *ICL*2 and *ACS* (Fig. 6b). This paper is the first to study how the transcription factor SpGAT1 regulated lipid metabolism and the interaction between SpGAT1 and SpMIG1, which might serve as a catalyst for much more research into lipid metabolism regulated by nitrogen and carbon metabolism crosstalk.

Conclusion
In conclusion, SpGAT1, a vital transcription factor of the NCR and TOR signaling pathway, could regulate lipid and carbon metabolism in *S. podzolica* zwy-2-3. On the one hand, SpGAT1 displayed divergent lipid metabolism regulatory patterns. *SpGAT*1 deletion reduced lipid yield by about 50 % in the high C/N ratio medium, but *SpGAT*1 overexpression boosted lipid yield by around 200 % in the low C/N ratio medium.

On the other hand, the transcription factor SpGAT1 regulated carbon uptake and utilization to promote lipid formation via interacting with SpMIG1, an important transcription factor of carbon metabolism. SpGAT1 also increased cytoplasmic acetyl-CoA level to promote lipid accumulation through positively regulating *ACS* and *ICL*1/*ICL*2 in the pyruvate bypass and glyoxylate shunt pathways, respectively. However, further research is necessary to confirm how SpGAT1 influences the utilization of carbon sources via SpMIG1. Overall, these findings add to our knowledge of nonconventional oleaginous yeast lipid metabolism.

Methods
Preparation of strains and inoculum
All experiments were performed using the yeast strain *S. podzolica* zwy-2-3 SCTCC300292 obtained from the Sichuan Province Typical Culture Collection center. YPD medium (called B2:3 medium in this paper, glucose 20 g/L, yeast extract 10 g/L, peptone 20 g/L) and nitrogen-limit medium (called B80:3 medium in this paper, glucose 80 g/L, yeast extract 3 g/L, $MgSO_4 \cdot 7H_2O$ 1.5 g/L) were used for cultivation. 25 mg/mL Rapamycin (Coolaber, BC grade) was dissolved in DMSO.
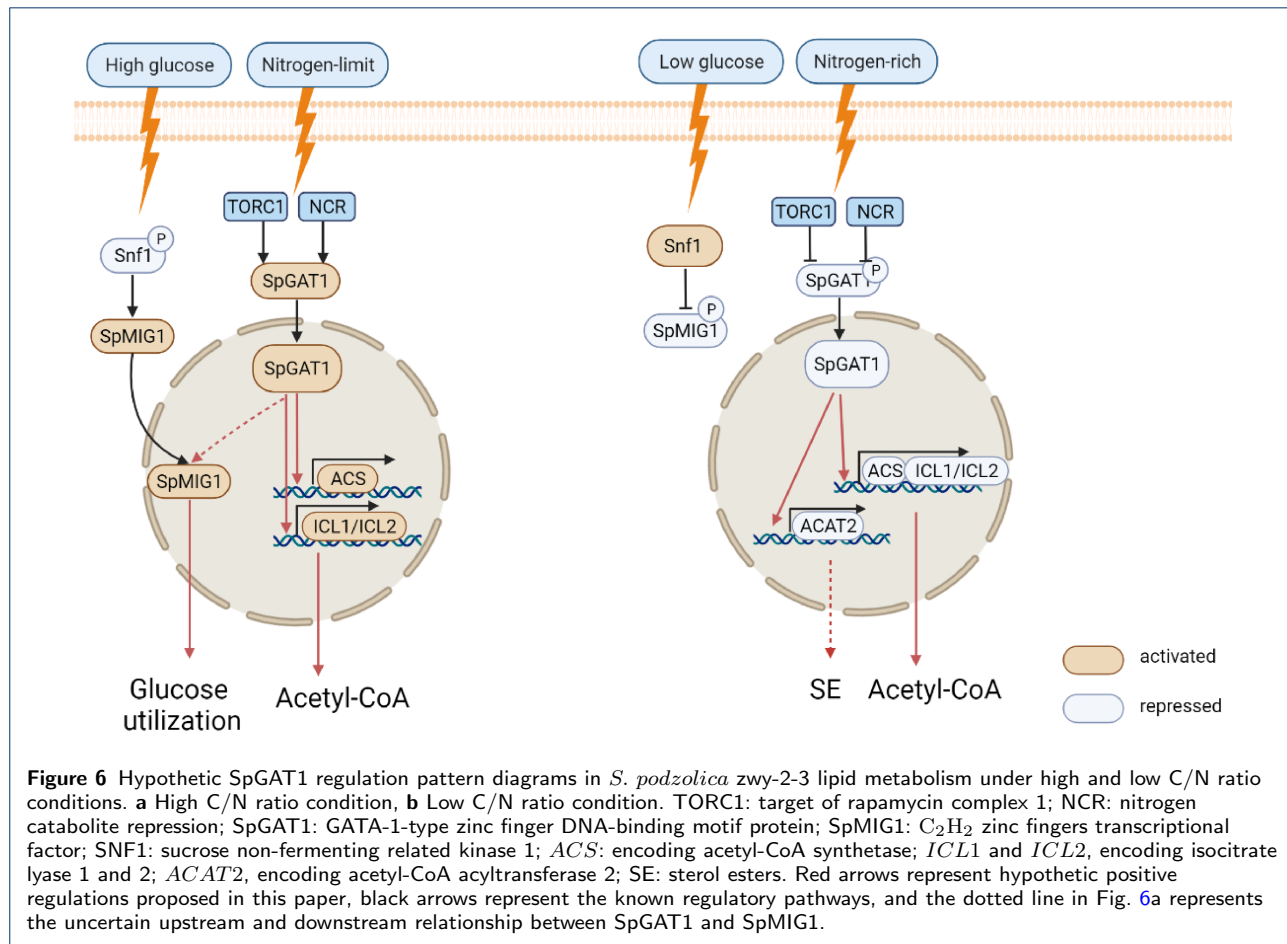
For pre-culture, seed cells were inoculated in YPD medium for 2 d at 28 °C, 180 rpm, then re-inoculated at a 1 % ratio into 50 mL YPD medium and cultured for 24 h. For fermentation, seed cells were inoculated in YPD medium and nitrogen-limit medium at a ratio of 2 % and cultured for 5 d in 500 mL baffled shake flasks. All the equipment was steam-sterilized at 121 °C for 20 min, and the medium was steam-sterilized at 115 °C for 15 min.

Determination of biomass and lipid
Cells were collected after fermentation by centrifugation (12000 rpm, 5 min) and dried to constant weight. Cellular total lipid was extracted by chloroform and methanol with the modified Folch method [12]. Drying cell pellets were pulverized and shaken for 12 h with 15 mL 4 mol/L HCl. Then, the mixture was then treated three times for ten minutes each in an ice bath and a boiling water bath. Subsequently, cell lysate was mixed with 10mL Folch reagent (2:1 chloroform/methanol) in a 50 mL tube and treated for 6 h. Finally, after centrifuging at 8000 rpm for 6 min, transferred chloroform layer into a clean tube and dried for 2-3 d.

Analysis of fatty acid composition by GC-MS
Methyl esterification of fatty acid: extracted yeast lipid(1 ul) was mixed with 2 mL hexane and 2 mL 0.6 mol/L $KOH-CH_3OH$ in a tube. Then, vortexed samples for 2 min and incubated for 15 min. Next, added

Figure 6 Hypothetic SpGAT1 regulation pattern diagrams in *S. podzolica* zwy-2-3 lipid metabolism under high and low C/N ratio conditions. **a** High C/N ratio condition, **b** Low C/N ratio condition. TORC1: target of rapamycin complex 1; NCR: nitrogen catabolite repression; SpGAT1: GATA-1-type zinc finger DNA-binding motif protein; SpMIG1: C_2H_2 zinc fingers transcriptional factor; SNF1: sucrose non-fermenting related kinase 1; ACS: encoding acetyl-CoA synthetase; $ICL1$ and $ICL2$, encoding isocitrate lyase 1 and 2; $ACAT2$, encoding acetyl-CoA acyltransferase 2; SE: sterol esters. Red arrows represent hypothetic positive regulations proposed in this paper, black arrows represent the known regulatory pathways, and the dotted line in Fig. 6a represents the uncertain upstream and downstream relationship between SpGAT1 and SpMIG1.

5 mL ddH$_2$O to allow the layers to separate, then hexane layers were transferred into a clean tube. GC-MS analysis: fatty acid methyl esters were analyzed on GC-MS-QP2010 and HP-88.

RNA extract and qRT-PCR gene expression

RNA extracted with TRIzol method. RNA reverse transcription method was according to Takara Primer ScriptTM RT reagent Kit with gDNA Eraser Reverse Transcription Kit instructions. The real-time RT-PCR method was according to Takara TB Green™ Premix Ex Taq™ II instructions and the CFX96 Real-Time PCR Detection System User Guide.

Identification, cloning, and bioinformatics analysis of SpGAT1

SpGAT1 was identified from the *S. podzolica* reference genome (DSM27192) because *S. podzolica* zwy-2-3 was earlier isolated from our laboratory without genomic data, its RNA-seq data haven't annotated and spliced the full length of *SpGAT1*. Then, RSH87488.1 in the DSM27192 strain was identified as SpGAT1. Using the CDS sequence of gene which encoded RSH87488.1 protein as a reference, primers were designed to clone the full-length SpGAT1 in *S. podzolica* zwy-2-3. Sp-GAT1 sequence was analyzed by bioinformatics after amplification, sequencing, and blast with RSH87488.1. The open reading frame of *SpGAT1* was predicted using ORF-Finder, the conserved structural domain was predicted using Pfam, and the isoelectric point and molecular weight of SpGAT1 were analyzed using the Expasy.

Construction of $\Delta gat1$ and OE::$gat1$ strains

To knockout *SpGAT1*, a 3373 bp fragment of *SpGAT1* extending from positions +270 bp to +3643 bp was replaced with hygromycin resistance cassette HYG based on a homologous recombination method. First, we generated the *SpGAT1* knockout cassette by overlapping the upstream and downstream homologous arms as well as the hygromycin resistance gene; then, ligating the *SpGAT1* knockout cassette to the pMD19T vector to construct the knockout vector KO-SpGAT1. After that, KO-SpGAT1 was transformed into *S. podzolica* zwy-2-3 competent cells by elec-

troporation. To construct the *SpGAT*1 overexpression strain, we cloned the *S. podzolica* zwy-2-3 own glyceraldehyde-3-phosphate dehydrogenase promoter and combined the promoter, the tCYC1 terminator, and the full-length of the *SpGAT*1 gene by overlapping PCR to construct the *SpGAT*1 overexpression cassette. Inserting the *SpGAT*1 overexpression cassette between the *Spe*I and *Hind*III sites of the prf-HU2 plasmid and transforming DH5α after recombinase ligation, then the successfully generated overexpression vector prf-HU2-OE-SpGAT1 transformed into the *S. podzolica* zwy-2-3 through Agrobacterium tumefaciens Mediated Transformation (ATMT). Both OE::*gat*1 and Δgat1 strains were screened on YPD with 100 μg/mL hygromycin. Verified right *SpGAT*1 knockout and overexpression strains with genomic PCR, the knockout and overexpression transformants were purified and rescreened on YPD (200 μg/mL hygromycin) medium.

Detection utilization abilities of different carbon and nitrogen sources

Cell suspensions of constructed mutant strains OE::*gat*1 and Δgat1 as well as WT strains were diluted at 1, 0.1, and 0.001 for serial dilution spot tests. For carbon utilization tests, cell suspensions were spotted on a B80:3 medium containing different carbon sources (glucose, sucrose, mannose, xylose, xylan) for 2 d; To test utilization of different nitrogen sources, cell suspensions were spotted on 10 % glucose YCB medium containing different amino acids and incubated for 3 d with 2 mM of each amino acid, 20 mM urea and 37 mM $(NH_4)_2SO_4$.

Subcellular localization of SpGAT1 and SpMIG1

To construct subcellular localization vectors, the full length of the *SpGAT*1 and *SpMIG*1 were amplified using the primers in Table. S2 and inserted between the pYES2-GFP plasmid *Kpn*I and the *Bam*HI digestion sites. Then constructed pYES2-SpGAT1-GFP and pYES2-SpMIG1-GFP vectors were transformed into BY4741 ($MAT\alpha$ $his3\Delta1$ $leu2$ $met15\Delta$ $ura3-52$) using the PEG/LiAc transformation method and spread on SD/-Ura plate to screen positive transformants.

To study the subcellular localization of transcription factor SpGAT1 and SpMIG1, BY4741-pYES2-SpGAT1 and BY4741-pYES2-SpMIG1-GFP were inoculated into SD/-Ura containing 2 % glucose until OD600 = 1.2-1.4 at 30 °C, 180 rpm; collecting cell pellet with 1000 rpm, for 5 min and incubating cells with SD/-Ura without sugar until intracellular glucose has been exhausted; then transferring cells to SD/-Ura containing 2 % galactose and incubated for 5 h.

Subcellular localization of SpGAT1 and SpMIG1 were detected under fluorescence microscopy after DAPI staining.

TFBS Tools predicts lipid metabolism genes regulated by transcription factor SpGAT1

Installed the R packages TFBS Tools, JASPAR2018, and Biostrings firstly, then used TBtools to extract gene promoter sequences from the reference genome DSM27192. The promoter sequences of genes with high scores (set score = "90 % ", strand = "+") were then screened as candidate genes; lastly, key lipid metabolism genes were rescreened from these candidate genes using the dynamic GO enrichment analysis tool on omicShare website.

Yeast one-hybrid and yeast two-hybrid assays

For yeast two-hybrid assays, refer to the Matchmaker® Gold Yeast Two-Hybrid System User Manual for operating instructions. First, *SpGAT*1 and *SpMIG*1 were cloned using the primers in Table. S2; then, *SpGAT*1 was inserted into the pGADT7 vector between the *EcoR*I and *Bam*HI digestion sites and *SpMIG*1 was inserted into the pGBKT7 vector between the *Nde*I and *Bam*HI digestion sites to form the bait and prey constructs. The negative control (pGADT7-SpGAT1 and pGBKT7) and (pGADT7 and pGBKT7-SpMIG1) and the experimental group (pGADT7-SpGAT1 and pGBKT7-SpMIG1) were co-transformed into Y2H according to the manufacturer's instructions (Clontech), and spread on SD/-Leu/-Trp to screen positive transformants; Finally, Y2H [pGADT7-SpGAT1+pGBKT7], Y2H [pGADT7 +pGBKT7-SpMIG1] and Y2H [pGADT7-SpGAT1+pGBKT7-SpMIG1] were diluted and spotted on DDO, TDO, QDO, and QDO+30mM 3-AT plates to detect the interaction of SpGAT1 with SpMIG1, respectively.

For yeast one-hybrid assays, refer to the Matchmaker® Gold Yeast one-hybrid Library Screening System User Manual instructions. pGADT7-GAT1 vector has been constructed in yeast two-hybrid assays, the promoter sequences of *PDC*, *ICL*1, *ICL*2, *ACAT*1, *ACS* and *ACAT*2 were inserted into the pAbAi vector. The negative control (pGADT7 and pAbAi-promoter) and the experimental group (pGADT7-GAT1 with pAbAi-promoter) were transformed into Y1H according to the manufacturer's instructions (Clontech); finally, the positive transformants were gradiently diluted and spotted on SD/-Leu and SD/-Leu/AbA to detect whether SpGAT1 bound to the promoter.

Appendix

Abbreviations
GAT1: GATA-1-type zinc finger DNA-binding motif protein, homologous protein of AreA; MIG1: C_2H_2 zinc fingers transcriptional factor, homologous protein of CreA; SNF1: Sucrose non-fermenting related kinase 1; TOR: Target of rapamycin; FAS1: Fatty acid synthase; ZWF1: Glucose-6-phosphate dehydrogenase; G3PDH: Glycerol-3-phosphate dehydrogenase; ACS: Acetyl-CoA-synthetase; ACC: Acetyl-CoA carboxylase; SIT4: Serine/threonine-protein phosphatase; ACAT1: Acetyl-CoA acetyltransferase 1; ACAT2: Acetyl-CoA acetyltransferase 2; ICL1: Isocitrate lyase 1; ICL1: Isocitrate lyase 1; PDC: Pyruvate decarboxylase; TAG: Triacylglycerol; SE: Sterol esters; NCR: Nitrogen catabolite repression; CCR: Carbon catabolite repression.

Availability of data and materials
All data generated or analyzed during this study are included in this published article and its Additional files.

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
All authors gave consent to publish the research in **Biotechnology for Biofuels**.

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Additional Files

Additional file 1

Table. S1 Strains and plasmids. **Table. S2** Primers for gene amplification and qRT-PCR. **Table. S3** cDNA sequence of $SpGAT1$ and $SpMIG1$.

Additional file 2

Fig. S1 Single cell lipid yield and fatty acid profile of $S. \, podzolica$ zwy-2-3 cultivated in rapamycin treatment and different C/N ratio mediums. **Fig. S2** Results of $SpGAT1$ mutant identification. **Fig. S3** Effects of SpGAT1 on fatty acid profiles and different carbon/nitrogen sources utilization in $S. \, podzolica$ zwy-2-3. **Fig. S4** Results of subcellular localization observed by fluorescence microscopy. **Fig. S5** Prediction of SpGAT1 interacting protein.

Supplementary Files

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