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Enhancement of biomass and lipid productivity by overexpression of a bZIP transcription factor in *Nannochloropsis salina*

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Abstract

Microalgae are considered as excellent platforms for biomaterial production that can replace conventional fossil fuel-based fuels and chemicals. Genetic engineering of microalgae is prerequisite to maximize production of materials and to reduce costs for the production. Transcription factors (TFs) are emerging as key regulators of metabolic pathways to enhance production of molecules for biofuels and other materials. TFs with the basic leucine zipper (bZIP) domain have been known as stress regulators and are associated with lipid metabolism in plants. We overexpressed a bZIP TF, NsbZIP1, in *Nannochloropsis salina*, and found that transformants showed enhanced growth with concomitant increase in lipid contents. The improved phenotypes were also notable under stress conditions including N limitation and high salt. To understand the mechanism underlying improved phenotypes, we analyzed expression patterns of predicted target genes involved in lipid metabolism via quantitative RT-PCR, confirming increases transcript levels. NsbZIP1 appeared to be one of type C bZIPs in plants that has been known to regulate lipid metabolism under stress. Taken together, we demonstrated that NsbZIP1 could improve both growth and lipid production, and TF engineering can serve as an excellent genetic engineering tool for production of biofuels and biomaterials in microalgae.

KEYWORDS
biofuels, bZIP transcription factor, lipids, microalgae, *Nannochloropsis salina*, TF engineering

1 | INTRODUCTION

Extensive utilization of petroleum-based fuels not only has caused extensive environmental harm in the form of pollution and climate change, it also poses a hazard to human health. With the growing realization that limited oil deposits are being depleted, development of alternative energy source is necessary. Such alternatives are biofuels produced by plants that fix carbon dioxide in the process leading to carbon neutral production. However, these first and second generation biofuels impose problems of agricultural and economic. In this regards, microalgae have emerged as the third generation biofuel feedstocks, which have much higher photosynthetic efficiency and biomass productivity (Amaro, Guedes, & Malcata, 2011; Scott et al., 2010).

Nannochloropsis spp. are photosynthetic unicellular microalgae in the class Eustigmatophyceae, and have received considerable attention as an industrial strain for the production of biodiesel (Ma, Chen, Yang, Liu, & Chen 2016). These strains have shown high lipid contents,

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rapid growth rate, and broad environmental tolerance (Wang et al., 2014). Research progress has been made in genomic (Radakovits et al., 2012), transcriptomic (Liang et al., 2013; Zheng et al., 2013), and lipidomic fields (Li et al., 2014). In addition, genetic transformation tools have been established employing homologous recombination, particle bombardment, and overexpression of genes related to lipid biosynthesis (Kang et al., 2015a; Kaye et al., 2015; Kilian, Benemann, Niyogi, & Vick, 2011). Genetic engineering of microalgae to improve production of biofuels and other biomaterials is being actively pursued. Currently most efforts are focused on metabolic enzymes, but overexpression of individual genes has not been successful to be employed in industrial production of biofuels from microalgae (Dunahay, Jarvis, & Roessler, 1995; La et al., 2012; Yao et al., 2014). This problem necessitates new approaches of microalgal genetic engineering for successful production of biofuels.

Recently, transcription factors (TFs) are emerging as key regulators of metabolism and stress tolerance, which can regulate multiple genes in metabolic and stress response pathways (Courchesne, Parisien, Wang, & Lan, 2009; Riano-Pachon, Correa, Trejos-Espinosa, & Mueller-Roeber, 2008). Among different types of TFs, those with the basic leucine zipper (bZIP) domain are potentially valuable for production of biofuels in microalgae. The bZIP domain is 60–80 amino acids long, and consists of a basic region for binding DNA and heptad repeats of leucine or other hydrophobic amino acids for dimerization (Jakoby et al., 2002). bZIP TFs are present in all eukaryotes, and play important roles in stress response and cell proliferation in lower eukaryotes including fungi and algae (Correa et al., 2008; Jindrich & Degnan, 2016). They can regulate underlying metabolic pathways of carbohydrates and lipids (Broeckx, Hulsmans, & Rolland, 2016), enabling them as prime targets for genetic engineering. Such "TF engineering" has advantage over the traditional engineering of metabolic enzymes in that the whole metabolic pathway can be regulated (Capell & Christou, 2004; Sornaraj, Luang, Lopato, & Hrmova, 2016; Wang, Wang, Shao, & Tang, 2016).

In fact, we have reported that overexpression of a basic helix loop helix (bHLH) TF can improve production of biomass and lipids in *Nannochloropsis salina* (Kang et al., 2015b). In an effort to find a better TF, we identified a bZIP TF postulated to be involved in regulation of multiple lipid metabolic genes in *Nannochloropsis oceanica* through elegant bioinformatics analyses by Jian Xu's group (Hu et al., 2014). We cloned a homolog of this bZIP TF in *N. salina*, named *NsbZIP1*, and overexpressed it in *N. salina* CCMP1776. As expected from the bioinformatic hypothesis (Hu et al., 2014), NsbZIP1 transformants showed improved lipid accumulation, and excitingly their growth was also increased. This kind of concomitant increase in growth and lipid content is desirable improvements that can offer greater potential for genetic engineering of microalgae for industrial production of biofuels and other biomaterials.

2 | MATERIALS AND METHODS

2.1 | Microalgal strain and culture conditions

N. salina CCMP1776 was obtained from National Center for Marine Algae and Microbiota (formerly Culture Collection of Marine Phytoplankton, CCMP). This strain was cultivated in filter-sterilized modified F2N medium (Kilian et al., 2011), containing sea salt (15 g/L; Sigma–Aldrich, St. Louis, MO), $NaNO_3$ (427.5 mg/L), $NaH_2PO_4 \cdot 2H_2O$ (30 mg/L), 5 ml/L trace metal mixture composed of Na_2 EDTA $\cdot 2H_2O$ (4.36 g/L), $FeCl_3 \cdot 6H_2O$ (3.15 g/L), $CoCl_2 \cdot 6H_2O$ (10 mg/L), $ZnSO4 \cdot 7H_2O$ (22 mg/L), $MnCl_2 \cdot 4H_2O$ (180 mg/L), $CuSO4 \cdot 5H_2O$ (9.8 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (6.3 mg/L), 10 mM Tris-HCl to maintain pH 7.6, and 2.5 ml/L vitamin stock containing vitamin B_{12} (1 mg/L), Biotin (1 mg/L), thiamine · HCl (200 mg/L) (Guillard & Ryther, 1962). WT and *NsbZIP1*-transformed *N. salina* were cultured under normal conditions (F2N medium), nitrogen limitation conditions (F2N medium made as reduction of $NaNO_3$ concentration to 75 mg/L), and high salt stress conditions (F2N medium with sea salt concentration increased to 50 g/L). Cells were grown at 25°C under fluorescent lights (120 µmol photons/m^2/s) with shaking at 120 rpm in 250-mL Erlenmeyer baffled flasks. Broth cultures were mixed by direct aeration with air containing 2% CO_2 at a constant flow of 0.5 vvm (volume gas per volume medium per minute).

2.2 | Growth analysis of batch cultures

Cells were inoculated at an optical density at 680 nm (OD_{680}) of 0.1, determined using a UV/Vis spectrophotometer (DU 730; Beckman Coulter, Brea, CA). Cell growth was analyzed by measuring cell density (in cells/ml) and dry cell weight (DCW). A hemocytometer was used to determine cell density. DCW was determined by weighing cells after filtering on GF/C filter papers (47 mm; Whatman, England), washing with deionized water, and drying at 105°C overnight.

2.3 | Construction of the pNsbZIP1 vector

For gene cloning, the coding sequence of *NsbZIP1* was amplified from *N. salina* cDNA with Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) using bZ1-F and bZ2-R primers (Table SI). PCR amplification was performed using the following conditions: 35 cycles of 98°C for 10 s, 58°C for 30 s, and 72°C for 1 min (30 s/Kb). After confirming the sequence of gel-purified DNA, the PCR product was cloned into the pNs301 vector backbone, amplified by bZ3-F and bZ4-R primers (Table SI). The pNsbZIP1 vector includes an endogenous TUB promoter and terminator necessary for *NsbZIP1* gene expression. The promoter and terminator of *UEP*, encoding the ubiquitin extension protein, were used for driving the expression of the Sh*ble* gene, a selection marker that confers resistance to Zeocin (Invitrogen, Carlsbad, CA). These vector was constructed using the Gibson assembly technique (Gibson et al., 2009).

2.4 | Particle bombardment transformation

Particle bombardment was performed following protocols reported earlier (Kang et al., 2015b; Zhang, Wu-Scharf, Jeong, & Cerutti, 2002). Briefly, the pNsbZIP1 plasmid was linearized using *Nco*I-HF, and coated onto microcarrier gold particles (Bio-Rad, Hercules, CA). For bombardment, 25 µl gold particles in 50% glycerol were mixed with 6 µl of concentrated DNA, 50 µl of 2.5 M $CaCl_2$, and 20 µl of 0.1 M

spermidine by vortexing. DNA-coated gold particles were washed with 70% ethanol and re-suspended in 100% ethanol. *N. salina* cells were cultivated to the mid-exponential phase, and 2×10^9 cells were layered on cellulose acetate membrane filters, which were placed on F2N agar medium containing ampicillin (1 µl/ml). Particle bombardment was conducted at a helium pressure of 700 psi and a target distance of 3 cm using a GDS-80 low-pressure gene-delivery system (Wealtec, Sparks, NV). After bombardment, cells on cellulose acetate membrane filters were released into F2N modified medium and incubated at 23°C under low-intensity light (10 µmol photons/m^2/s) for 1 day. Cells were harvested by centrifugation at 3500 rpm for 15 min and then plated on selective F2N agar medium containing Zeocin (2.5 µg/ml).

2.5 | Molecular assay of the transformants by genomic PCR and Western blotting

Molecular characterization of transformants were performed via genomic DNA PCR for the presence of the transgene and Western blotting for expression of the FLAG-tagged NsbZIP1 protein (Kang et al., 2015b). For genomic PCR, crude genomic DNA was prepared using Instagene Matrix (Bio-Rad) following manufacturer's manual. The Sh*ble* gene was detected using S1 (forward) and S2 (reverse) primers, and 18s ribosomal DNA was detected with SR6 (forward) and SR9 (reverse) primers (Table S1). The expected sizes of Sh*ble* and 18S rDNA PCR products were 357 and 380 bp, respectively. For Western blotting, 3×10^8 cells in the mid-exponential phase were used, and expression of FLAG-tagged NsbZIP1 was confirmed using the rabbit anti-FLAG-tag antibody (Cell signaling Technology, Danvers, MA) at a dilution of 1:1000, followed by horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Cell Signaling Technology). Signals were detected with enhanced chemiluminescence (ECL) and the ChemiDoc system (Bio-Rad).

2.6 | Quantification of neutral lipids and FAME

Neutral lipids in cells were stained with Nile Red and quantified following previously described protocols (Chen, Zhang, Song, Sommerfeld, & Hu, 2009). Briefly, cultures were diluted to the same OD_{680} (0.1) with deionized water, and 180 µl of cell suspensions were stained by incubating with 20 µl of a 5 mg/L solution of Nile Red in dimethyl sulfoxide (DMSO; Sigma–Aldrich) on a shaker at 37°C for 30 min in the dark. Relative fluorescence intensity was measured at excitation and emission wavelengths of 530 and 575 nm, respectively, using a multi-mode microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA). Neutral lipid was visualized by Leica DM2500 microscope equipped with EL6000 external light source (Leica Microsystems, Wetzlar, Germany) using I3 filter (excitation: BP 470/20 nm; emission: LP>515 nm). Quantitation of FAME was done using a modified Folch's method (Park et al., 2012), previously described in detail (Kang et al., 2015b).

2.7 | Quantitative real-time PCR (qRT-PCR)

Expression of NsbZIP1 and the target genes were quantitated with qRT-PCR following our previous report (Kang et al., 2015b). We analyzed

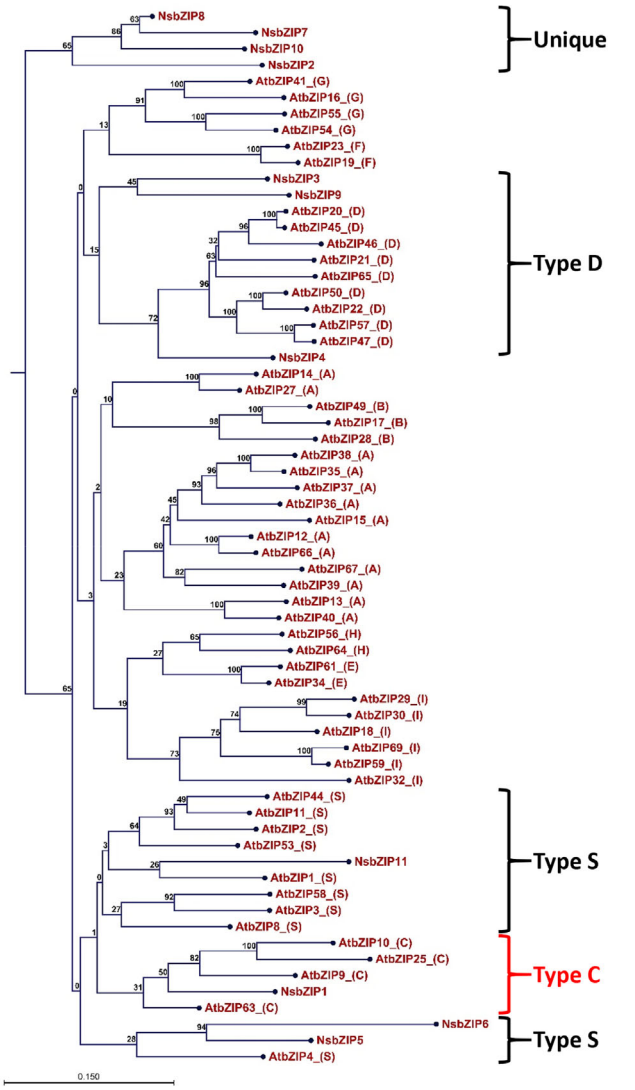

FIGURE 1 Phylogenetic tree of the *N. salina* and *Arabidopsis bZIP* genes. The phylogenetic tree of aligned sequences was constructed with the maximum likelihood method employing neighbor-joining provided in CLCbio Main Workbench. *NsbZIPs* used in this study are listed in Table S3, and *Arabidopsis* bZIPs were obtained from Jacoby et al (Jakoby et al., 2002) with corrections of some partial sequences

expression of transgenic *NsbZIP1* (using primers qFG-F and qFG-R), total *NsbZIP1* (primers qbZ1-F and qbZ1-R), and four target genes related to the lipid biosynthesis pathway. The four genes postulated to be the target of NsbZIP1 (Hu et al., 2014) were Acyl-CoA-binding proteins (ACBP), 3-ketoacyl-ACP synthase (KAS), long-chain acyl-CoA synthase (LC-FACS), and lysophospholipid acyltransferase (LPAAT) (Table S1). The housekeeping actin gene was used as a loading control.

3 | RESULTS

Identification of bZIP Genes in *N. salina* and phylogenetic analysis

TFs play important roles in regulating carbohydrate and lipid metabolism and stress responses, and elegant and pioneering work in

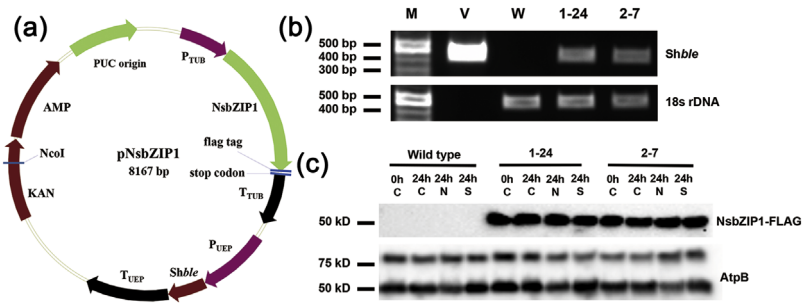

FIGURE 2 The pNsbZIP1 transformation vector and molecular identification of NsbZIP1 transformants. (a) Schematic map of the pNsbZIP1 vector. (b) Verification of Sh*ble* (357 bp) and 18s rDNA (380 bp) PCR products in the WT strain and NsbZIP1 transformants by agarose gel electrophoresis. (c) Western blot analysis of FLAG-tagged NsbZIP1 transformants. The band in NsbZIP1 transformants shows the expected molecular weight of 50 kD. The β-subunit of ATP synthase (ATP β, experimental control) was used as a loading control; expected sizes of 72.6 kD (F-type H-ATPase *ß* subunit) and 53.13 kD (CF_1 *ß* subunit of ATP synthase). WT, wild type; C, normal conditions; N, nitrogen-limitation conditions S, high salt conditions

Jian Xu's group postulated a few TFs that could regulate genes involved in lipid metabolic pathways in *N. oceanica* (Hu et al., 2014). A bZIP TF (s259.g7362) in particular was shown to presumably regulate multiple enzymes in the lipid biosynthetic pathway, and we decided to find the homolog of this gene in *N. salina* to test if this TF could improve lipid production. Due to lack of functional genomic resources in *N. salina*, we first searched the *Nannochloropsis gaditana* genomic resource CRIBI Genomics *Nannochloropsis* genome portal (http://www.nannochloropsis.org/) (Corteggiani et al., 2014), and identified 11 bZIPs. Due to close phylogenetic relationship between *N. gaditana* and *N. salina* (Radakovits et al., 2012), we were able to identify all 11 bZIP homologs in *N. salina*. We named these *NsbZIP1* through *NsbZIP11*, and *NsbZIP1* was closest to the *bZIP* (s259.g7362) in *N. oceanica* as summarized in Table S2.

bZIP TFs include multiple types with distinctive functions in *Arabidopsis thaliana* (Correa et al., 2008; Jakoby et al., 2002); we thus compared NsbZIPs with AtbZIPs to reveal their evolutionary relationship and possible functional mechanisms of NsbZIPs. Phylogenetic analyses were performed using AtbZIPs identified in *Arabidopsis* (Jakoby et al., 2002) together with our NsbZIPs identified in *N. salina* as shown in Figure 1. Probably due to heterogeneity of bZIPs in *Arabidopsis* and *Nannochloropsis*, we were not able to obtain high confidence bootstrap values for all branches of bZIPs. However, tentative grouping of NsbZIPs revealed four NsbZIPs unique in *N. salina*, three type D, three type S NsbZIPs, and NsbZIP1 belonged to the type C (Figure 1 and Table SII). Types C and S bZIPs are supposed to be evolutionarily related and they have been suggested to be originated from an ancient proto-C type bZIPs (Correa et al., 2008), and these bZIPs are known to regulate carbohydrate and lipid metabolism under stress conditions (Broeckx et al., 2016).

3.1 | Characterization of transgenic cells expressing NsbZIP1

We transformed the *NsbZIP1* expression vector (Figure 2a), pNsbZIP1, into *N. salina* by particle bombardment. Transformed cells were selected based on resistance to Zeocin conferred by the Sh*ble* marker gene, and the presence of Sh*ble* was confirmed by genomic PCR (Figure 2b). The NsbZIP1 coding sequence was fused with the FLAG tag (about 50 kD), and expression of NsbZIP1 was confirmed by Western blotting using an anti-FLAG antibody, which showed similar expression pattern (Figure S1). We also screened these transformants for their growth and FAME contents (Figure S2), and selected 1–24 and 2–7 for further characterization. Western blot analyses were performed with various conditions for the selected ones (Figure 2c), but we did not find any significant variation in expression pattern. We used these two transformants for further characterization, including their growth, lipid contents, and expression of their presumed lipid metabolic target genes.

3.2 | Growth of NsbZIP1 transformants under various culture conditions

To determine the effects of *NsbZIP1* overexpression on growth, we counted cells every day after inoculating cells at OD_{680} of 0.1 under normal, N limitation and high salt conditions (Figure 3). Overall, NsbZIP1 1–24 and 2–7 transformants showed better growth than the WT strain under all culture conditions, increasing by more than 25% compared to WT at day 12 under normal and high salt conditions (Figures 3a and 3c). The greatest difference in cell density between WT and the transformants was observed under N limitation conditions (Figure 3b), where the cell density of NsbZIP1 1–24 and 2–7 at day 8 increased by 81% and 44%, respectively.

This increased growth in the transformants positively contributed to biomass productivity (Table 1). Under normal condition, biomass productivity of NsbZIP1 1–24 and 2–7 was 22% and 17% higher than that of WT at day 12, respectively. Under N limitation condition, 1–24 and 2–7 showed 70% and 35% increase in biomass productivity on day 8, and 39% and 29%, respectively, on day 12 compared with those of WT. Under high salt condition at day 8, biomass productivity of the transformants was increased by more than 35%. Even though there was no statistical significance, transformants were higher than WT on day 12.

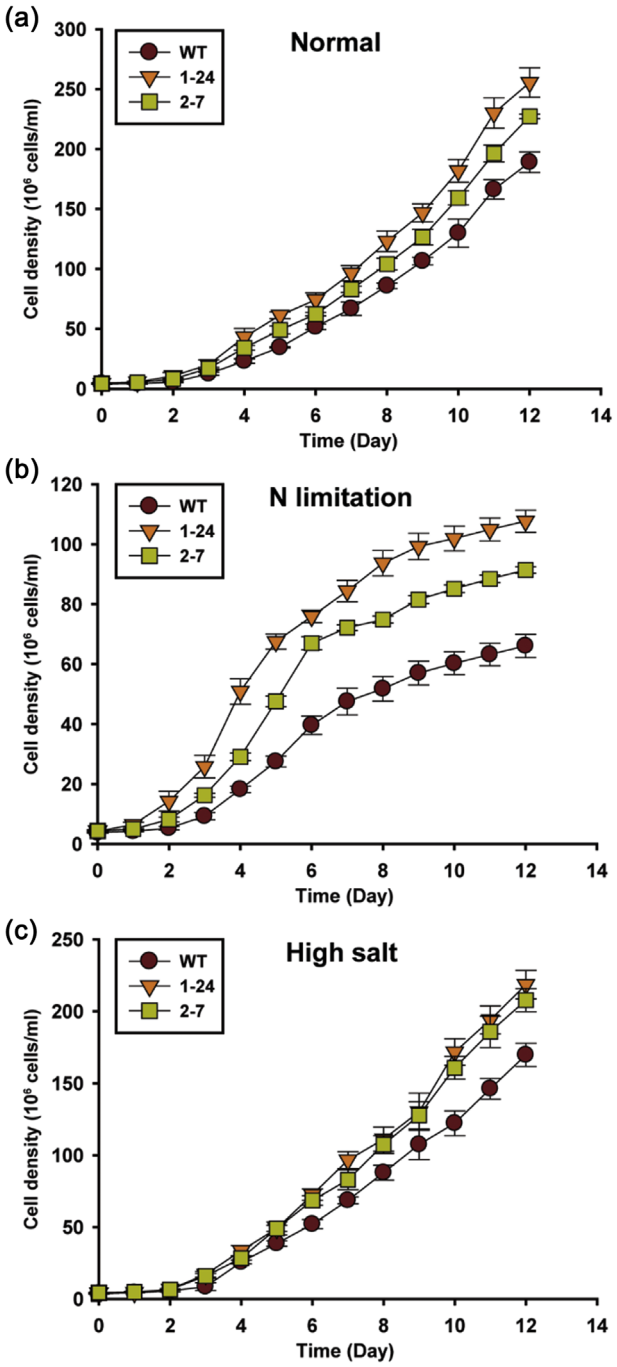

FIGURE 3 Growth of NsbZIP1 transformants based on the cell density. Cells were grown under (a) normal conditions, (b) N limitation conditions, and (c) high salt conditions at a light intensity of 120 µmol photons/m^2/s at 25°C with 2% CO_2. The data points represent the average of samples and error bars indicate standard error ($n = 4$)

To understand why NsbZIP1 transformants showed improved growth, we analyzed photosynthetic efficiency by estimating maximum quantum yield (Fv/Fm). NsbZIP1 transformants showed higher Fv/Fm (Figure S3) under all culture conditions, indicating that better photosynthetic efficiency compared to WT. It seems that NsbZIP1 improved photosynthetic efficiency resulting in enhanced growth of the transformants as reported for other microalgae (Perin et al., 2015; Shin, Lee, Jeong, Chang, & Kwon, 2016).

3.3 | Improvement in lipid contents in NsbZIP1 transformants

We analyzed relative contents of neutral lipids by staining cells with Nile Red. As shown in Figure 4, relative neutral lipid contents of NsbZIP1 transformants were increased in most conditions on day 12, while improvements were variable on days 4 and 8. Most consistent improvements were observed with the N limitation condition, in which neutral lipid contents were significant increased at all time points, even though increments were moderate on day 4. Under normal condition, only 2–7 showed significant increase (by 33%) at day 12 (Figure 4a), while both transformants showed significant increase at day 12 under high salt condition up to 203% increase (Figure 4c). Under the N limitation condition, WT and transformants showed robust accumulation of neutral lipids, and both transformants showed increase of up to 88% on day 12 (Figure 4b).

Individual classes of lipids were also analyzed by thin layer chromatography (TLC) mainly for triacylglycerol (TAG), followed by HPLC for different classes of lipids. Relative TAG contents by TLC were increased in both transformants compared to WT (Figure S4). Increased TAG contents were observed under all culture conditions, mostly under the high salt condition. We also fractionated and quantitated different classes of lipids including hydrocarbons, membrane lipids, and other storage lipids by using HPLC as summarized in Table S3. Under the normal condition, we were able to identify small fractions of hydrocarbons, free fatty acids (FFA), diacylglycerol (DAG), monoacylglycerol (MAG) and phospholipids, which did not seem to be affected in transformants. Glycolipids including mono- and digalactosyldiacylglycerol (MGDG and DGDG) were present at higher level, and transformants did not show significant difference compared to WT. In contrast, TAG contents were increased in transformants compared to WT, consistent with the TLC data. Interestingly, under nitrogen and high salt conditions, most classes of lipids disappeared, and we were able to detect only hydrocarbons and TAG. TAG contents were increased in transformants under only high salt conditions. In addition, we visualized neutral lipids in lipid bodies by staining cells with Nile red (Figure S5). We did not find any morphological alterations in the transformants, and NsbZIP1 transformants showed increased lipid bodies compared to WT.

We also measured FAME contents that can represent biodiesel contents if they were to be used for production of biofuels (Figure 5). Overall, consistent with neutral lipid contents as shown in Figure 4, increase in FAME contents of transformants were noticeable on day 12 (Figure 5b). Under normal conditions, NsbZIP1 1–24 and 2–7 showed 21% and 13% increase, respectively, in FAME contents on day 12. The difference in FAME content was more conspicuous under N limitation and high salt conditions. Under the N limitation condition, the FAME content of NsbZIP1 1–24 and 2–7 on day 8 was 39% and 18% higher relative to that of WT (Figure 5a), and were also higher on day 12 (Figure 5b). Under high salt condition, FAME content was

TABLE 1 Biomass and FAME productivity of NsbZIP1 transformants and *N. salina* WT

		Biomass productivity (mg/L/d)		FAME productivity (mg/L/d)	
Culture condition	**Strain**	**8 day**	**12 day**	**8 day**	**12 day**
Normal	WT	91.6 ± 8.5	151.1 ± 5.7	13.8 ± 1.9	29.6 ± 2.7
	1-24	115.6 ± 10.7	184.4 ± 9.1*	19.9 ± 1.1*	43.6 ± 3.6*
	2-7	102.1 ± 6.5	178.1 ± 7.1*	16.9 ± 1.3	39.3 ± 2.1*
N limitation	WT	70.8 ± 4.8	76.5 ± 2.5	26.1 ± 1.6	35.5 ± 0.9
	1-24	120.8 ± 1.7***	106.9 ± 2.4***	62.0 ± 2.1***	56.7 ± 2.1**
	2-7	98.9 ± 6.2**	99.3 ± 4.3**	43.4 ± 3.2**	48.9 ± 2.1***
High salt	WT	101.1 ± 9.9	167.7 ± 18.2	21.3 ± 3.3	41.0 ± 6.3
	1-24	134.4 ± 12.7*	205.2 ± 12.2	26.3 ± 1.6	60.9 ± 4.5*
	2-7	137.5 ± 11.2*	195.8 ± 16.0	26.7 ± 3.7	77.2 ± 11.7*

The data points represent the average of samples and error bars indicate standard error ($n = 4$). Significant differences, indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), were determined by Student's *t*-test.

higher in NsbZIP1 1–24 (23.2%) and NsbZIP1 2–7 (60%) at day 12 compared with WT (Figure 5b).

To simulate biodiesel production using NsbZIP1 transformants, we estimated FAME productivity by multiplying FAME contents by biomass (Table 1). Although FAME contents of transformants were not significantly increased under normal condition, FAME productivity was increased by 30% compared to WT at day 12 due to increased biomass productivity. Under N limitation conditions, NsbZIP1 1–24 showed remarkable increase in FAME productivity up to 137% and 59% on days 8 and 12, respectively. Transformants 1–24 and 2–7 also showed improvements in FAME productivity by 48% and 88% increase, respectively, on day 12 under high salt condition. We also assessed FAME composition (Table S4), and found no substantial differences between WT and transformants, suggesting that the total lipid contents were increased in transformants without changes in fatty acid profile.

3.4 | Expression of *NabZIP1* and target candidates involved in lipid biosynthesis

We analyzed transcript levels of *NsbZIP1* and its target genes predicted by previous report (Hu et al., 2014) with qRT-PCR (Figure 6) using primers listed in Table S1. RNA samples were harvested before (0 hr) and 24 hr after inoculation into normal, N limitation and high salt conditions. Expression of *NsbZIP1* was divided into total and transgenic by using primers that are common and unique to the transgene, respectively. Transgenic *NsbZIP1* was not detectable in WT, and all transformant samples showed expression of *NsbZIP1* (Figure 6A). Interestingly, transgenic *NsbZIP1* expression was highest under the N limitation condition, even though its expression was driven by the endogenous *TUB* promoter that is supposed to be a constitutive promoter. Total NsbZIP1 expression was detectable in WT, and NsbZIP1 transformants showed increased expression in all conditions (Figure 6B).

The bZIP homolog in *N. oceanica* was postulated to regulate ACBP (s080.g2727), KAS (s330.g9573), LC-FACS (s185.g5786), and LPAAT (s043.g2077), based on bioinformatics analyses of TF binding sequences (Hu et al., 2014). We identified homologs of the target candidates in *N. salina*, and analyzed expression of these genes with qRT-PCR as shown in Figure 6c–f. As expected with the presence of transgenic NsbZIP1, all four genes showed higher level of expression in the transformants than in WT. This increased expression of the target genes is consistent with the excellent bioinformatics predictions postulated by (Hu et al., 2014). Interestingly, target genes also showed highest expression under the N limitation condition in most cases, consistent with the expression pattern of transgenic *NsbZIP1*, which may have resulted in increased lipid accumulation under the same condition.

4 | DISCUSSION

We have successfully demonstrated overexpression of a bZIP TF can increase lipid contents without compromising growth. In fact, our *NsbZIP1* transformants showed enhanced growth under normal and stress conditions, which synergistically increased lipid productivity (Table 1). The increase in lipid contents was accompanied with increased expression of *NsbZIP1* transgene and its target genes that are involved in lipid biosynthetic pathways. This is a good example of TF engineering that can be employed in genetic engineering of microalgae for production of biofuels and other biomaterials. This TF engineering clearly showed correlation between solid increase in lipid contents and induction of multiple lipid metabolic genes, which distinguishes previous results of overexpression of individual metabolic genes.

TF engineering is an attractive approach for genetic improvements for production of biomaterials, which has been established in plants (Rasineni, Loh, & Lim, 2017; Shen et al., 2010). It was also postulated in the microalgal field (Bajhaiya, Ziehe Moreira, & Pittman, 2017; Courchesne et al., 2009; Hu et al., 2014), but the progress has been slow. We thus took the step into employment of TF engineering for production of lipids for possible production of biofuels and other high value product. We reported that overexpression of a bHLH TF, NsbHLH2, for improvement of biomass and lipid productivity in *N. salina* (Kang et al., 2015b). Such employment of TFs is better than

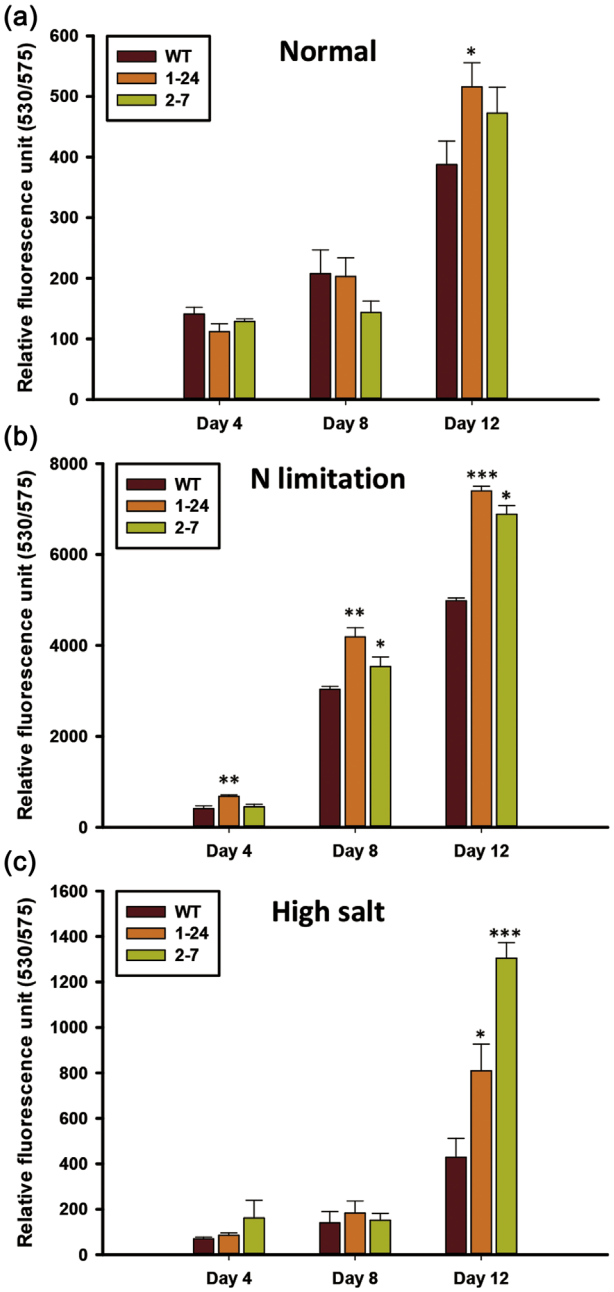

FIGURE 4 Analyses of neutral lipids of NsbZIP1 transformants. Cells were grown under (a) normal conditions, (b) N limitation conditions, and (c) high salt conditions at a light intensity of 120 µmol photons/m^2/s at 25°C with 2% CO_2. The lipid analyses were carried out days 4, 8, and 12. Relative Neutral lipid contents were determined by Nile red staining. The data points represent the average of samples and error bars indicate standard error ($n = 4$). Significant differences, indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), were determined by Student's *t*-test

overexpression of individual metabolic genes, because TFs can regulate the whole metabolic and other pathways by regulating involved genes. There are examples of overexpression of key metabolic genes failing to achieve improvements in production of the target molecules, which include acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS) (Blatti, Michaud, & Burkart, 2013; Gimpel, Henriquez, & Mayfield, 2015). The failure may be caused by complicated metabolic feedback mechanisms that can revert changes made by overexpression of a single metabolic gene. TF engineering is thus expected to overcome such difficulties in microalgae, and our data provide an excellent proof-of-concept that TFs can be used for genetic engineering of microalgae for production of sustainable biomaterials and biofuels.

NsbZIP1 was similar to the C type of bZIPs in *Arabidopsis* (Figure 1) (Jakoby et al., 2002), even though we are cautious about the assignment because of difficulties in our phylogenetic analyses. Heterogeneous bZIP sequences from far-related organisms were difficult to align and most of bootstrap values for the phylogenetic tree were low. However, our tentative classification of NsbZIP1 to type C was exciting in that type C bZIP TFs are involved in carbohydrate and lipid metabolism, stress responses, and other developmental processes in plants (Broeckx et al., 2016; Kusano, Berberich, Harada, Suzuki, & Sugawara, 1995; Peviani, Lastdrager, Hanson, & Snel, 2016). Actually, type C bZIPs are evolutionarily and functionally related to type S, and they are hypothesized to be originated from a proto-C type bZIPs from ancient algae (Correa et al., 2008). These C/S bZIPs are known to regulate metabolic regulation under stress conditions (Jakoby et al., 2002). Consistent with the functions in plants, NsbZIP1 enhanced lipid metabolic processes, and its effects were more pronounced under N limitation and high salt conditions in terms of not only growth/lipid contents (Figure 3–5) but also target gene regulation (Figure 6).

To confirm possibilities of NsbZIP1 for biofuel production, we analyzed biomass and FAME productivity. To our surprise, both *NsbZIP1* transformants showed improved growth and lipid contents. Previous studies of microalgae have reported inverse relationship between growth and lipid content, since stresses are in general required for lipid accumulation, but they suppress growth of microalgae (Pal, Khozin-Goldberg, Cohen, & Boussiba, 2011). We showed improved photosynthesis in transformants by measuring quantum yield (Figure S3), which can contribute to better growth. It is thus notable that NsbZIP1 increased lipid contents without sacrificing growth and biomass productivity, resulting in synergistically enhanced FAME productivity as summarized in Table 1.

In this study, we confirmed that NsbZIP1 plays important roles in regulating genes (*KAS1*, *LC-FACS*, *ACBP*, and *LPAAT*) involved in the lipid biosynthesis. These genes play an important role in lipid metabolism of microalgae (Bellou et al., 2014). It has been also demonstrated that the four genes in *N. oceanica* are up-regulated under N deprivation condition with concomitant increase in lipid accumulation (Li et al., 2014), consistent with our results. Our results showed that overexpression of NsbZIP1 increased expression levels of lipid biosynthetic genes, leading to improved lipid accumulation.

FAME contents in NsbZIP1 transformants did not show any difference to WT under normal and high salt condition on day 8 (Figure 5a), and showed delayed increase in FAME contents on day 12 (Figure 5b). This phenotype might be contradictory to the RNA expression of target genes that showed significantly increased accumulation of RNAs in both transformants under salt stress on day 1. However, many transcriptomic and RT-PCR data have shown

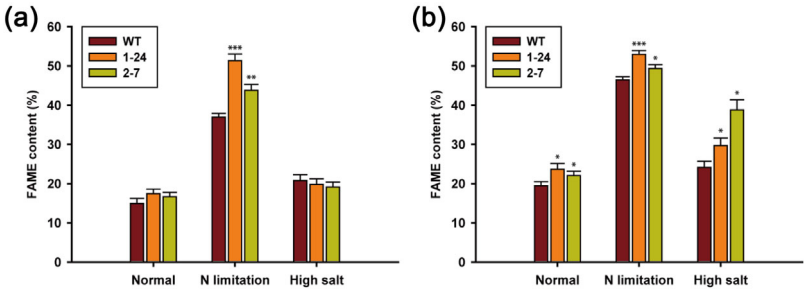

FIGURE 5 FAME contents in NsbZIP1 transformants under various culture conditions. FAME content and composition were obtained at day 8 (a) and day 12 (b). The data points represent the average of samples and error bars indicate standard error ($n = 4$). Significant differences compared to WT, indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), were determined by Student's t-test

that earlier expression of lipid metabolic genes, followed by delayed accumulation of lipids (Huang et al., 2016; Li et al., 2014; Wang et al., 2014). Our NsbZIP1 transformants showed delayed induction of FAME contents up to 12 days, which may be the characteristics of NsbZIP1 regulation. It should be noted that cultures did not reach the stationary phase by day 12 (Figure 3), but we did not continue experiments for longer cultivation time. We focused on optimum biomass and lipid productivities, which could be achieved by day 12.

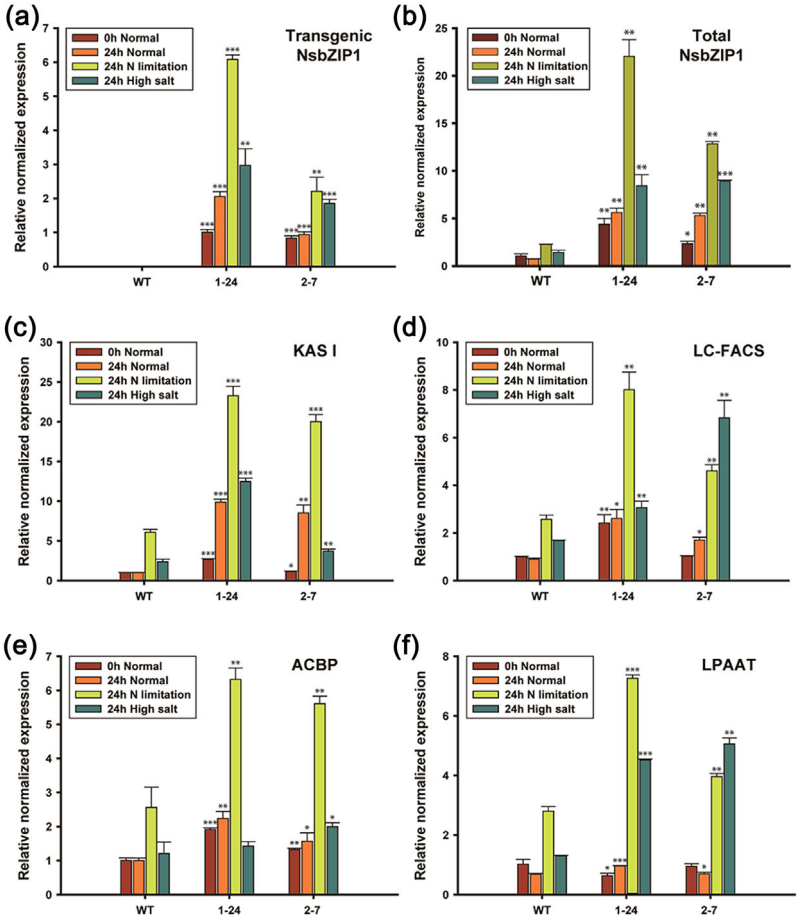

FIGURE 6 Expression of *NsbZIP1* and lipid metabolizing genes in NsbZIP1-overexpressing strains 1–24, 2–7 and the WT strain under different conditions. Gene expression in *N. salina* was measured by qRT-PCR. (a) Transgenic NsbZIP1 mRNA expression, determined using qFG-F and qFG-R primers. (b) Total NsbZIP1 mRNA expression, including both endogenous and transgenic, determined using qbZ1-F and qbZ1-R primers (c) Expression of KAS1 mRNA using qKAS-F and qKAS-R primers. (d) Expression of LCFACS mRNA using qLCFACS-F and qLCFACS-R primers. (e) Expression of ACBP mRNA using qACBP-F and qACBP-R primers. (f) Expression of LPAAT mRNA using qLPAAT-F and qLPAAT-R primers. All primer information was described in Table S1. The data points represent the average of samples and error bars indicate standard error ($n = 3$). Significant differences compared with WT, as indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), were determined by Student's t-test. KAS1, 3-ketoacyl-ACP synthase; LC-FACS, long-chain acyl-CoA synthase; ACBP, acyl-CoA-binding proteins; LPAAT, lysophospholipid acyltransferase

It is also noticeable that even though we overexpressed the *NsbZIP1* TF using constitutive TUB promoter, the expression levels were highest under N limitation condition. This phenomenon could be caused by integration sites of transgenes and culture conditions (Cao et al., 2012; Kang et al., 2015b; Nicot, Hausman, Hoffmann, & Evers, 2005; Rosic, Pernice, Rodriguez-Lanetty, & Hoegh-Guldberg, 2011; Silveira, Alves-Ferreira, Guimaraes, da Silva, & Carneiro, 2009). It was thus likely that increased NsbZIP1 expression levels affected expressions of the four target genes involved in lipid synthesis, resulting in enhanced lipid accumulation, especially under N limitation condition. Taken together, NsbZIP1 expression improved not only biomass productivity probably by improving photosynthetic efficiency, but lipid content by regulating lipid metabolic genes. This synergistic improvement of biomass and lipid productivity provides excellent proof-of-concept for genetic improvement of industrial microalgae, in which homologs of *NsbZIP1* can be employed in TF engineering for production of biofuels and other biomaterials.

5 | CONCLUSIONS

This study demonstrated that overexpression of NsbZIP1 TF in *N. salina* enhanced lipid production with increased growth. We showed that the increase in lipid contents were accompanied by increased expression of lipid biosynthetic genes that has been postulated to be the targets of the bZIP homolog identified in *N. oceanica* based on functional genomic analyses. Overexpression of NsbZIP1 can serve as a good example of TF engineering of other industrial microalgae for improvement of biomass and lipid productivities. This study also revealed importance of coordinated expression of multiple metabolic genes involved in certain pathways, rather than overexpression of individual genes.

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CONFLICTS OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

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