Received: 30 May 2020 | Accepted: 10 July 2020

DOI: 10.1111/gcbb.12746

ORIGINAL RESEARCH

GCB-BIOENERGY BIOPRODUCTS FOR A SUSTAINABLE BIOECONOMY Open Access

WILEY

A submerged duckweed mutant with abundant starch accumulation for bioethanol production

Yu Liu[1,2] | **Hua Xu**[3] | **Yu Wang**[3] | **Xianfeng Tang**[3] | **Guo He**[3] | **Shumin Wang**[3] | **Yubin Ma**[4] | **Yingzhen Kong**[5] | **Changjiang Yu**[3] | **Gongke Zhou**[1,2]

[1]College of Resources and Environment, Qingdao Agricultural University, Qingdao, China

[2]Academy of Dongying Efficient Agricultural Technology and Industry on Saline and Alkaline Land in Collaboration with Qingdao Agricultural University, Qingdao Agricultural University, Qingdao, China

[3]Key Laboratory of Biofuels, Qingdao Engineering Research Center of Biomass Resources and Environment, Shandong Provincial Key Laboratory of Energy Genetics, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao, China

[4]College of Marine Life Sciences, Ocean University of China, Qingdao, China

[5]College of Agronomy, Qingdao Agricultural University, Qingdao, China

Correspondence
Changjiang Yu, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, No. 189 Songling Road, Laoshan District, Qingdao 266101, Shandong, China.
Email: yucj@qibebt.ac.cn

Gongke Zhou, College of Resources and Environment, Qingdao Agricultural University, No. 700 Changcheng Road, Chengyang District, Qingdao 266109, Shandong, China.
Email: Zhougk@qau.edu.cn

Funding information
This research was funded by grants from the National Natural Science Foundation of China (NSFC) (No. 31700308, No. 51509237), "First class grassland science discipline" program of Shandong Province, Shandong Provincial Natural Science Foundation, China (No. ZR2017BC089, No. ZR2015PE012), Key project of STS in Chinese Academy of Sciences, Key projects of the Chinese Academy of Sciences (No. ZDRW-ZS-2017-2-1), and the Taishan Scholar Program of Shandong (to G.Z.).

Abstract

Duckweed is one kind of promising bioenergy plant with prominent advantages such as fast growth rate and high starch content. However, almost all previous studies focused on the natural duckweed germplasms. In this study, heavy-ion irradiation was used to establish a mutant library of *Lemna aequinoctialis* 6002, and one mutant named submarine-1 (*sub-1*) was screened, which could accumulate more starch but with smaller granules. Unexpectedly, under proper external growth conditions such as poor nutritional status and insufficient growth space, *sub-1* mutant would sink underwater due to formation of dense tissue structure and large amount of fine starch particles with the extension of cultivation time. The starch content in the sinking *sub-1* increased to over 45% (dry weight) and was 12% higher than the floating *sub-1*, highlighting that submergence can be considered as a spontaneous and efficient indicator for screening of high-starch duckweed. Additionally, the saccharification efficiency of starch and ethanol yield had increased in *sub-1* mutant compared to the wild type. Based on the unique characteristics of *sub-1* mutant, a cultivation model of submerged duckweed in a simulated aquaculture pond was designed to get more starch-rich biomass, enabling effective production of renewable bioenergy.

KEYWORDS

bioethanol, duckweed, heavy-ion irradiation, starch, *sub-1* mutant, submergence

Yu Liu and Hua Xu have contributed equally to this work.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2020 The Authors. *GCB Bioenergy* Published by John Wiley & Sons Ltd

1 | INTRODUCTION

Duckweed is a kind of aquatic plant commonly used for wastewater remediation. Various studies have reported that duckweed can efficiently remove nitrogen, phosphorus, and heavy metals in the water (Mohedano, Costa, Tavares, & Beli, 2012; Xu, Fang, et al., 2018; Xu, Yu, et al., 2018). In addition to its application in phytoremediation, recently the economic value of duckweed has attracted increasing attention. Duckweeds with high starch, high protein, or high flavonoid are screened from various species in the natural germplasms. Their applications in bioenergy, feed, and pharmaceutical production have been gradually exploited (Rival et al., 2008; Sonta, Rekiel, & Batorska, 2019; Tao et al., 2017; Yu et al., 2014). Undoubtedly, duckweed is becoming a novel and multipurpose crop in terms of resource providing and environment remediation.

As a renewable energy resource, bioenergy is considered as one of the most effective ways to ameliorate the climate change caused by carbon emission and reduce the dependence on fossil fuels (Ragauskas et al., 2006). Compared with the other potential feedstocks for bioethanol production, duckweed has several advantages, such as rapid biomass accumulation, high starch content and no demand for land used for food crops. Compared with lignocellulosic plants, the duckweed biomass is much less recalcitrant to saccharification because it contains low amounts of cellulose, hemicellulose, and lignin (Ge, Zhang, Phillips, & Xu, 2012; Zhao et al., 2014). Improvement of starch accumulation in duckweed biomass would make the biomass-to-ethanol conversion process more cost-effective. So far, significant progress has been made on the understanding of the mechanism of duckweed starch accumulation. The starch content is increased to 45.4% (dry weight, DW) after nutrient starvation for 7 days in *Landoltia punctata* (Tao et al., 2013). Light intensity and photoperiod significantly influence starch accumulation in *Lemna aequinoctialis* 6000 (Yin et al., 2015). Integrated analysis of transcriptome and metabolites revealed that most of metabolic pathways are inhibited except for starch metabolism under the N starvation treatment. The fixed CO_2 fluxes into the starch synthesis pathways, leading to the rapid accumulation of starch-rich biomass (Yu et al., 2017). The phytohormone abscisic acid dramatically promotes starch accumulation for 3.3-fold in *L. punctata* (Liu, Chen, et al., 2019). *Spirodela polyrhiza* turion, in place of seeds of terrestrial plants, generally contains over 65% starch (DW) and its formation is influenced or affected by phytohormone, spectral composition, and nutrients status (Wang & Messing, 2012; Xu et al., 2019). However, only natural duckweed species were used in all above researches.

As a supplement to the screening of natural germplasms, the transgenic technology and DNA mutagenesis technology are efficient and frequently used methods to obtain better genetic traits. Genetic manipulation has already been performed in some species in the duckweed family (Chhabra, Chaudhary, Sainger, & Jaiwal, 2011; Vunsh et al., 2007; Yang et al., 2018) including gene overexpressing, artificial microRNA-induced posttranscriptional gene silencing, and CRISPR/Cas9-mediated genome editing (Cantó-Pastor et al., 2015; Liu, Wang, et al., 2019; Yang et al., 2017). It can be anticipated that these approaches will definitely help us to uncover the mechanisms underlying starch accumulation in duckweed. The heavy-ion beam, as a kind of ionizing radiation, is able to induce double-stranded breaks of DNA in vivo, which mainly causes base substitutions, deletions, and insertions (Kazama et al., 2011). Compared with other physical mutagenesis, heavy-ion beams have high linear energy transfer (LET) and relative biological effectiveness, which will widen the mutation spectrum and increase the mutation frequency for plant breeding (Kazama et al., 2011). Besides, mutagenesis induced by the heavy-ion beam is nearly random in the rice genome and displays a moderate preference for cytosine and guanine nucleotides (Ichide, Morita, Shirakawa, Hayashi, & Abe, 2019). Recently, many new cultivars have already been developed through heavy-ion irradiation in microalgae (Ma et al., 2013), macroalgae (Hirano et al., 2019), ornamentals (Okamura et al., 2003), trees (Sasaki et al., 2018), and crops (Ichide et al., 2019). Moreover, new varieties from embryogenic calluses irradiated by heavy-ion beams have also been gained in various higher plants (Nakano et al., 2010). However, ion beam-irradiation has been rarely reported in duckweed.

In this study, heavy-ion irradiation mutagenesis was first performed on the embryogenic calli of *L. aequinoctialis* 6002, and the new plants regenerated were collected for screening. As expected, a high-starch duckweed mutant submarine-1 (*sub-1*) was obtained. The morphological variation, growth rate, photosynthesis efficiency, starch properties, saccharification, and ethanol yield of *sub-1* mutant were investigated. Based on the characteristics of *sub-1* mutant, we proposed a new model for the cultivating and screening of high-starch duckweed cultivars to improve the conversion efficiency and reduce costs for the bioethanol production.

2 | MATERIALS AND METHODS

2.1 | Plant and callus culture conditions

The duckweed used in this study was previously identified and named *L. aequinoctialis* 6002 (Liu, Wang, et al., 2019). *L. aequinoctialis* 6002 was cultured in Schenk & Hildebrandt (SH) liquid medium supplemented with 10 g/L sucrose, and maintained in a growth chamber at 24°C under a light intensity of 110 μmol m^{-2} s^{-1} and a photoperiod of 16 hr (light):8 hr (dark). Callus was induced from *L. aequinoctialis*

6002 fronds on the Murashige and Skoog medium containing 3% (w/v) sucrose, 0.1 mg/L thidiazuron and 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Embryogenic calli were produced after dark cultivation for 4 weeks, and developed calli were transferred to fresh induction medium every 4–5 weeks for sub-cultivation.

2.2 | Heavy-ion irradiation mutagenesis and mutant collection

The 3-week old duckweed callus was irradiated by 80 MeV/μ carbon ion, which was supplied by the Heavy-Ion Research Facility in Lanzhou, Institute of Modern Physics, Chinese Academy of Sciences. The average LET value was 31 keV/μm, and a series of irradiation dosages, including 10, 25, 50 and 75 Gy, were used for mutagenesis in vivo. Afterwards, the irradiated callus were transferred to the regeneration medium (Gamborg's B5 basal medium supplemented with 4.5 mg/L indole-3-acetic acid, 1 mg/L kinetin, 10 g/L sucrose, and solidified with 7.9 g/L agar), and cultivated under white light with a photoperiod of 16 (light):8 (dark). The regenerated fronds were transferred to SH solid medium supplemented with 10 g/L sucrose for conservation, and then newly grown fronds were proliferated on liquid SH medium. After 2 months of regeneration, collection, and propagation, a heavy-ion mutagenized duckweed mutant library was established.

2.3 | Growth performance of *sub-1* mutant

To determine the biomass accumulation, one colony (one plant formed by three fronds) of duckweed was put into SH liquid medium, and the fresh weight was determined after continuous cultivation for 2 weeks. Meanwhile, the number of duckweed colonies was recorded. The weight of one plant was calculated by dividing the total fresh weight by the number of plants. In a culture flask with a diameter of 6 cm, the surface of the SH medium was covered with a layer of duckweed. After weighing the total weight of duckweed, the output of each unit area was calculated.

To study the effect of nutritional status on the submergence of *sub-1* mutant, 4 g of duckweed freshly cultivated for 2 weeks was inoculated into 100 ml of SH medium, 1/4 SH medium, 1/16 SH medium, and distilled water, respectively, for 1 week. The fresh weight of the floating and submerged parts was measured separately. On the other hand, a similar approach was adopted regarding the growth space test. Four grams of duckweed freshly cultivated for 2 weeks was inoculated into a 100 ml or 1 L Erlenmeyer flask containing 100 ml of 1/4 SH medium for 1 week, and then those two parts of duckweed were separately weighed.

2.4 | Chlorophyll fluorescence parameter monitoring and photosynthetic pigment content determination

MAXI-Version of the IMAGING-PAM (Heinz Walz) was used to determine the duckweed chlorophyll fluorescence parameters including Fv/Fm (potential maximum quantum efficiency), Y (II) (quantum yield of PSII), and qN (non-photochemical quenching coefficient) following a reported method (Maxwell & Johnson, 2000). The photosynthetic pigments in the duckweed fronds were extracted with 80% acetone, and the absorbance at 470, 646, and 663 nm was measured by a spectrophotometry (GeneQuant 1300; GE Healthcare). The contents of chlorophyll *a*, chlorophyll *b*, and total carotenoids were calculated by the equations built up by Wellburn (1994).

2.5 | Starch content, morphology, and composition analysis

A modified method was used to determine the starch content in duckweed (Smith & Zeeman, 2006). Briefly, 50 mg freeze-dried powder of biomass was mixed with 1.5 ml 80% ethanol and then placed in a 70°C water bath for 15 min. The supernatant was removed by centrifugation at high-speed centrifuge. After repeating the above steps twice, the precipitate was transferred to volumetric flask and made up to 10 ml with deionized water. Then, 0.5 ml homogenate was put into a 2 ml EP tube and heated in a boiling water bath for 10 min. When cooled to room temperature, 0.5 ml 200 mM sodium acetate (pH 4.8) mixed with 1 μl α-amylase (Sigma A4582), and 1 μl amyloglucosidase (Sigma A7095) was added, and then incubated at 37°C for 4 hr for starch hydrolysis. After centrifugation, the glucose in the supernatant was measured with a glucose assay kit (Megazyme International), and the starch content was calculated by the equation: starch content = glucose content × 0.909; Zhang et al., 2011).

For extraction of starch granules, about 5 g fresh duckweeds was fully grounded in liquid nitrogen, and the powder was suspended in deionized water. The turbid liquid was filtered into a 50 ml centrifuge tube through four layers of gauze. Subsequently, Miracloth (Merck KGaA) was used to filter it again into another new 50 ml centrifuge tube. After standing for 1 hr, a white precipitate would appear at the bottom of the centrifuge tube. And then the supernatant was removed and the pellets containing over 70% starch granules were dried in a 50°C oven. The morphology of starch granules was observed by a scanning electron microscope (S-4800; Hitachi).

The Amylose/Amylopectin Assay Kit (Megazyme International) was used to determine the content of amylopectin and amylose following the manufacturer's instruction. The

amylopectin content was calculated by subtracting amylose from the total starch.

2.6 | Visualization of stomata in mutant

The fresh duckweed fronds of floating *sub-1* mutant (*f-sub1*) and sinking *sub-1* mutant (*s-sub1*) were carefully mounted on the copper stage with glue, immediately frozen with liquid nitrogen, sprayed with gold, and observed under low vacuum mode used a scanning electron microscope JSM-6360LV (JEOL).

2.7 | Total glucose assay, enzymatic saccharification, and ethanol fermentation

Freeze-dried duckweed biomass powder was subjected to a series of organic reagent extraction before acid hydrolysis. Briefly, about 60 mg powder was extracted in turn by 80% ethanol, chloroform:methanol (2:1 v/v:), 65% ethanol, 80% ethanol, 100% ethanol, and 100% acetone, and the remaining material is considered as alcohol insoluble residue (AIR). Subsequently, 72% (w/w) H_2SO_4 was used for acid hydrolysis of AIR, according to the previously reported method (Moxley & Zhang, 2007). After neutralizing the hydrolysate to pH 6 using calcium carbonate, the glucose in hydrolysate was determined with a D-Glucose Assay Kit (Megazyme International).

According to a modified method (Yu et al., 2014), one-step hydrolysis was used for enzymatic saccharification. Briefly, 50 mg lyophilized powder of duckweed was mixed with 1 ml 25 mM NaOAc (pH4.8), and incubated at 100°C for 10 min. After cooling down on ice, 0.5 μl α-amylase (Sigma A4582), 0.5 μl -amyloglucosidase (Sigma A7095), and 0.5 μl pullulanase (Sigma P1067) were added into the mixture, and then incubated at 50°C with shaking at 250 rpm for 30 hr. The released glucose was determined with a D-Glucose Assay Kit (Megazyme International).

Ethanol fermentation was carried out in micro-bioreactors using a yeast stain reported by our previous work and the micro-bioreactor was designed in the way reported previously (Ge et al., 2012; Yu et al., 2014). Yeast cells were inoculated into 500 μl each of hydrolysates (from enzymatic saccharification) in the micro-bioreactors without additional nutrients. The micro-bioreactors were placed in an incubator and fermented at 30°C with shaking at 300 rpm for 30 hr. Ethanol in the fermentation solution was quantified by gas chromatography (7890A; Agilent Technologies; Chen et al., 2012).

2.8 | Statistical analysis

The data were presented as the mean ± *SD* triplicate. SPSS 19.0 (SPSS Inc.) was used for statistical analyses, and *p* values were calculated using one-way ANOVA. Asterisks denote significant differences between two groups of data (*$p < .05$; **$p < .01$). Significant differences are shown by different letters in the amylose/amylopectin content assay, enzymatic saccharification, and ethanol fermentation analysis ($p < .05$).

3 | RESULTS

3.1 | Heavy-ion irradiation mutagenesis and mutant screening

Heavy carbon ion beam was used to irradiate the embryogenic calli originally induced from fronds of *L. aequinoctialis* 6002 (Figure 1a). Afterwards, the irradiated calli were transferred to the fresh regeneration medium for new plant production. There was no obvious difference in appearance of the duckweed callus under the 25 Gy irradiation dosage initially (Figure 1a,b). However, during cultivation in the regeneration medium, the pale-yellow callus turned green and swelled within 1 week (Figure 1c), and then the regenerated fronds emerged from the third week to the fifth week, depending to the exposed intensity of the heavy-ion beam (Figure 1d). Whenever a complete frond appeared, it was transferred to the SH medium for propagation and phenotype identification (Figure 1e). The number of regenerated fronds gradually decreased with increased irradiation intensity, suggesting that genomic DNA damage caused by heavy-ion irradiation significantly affect the differentiation of duckweed callus. As shown in Figure 1f, when the irradiation intensity reached 100 Gy, the mortality rate exceeded 80%, and no new regenerated plants appeared. Finally, all the new varieties were collected and a *L. aequinoctialis* 6002 mutant pool was formed.

After heavy-ion mutagenesis, 83% of the mutants in the pool changed regarding to the starch content. The number of mutants with significantly increased starch contents exceeded half of the total mutants (Table 1). Moreover, new varieties with an over 30% increase in starch content were obtained under each irradiation intensity, suggesting that heavy-ion radiation could be an efficient method at least for producing duckweed of high starch level. Although higher irradiation intensity will cause more dramatic mutations, irradiation intensity below 100 Gy was more suitable for *L. aequinoctialis* 6002 based on our data (Figure 1f). Fortunately, a new variety with a starch content more than two times higher was obtained under the 75 Gy irradiation dosage, which was named as *sub-1* and characterized in the following analyses (Table 1).

3.2 | Biological characteristics of *sub-1* mutant

There are clear phenotypic differences between *sub-1* mutant and wild-type *L. aequinoctialis* 6002 (Figure 2a,b).

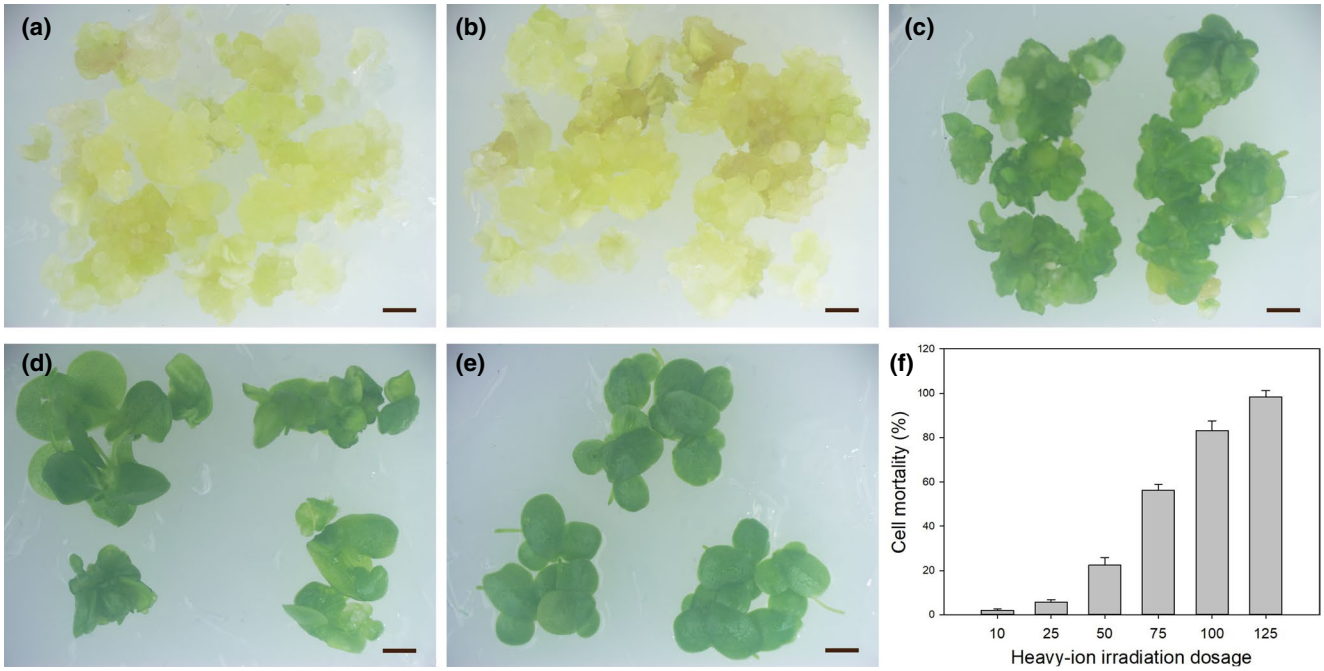


FIGURE 1 Regenerated mutants of *Lemna aequinoctialis* 6002 by heavy-ion irradiation. (a) Three-week-old callus before irradiation; (b) callus irradiated by 75 Gy carbon irons; (c) callus differentiation for 1 week; (d) frond regeneration; (e) cultivation of new varieties; (f) cell mortality with different radiation dosage. Scale bars = 1 mm

TABLE 1 Statistical analysis of the starch contents in the mutants regenerated from heavy-ion irradiated callus

	Number of mutants					
			Increase			
Irradiation intensity (Gy)	No change	Decrease	<10%	10%–30%	30%–60%	>60%
10	6	11	12	7	2	0
25	4	18	2	4	1	0
50	6	5	5	3	1	0
75	0	2	2	2	0	1

Note: The starch content was determined as described in Section 2, and then the differences of the starch contents in these mutants with that in wild-type *Lemna aequinoctialis* 6002 were calculated.

Compared with the wild type, the fronds of *sub-1* mutant were significantly smaller. Moreover, the fronds of *sub-1* mutant became light green, no matter whether they were parent or daughter fronds. *L. aequinoctialis* 6002 usually has one root on the abaxial surface of the mature frond. However, the root length of *sub-1* mutant was remarkably shorter than the wild type, and the root was almost disappeared in some fronds of *sub-1* mutant (Figure 4c), suggesting that heavy-ion irradiation dramatically affected the root occurrence and elongation of duckweed. In addition, asexual reproduction of duckweed also substantially changed due to the irradiation. As shown in Figure 2a, when grown in the SH medium for 2 weeks, one plant of *sub-1* mutant reproduced only 20 offsprings, which was much less than the wide-type control (~66; Figure 2c). Nevertheless, the gap between the mutant and control in biomass accumulation was nearly comparable (Figure 2c), because *sub-1* mutant is slightly higher in weight per plant than the wild type (Figure 2d). Meanwhile, the biological yield per unit area of *sub-1* mutant was nearly 1.8 times of that of the wild type, indicating that the mutant is able to use the growth space more efficiently.

Starch is a high-molecular carbohydrate and is formed by polymerization of the glucose molecules, which are mainly derived from the carbon assimilation of photosynthesis in plants. To investigate the reason of high starch accumulation in *sub-1* mutant, the chlorophyll fluorescence parameters and photosynthetic pigment content were determined. The Fv/Fm value indicating potential maximum quantum efficiency showed no difference between the wild type and *sub-1* mutant (Figure 3a), while the quantum yield of PSII (Y (II)) in *sub-1* mutant, which represents the proportion of PSII reaction centers for converting absorbed radiant energy to chemically fixed

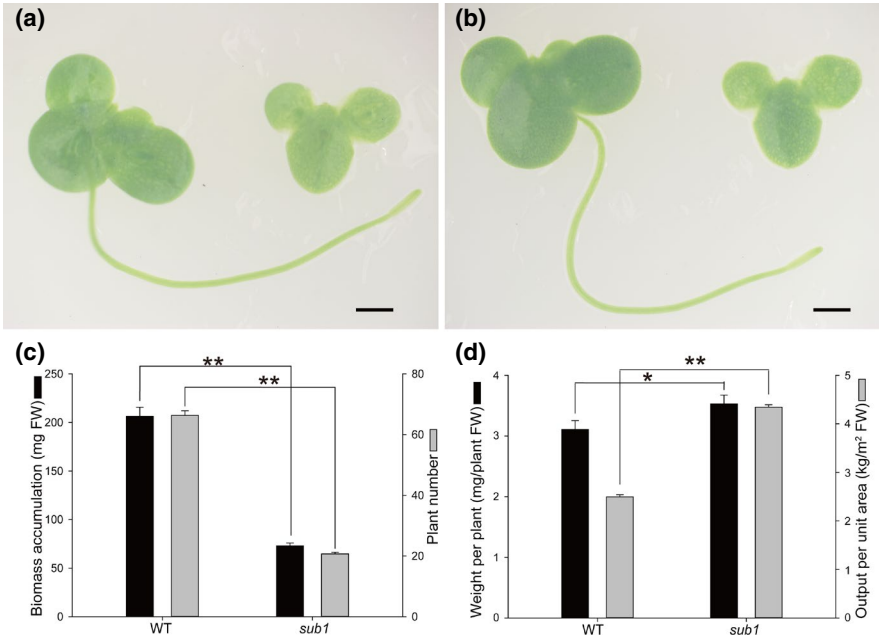


FIGURE 2 Morphology and growth of wild type and *sub-1* mutant. (a) Adaxial surface of wild type (left) and *sub-1* frond (right); (b) abaxial surface of wild type (left) and *sub-1* frond (right); (c) biomass accumulation and plant numbers of wild type and *sub-1* mutant; (d) Weight per plant and output per unit area of wild type and *sub-1* mutant. Bars represent *SD*s from three biological repeats. Asterisks indicate the significant difference between two groups (*$p < .05$; **$p < .01$) using Duncan's test. Scale bars = 1 mm

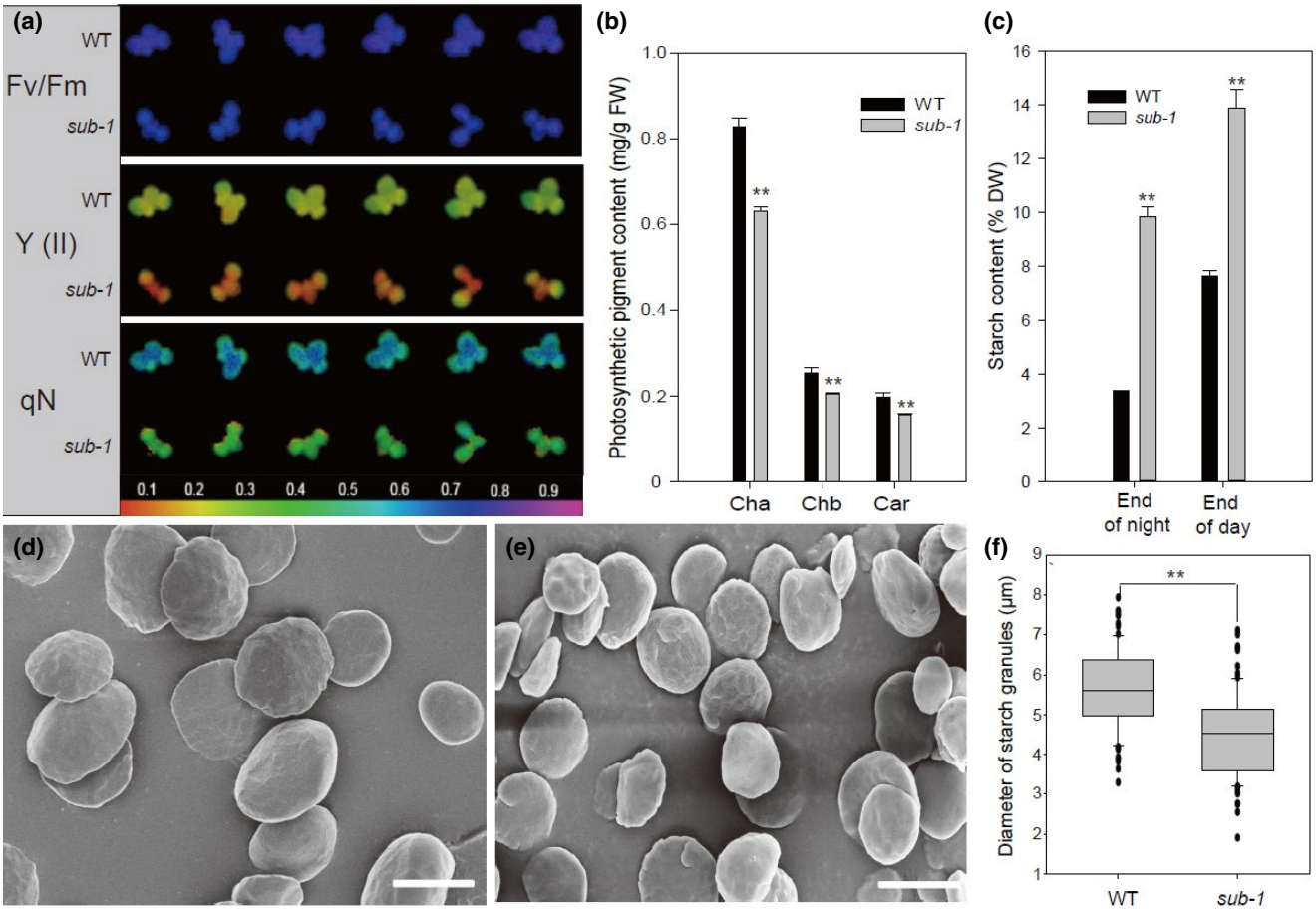


FIGURE 3 Photosynthesis and starch properties of wild type and *sub-1*. (a) Chlorophyll fluorescence parameters including Fv/Fm (potential maximum quantum efficiency), Y(II) (quantum yield of PSII) at light intensity of 185 μmol m^{-2} s^{-1} and qN (non-photochemical quenching coefficient) at light intensity of 185 μmol/m^2 s^{-1}; (b) photosynthetic pigment content of wild type and *sub-1*; (c) starch content of wild type and *sub-1*; (d, e) scanning electron microscope observation of starch granules from wild type (d) and *sub-1* (e); (f) statistical analysis of starch granule diameter. The diameter of more than 150 starch granules was measured using ImageJ 1.52v (National Institutes of Health). Bars represent *SD*s from three biological repeats. Asterisks indicate the significant difference between two groups (** $p < .01$) using Duncan's test. Scale bars = 5 μm (d, e)

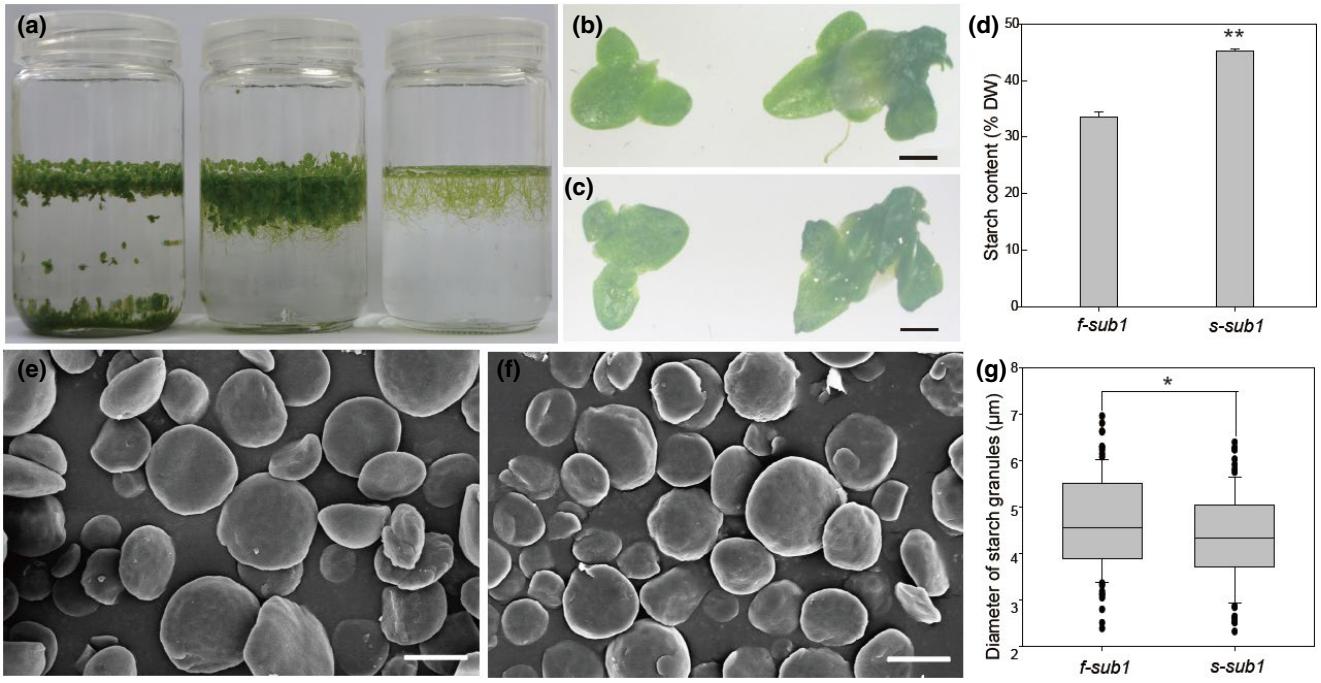


FIGURE 4 Comparison of the morphology and characteristic of starch between *f-sub1* mutant and *s-sub1* mutant. (a) The overcrowded *sub-1* submerged at the bottom (left: overcrowded *sub-1*, middle: overcrowded wild type, right: released wild type); (b) adaxial surface of *f-sub1* (left) and *s-sub1* (right); (c) abaxial surface of floating *sub-1* (left) and sinking *sub-1* (right); (d) starch content of *f-sub1* and *s-sub1*; (e, f) scanning electron microscope observation of starch granules from *f-sub1* (e) and *s-sub1* (f); (g) statistical analysis of starch granule diameter. The diameter of more than 150 starch granules was measured using ImageJ 1.52v (National Institutes of Health). Bars represent *SD*s from three biological repeats. Asterisks indicate the significant difference between two groups (**$p < .01$) using Duncan's test. *Starch granule diameter in the f-sub1 mutant was higher compared with the s-sub1 mutant. Scale bars = 2 cm (a), bars = 1 mm (b, c), bars = 4 μm (e, f)

energy through photochemical charge separation (Maxwell & Johnson, 2000), was distinctly lower than that in wild type (Figure 3a). Moreover, the qN value, as an index of displaying thermal dissipation of excess light energy that could not be used for photosynthesis (Muller, Li, & Niyogi, 2001), was decreased in *sub-1* mutant as well (Figure 3a). In addition, heavy-ion irradiation also caused a significant reduction of the photosynthetic pigment content in *sub-1* mutant (Figure 3b). Taken together, these results suggest that heavy-ion irradiation induced a reduction of the light capturing and conversion efficiency probably by affecting the content of the photosynthetic pigments in *sub-1* mutant. Subsequently, from the scanning electron microscopy (SEM) images of starch granules, we found that higher proportion of small-grain starch was present in *sub-1* mutant. The starch granule size was about 5.6 μm in wild type, while it was 4.5 μm in *sub-1* mutant (Figure 3d–f). The small starch size would help store more starch granules in vivo in *sub-1* mutant.

3.3 | Submergence as a new screening method for high-starch duckweed

As an aquatic plant, duckweed usually floats on the water surface. When it encounters a harsh environment, duckweed, especially *Spirodela*, will form a turion with high starch accumulation and then sink to the bottom. Interestingly, the *sub-1* mutant was prone to sink underwater, usually after 2–3 weeks of cultivation. As shown in Figure 4a, the *sub-1* mutant submerged in the left culture flask, whereas submergence of wild type in the middle flask was caused by overcrowding and remained floating on the surface after releasing to the right flask, indicating that the *sub-1* mutant gained this unexpected characteristic after heavy-ion mutagenesis. Compared to the *f-sub1*, the *s-sub1* exhibited significant differences in morphology, including dark green leaf and denser tissues just like callus in the differentiation process (Figure 4b,c). The apparently elongated roots of duckweed (Figure 4a,b) is an indicator of nutritional deficiency in the liquid medium over 2-week cultivation. After 2-week cultivation, the starch content in *sub-1* mutant was increased significantly (Figure 4d). Moreover, the starch content of the *s-sub1* reached 45.13%, which was nearly 12% higher than *f-sub1*, indicating that duckweed with higher starch content can be spontaneously selected by submergence. Subsequently, SEM observations showed that the proportion of the small granular starch in the *s-sub1* mutant was higher compared with the *f-sub1* mutant (Figure 4e–g), implying that accumulation of smaller and denser starch granules in the body should be a necessary condition for duckweed submergence. To verify this hypothesis, stomata morphology of *s-sub1* and *f-sub1*

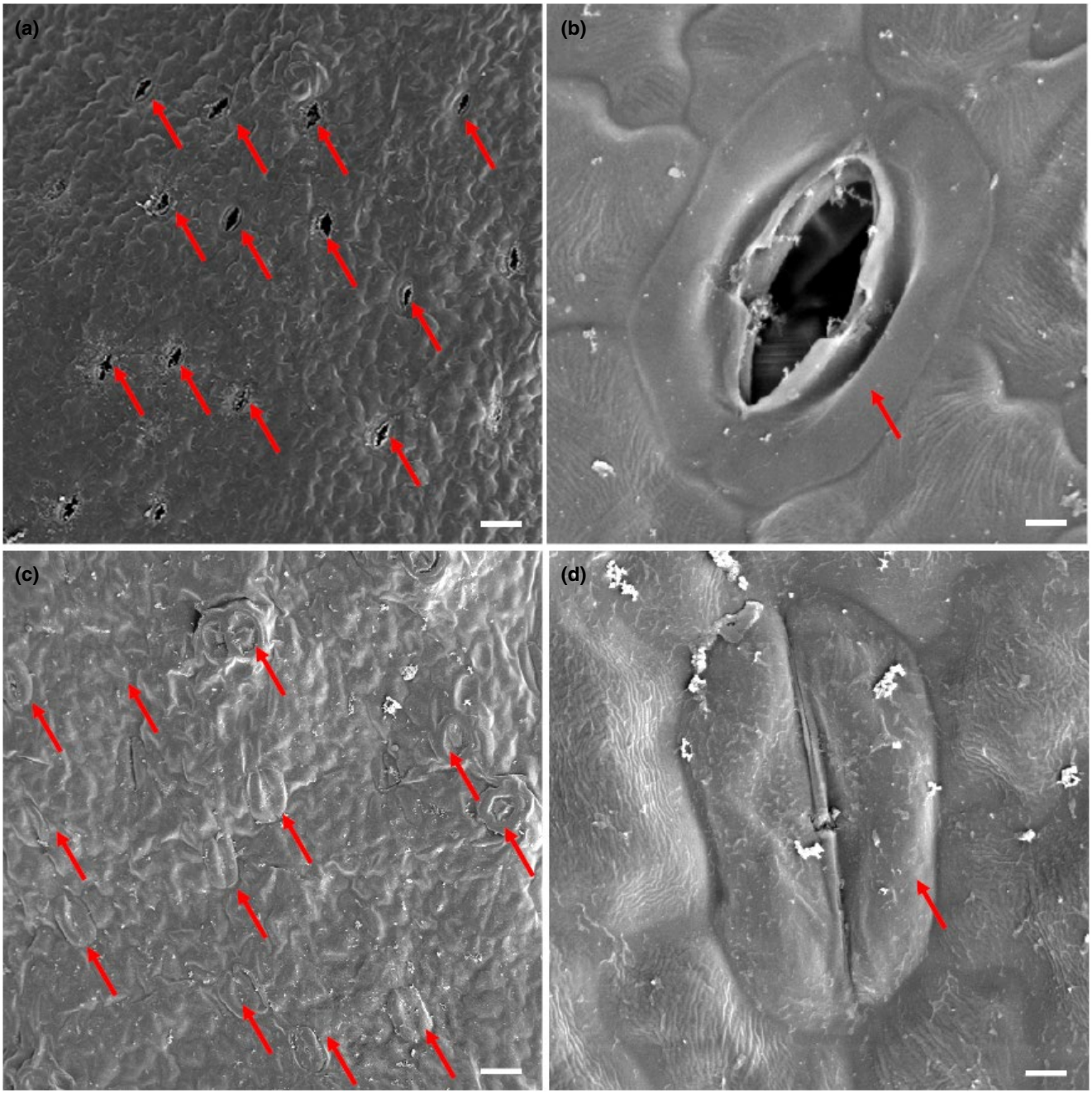


FIGURE 5 Stomatal phenotypes of *f-sub1* and *s-sub1*. Scanning electron microscopy images of adaxial stomata of Floating mutant and sinking mutant. Red arrows in images indicate stomata. Scale bars = 20 μm (a, c), bars = 70 μm (b, d). Stomata were opened in floating mutant (a, b), which were closed in sinking mutant (c, d)

was observed with SEM. Stomata mediate gas and water exchange through transpiration, which are of important significance for water maintaining and floating in duckweed. The SEM results showed that the stomata were opened in the *f-sub1* mutant but were closed in the *s-sub1* mutant (Figure 5).

To investigate the impact of nutritional status on the submergence habitat of *sub-1* mutant, four types of media including SH medium, 1/4 SH medium, 1/16 SH medium, and distilled water were used in the cultivation. After 1 week, duckweeds started sinking under all four treatment conditions, the sinking proportions were 6.90% in SH medium, 16.82% in 1/4 SH medium, 12.19% in 1/16 SH medium, and 12.22% in distilled water, respectively (Figure 6a). When the nutritional level was extremely low (i.e. 1/16 SH medium and distilled water), the accumulation of duckweed biomass was significantly inhibited (Figure 6a). However, the proportion of sinking duckweed was also lower than that in 1/4 SH medium (Figure 6a), suggesting that only nutrient starvation may not be the ideal way to obtain sinking duckweeds with high starch content. On the other hand, the growth space was

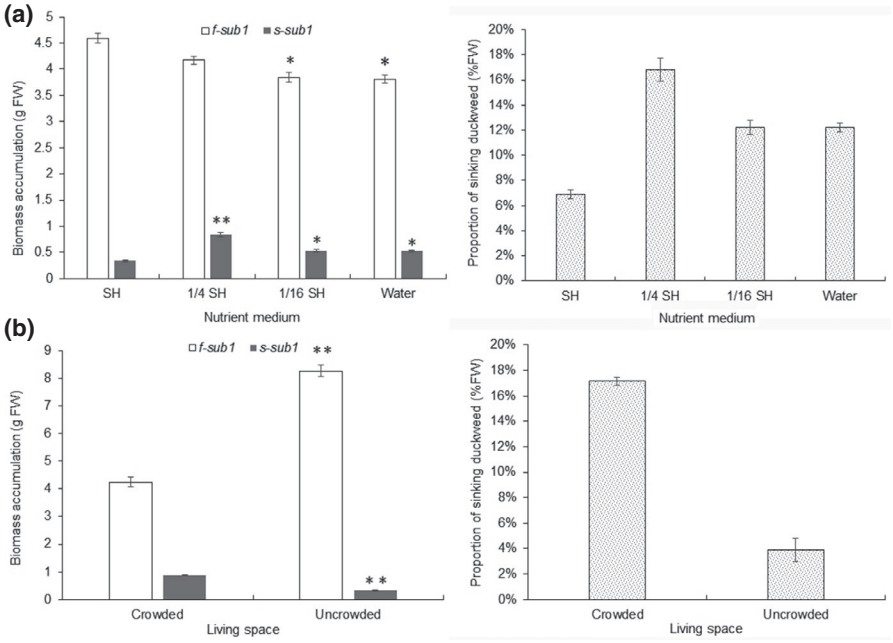


FIGURE 6 Effects of nutrition and growth space on the submergence of *sub-1*. (a) Effect of nutrition on *sub-1* mutant submergence: Four grams of duckweed freshly cultivated for 2 weeks was individually inoculated into 100 ml of Schenk & Hildebrandt (SH) medium, 1/4 SH medium, 1/16 SH medium, and distilled water for 1 week, and then the fresh weights of the floating and submerged parts were measured, respectively. (b) Effect of growth space on *sub-1* submergence: Four grams of duckweed freshly cultivated for 2 weeks was inoculated into a 100 ml (Crowded) or a 1 L (Uncrowded) Erlenmeyer flask containing 100 ml of 1/4 SH medium for 1 week, and then those two parts of duckweed were separately weighed. Bars represent *SD*s from three biological repeats. Asterisks indicate the significant difference between two groups (*$p < .05$; **$p < .01$) using Duncan's test

TABLE 2 The proportion of amylose and amylopectin in the total starch in wild type and *sub-1* mutant

Starch characteristics	Species		
	Wild type	***f-sub1***	***s-sub1***
Amylose (%)	20.55 ± 1.09a	16.99 ± 0.16b	16.94 ± 0.45b
Amylopectin (%)	79.45 ± 1.09a	83.01 ± 0.16b	83.06 ± 0.45b
Amylose/Amylopectin	0.26 ± 0.02a	0.20 ± 0.01b	0.20 ± 0.01b

Note: Values are mean ± *SD* ($n = 3$). Means with different letters are significantly different (one-way ANOVA, Duncan's test, $p < .05$).

considered as another factor that might modulate the *sub-1* mutant submergence. Obviously, ample growth space was more conducive to the accumulation of duckweed biomass. However, compared to the proportion of sinking duckweed in uncrowded space (3.88%), the proportion of sinking duckweed in a 100 ml Erlenmeyer flask (17.15%), which offered a relative crowded space, was much higher than uncrowded condition (Figure 6b), implying that overcrowding is a stimulating factor to induce *sub-1* mutant submergence.

3.4 | Starch composition and enzymatic saccharification

The composition of starch is an important factor in determining the efficiency of enzymatic saccharification. More specifically, the lower the ratio of amylose/amylopectin in starch, the higher the conversion efficiency will be. As shown in Table 2, the amylose content of wild-type *L. aequinoctialis* 6002 was 20.55% and the content of amylopectin was 79.45%. However, the amylose content of *f-sub1* mutant was decreased to 16.99% and declined to 16.94% in the *s-sub1* mutant, respectively (Table 2), which were significantly lower than wild type. The ratios of amylose/amylopectin in *f-sub1* (0.2) and *s-sub1* (0.2) were both lower than that in wild type (0.26), suggesting that two types of *sub-1* mutant (*f-sub1* mutant and *s-sub1* mutant) are more inclined to synthesize amylopectin as the starch content increased which is a favorable feature of *sub-1* mutant for enzymatic saccharification. The released glucose was 4.73 g/L in wild type, 18.94 g/L in the *f-sub1* mutant, and 24.82 g/L in the *s-sub1* mutant, and was consistent with the starch content in these samples (Table 3).

TABLE 3 Glucose released from duckweed biomass after enzymatic saccharification and acid hydrolysis, and ethanol yield by yeast fermentation using duckweed biomass hydrolysate

Saccharification and fermentation	Species		
	Wild type	*f-sub1*	*s-sub1*
Biomass input (g DW/L)	49.79 ± 0.39	49.98 ± 0.19	49.99 ± 0.21
Total glucose (g/L)	12.59 ± 0.36a	26.24 ± 0.89b	30.52 ± 1.01c
Released glucose (g/L)	4.73 ± 0.10a	18.49 ± 0.21b	24.82 ± 0.03c
Release percentage (%)	37.56 ± 0.83a	70.47 ± 0.81b	81.31 ± 0.96c
Y_E (g/L)	2.12 ± 0.05a	8.39 ± 0.22b	11.59 ± 0.22c
$Y_{E/G}$ (g/g)	0.448 ± 0.010a	0.454 ± 0.012ab	0.467 ± 0.009b
$Y_{E/B}$ (g/g)	0.043 ± 0.002a	0.168 ± 0.004b	0.232 ± 0.004c

Note: "Total glucose" was obtained by acid hydrolysis, while "released glucose" represented the glucose released from starch by enzymatic saccharification. The release percentage was calculated by dividing "released glucose" by "total glucose." Values are mean ± *SD* ($n = 3$). Means with different letters are significantly different (one-way ANOVA, Duncan's test, $p < .05$).

Abbreviation: DW, dry weight.

In the *s-sub1* mutant, the glucose release percentage was increased to 81.31%, which was much higher than the *f-sub1* mutant (70.47%) and wild-type plant (37.56%) (Table 3). The results of ethanol fermentation showed that the *s-sub1* had the highest ethanol yield (0.232 g/g DW), implying that the *sub-1* mutant biomass is more suitable for bioethanol production (Table 3).

4 | DISCUSSION

4.1 | Heavy-ion irradiation is an effective breeding method for duckweed

Although duckweed is recognized as a promising feedstock to produce bioethanol or biogas, there are still a few problems, such as lacking of high-quality varieties for commercial application and cost-effective methods for duckweed biorefinery, hampering its applications in bioenergy production (Souto et al., 2019). Currently, almost all studies focused on screening of specific traits (i.e., high starch content) from natural duckweed using wild germplasms collected from different geography locations (Ge et al., 2012; Xu et al., 2019; Yu et al., 2014). Therefore, it is necessary to develop more duckweed breeding approaches to obtain proper duckweed variants for industrial application. As duckweed usually proliferates through asexual reproduction, the classic hybrid breeding method is difficult to be applied on duckweed. Thus, artificial mutagenesis and molecular breeding are preferable for duckweed breeding, which dramatically shorten the time for acquirement of new varieties. Compared with other physical mutagenesis approaches such as γ- and X-rays, heavy-ion beam makes more intensive biological effects due to its higher LET values (Zhou et al., 2006). Heavy-ion irradiation has been successfully used in the mutagenesis of lots of plant species, such as *Arabidopsis thaliana* (Kazama et al., 2011), wheat (*Triticum aestivum*; Fitzgerald et al., 2015), and *Oryza sativa* (Ishikawa et al., 2012). To the best of our knowledge, this work was the first utilization of heavy-ion irradiation in bioenergy crop duckweed breeding.

4.2 | Variations of starch content and properties in *sub-1* mutant

Duckweed is considered to be a promising feedstock to produce bioethanol or biogas, and high starch content and fast-growing rate are the most superior characteristics of duckweed, which are the major reasons making it an ideal energy plant. However, the content of starch in duckweed changes substantially under the influence of itself and the external environment. The starch content of duckweed decreases during the quick growth period, whereas increases rapidly at the stationary growth phase (Ge et al., 2012), implying that the slowdown of growth rate would promote starch accumulation in the body (Figure 2c). Normally, plants accumulate starch for energy storage through photosynthesis during the day and consume starch to provide energy at night, making nightfall a better time for duckweed collection during bioenergy production. As shown in Figure 3c, the starch content in *sub-1* mutant was significantly higher at both the beginning and ending of the photoperiod than that in wild type. However, the diurnal variation of the starch content was larger in the wild type (2.26 times) compared with *sub-1* mutant (1.41 times), implying that less consumption at night favored starch accumulation in *sub-1* mutant. Therefore, it would be more flexible for duckweed harvest for *sub-1* mutant. In addition, the content of starch can also be regulated by manipulating the growth environment of duckweed, such as nutrient levels, hormone addition, light intensity, and photoperiod (Liu, Chen, et al., 2019; Tao et al., 2013;

Yin et al., 2015). However, these external conditions do not only affect the duckweed biomass production but also increase the bioethanol production cost, and even influence the environment safety. So, screening of a duckweed germplasm with quality traits (i.e., stable content of high starch) is crucial to solve these problems. Therefore, the starch content was used as a criterion to screen suitable candidates from the obtained *L. aequinoctialis* 6002 mutant libraries. By screening a mutant library generated by heavy-ion radiation, a *sub-1* mutant with high starch content was obtained (Table 1). Nutrient starvation after an over 2-week cultivation resulted in increasing of the starch content in *sub-1* mutant (Figure 4d), which was consistent with previous studies, suggesting that heavy-ion irradiation mutagenesis did not disturb the internal regulation mechanism of starch accumulation in duckweed. However, heavy-ion irradiation caused rising of small starch granules in *sub-1* mutant (Figure 3d–f), accompanied with e starch composition change (Table 2). In our early report, *L. aequinoctialis* (not the same variety used in this work) had lower amylose content (20.12%) and higher amylopectin content (79.88%), which was very close to wild-type *L. aequinoctialis* 6002 (Yu et al., 2014). After a 30-day cultivation in the SH medium, the starch content increased by 11% and the amylopectin content reached 86.60%, suggesting that the increased starch content correlated with the increased amylopectin content (Yu et al., 2014). Consistent with previous result, the amylose content of *f-sub1* mutant and *s-sub1* mutant were both significantly lower than wild type, which lead to the ratios of amylose/amylopectin in *f-sub1* (0.2) and *s-sub1* (0.2) lower than that in wild type (0.26; Table 2). It suggested that *sub-1* mutant will synthesis more amylopectin than amylose as the starch content increased. The lower ratio of amylose/amylopectin in *sub-1* mutant will improve enzymatic saccharification efficiency and reduce costs for bioethanol production.

4.3 | Spontaneous screening of high-starch duckweed with submergence phenotype

As we all know, nearly all duckweeds are propagated by asexual reproduction during their growth, so seeds and pollens are difficult to collect for mutagenesis. Alternatively, callus, as a genetic manipulation target, can be treated with mutagens, ionizing radiation (e.g., γ-rays), or chemical mutagens (e.g., ethyl mesylate), generating new individuals with new traits (Asif & Ansari, 2019; Nikam et al., 2014). Therefore, duckweeds were usually collected and analyzed in bulk in many studies, regardless of different individuals or tissues. However, there is no doubt that the starch content varies between old and young duckweed or between the parent and daughter fronds. Submerged turion can be regarded as a dormant daughter frond of *Spirodela polyrrhiza*. The starch content of turion was as high as 65%, while the mother frond only contained less than 20% starch (Xu, Fang, et al., 2018; Xu, Yu, et al., 2018). If not harvested separately, it will seriously lower the quality of biomass used for bioenergy production. Research on promoting formation of turion has been reported (Appenroth, 2002; Xu et al., 2019), but it is not clear how to induce turion to separate from the mother frond and sink to the bottom, which will directly affect the convenience of turion collection, especially in large-scale cultivation. Obviously, an efficient and spontaneous screening approach of high-starch duckweed will be very valuable for practical operations in bioenergy production. This heavy-ion irradiated *sub-1* mutant, produced individuals with higher starch content and sank to the bottom with closed stomata under certain growth stage and culture conditions (Figures 4 and 5), portending enhancement of tissue compactness as well as improvement of starch accumulation. There were two possible reasons for closing of the stomata in the *s-sub1* mutant. First, the starch content was highly accumulated in the *s-sub1* mutant, and then lots of starch granules led to cell extrusion and smaller intercellular spaces. The stomata were ultimately closed by a series of reactions above. Second, high starch content will change the density of *sub-1* mutant and when the density of *sub-1* mutant exceeds that of water, *sub-1* mutant will sink into the water bottom and close the stomata. These results laid a foundation for duckweed molecular breeding in future studies.

4.4 | Beneficial performances of *sub-1* mutant for bioethanol production

It is well known that glucose hydrolyzed from plant biomass is mainly derived from the cell wall polysaccharides and starch. Compared to cell wall polysaccharides, starch could be hydrolyzed more easily by enzymes due to its single component and uncomplicated structure, and glucose released from starch could be considered as easy-to-release glucose. In addition, the starch proportion had an important influence on the enzymatic saccharification efficiency. Higher proportion of amylopectin would be better. Compared with wild-type *L. aequinoctialis* 6002, heavy-ion irradiated *sub-1* mutant has a smaller body but a larger biomass yield (Figure 2), suggesting that the mutant cells should be more densely organized. Apart from the increased starch content, the starch particles were smaller in *sub-1* mutant (Figures 3 and 4), which might be conducive to starch accumulation and submergence triggering. In addition, higher proportion of amylopectin in *sub-1* mutant would help improve the enzymatic saccharification efficiency (Table 2). Based on these characteristics, both *f-sub1* (18.49 g/L) and *s-sub1*

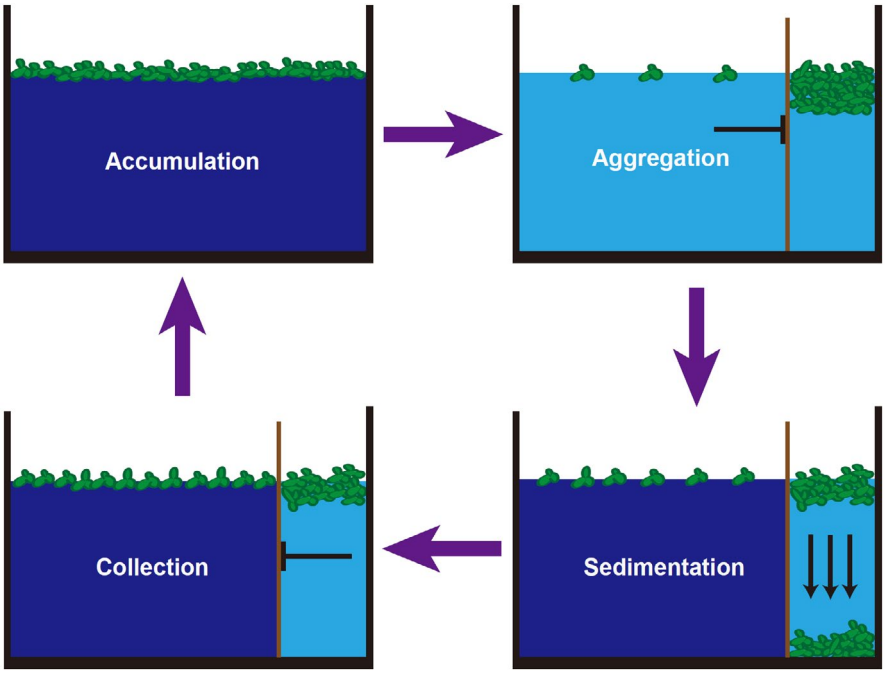


FIGURE 7 Cultivation model of duckweed for starch-rich biomass in a simulated aquaculture pond. The cultivation process consists of four main steps including biomass accumulation, space compression, high-starch frond deposition, and harvest, forming a cycle to maintain the production continuity in a pond. Dark blue color:complete Schenk & Hildebrandt (SH) culture medium. Light blue color:nutrition deficient medium. Duckweeds were first cultivated in the complete SH medium and then aggregated at one side to induce the duckweed sink into bottom when the nutrients were nearly used up. Meanwhile, fresh SH medium was supplied on the other side for renewed duckweed cultivation. Finally, the submerged duckweeds were harvested and a new cycle was started. According to our results, the best condition for inducing duckweed submergence is using 1/4 SH culture medium

(24.82 g/L) had higher released glucose content than wild type (4.73 g/L) after enzymatic saccharification which led to a higher yield in bioethanol production. The bioethanol yields in *f-sub1* mutant and *s-sub1* mutant were 3.9 and 5.4 times higher than wild type, respectively (Table 3). It suggested that the s-*sub1* mutant biomass is more suitable for bioethanol production. These properties showed *sub-1* mutant good advantages and potential for the commercial bioethanol application in future. As *s-sub1* mutant had the highest ethanol yield (0.232 g/g DW), a cultivation model of *sub-1* mutant in a simulated aquaculture pond was proposed (Figure 7). The four-step cultivation process formed a cycle to maintain the production continuity in a pond. Duckweeds were first cultivated in the complete SH medium for rapid biomass accumulation. With growing of duckweed, the nutrients in the pond were gradually consumed. Since nutrient-free treatment was not the best way to trigger sinking (Figure 6a), an appropriate time point for aggregation could be chosen during gradual decline of the nutrient levels in the cultivation process. The aggregated duckweed accumulated more starch and then sank into bottom. Meanwhile, the nutrients were re-infused into the idle side of the pond for cultivation of duckweed which were not for immediate harvest. Finally, the submerged duckweeds were harvested and a new cycle started.

In summary, artificial mutagenesis will enrich the germplasm resources of duckweed and facilitate discovery of more excellent mutants. It is expected that heavy-ion irradiation as well as other artificial mutagenesis should be more frequently applied to improve duckweed varieties to better serve bioenergy production. Due to the continuous release and update of duckweed genome sequence, genome re-sequencing technique will be used in the near future to clarify the key genes that cause submergence and starch-rich habitats of *sub-1* mutant, and then reveal the corresponding molecular mechanism. Based on the *sub-1* mutant properties, a continuous screening and cultivation mode for high starch production of duckweed has been developed, which will be further optimized and expanded to offer a foundation for large-scale culture of starch-rich duckweed with low cost and high yield for the bioethanol production.

DATA AVAILABILITY STATEMENT

Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Yu Liu https://orcid.org/0000-0002-2873-8221
Hua Xu https://orcid.org/0000-0003-0383-5305

Changjiang Yu https://orcid.org/0000-0002-2735-5691
Gongke Zhou https://orcid.org/0000-0002-0771-7165

REFERENCES

Appenroth, K. J. (2002). Co-action of temperature and phosphate in inducing turion formation in *Spirodela polyrhiza* (Great duckweed). *Plant Cell and Environment*, *25*(9), 1079–1085. https://doi.org/10.1046/j.1365-3040.2002.00885.x

Asif, A., & Ansari, M. Y. K. (2019). Generation of mutant lines of *Nigella sativa* L. by induced mutagenesis for improved seed yield. *Industrial Crops and Products*, *139*, UNSP 111552. https://doi.org/10.1016/j.indcrop.2019.111552

Cantó-Pastor, A., Mollá-Morales, A., Ernst, E., Dahl, W., Zhai, J. X., Yan, Y. H., … Martienssen, R. (2015). Efficient transformation and artificial miRNA gene silencing in *Lemna minor*. *Plant Biology*, *17*, 59–65. https://doi.org/10.1111/plb.12215

Chen, Q., Jin, Y. L., Zhang, G. H., Fang, Y., Xiao, Y., & Zhao, H. (2012). Improving production of bioethanol from duckweed (*Landoltia punctata*) by pectinase pretreatment. *Energies*, *5*(8), 3019–3032. https://doi.org/10.3390/en5083019

Chhabra, G., Chaudhary, D., Sainger, M., & Jaiwal, P. K. (2011). Genetic transformation of Indian isolate of *Lemna minor* mediated by *Agrobacterium tumefaciens* and recovery of transgenic plants. *Physiology and Molecular Biology of Plants*, *17*(2), 129–136. https://doi.org/10.1007/s12298-011-0059-5

Fitzgerald, T. L., Powell, J. J., Stiller, J., Weese, T. L., Abe, T., Zhao, G., … Kazan, K. (2015). An assessment of heavy ion irradiation mutagenesis for reverse genetics in wheat (*Triticum aestivum L.*). *PLoS One*, *10*(2), e0117369. https://doi.org/10.1371/journal.pone.0117369

Ge, X. M., Zhang, N. N., Phillips, G. C., & Xu, J. F. (2012). Growing *Lemna minor* in agricultural wastewater and converting the duckweed biomass to ethanol. *Bioresource Technology*, *124*, 485–488. https://doi.org/10.1016/j.biortech.2012.08.050

Hirano, T., Sato, Y., Ichinose, K., Yamada, M., Hayashi, Y., Fukunishi, N., & Abe, T. (2019). Mutant induction in gametophytes of *Undaria pinnatifida* (Phaeophyceae) by heavy ion beam irradiation. *Phycological Research*, *68*(1), 63–69. https://doi.org/10.1111/pre.12397

Ichide, H., Morita, R., Shirakawa, Y., Hayashi, Y., & Abe, T. (2019). Targeted exome sequencing of unselected heavy-ion beam-irradiated populations reveals less-biased mutation characteristics in the rice genome. *The Plant Journal*, *98*(2), 301–314. https://doi.org/10.1111/tpj.14213

Ishikawa, S., Ishimaru, Y., Igura, M., Kuramata, M., Abe, T., Senoura, T., … Nakanishi, H. (2012). Ion-beam irradiation, gene identification, and marker-assisted breeding in the development of low-cadmium rice. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(47), 19166–19171. https://doi.org/10.1073/pnas.1211132109

Kazama, Y., Hirano, T., Saito, H., Liu, Y., Ohbu, S., Hayashi, Y., & Abe, T. (2011). Characterization of highly efficient heavy-ion mutagenesis in *Arabidopsis thaliana*. *BMC Plant Biology*, *11*, 161. https://doi.org/10.1186/1471-2229-11-161

Liu, Y., Chen, X. Y., Wang, X. H., Fang, Y., Zhang, Y., Huang, M. J., & Zhao, H. (2019). The influence of different plant hormones on biomass and starch accumulation of duckweed: A renewable feedstock for bioethanol production. *Renewable Energy*, *138*, 659–665. https://doi.org/10.1016/j.renene.2019.01.128

Liu, Y., Wang, Y., Xu, S. Q., Tang, X. F., Zhao, J. S., Yu, C. J., … Zhou, G. K. (2019). Efficient genetic transformation and CRISPR/Cas9-mediated genome editing in *Lemna aequinoctialis*. *Plant Biotechnology Journal*, *17*(11), 2143–2152. https://doi.org/10.1111/pbi.13128

Ma, Y. B., Wang, Z. Y., Zhu, M., Yu, C. J., Cao, Y. P., Zhang, D. Y., & Zhou, G. K. (2013). Increased lipid productivity and TAG content in Nannochloropsis by heavy-ion irradiation mutagenesis. *Bioresource Technology*, *136*, 360–367. https://doi.org/10.1016/j.biortech.2013.03.020

Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence – A practical guide. *Journal of Experimental Botany*, *51*(345), 659–668. https://doi.org/10.1093/jexbot/51.345.659

Mohedano, R. A., Costa, R. H. R., Tavares, F. A., & Beli, P. (2012). High nutrient removal rate from swine wastes and protein biomass production by full-scale duckweed ponds. *Bioresource Technology*, *112*, 98–104. https://doi.org/10.1016/j.biortech.2012.02.083

Moxley, G., & Zhang, Y. H. P. (2007). More accurate determination of acid-labile carbohydrates in lignocellulose by modified quantitative saccharification. *Energy & Fuels*, *21*(6), 3684–3688. https://doi.org/10.1021/ef7003893

Muller, P., Li, X. P., & Niyogi, K. K. (2001). Non-photochemical quenching. A response to excess light energy. *Plant Physiology*, *125*(4), 1558–1566. https://doi.org/10.1104/pp.125.4.1558

Nakano, M., Amano, J., Watanabe, Y., Nomizu, T., Suzuki, M., Mizunashi, K., … Abe, T. (2010). Morphological variation in *Tricyrtis hirta* plants regenerated from heavy ion beam-irradiated embryogenic calluses. *Plant Biotechnology*, *27*(2), 155–160. https://doi.org/10.5511/plantbiotechnology.27.155

Nikam, A. A., Devarumath, R. M., Shitole, M. G., Ghole, V. S., Tawar, P. N., & Suprasanna, P. (2014). Gamma radiation, in vitro selection for salt (NaCl) tolerance, and characterization of mutants in sugarcane (*Saccharum officinarum* L.). *In Vitro Cellular & Developmental Biology – Plant*, *50*(6), 766–776. https://doi.org/10.1007/s11627-014-9630-4

Okamura, M., Yasuno, N., Ohtsuka, M., Tanaka, A., Shikazono, N., & Hase, Y. (2003). Wide variety of flower-color and -shape mutants regenerated from leaf cultures irradiated with ion beams. *Nuclear Instruments & Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*, *206*, 574–578. https://doi.org/10.1016/S0168-583X(03)00835-8

Ragauskas, A. J., Williams, C. K., Davison, B. H., Britovsek, G., Cairney, J., Eckert, C. A., … Tschaplinski, T. (2006). The path forward for biofuels and biomaterials. *Science*, *311*(5760), 484–489. https://doi.org/10.1126/science.1114736

Rival, S., Wisniewski, J.-P., Langlais, A., Kaplan, H., Freyssinet, G., Vancanneyt, G., … Edelman, M. (2008). Spirodela (duckweed) as an alternative production system for pharmaceuticals: A case study, aprotinin. *Transgenic Reseach*, *17*(4), 503–513. https://doi.org/10.1007/s11248-007-9123-x

Sasaki, N., Watanabe, A., Asakawa, T., Sasaki, M., Hoshi, N., Naito, Z., … Nishihara, M. (2018). Biological effects of ion beam irradiation on perennial gentian and apple. *Plant Biotechnology*, *35*(3), 249–257. https://doi.org/10.5511/plantbiotechnology.18.0612a

Smith, A. M., & Zeeman, S. C. (2006). Quantification of starch in plant tissues. *Nature Protocols*, *1*(3), 1342–1345. https://doi.org/10.1038/nprot.2006.232

Sonta, M., Rekiel, A., & Batorska, M. (2019). Use of duckweed (*Lemna* L.) in sustainable livestock production and aquaculture – A review.

Annals of Animal Science, *19*(2), 257–271. https://doi.org/10.2478/aoas-2018-0048

Souto, L. R. F., da Silva, I. F., Ninow, J. L., Collins, S. R., Elliston, A., & Waldron, K. W. (2019). Effect of hydrothermal pre-treatment on duckweed (*Landoltia punctata*) biomass for simultaneous saccharification and fermentation process. *Biomass and Bioenergy*, *127*, 105259. https://doi.org/10.1016/j.biombioe.2019.105259

Tao, X., Fang, Y., Huang, M. J., Xiao, Y., Liu, Y., Ma, X. R., & Zhao, H. (2017). High flavonoid accompanied with high starch accumulation triggered by nutrient starvation in bioenergy crop duckweed (*Landoltia punctata*). *BMC Genomics*, *18*, 166. https://doi.org/10.1186/s12864-017-3559-z

Tao, X., Fang, Y., Xiao, Y., Jin, Y.-L., Ma, X.-R., Zhao, Y., … Wang, H.-Y. (2013). Comparative transcriptome analysis to investigate the high starch accumulation of duckweed (*Landoltia punctata*) under nutrient starvation. *Biotechnology for Biofules*, *6*, 72. https://doi.org/10.1186/1754-6834-6-72

Vunsh, R., Li, J., Hanania, U., Edelman, M., Flaishman, M., Perl, A., … Freyssinet, G. (2007). High expression of transgene protein in *Spirodela*. *Plant Cell Reports*, *26*(9), 1511–1519. https://doi.org/10.1007/s00299-007-0361-4

Wang, W. D., & Messing, J. (2012). Analysis of ADP-glucose pyrophosphorylase expression during turion formation induced by abscisic acid in *Spirodela polyrhiza* (greater duckweed). *BMC Plant Biology*, *12*, 5. https://doi.org/10.1186/1471-2229-12-5

Wellburn, A. R. (1994). The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *Journal of Plant Physiology*, *144*(3), 307–313. https://doi.org/10.1016/S0176-1617(11)81192-2

Xu, H., Yu, C., Xia, X., Li, M., Li, H., Wang, Y. U., … Zhou, G. (2018). Comparative transcriptome analysis of duckweed (*Landoltia punctata*) in response to cadmium provides insights into molecular mechanisms underlying hyperaccumulation. *Chemospere*, *190*, 154–165. https://doi.org/10.1016/j.chemosphere.2017.09.146

Xu, Y.-L., Fang, Y., Li, Q. I., Yang, G.-L., Guo, L., Chen, G.-K., … Zhao, H. (2018). Turion, an innovative duckweed-based starch production system for economical biofuel manufacture. *Industrial Crops and Products*, *124*, 108–114. https://doi.org/10.1016/j.indcrop.2018.07.061

Xu, Y.-L., Tan, L. I., Guo, L., Yang, G.-L., Li, Q. I., Lai, F., … Zhao, H. (2019). Increasing starch productivity of *Spirodela polyrhiza* by precisely control the spectral composition and nutrients status. *Industrial Crops and Products*, *134*, 284–291. https://doi.org/10.1016/j.indcrop.2019.04.002

Yang, J., Li, G., Hu, S., Bishopp, A., Heenatigala, P., Kumar, S., … Hou, H. (2018). A protocol for efficient callus induction and stable transformation of *Spirodela polyrhiza* (L.) Schleiden using *Agrobacterium tumefaciens*. *Aquatic Botany*, *151*, 80–86. https://doi.org/10.1016/j.aquabot.2018.08.004

Yang, L., Han, Y., Wu, D. I., Yong, W., Liu, M., Wang, S., … Sun, J. (2017). Salt and cadmium stress tolerance caused by overexpression of the *Glycine max* Na^+/H^+ antiporter (*GmNHX1*) gene in duckweed (*Lemna turionifera* 5511). *Aquatic Toxicology*, *192*, 127–135. https://doi.org/10.1016/j.aquatox.2017.08.010

Yin, Y. H., Yu, C. J., Yu, L., Zhao, J. S., Sun, C. J., Ma, Y. B., & Zhou, G. K. (2015). The influence of light intensity and photoperiod on duckweed biomass and starch accumulation for bioethanol production. *Bioresource Technology*, *187*, 84–90. https://doi.org/10.1016/j.biortech.2015.03.097

Yu, C., Sun, C., Yu, L. I., Zhu, M., Xu, H., Zhao, J., … Zhou, G. (2014). Comparative analysis of duckweed cultivation with sewage water and SH media for production of fuel ethanol. *PLoS One*, *9*(12), e115023. https://doi.org/10.1371/journal.pone.0115023

Yu, C., Zhao, X., Qi, G., Bai, Z., Wang, Y. U., Wang, S., … Zhou, G. (2017). Integrated analysis of transcriptome and metabolites reveals an essential role of metabolic flux in starch accumulation under nitrogen starvation in duckweed. *Biotechnology for Biofuels*, *10*(1). https://doi.org/10.1186/s13068-017-0851-8

Zhang, L., Zhao, H., Gan, M., Jin, Y., Gao, X., Chen, Q., … Wang, Z. (2011). Application of simultaneous saccharification and fermentation (SSF) from viscosity reducing of raw sweet potato for bioethanol production at laboratory, pilot and industrial scales. *Bioresource Technology*, *102*(6), 4573–4579. https://doi.org/10.1016/j.biortech.2010.12.115

Zhao, X., Moates, G. K., Wellner, N., Collins, S. R. A., Coleman, M. J., & Waldron, K. W. (2014). Chemical characterisation and analysis of the cell wall polysaccharides of duckweed (*Lemna minor*). *Carbohydrate Polymers*, *111*, 410–418. https://doi.org/10.1016/j.carbpol.2014.04.079

Zhou, L. B., Li, W. J., Yu, L. X., Li, P., Li, Q., Ma, S., … Leloup, C. (2006). Linear energy transfer dependence of the effects of carbon ion beams on adventitious shoot regeneration from in vitro leaf explants of *Saintpaulia ionahta*. *International Journal of Radiation Biology*, *82*(7), 473–481. https://doi.org/10.1080/09553000600863080

How to cite this article: Liu Y, Xu H, Wang Y, et al. A submerged duckweed mutant with abundant starch accumulation for bioethanol production. *GCB Bioenergy*. 2020;12:1078–1091. https://doi.org/10.1111/gcbb.12746