A submerged duckweed mutant with abundant starch accumulation for bioethanol production

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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
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 *Landolitia punctata* (Tao et al., 2013). Light intensity and photoperiod significantly influence starch accumulation in *Lemma 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 (Liu, Wang, et al., 2019). However, only natural duckweed species were used in all above researches.

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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
amylpectin content was calculated by subtracting amyllose 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 using 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) H2SO4 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 amyllose/amylpectin 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 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).
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 SDs 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/m² 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 SDs from three biological repeats. Asterisks indicate the significant difference between two groups (**p < .01) using Duncan’s test. Scale bars = 5 μm (d, e)
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 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 SDs 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).

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...
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 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)
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).

![Figure 6](image)

**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 SDs from three biological repeats. Asterisks indicate the significant difference between two groups (*p < .05; **p < .01) using Duncan’s test.

| 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).
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       | 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)                      | 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 $sub$-$I$ 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 acquisition of new varieties. Compared with other physical mutagenesis approaches such as $\gamma$- 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 $sub$-$I$ 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 $sub$-$I$ 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 $sub$-$I$ mutant (1.41 times), implying that less consumption at night favored starch accumulation in $sub$-$I$ mutant. Therefore, it would be more flexible for duckweed harvest for $sub$-$I$ 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 a 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 pollen 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 polyrhiza. 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...
(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 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.

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.

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