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Draft genomes and phenotypic characterization of *Tisochrysis lutea* strains. Toward the production of domesticated strains with high added value

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ABSTRACT

Tisochrysis lutea is a microalga species currently used in aquaculture as a feed for shellfish, oysters and shrimps. It also has many other potential industrial applications, such as the production of neutral lipids for biofuels or the production of ω-3 fatty acids for nutraceuticals (human food complements). To efficiently exploit the potential of this microalga, however, higher lipid productivities are needed. To this end, improvement programs need to be developed and optimized. The diversity of strains available in microalgae has not yet been exploited in such improvement programs.

In this study, the intra-strain diversity was observed and exploited to increase neutral lipid productivity. New clonal strains with higher neutral lipid productivity were successfully selected. The best clonal strain selected accumulated 520% more triacylglycerols, with a similar growth rate to the wild-type strain in continuous light and nitrogen starvation conditions. In a photoperiod culture condition, this clonal stain also accumulated 84% more storage lipids and 30% less carbohydrates, compared to the wild-type strain. This clonal strain thus had a higher productivity which is of great interest for feed or biofuel applications.

This study also focused on identifying the genomic mechanisms responsible for the improvements in these clonal strains. With this objective, the genome of *Tisochrysis lutea* was sequenced for the first time. It is the third genome of a Haptophyte microalga sequenced so far. Different genetic polymorphisms were identified between the sequenced genomes of the wild-type strain and clonal strains. Activity of transposable elements seems to have been involved in the genome reshuffling obtained through the improvement program. The contribution of transposable elements to the adaptive capacity of microalgae remains to be demonstrated.

1. Background

Marine microalgae are unicellular photosynthetic eukaryotes. They form the first link in the food chain for aquatic organisms, and are responsible for 35% of the Earth's primary production in the ocean [1,2]. We have only just begun to explore the world of microalgae [3]. Currently, around 140,000 algae species have been inventoried (http://www.algaebase.org/), but their true number is estimated to be around 1,000,000 species [4]. With the depletion of terrestrial resources, microalgae are attracting a great deal of interest. They can be exploited in many areas such as food, feed, cosmetics, bioremediation and production of third-generation biofuels [5,6]. However, in many domains, there are obstacles that need to be solved to develop economically and environmentally-friendly processes.

The microalgae research and development community agrees that the improvement of algae strains is one of the major factors that must be addressed [7,8]. Currently, all cultivated microalgae strains are directly derived from their natural environment and are considered as wild-type strains. As with modern agriculture, microalgae exploitation requires improved strains to be obtained in order to become economically viable [9]. The improvement of microalgae is a recent idea, since the first microalgae improvement programs were set up in the last decade. However, at present, fragmented knowledge on the biology of microalgae species (biodiversity, physiology, intracellular mechanisms, phenotypes, lifecycle, *etc.*) limits the use of improvement strategies. The current challenge is thus to develop and optimize improvement methodologies to reduce the gap between the methods available for microalgae and practices already in use for higher plants and animals.

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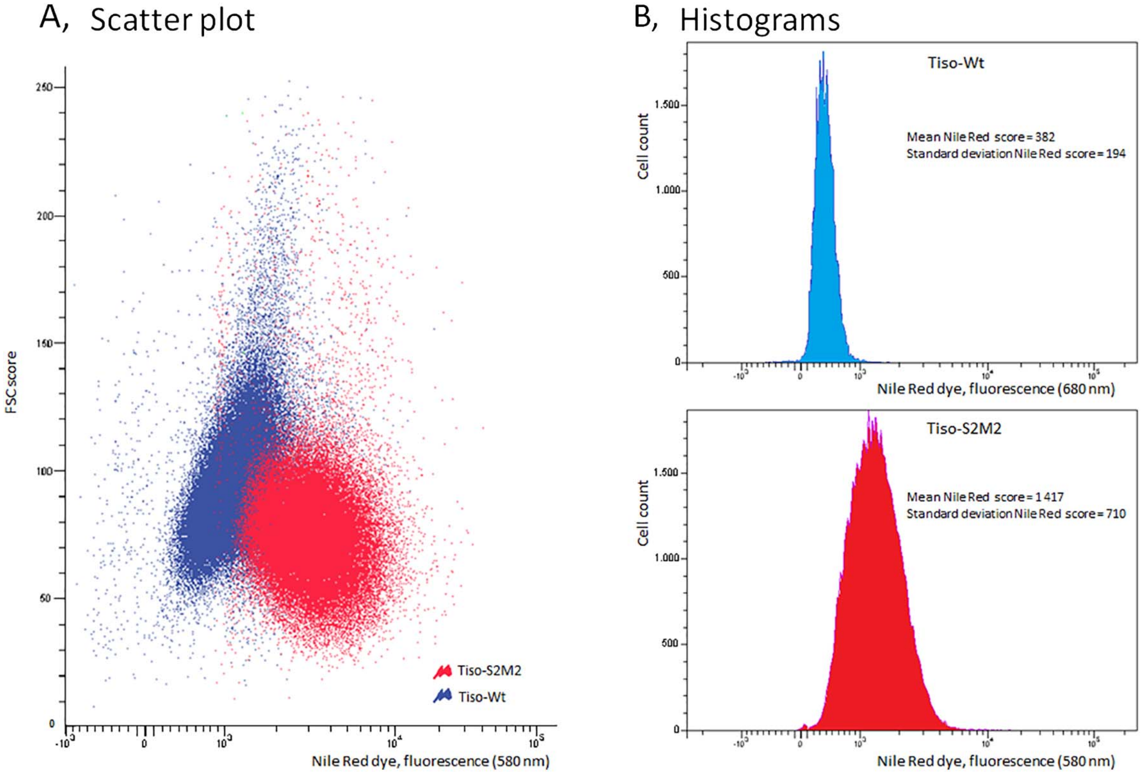

Fig. 1. Neutral lipid content of the cells of Tiso-WT and Tiso-S2M2 strains. Fig. A: scatterplot where each point represents an algae cells; blue: Tiso-WT strain; red: Tiso-S2M2 strain. All algal cells were dyed with Nile Red dye. Nile Red fluoresces at 560 nm and is linearly related to the neutral lipid content of the cell [12]. A high heterogeneity in neutral lipid content was observed in both strains. Fig. B: histograms showing the distribution of algae cell numbers as a function of Nile Red fluorescence. The mean neutral lipid content was higher for Tiso-S2M2 than for Tiso-Wt. Additionally, the standard deviation was higher for Tiso-S2M2 than Tiso-Wt, demonstrating a higher heterogeneity for neutral lipids in Tiso-S2M2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Tisochrysis lutea (Tiso) was chosen for this study because it has been used for decades in aquaculture as feedstock for shellfish, oyster and shrimps and has a promising future in the production of human food complements. Indeed, this microalga contains many antioxidant molecules of interest for health. In addition, it has been demonstrated to have a high content of long-chain polyunsaturated fatty acids such as docosahexaenoic acid (DHA) [10] and stearidonic acid (SA) [11].

An improvement program to increase lipid productivity was conducted by Bougaran et al. [12] using an empirical selection strategy based on a sequential mutation-selection procedure performed on the Tiso wild type strain. Mutagenesis was performed on Tiso-Wt with UV-c radiation treatments to increase the probability of obtaining individuals with interesting lipid profiles. To select these, a selection procedure was then applied using a flow cytometer with a cell sorter. The sorting of the best candidates according to their lipid traits was assisted by neutral lipid staining (Nile Red dye). Among the algal cells, the 10% most lipid-rich individuals were selected. Two successive rounds of mutation-selection were applied, through which the Tiso-S2M2 strain was obtained. The improved strain, demonstrated an increase in neutral lipid productivity of 80% compared to the initial Tiso-Wt strain [13]. In addition, the ability for lipid over-accumulation was proven to remain stable under our culture conditions for more than 6 years (until present).

After this first improvement program, molecular approaches were used to characterize the improved strain (Tiso-S2M2) and to guide further improvement strategies. An RNAseq approach was performed on cultures under nitrogen deficiency to compare coding sequences and their expression levels between Tiso-Wt and Tiso-S2M2 strains [14]. Through this work, the first reference transcriptome of the *Tisochrysis lutea* species was built. Variations in RNA and protein expression levels were measured to better characterize the metabolic differences between Tiso-S2M2 and Tiso-Wt [14,15]. At the different molecular levels (protein and RNA), many variations were identified associated with candidate genes (more than a hundred genes). This high candidate number suggested a large genome alteration during the improvement program leading to the Tiso-S2M2 strain. Overall, lipid synthesis in Tiso-S2M2 seemed similar to that in Tiso-Wt, whereas lipid catabolism, carbon assimilation, carbohydrate metabolism and many regulation proteins seemed to be more affected. Additionally, coding sequence analysis in the two strains revealed polymorphism (SNPs). Comparison of this genetic polymorphism between Tiso-Wt and Tiso-S2M2 revealed that, quantitatively, the amount of molecular variation was maintained in the improved strain Tiso-S2M2. These results suggested a conservation of algal cell diversity in Tiso-S2M2 despite the mutation-selection procedures. This diversity could be exploited for subsequent improvement programs. Several studies show that screening microalgal diversity is a good approach for selecting the most interesting strains [16–19].

In the present study, following the results from earlier research [12,14], the extent of intra-strain diversity in the wild-type strain and improved strain (Tiso-S2M2) were qualified on the neutral lipid content. Then, intra-strain diversity of Tiso-S2M2 was exploited to obtain new strains further improved for their neutral lipid content. Obtaining domesticated strains with high neutral lipid content is of interest for feed and biodiesel applications. To this end, cells with a high content of neutral lipids were isolated from Tiso-S2M2. The two best clonal strains (Tiso-S2M2-CL1 and Tiso-S2M2-CL2) in terms of neutral lipid content were examined to obtain details at the genomic and phenotypic level that would improve our understanding of the impact of the improvement program and the isolation procedure. To perform this step, the genome of the wild-type strain and the genome of the two new clonal strains were sequenced and their genetic polymorphism was assessed. A first draft reference genome of Tiso was reconstructed from the sequenced data. We thus also report the first sequenced genome of