Universidade de Aveiro Departamento de Biologia
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Joana Sofia Constantino Amaral

Famílias SnRK em Chlamydomonas: alvos promissores para bioprodução

SnRK families in Chlamydomonas: promising targets for bioproduction

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SnRK families in Chlamydomonas: promising targets for bioproduction

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor Luis Valledor González, Bolseiro de Pós-doutoramento do Departamento de Biologia da Universidade de Aveiro e coorientação da Doutora Glória Catarina Cintra da Costa Pinto, Bolseira de Pós-doutoramento do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro da FCT – Fundação para a Ciência e Tecnologia, através do projeto Chlamy4future (EXPL/AAG-TEC /2056/2013).

À avó Nita.

o júri

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agradecimentos

Gostaria de agradecer a todos aqueles que apoiaram a realização deste trabalho, quer em termos pessoais, quer em temos profissionais. Sem a vossa ajuda seria tão mais difícil.

Como não poderia deixar de ser, agradeço à minha família. À mãe, à mana, à avó, aos tios e primos. Pelo constante apoio e preocupação e por acreditarem sempre que tudo ia correr, mesmo quando eu tinha dúvidas. Foi muitas vezes complicado lidar com a saudade mas voltar a casa enchia-me sempre o coração. Sou, de facto, abençoada.

Agradeço ao Dr. Luis. Por partilhar comigo um pouco do seu mundo de génio. Pela paciência e tempo dispensados. Pela compreensão e ânimo quando as coisas não corriam tão bem! E por me receber tão bem no seu laboratório. Também à Dra. Glória. Pela preocupação e apoio e por acreditar no meu potencial ao longo dos últimos anos, propondo-me sempre novos desafios que me permitem desenvolver as minhas capacidades científicas.

Às equipas de trabalho de Oviedo e de Aveiro. Mais do que pela partilha de experiências e conhecimentos científicos, agradeço a vossa amizade. Em especial, agradeço ao Fran, por partilhar comigo o seu enorme conhecimento sobre Chlamydomonas e por me iniciar neste mundo das microalgas, sempre acompanhado de momentos de boa disposição. Não poderia também deixar de agradecer à Barbara e à Cláudia, que têm vindo a acompanhar a minha viagem no mundo cientifico. Obrigada por me terem acolhido desde o primeiro momento e por me ensinarem coisas novas a cada dia. É um privilégio poder trabalhar com pessoas como vocês.

Aos amigos. Aos de sempre e aos de agora. Obrigada por tudo.

Palavras-chave Microalgas, resposta a stress, biorefinaria, bioengenharia, biocombustível

Resumo

Dada a demanda mundial por energia e os custos ambientais associados ao uso de combustíveis fósseis, é crucial encontrar uma fonte de energia livre de emissões de CO_2, sustentável e renovável. As microalgas são das matérias-primas mais estudadas para a produção de biocombustíveis, especialmente por produzirem grandes quantidades de compostos energéticos (TAG e amido) e metabolitos secundários valiosos (como pigmentos, vitaminas e bioplástico). Atualmente utiliza-se uma estratégia de produção de duas fases que envolve um passo de imposição de stresse para acumular compostos interessantes para a produção de biocombustíveis. No entanto, o crescimento celular das microalgas é frequentemente reduzido, exigindo tempos de cultivo mais longos, e as técnicas de imposição de stresse são ainda caras, representando custos elevados para este processo. De forma a torná-lo rentável, é necessário usar uma abordagem de biorefinaria, combinando a extração de moléculas energéticas e de subprodutos de elevado valor económico. No entanto, a obtenção de biomassa continua a ser uma importante fator limitante. Para o ultrapassar é essencial estudar as redes metabólicas e regulatórias envolvidas na resposta a stresse de forma a identificar potenciais alvos para bioengenharia. Isto permitiria manter o crescimento celular em condições de stresse ou mimetizar um cenário de stresse através da ligação de um gene de interesse a um promotor induzido por um estímulo simples, reduzindo os custos de produção. A microalga modelo *Chlamydomonas reinhardtti* foi usada para estudar o envolvimento das proteinas quinases SnRK na resposta a stresse. Esta família está altamente associada à resposta a stresse em plantas. Alguns estudos relatam o seu envolvimento na resposta a stresse em Chlamydomonas, embora se saiba pouco sobre este fenómeno. As SnRK de Chalydomonas foram identificadas e classificadas com base nas semelhanças com as sequências e estruturas de domínios previamente descritas em Arabidopsis. Os seus padrões de expressão foram avaliados por RT-qPCR sob uma ampla gama de condições de stresse para procurar genes alvo envolvidos em vias de resposta a stresse em Chlamydomonas. Ao utilizar ferramentas bioinformáticas, foram identificadas 20 SnRK de 4 subfamílias (SnRK1 e as suas subunidades reguladoras e dois grupos de SnRK2). Surpreendentemente, a subfamília SnRK3, específica de plantas, não foi encontrada em Chlamydomonas. A análise dos padrões de expressão das SnRK por RT-qPCR identificou a SnRK2.9 como potencial candidata para estudos futuros, dado que esta mostrou uma resposta específica sob calor. De acordo com a análise dos dados de RNA-seq disponíveis, as SnRK2.12 e SnRK2.7 parecem ter um papel na mediação da deficiência em ferro e do stresse oxidativo, respetivamente. A radiação UV, que pode ser aplicada de forma barata, levou à acumulação de lípidos. Este trabalho representa um grande avanço para a investigação em microalgas e biologia do stresse, uma vez que, apesar de as SnRK serem um grupo chave de proteínas quinase em biotecnologia, esta família nunca foi descrita em microalgas.

Keywords Microalgae, stress response, biorefinery, bioengineering, biofuel

Abstract

Given the great world energy demand and the environmental costs associated to fossil fuels use, it is imperative to find a CO_2 neutral, sustainable, and renewable energy source. Microalgae are one of the most studied biofuel feedstock, mainly because they produce considerable amounts of energetic compounds (TAG and starch) and other valuable secondary metabolites (such as pigments, vitamins, and bioplastic). Currently, a two-phase cultivation strategy including a stress imposition step is used to accumulate interesting compounds for biofuel production. However, microalgae cell growth is often reduced, requiring longer cultivation times, and stress imposition techniques are still expensive, which represent high costs for the microalgal biofuel production process. In order to make it profitable, a biorefinery approach must be used, combining the extraction of energetic molecules and high value-added by-products. However, biomass supply continues to represent a major limiting factor. To overcome this limitation, the study of the metabolic and regulatory networks involved in stress response is essential so that potential targets for bioengineering can be identified. This would allow either the maintenance of cell growth under stress conditions or the mimicking of a stress condition by coupling a gene of interest to a promoter induced by a simple stimulus, reducing production costs. Therefore, the model microalga *Chlamydomonas reinhardtti* was used to study the involvement of SnRK protein kinases in stress response. This family is highly associated to plant stress response mechanisms. A few studies also report its involvement in Chlamydomonas stress response, although little is known about it. We identified and classified Chlamydomonas SnRK based on sequence and domain structure similarities with the SnRK sequences described in Arabidopsis using bioinformatic tools. Moreover, its expression patterns were evaluated by RT-qPCR under a wide range of stress conditions in order to look for target genes that might be involved in Chlamydomonas stress response pathways. By using bioinformatic tools 19 SnRK genes coding for 20 proteins from 4 subfamilies (SnRK1, its regulatory subunits, and two groups of SnRK2 proteins) were identified. Surprisingly, the plant-specific SnRK3 subfamily was not found in Chlamydomonas. The analysis of SnRK expression patterns under a wide range of stresses by RT-qPCR identified SnRK2.9 as a potential candidate for future studies as its response was specific to heat stress. Also SnRK2.12 and SnRK2.7 seem to have an important role in mediating Iron deficiency and oxidative stress, respectively, according to the mining of available RNA-seq data. Furthermore, from the stresses studied, UV radiation showed interesting results as it led to lipid accumulation and it is a stimulus that can be applied inexpensively. This work represents a great advance in microalgal and stress biology research since that, although SnRK are a key group of protein kinases for biotechnology, this family was never described before in microalgae.

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Part I

Current world energy demand requires alternative and sustainable energy sources

Since the beginning of times, man has been using natural resources in order to obtain energy for its own good. With the industrial revolution, this need became more obvious and man turned entirely dependent on energy use for its everyday life. Nowadays, the access to high quality energy sources is highly associated to prosperity and human well-being (Dale et al. 2014), as it is usual in developed countries. The report published last year by the International Energy Agency (IEA) give us a clear view of the current and worrying energy demand. In only about 40 years the total consumption of energy worldwide almost doubled, reaching 8 979 Million tonnes of oil equivalent in 2012 (IEA 2014) (Fig. 1A). Transportation and industry represent the major sectors responsible for energy consumption and rely mostly on oil, natural gas and coal, that represent more than 50% of the total energy used (IEA 2014) (Fig. 1A). These fossil fuels have been submitted to an enormous exploitation along the years. It is predicted that its prices rise (EC 2014) with the increasing consumption of its reserves, which will eventually run-off. Therefore, regarding the uncertain future of fossil fuels, the economic activities based on its consumption become unsustainable (Dale et al. 2014), making this problem a focus of big concern worldwide.

Moreover, the massive consumption of fossil fuels is intimately related with the increasing emission of greenhouse gas (GHG), especially carbon dioxide (CO_2) (IPCC 2014) (Fig. 1B). Total CO_2 emissions have been increasing since the industrial revolution and almost doubled in the last 40 years (IEA 2014). The high concentration of CO_2 in the atmosphere is one of the major impact factors to climate change, leading to the increase of the earth surface mean temperature. This increase leads to important changes in physical, biological, and human and managed systems globally (IPCC 2014) (Fig. 1C). Glacier melting, precipitation patterns changes, and ocean acidification are some of the most known phenomena that can have a strong impact on ecosystems biodiversity with consequences on ocean and earth life.

A great effort has been made in order to preserve the existent natural resources and biodiversity. The Kyoto Protocol and the RIO+20 United Nations Conference on Sustainable Development are good examples of that, bringing together world leaders to discuss this topic. However, besides the implementation of climate change mitigation policies and the sustainable practices associated to it, it is likely that GHG emissions continue to increase. According to the EC (2014) a great population is predicted in the next 25 years, which implies a greater energy

demand that comes with all the costs associated to it. It is therefore urgent to find alternative renewable energy sources able to respond to the current energy needs with fewer impacts in ecosystems.

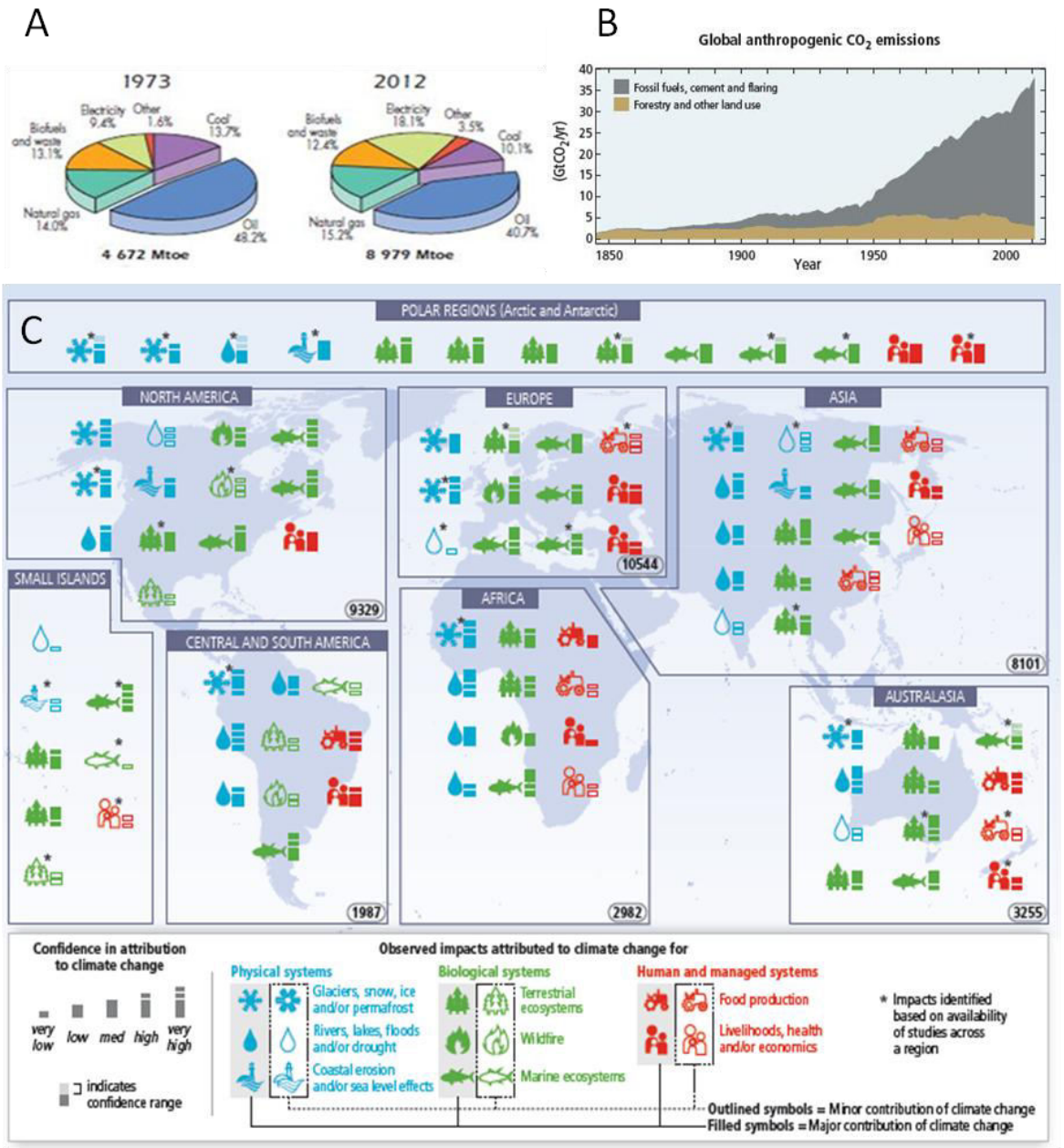

Fig. 1 Current energy demand and its consequences. **(A)** Total energy consumption evolution from 1973 to 2012 in million tonnes of oil equivalent (Mtoe) and respective percentage of energy sources used. Adapted from IEA (2014). **(B)** Evolution of global anthropogenic CO_2 emissions (gigatonnes/year) from forestry and other land use and from burning of fossil fuels, cement production and flaring. Adapted from IPCC (2014). **(C)** Impacts attributed to climate change based on scientific evidences from climate change publications from 2001 to 2010 (numbers in ovals in each region). Symbols indicate the categories of the attributed impacts, the relative contribution of climate change (major or minor) to the observed impact and the confidence of its attribution. Adapted from IPCC (2014).

Searching for alternative energy sources – Biofuels as a solution

As fossil fuels come along with great environmental costs associated, it is important to conserve energy, enhance efficiencies and look for alternative energy sources so to prepare a sustainable future (Dale et al. 2014). We could say that one of the biggest challenges of our times is to find a clean and renewable energy source that fulfils man needs. A great effort is being made to develop and improve alternative energy sources, such as solar energy, hydroelectric, geothermal, wind, biofuels, and carbon sequestration, among others (Dewulf and Van Langenhove 2006). However, biofuel, waste and other energy sources (geothermal, solar, wind, heat, etc.) still represent a minority in the global scenario (15.9% of the total energy consumed) (IEA 2014).

The most promising sustainable alternative to fossil fuels are biofuels, i.e., technologies that produce fuel with at least one component based on a biological system (Hannon et al. 2010), since they are non-toxic, biodegradable, and renewable energy sources that lead to the reduction of GHG emissions (Gouveia and Oliveira 2009). The most commons are biodiesel and bioethanol that replace diesel and gasoline, respectively (Mata et al. 2010). Ideally, these biofuels would be ready to use in the current infrastructures and vehicles or could be introduced into the processing chain of the existing refineries, having the same performance of fossil fuels (Savage 2011). Considering the particularly acute situation of the transport sector, with no relevant alternatives to fossil fuels, biofuels can easily replace current fuels with little or no modifications of vehicle engines (Mata et al. 2010). Also, biofuels can be produced using existing technologies and distributed by the available distribution system (Mata et al. 2010).

Biofuel production research is mainly focused on photosynthetic organisms since they naturally convert solar energy into chemical energy, stored in molecules such as lignin, cellulose, starch, and oils (Schenk et al. 2008; Merchant et al. 2012a). These molecules can be used for biofuel production, either directly or after conversion. In fact, research on green organism-based biofuel production is a main topic for the Portuguese government (ENE 2020) and the European Union (FP7 and Horizon 2020), aiming to increase their energy independence and competitiveness while promoting environmental and economic sustainability. Several possible biological systems suitable for biofuel production have been studied in order to achieve this goal. Based on the feedstock source biofuels can be classified into (a) first generation biofuels - prepared directly from food crops, mainly oleaginous crops, such as sugarcane, corn, and sunflower; (b) second generation biofuels - produced from non-edible plants and crop by-products; (c) and third

generation biofuels - produced using non-food feedstock, such as algae and photosynthetic bacteria (Abideen et al. 2014).

Crop plants biomass and oils are largely used for bioethanol and biodiesel production, respectively (Hannon et al. 2010). However, its performance is lower than that of fossil fuels. Also, its price is greatly increased because it is produced from food feedstock (Mata et al. 2010) and it requires extensive plantations, which will probably lead to land competition and biodiversity loss due to deforestation and use of ecological important areas (Gallagher 2008). Therefore, the use of crop plants for biofuel production is only feasible at small scale (Hannon et al. 2010) and, even if the current biofuel-producing crops occupied all the arable land worldwide, it would not be enough to respond to the current energy demand (Schenk et al. 2008). Furthermore, the intensive use of land leads to high fertilizer and pesticide applications and water use, which can represent significant environmental problems (Schenk et al. 2008).

Second and further generation biofuel-production systems upsurge as an alternative to overcome first generation biofuel production limitations as they have higher energy yields per hectare, are more water-efficient, and do not require agricultural land (Schenk et al. 2008; Mata et al. 2010). E.g. the extensive sunny western Iberian Peninsula, occupied by the *dehesa* (Spain) and the *montado* (Portugal) with soil and climate characteristics that make them unsuitable for intensive farming (Marañón 1988), could be more efficiently used if biofuel production facilities were integrated in these agro-sylvo-pastoral systems. Investigation on biofuels is still on its infancy but microalgae seem to be one of the feedstocks with more potential (Mata et al. 2010).

Potential of using microalgae for biofuel production – the biorefinery concept

Microalgae are fast growing photosynthetic microorganism (prokaryotic or eukaryotic) that are able to live even under harsh conditions (Mata et al. 2010). These are among the most diverse of all organism and are present in every aquatic and terrestrial ecosystem (Reijnders et al. 2014), being represented by more than 50 000 species (Richmond 2008). As reviewed by Tirichine and Bowler (2011), algae are extensively studied especially because they (a) are photosynthetic organisms, providing food to other organisms and sequestering great amounts of atmospheric CO_2 into the ocean, which represents an important mechanism against global warming; (b) have a primitive origin, allowing tracking back the evolution of life on Earth; (c) have unique structural, mechanical, biochemical and optical features that can be used to build devices for drug delivery,

biomolecule separation, and computer chip manufacturing; (d) can be used as model systems to study cellular functions; and (e) have a great potential to become a biofuel source.

Although Meier (1955) and Oswald and Golueke (1960) had already proposed the use of microalgae for methane gas production, the idea of using algae for biofuel production was first discussed only during the oil crises in the 1970s (Hu et al. 2008). Later on, the increase of fossil fuel prices and the request to reduce GHG emissions renewed the interest on using microalgae for biofuel production (Mata et al. 2010). In fact, according to Konur (2011) scientometric evaluation of the literature on algae and bio-energy published over the last 30 years, there was an exponential increase on this topic publications that visibly reflects this renewed interest. Microalgae are one of the most studied third generation systems for biofuel production. If its metabolism is engineered, microalgae-based biofuels are also named as fourth generation biofuels (Lü et al. 2011).

Microalgae are easy to cultivate, have a rapid life cycle and a fast growth rate, require little or no attention while growing, and are able to live in several environmental conditions (Mata et al. 2010). These organisms are able to accumulate considerable amounts of cellulose, starch and oils that might be used to produce bioethanol (cellulose and starch) and biodiesel (oils) (Schenk et al. 2008). Most studies are focused on studying microalgae lipid accumulation since biodiesel could easily respond to the high energy demand than bioethanol. Microalgal triacylglycerides (TAG; triple esters: three fatty acids esterified to a glycerol bone) are powerful energy storage molecules of great interest since they can be extracted and easily converted into biodiesel by transesterification reactions that displace glycerol with small alcohols, such as methanol (Waltz 2009; Sharma et al. 2012) (Fig. 2). Also, there is a great potential for other economical and environmental uses related to microalgae-based biofuel production, such as: (a) fixation of CO_2, leading to the reduction of GHG effects; (b) wastewater treatment by using water contaminants as nutrients (NH_4^+, NO_3^-, and PO_4^{3-}); (c) use of the biomass remaining from oil extraction as fertilizer or to produce ethanol, methane, electricity or heat; (d) extraction of fine chemical and bulk products such as fats, polyunsaturated fatty acids, oils, natural dyes, sugars, pigments, antioxidants, and high-value bioactive compounds (reviewed by Mata et al. (2010)).

However, besides all of the advantages pointed out for microalgae-based biofuels, it is still a matter of debate whether they can perform as good as other biofuels. The discussion between Chisti (2008a; 2008b) and Reijnders (2008) about if “Biodiesel from microalgae beats bioethanol” from crop plants is a great example of that, with Chisti (2008b) concluding that microalgal biofuels

have the potential to be far superior than traditional crop-based ones and to be produced sustainably. Also, cyanobacteria (prokaryotic bluegreen algae) are extensively studied as they have higher solar energy into biomass conversion efficiencies than food-crops and algae, well-established methods for genetic engineering, and secrete free fatty acids (FFA), precursors of biodiesel (Parmar et al. 2011). However, unlike microalgae, cyanobacteria do not naturally synthesise and accumulate significant amounts of TAG (Radakovits et al. 2010). Despite the distrust of many authors on microalgae-based biofuel and the hot discussion on it, this research field has been growing over the last years and there is a lot of hope on the development of this alternative energy source.

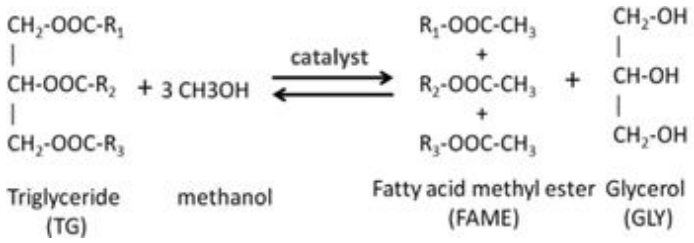

Fig. 2 Transesterification of TAG (TG) using methanol in order to produce biodiesel (FAME). R1-3 represent hydrocarbon groups. (Source: Shi et al. 2012).

Moreover, although it has been shown that microalgae productivity is higher than that of traditional crops (e.g. yields 8-25 fold higher than oil palm, 6 000 vs. 50 000-150 000 L/Ha per year), their cultivation for sole biofuel production (Fig. 3A) at commercial scale appears not yet to be economically feasible and sustainable (Markou and Nerantzis 2013). Currently, the most suggested production strategy for microalgae is a two-stage cultivation. Cells are grown in optimal conditions to produce biomass in a first stage and, later, stress conditions are applied aiming to accumulate desired compounds (Stephenson et al. 2010), such as sugars and lipids known to accumulate as a part of microalgae stress response mechanisms. However, it decreases cultures growth rate and, therefore, biomass production, increasing cultivation times and leading to productivity and profitability decrease. Moreover, in most cases harvesting of biomass is essential to move from first to second stage (i.e. when a nutrient must be completely removed from media or when culture densities needs to be artificially increased), increasing the cost of applying external stresses to production ponds or photobioreactors.

As researchers believe that the stress imposition step cannot be avoided, recent works aimed to mitigate its costs by increasing the cellular capability of accumulating biomolecules or by applying different stress gradients to partially overlap growth and stress periods (e.g. Ho et al. (2014)). Several genes have been proposed as potential targets for bioengineering, especially the ones related to lipid (TAG biosynthesis, lipid bodies formation and metabolism) (Merchant et al.

2012b) or sugars/starch (hexoses and starch synthases) (Blaby et al. 2013) biosynthetic pathways since they are directly related to the accumulation of energetic biomolecules. However, the increase of productivity observed was modest. This fact, together with the available system-wide studies of stress response in microalgae, points that the accumulation of these molecules is the result of a system metabolic adjustment rather than the overexpression of a certain pathway, showing the complexity of increasing biofuel production by following classic approaches.

In fact, a lot of effort is still required to improve microalgal biofuel production and make it economically competitive. These are highly attractive organisms for green technology (Wobbe and Remacle 2015), being one of the most interesting green biofactories that have been used for centuries as fertilizer, fodder, food, medicine, and, more recently, for biodiesel production (Sarmidi and El Enshasy 2012). Thus, its natural capacity to produce several compounds creates the opportunity to develop a sustainable process for biofuel production (Fig. 3B): the novel biorefinery approach concept (Fig. 3C), named after the petroleum refinery. It aims to increase production profitability by designing a combined process for extracting not only energetic molecules from biomass, but also secondary metabolites of high added-value such as pigments (astaxanthin, β-carotene, lutein), vitamins, or bioplastics (polyhydroxyalkanoate) (Markou and Nerantzis 2013). E.g. Adarme-Vega et al. (2012) proposed a bioprocess production chain that results in biodiesel, omega-3 fatty acids (ω-3), and protein-rich animal feed production. This biorefinery approach will undoubtedly be the future for microalgae-based biofuel production since that a sustainable production is achieved when all the products obtained represent an economical income. Despite biorefineries have the potential to be profitable in a near future, since the processes for co-purification of molecules are well-defined, the drawbacks related to biomass supply, namely the two-stages culture system and the development of strategies to maximized the co-accumulation of interesting molecules, must also be solved at short/mid-term to allow an adequate expansion of this technology in the current scenario of low prices mineral oils.

Therefore, studying the metabolic and regulatory networks involved in microalgae stress response is imperative to identify targets for future genetic manipulation that allow the accumulation of valuable compounds while maintaining cell growth or that mimic stress conditions, instead of applying them. Both possibilities would result in a considerable reduction of microalgal biofuel production costs, making it economically competitive when using a biorefinery production strategy.

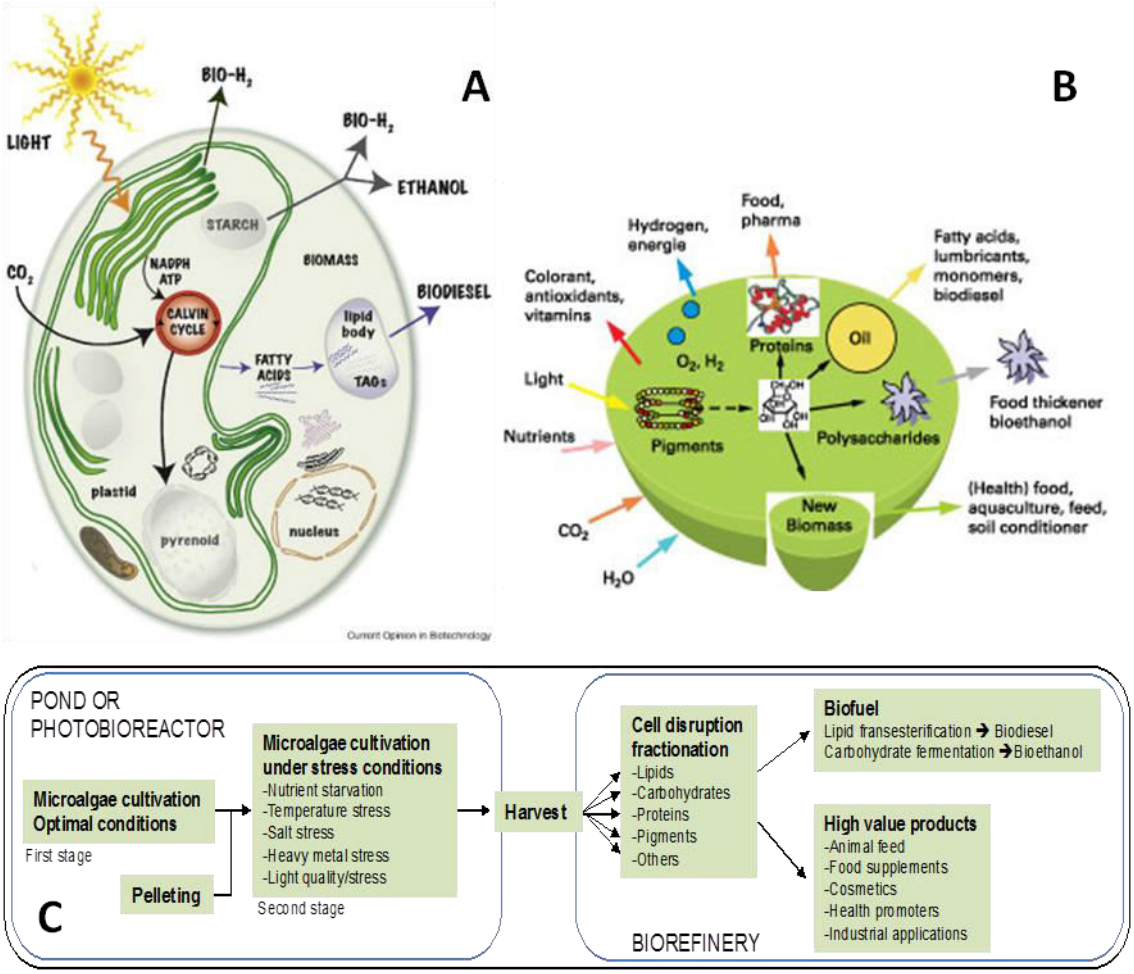

Fig. 3 Microalgae for a sustainable bioproduction of biofuel: the biorefinery concept. Instead of looking at microalgae as a system directed to biofuel production only (Source: Beer et al. 2009) **(B)** a sustainable bioprocess can be developed considering the natural ability of microalgae to produce several products with application in different fields (Source: Posten and Walter 2012). **(C)** This novel process is named biorefinery, after the current petroleum refineries, and is present in the scheme cultivation flow (Adapted from Markou and Nerantzis 2013).

Chlamydomonas reinhardttii, a model organism

The Chlorophycea is probably the most studied green algae group due to the establishment of *Chlamydomonas reinhardtii* as a model organism in plant biology (Hannon et al. 2010; Neupert et al. 2012). *C. reinhardtii*, also referred to as the *Eschericia coli* of algae (Waltz 2009) or the photosynthetic yeast (Rochaix 1995), is the only alga that attained the status of model organism over the last decades (Tirichine and Bowler 2011). It has been mainly used to study flagellar structure and function, genetics, basal bodies (centrioles), chloroplast biogenesis, photosynthesis, light perception, cell-cell recognition, and cell cycle control (Harris 2001). Chlamydomonas is a pioneer organism in several scientific discoveries (tetrad analysis, existence

and transformation of the chloroplastic genome, and transformation of the mitochondrial genome of a photosynthetic organism) (Lefebvre and Silflow 1999) and it is currently considered an attractive host for bioproduction in biotechnology (Neupert et al. 2012).

C. reinhardtii is an unicellular motile green algae with two flagella at its interior end, a hydroxyproline-rich glycoprotein wall, a single cup-shaped chloroplast that occupies approximately two-thirds of its volume, and several mitochondria (Rochaix 1995; Harris 2001; Neupert et al. 2012) (Fig. 3). Wild-type *C. reinhardtii* cells are oval-shaped and approximately 10 µm length and 3 µm width (Rochaix 1995). It is able to grow autotrophically, mixotrophically or heterotrophically (Rochaix 1995). Thus, wild-type *C. reinhardtii* is easily grown in neutral liquid or agar medium without any supplementary vitamins or co-factors, both with or without acetate and light, although their growth rates are increased in the presence of light (Harris 2001). Its optimal growth temperature is 20-25 °C and at 25 °C in a minimal medium with light (200-400 µE m^{-2} s^{-1} PAR (photosynthethically active radiation)) in average cell number doubles each 6-8 h (Harris 2001). When growth at light-dark cycles, cells remain in G1 during the light phase and divide throughout the dark phase (Harris 2001). Usually Chlamydomonas reproduce asexually by mitotic division (Rochaix 1995; Neupert et al. 2012). However, under certain conditions, such as Nitrogen deprivation, *C. reinhardtii* haploid cells (*mt+* or *mt-* mating type) start sexual reproduction (Rochaix 1995; Harris 2001; Neupert et al. 2012). The fact that *C. reinhardtii* is haploid during vegetative growth allows the immediately expression of mutations and the readily observation of specific mutant phenotypes as colonies on solid medium (Shrager et al. 2003).

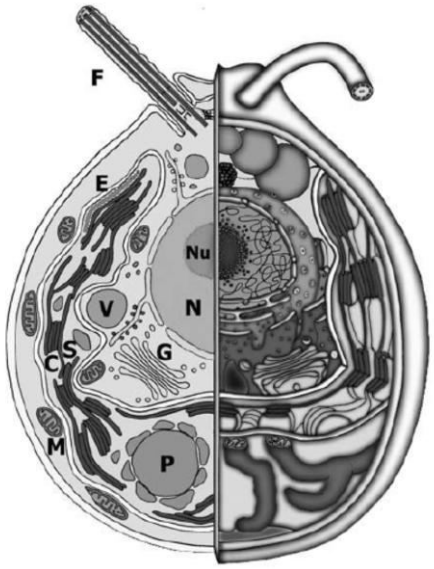

Fig. 4 *Chlamydomonas reinhardtii* cell structure: central nucleus (N) with the nucleolus (Nu), the two flagella (F), the cup-shaped chloroplast (C) with the eyestpot (E), the pyrenoid (P) and the mitochondria (M). Golgi vesicles (G), starch grains (S), and vacuoles (V) are also represented (Source: Harris 2009).

The *C. reinhardtii* genome is 121 Mb size, with 17 chromosomes (Tirichine and Bowler 2011) and approximately 15 000 genes (Grossman et al. 2007), comprising three autonomous genomes (nuclear, chloroplastic, and mitochondrial) that are fully sequenced and suitable of genetic transformation (Rochaix 1995; Harris 2001; Neupert et al. 2012). It is the only biological system where all the genomes have been successfully transformed (Lefebvre and Silflow 1999). The existence of the sequenced genome possibly allows to reveal new metabolic routes and their regulation mechanisms, enhances our knowledge about previously known metabolic pathways (Grossman et al. 2007), and facilitates genetic manipulation (Radakovits et al. 2010).

The Chlamydomonas simple and easily controlled cell life cycle, the ease of isolation of mutants, and the availability of tools for molecular genetic research (wild-type and mutant strains, plasmid constructs, and a large on-line database) makes it an ideal model system (Harris 2001). As a model organism it represents many features of its near relatives and may show facts or principles applicable to an entire domain of life (Davis 2004). Thus, although *C. reinhardtii* might not be the ideal species for biofuel production, as it does not produce much oil under natural conditions (Waltz 2009), the technologies established for this species have great potential for application in other promising oleaginous algae species (Hannon et al. 2010), such as its close relatives green algae Chlorella and Dunaliella. Besides its great lipid production they are also promising candidates for bioproduction of other high-valuable secondary metabolites, such as astaxanthin and β-carotene (Markou and Nerantzis 2013).

Stress imposition as a strategy to induce TAG production in Chlamydomonas

Lipid induction in microalgae has been studied along the last few decades thanks to its potential to enhance biofuel production. Currently, microalgae are submitted to stress conditions to enhance its TAG production (Reijnders et al. 2014). Physiologically, microalgae TAG not only have a storage function but also an adaptive function (Solovchenko 2012). It is thought that its accumulation under stress occurs because TAG (a) are the source of long-chain fatty acids for the membranes needed to photosynthetic apparatus rearrangements, (b) prevent photo-oxidative damage and subsequent reduction of cell capacity to use photosynthesis products in other biosynthetic processes, as its biosynthesis consumes excessive photoassimilates, and (c) in carotenogenic microalgae, are deposited as cytoplasmatic oil bodies, creating a deposit for secondary carotenoids forming an optical screen that protects cells against photodamage by excessive PAR (Solovchenko 2012).

Several studies were carried out exposing microalgae to different stress scenarios in order to evaluate lipid accumulation in such conditions. Nitrogen (N) starvation is one of the most widely investigated stresses as it is the most critical nutrient affecting lipid metabolism in algae (Sharma et al. 2012). It was stated by several authors that under N depletion *C. reinhardtii* accumulates considerable amounts of TAG (e.g. Wang et al. 2009; Siaut et al. 2011; Boyle et al. 2012; Valledor et al. 2014). Studies about other nutrient limitations are, however, scarcer. Regarding Phosphorous (P) limitation in *C. reinhardtii*, Weers and Gulati (1997) observed an increase in the amount of saturated, monounsaturated and diunsaturated fatty acids and a fatty acid composition similar to the N limited cells. Iwai et al. (2014) also compared the effect of N and P limitation in *C. reinhardtii* finding that both stresses induced oil droplets accumulation but that only under P deprivation the thylakoid membranes were maintained, which allows a higher accumulation of TAG. Similarly, under Sulphur (S) deprivation *C. reinhardtii* showed to accumulate large amounts of TAG (Matthew et al. 2009). Moreover, according to Cakmak et al. (2012), TAG content increased under S starvation reaching its peak after 4 days of starvation and, unlike N-starved cells, maintaining it afterwards, which demonstrates that S starvation could be a better strategy to enhance TAG production in *C. reinhardtii* than N starvation. Acetate was also described as a limiting factor and the central molecule in lipid droplet synthesis in *C. reinhardtii* (Ramanan et al. 2013) and it is well-known that this organism does not assimilate nitrate or ammonium if a carbon source, like acetate, is not available (Thacker and Syrett 1972).

Besides nutrient limitation studies, also some temperature shift experiments were already performed. Knowing that lipid profiles change with temperature, microalgae-based biodiesel properties would also change with different climates and seasons (Sharma et al. 2012), which makes this an important field of research. Sato et al. (2000) evaluated the effect of temperature (15 °C or 35 °C) on the *C. reinhardtii* thylakoid membranes acidic lipids content, concluding that it was not affected by this environmental factor. Valledor et al. (2013) unravelled for the first time cold stress adaptation in this organism reporting a reduction on total lipid content but an increase in desaturation of FAME (fatty acid methyl esters). FAME molecules in most cases represent biodiesel produced from vegetable oil or animal fat (Knothe 2005). In fact, although it is well-known that temperature influences fatty acid composition and total lipid content in algae, a general trend cannot be established as little information is available on this topic (Hu et al. 2008).

Salt stress induced TAG accumulation in *C. reinhardtii* as well (Siaut et al. 2011; Fan et al. 2011). Munnik et al. (2000) reported the increase in PA (phosphatidic acid) and its conversion to

DGPP (diacyl-glycerol pyrophosphate) in *Chlamydomonas moewusii* grown under hyperosmotic stress conditions (NaCl, KCl, glycerol, sucrose, and mannitol). PA is an intermediate in lipid synthesis and produces DGPP, which is a potential signalling molecule involved in one of the several phospholipid-based signalling pathways activated by osmotic stress in plants (Munnik et al. 2000). Similarly, Meijer et al. (2001) observed the increase of phospholipids during hyperosmotic stress, mainly LPA (lyso-phosphatidic acid) that was in part generated from PA. Hema et al. (2007) also tested different PEG (polyethylene glycol) concentration and observed that C*. reinhardtii* cells suffer a severe reduction in growth and chlorophyll degradation when submitted to high levels of osmotic stress. Nonetheless, no lipid accumulation or lipid profile changes were accessed.

However, by exposing cells to stressful environments, its growth is reduced and the trade-off between TAG production and cell growth becomes an important limiting factor (Klok et al. 2013). Unrevealing the metabolic and regulatory networks involved in stress response would allow us to modify them in order to increase its TAG synthesis while maintaining cell growth (Fig. 5). Besides recent studies are focused in engineering microalgae to improve its productivity and energy value, few progresses were achieved until recently (Mata et al. 2010). The experiment performed by Iwai et al. (2014) is an example of a few successful cases. A P deprivation-inducible promoter was constructed and the transformant obtained strongly enhanced its TAG accumulation.

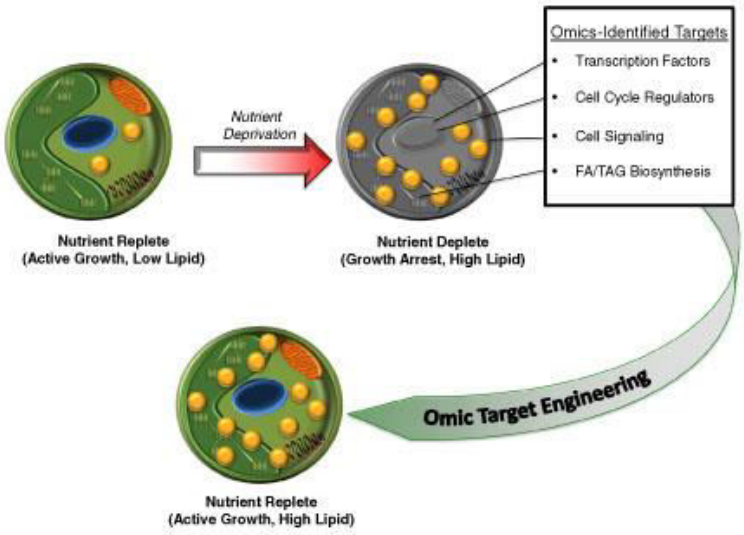

Fig. 5 Omic-driven strain engineering strategy. Nutrient deprivation increases the production of desirable products but sometimes cell growth is compromised. Knowing target genes and proteins involved in nutrient sensing, cell-cycle progression, and lipid accumulation allows the use of engineering strategies (gene silencing, knockout, and/or overexpression) to develop algal strains with relevant phenotypes, such as high lipid accumulation under optimal conditions and active growth (Source: Guarnieri and Pienkos 2015).

SnRK family and stress response – looking for target genes for bioengineering

Plants contain a large group of protein kinases related to the classical sucrose non-fermenting-1 (SNF1)-type kinases from yeasts. Rye RKIN1 was the first protein from this group to be described (Alderson et al. 1991). Later on, Halford and Hardie (1998) named this plant protein family as SNF1-related kinase (SnRK), recognizing three subfamilies (SnRK1, SnRK2, and SnRK3). In general, protein kinases play a key role on the regulation of cellular function (cell division, metabolism, and response to external signals) (Hrabak et al. 2003) as they promote protein phosphorylation/dephosphorylation, the major mechanism of post-translational regulation of protein activity and transduction of intracellular signals in eukaryotic organisms (Halford and Hey 2009). Yeast SNF1 is well known by its role in glucose repression, a mechanism that regulates carbon metabolism in yeast (Dickinson and Schweizer 2004). This mechanism affects the use of alternative carbon sources, gluconeogenesis, enzyme synthesis, respiration, and mitochondria and peroxisomes biogenesis and it ensures that glucose is always consumed first by fermentation to ethanol and that aerobic metabolism starts only when glucose levels are low (Halford and Hey 2009). SNF1 activity is greatly increased upon glucose removal (Woods et al. 1994; Wilson et al. 1996), being activated by the phosphorylation of its Thr^{210} residue after sensing of an elusive metabolite (Halford and Hey 2009).

SnRK represent one of the seven types of serine-threonine protein kinases from the CDPK-SnRK superfamily (Calcium-dependent protein kinase-SNF1-related kinase) (Hrabak et al. 2003). All CDPK-SnRK proteins contain a catalytic domain typical of eukaryotic Ser-Thr kinases that was first used for its classification into this superfamily (Hanks and Hunter 1995). Afterwards, proteins were assigned to different groups based on the sequence and function of their flanking domains. N-terminal domains are highly variable in length and sequence even within each subgroup but its function is not clear (Hrabak et al. 2003). Usually SnRK do not contain a putative N-terminal myristoylation sequence (like CDPK and CDPK-related kinases (CRK)) and thus it is unlikely to find them associated to the membrane thanks to this hydrophobic modification (Hrabak et al. 2003). In plants, only CDPK and SnRK3 contain an autoregulatory region immediately C-terminal to the kinase domain that is regulated by interaction with calcium-binding domain or protein, similarly to the animal CaMK (calmodulin-dependent protein kinase) and CCaMK (calcium and calmodulin-dependent protein kinase) (Hrabak et al. 2003). Most CDPK-SnRK have C-terminal domains that regulate kinase activity or mediate protein-protein interactions, as it is thought to happen in the highly variable SnRK C-terminus (Hrabak et al. 2003). Moreover, SnRK can be differentiated from

the other groups as it contains a Thr in their activation loop, a conserved subdomain (Hrabak et al. 2003).

Considering the premier model plant organism, 38 SnRK from three subgroups were found in Arabidopsis based on sequence similarity and domain structure (Hrabak et al. 2003). The three Arabidopsis SnRK1 sequences showed to be the most closely related to SNF1 from yeast and to AMP-activated protein kinases (AMPK) from animals (Hrabak et al. 2003). Both SNF1 and AMPK are involved in the regulation of the activity of key enzymes of carbon metabolism (Carling et al. 1987; Carling et al. 1989; Hardie et al. 1989) and reflect the cell energetic status based on the ATP/AMP balance and the level of stress that the cell is being submitted to, through the direct or indirect action of AMP over these enzymes (Hardie and Carling 1997). Likewise, as reviewed by Hrabak et al. (2003), several crop species studies suggest that SnRK1 are involved in metabolism regulation in response to nutritional or environmental stress through the interaction with other proteins on its C-terminal regulatory domain (Fig. 6).

The SnRK2 and SnRK3 subfamilies seem to be unique to plants (Halford et al. 2000), being larger and more diverse than the snRK1 subfamily (Halford and Hey 2009). In Arabidopsis SnRK2 and SnRK3 subfamilies comprise 10 and 25 genes, respectively (Halford et al. 2003; Hrabak et al. 2003). SnRK2 and SnRK3 catalytic domains are less similar to SnRK1 than SnRK1 is to SNF1 and AMPK (Halford et al. 2003; Hrabak et al. 2003). Halford and Hey (2009) propose that this happens because SnRK2 and SnRK3 emerged in plants by gene duplication of SnRK1 and diverged during plant evolution in order to play new roles that enable plants to link stress and ABA signalling with metabolic signalling. In fact, the members of these subfamilies play important roles in signalling pathways that regulate plant response to nutrient limitation, drought, cold, salt, and osmotic stress (Coello et al. 2011). Its involvement in response to stress in Arabidopsis was already reported e.g. under drought (SnRK2.8) (Umezawa et al. 2004), hyperosmolarity (Kobayashi et al. 2005), Sulphur starvation (SnRK2.3) (Kimura et al. 2006), and nutrient deprivation and growth reduction (SnRK2.8) (Shin et al. 2007). Also, the involvement of SnRK2 and SnRK3 in signalling cascades mediated by ABA (SnRK2.2, SnRK2.3 and SnRK2.6) (Fujii et al. 2009) or by auxins (Farrás et al. 2001) was already observed. Moreover, Halford and Hey (2009) explained that AREBP (ABA-responsive element-binding proteins) represent convergence points for signalling by the three plant SnRK families and probably by CDPK.

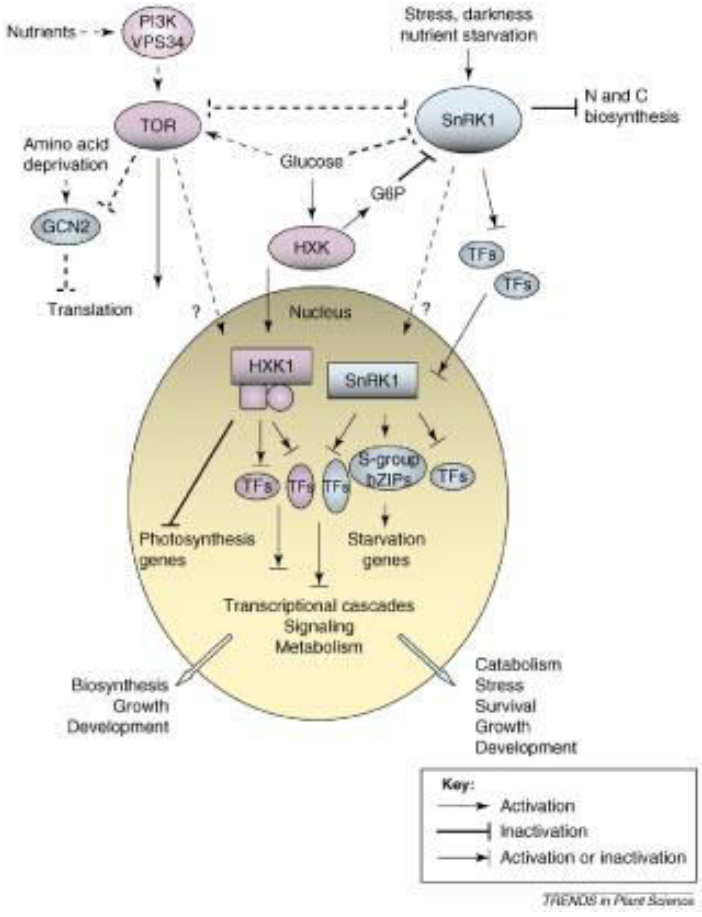

Fig. 6 A model of the interactions between nutrient and energy signalling components – Arabidopsis SnRK proteins mediate signalling networks (Source: Baena-Gonzalez and Sheen 2008).

Bioengineering SnRK in *C. reinhardtii* - potential to enhance bioproduction

The key role of SnRK on connecting metabolic and stress signalling makes them potential candidates for manipulation to improve crop performance in extreme environments (Coello et al. 2011). Studying this family in plants becomes a difficult challenge as they comprise a high number of members and there are compensatory capacities of the members from the different subfamilies (Dale et al. 1995). As *C. reinhardtii* shares common ancestry with vascular plants (Hannon et al. 2010), it is expected that many of their responses to limiting conditions are similar (Grossman 2000). By using less complex organisms to study this family of proteins, such as *C. reinhardtii*, the understanding and manipulation of the pathways in which this family is involved becomes easier. As said before, this green unicellular microalgae has its genome fully sequenced, which presents characteristics from both animals and superior plants but with an inferior number of genes and protein families and simpler pathways.

Furthermore, considering that it has been proved that stress conditions induce TAG and other high value-added biomolecules (e.g. astaxanthin, β-carotene or lutein) accumulation in *C.*

reinhardtii and the importance of TAG for biofuel production, a great interest rises for the study of SnRK and stress response in this model organism. There are only a few studies that suggest that, like in plants, SnRK are involved in stress response in *C. reinhardtii*, namely under Sulphur (Gonzalez-Ballester et al. 2008; Gonzalez-Ballester et al. 2010) and Nitrogen deprivation (Valledor et al. 2014) and cold stress (Valledor et al. 2013). Gonzalez-Ballester et al. (2008) started to define the SnRK2 family in Chlamydomonas and Valledor et al. (2013) found homologous sequences to the SnRK1.1/AKIN10 (CKIN1), SnRK1.2/AKIN11 (CKIN2), and other (CKIN3) sequences from Arabidopsis. CKIN1 expression has been diminished under cold stress (Valledor et al. 2013) and SnRK2.1 showed to be involved in Sulphur deprivation in Chlamydomonas (Gonzalez-Ballester et al. 2008).

However, a lot of effort is still needed in order to fully describe the SnRK family in *C. reinhardtii* and its involvement in stress response. Unravelling this will allow the future manipulation of genes of interest for biofuel production improvement. Using genetic engineering to manipulate target SnRK genes coupled to inducible promoters induced by simple stimulus represents a promising strategy for bioproduction in Chlamydomonas. This “stress mimicking” technology would allow cost reduction and time saving comparing to the process used nowadays for TAG accumulation. Together with the use of the valuable by-products produced from microalgae this would represent a great advance for micralgal biotechnology.

Thesis main purposes

Regarding the current energy demand and the urge to find a clean and renewable energy source, microalgae-based biofuels showed to be one of the most promising alternatives to fossil fuels. Microalgae accumulate considerable amounts of lipids and carbohydrates that can easily be used for biofuel production. Microalgal TAG are one of the most interesting compounds for biofuel production and it has been already shown that its levels greatly increase in microalgae submitted to several abiotic stresses. However, the current systems using stress to increase lipid accumulation in microalgae are not economically sustainable yet, especially because of the costs associated to stress imposition and cell growth reduction. To overcome these limitations a biorefinery approach must be used and the metabolic and regulatory networks involved in microalgae stress response should be investigated to find targets for microalgae bioengineering.

The SnRK protein kinases rise as a family with great potential to look for targets for bioengineering. In plants it is obvious the role of the SnRK in stress response and its involvement in carbon metabolism. However, in microalgae little is known about this family. Only a few studies showed that in *C. reinhardtii* there are also some SnRK involved in stress response. Studying SnRK and stress response in microalgae becomes a target of interest as it will allow further studies of bioengineering of metabolic pathways in order to enhance its bioproduction capacity. *C. reinhardtii* is used for this purpose as it is a well-established model organism and thus easier to study. The results obtained by using this organism can afterwards be applied to other close relatives with higher production rates.

Therefore, the thesis herein present as a research paper aims to define the SnRK family in Chlamydomonas. In order to do so, the following topics are explored:

1. Identification and classification of the SnRK family in Chlamydomonas;
2. Evaluation of physiological responses and expression profiles of Chlamydomonas SnRK genes under abiotic stresses.

Part II

SnRK family in Chlamydomonas: promising targets for bioproduction

Genome-wide description of the Chlamydomonas SNF1-related kinases protein family: Involvement in abiotic stress response.

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Abstract

Microalgae can be considered next generation biofactories since they exhibit a great capacity for accumulating energetic (TAG and sugars) and high value-added (such as astaxanthin, β-carotene, and lutein) biomolecules. However, their profitability is still questionable, mainly because of its elevated production costs. Its major limiting factor is that the accumulation of different biomolecules must be triggered by a specific stressful situation. SnRK have been widely studied in Arabidopsis and other model organisms as key regulators of abiotic stress response, including those that lead to the accumulation of lipids or sugars in microalgae. Herein we describe 20 SnRK protein kinases in the model species *Chlamydomonas reinhardtii* using a genome-wide approach. These were classified into four subfamilies (SnRK1, regulatory subunits of SnRK1, and two groups of SnRK2) according to their protein sequence and domain structure similarities. Unexpectedly, any member of the SnRK3 subfamily was found in this species. RT-qPCR expression analyses of all the members of this family under a wide set of abiotic stresses, together with the mining of available high-throughput data, allowed the identification of the stress responsiveness of SnRK family members such as SnRK2.7 (oxidative stress), SnRK2.9 (heat stress), or SnRK2.12 (Iron deficiency). These SnRK appear as interesting targets for further bioengineering-based studies that aim to improve the current microalgae strains. Therefore, these results represent a great advance for increasing our understanding on microalgal stress biology and its potential biotechnological applications.

Keywords: microalgae, stress response, abiotic stress, signalling, SnRK, bioengineering, biofuel

Introduction

Currently the world is facing great problems concerning energy supply and the increasing atmospheric CO_2 levels resulting from energy use, namely fossil fuels. Thus, one of the main challenges of our times is to find a sustainable clean and renewable energy source that is CO_2 neutral. Non-edible feedstock-based biofuels represent a promising alternative to fossil fuels. These have higher energy yields per hectare than crop-based biofuels, are more water-efficient, and do not require agricultural land to grow (Schenk et al. 2008; Mata et al. 2010). Microalgae are one of the most studied non-edible biological systems. Apart from its ease of cultivation, fast life cycle and growth rate, and capacity to live under several environmental conditions, other advantages can be taken from its use for biofuel production: (a) reduction of CO_2 effects by its fixation; (b) wastewater treatment by using water contaminants as nutrients; (c) use of biomass as fertilizer or to produce ethanol, methane, electricity or heat; (d) and extraction of chemical and bulk products and high-value bioactive compounds (reviewed by Mata et al. 2010).

The powerful microalgal energy storage TAG (triacylglycerides) molecule is highly interesting for biodiesel production (Merchant et al. 2012). Several studies report its accumulation under stress, mainly regarding nutrient limitation (e.g. Cakmak et al. 2012; Valledor et al. 2014b; Iwai et al. 2014). Stress imposition is, in fact, currently used as a strategy to enhance TAG production (Reijnders et al. 2014), specially using a two-stage cultivation process. I.e. on a first stage cells are grown under optimal conditions to produce biomass and then a stimulus is used to trigger the accumulation of the desired compounds (Stephenson et al. 2010). However, cell growth is often compromised under such conditions (Klok et al. 2013), requiring larger cultivation times, and the costs of applying a stress condition are still high (e.g. removing a nutrient from the media), making the cultivation of microalgae for sole biofuel production economically unsustainable. The novel biorefinery production approach rises as an alternative, aiming to combine the extraction of energetic molecules from biomass with the extraction of other valuable secondary metabolites, such as pigments (astaxanthin, β-carotene, and lutein) vitamins, and bioplastics (polyhydroxyalkanoate) in order to increase biofuel production profitability (Markou and Nerantzis 2013). However, the drawbacks related to biomass supply still need to be solved. Studying the metabolic and regulatory networks involved in stress response is the first step to identify targets for genetic engineering in order to obtain microalgae strains that are able to maintain its growth rates under stress conditions (Guarnieri and Pienkos 2015). Furthermore, using a “stress mimicking” technology that couples an inducible promoter triggered

by a simple stimulus to a gene involved in the response of a specific stress condition would greatly reduce valuable-biomolecules cost production as microalgae cells would mimic stressful scenarios with no need to apply it.

The cAMP-dependant SnRK [Sucrose non-fermenting-1 (SNF1)-related protein kinase] protein kinase family emerges as an interesting object of study concerning microalgae stress response. This protein family mediates the connexion between central metabolism, gene regulation, and stress response together with hexokinases (HXK) and sucrose phosphatases (SPP). SNF1 was initially described in yeast, being well-known by its role in glucose repression, regulating carbon metabolism (Dickinson and Schweizer 2004). However, the specific role of each SnRK is mostly unknown. These kinases concentrate divergent stress signals by activating different enzymes and transcription factors, not only related to metabolic regulation but also to protein biosynthesis and cell organization, being the key regulators of a complex system (Halford and Hey 2009). The SnRK family also controls lipid accumulation in yeast (Kamisaka et al. 2007) and has been linked to increased tolerance to Nitrogen stress (Aukerman et al. 2010) and to energy sensing and gene regulation (Robaglia et al. 2012) in Arabidopsis, being considered a potential target to improve plant performance under unfavourable conditions (Coello et al. 2011).

Studies in Arabidopsis showed that 38 members from 3 subfamilies compose this family: 3 SnRK1, 10 SnRK2, and 25 SnRK3 (Hrabak et al. 2003). Plant SnRK1 family is implied in plant response to starvation and energy deficit by coordinating the energetic balance (ATP/cAMP) and C/N ratios to specifically regulate broad branches of the metabolism, either directly by phosphorylation of enzymes or transcription factors (Halford and Hey 2009). SnRK2 and SnRK3 appear to be unique to plants (Halford et al. 2000) and there is strong evidence that they evolved after gene duplication of SnRK1 in order to enable plants to develop networks capable of linking stress and ABA signalling with metabolic signalling (Halford and Hey 2009). In fact, the SnRK2 and SnRK3 showed to have a key role in signalling pathways that regulate plant response to nutrient limitation, drought, cold, salt, and osmotic stress (Coello et al. 2011). The SnRK2 subfamily has an essential role in the control of gene expression through the activation of bZIP transcription factors and SWI/SNF/helicase complexes (Baena-González and Sheen 2008; Fujii et al. 2011) tightly connected to epigenetic mechanisms to perfectly control gene activation or repression. This system, together with the intervention of TOR, has been reported to be quickly responsive to cold stress and Nitrogen starvation, being correlated to lipid and starch accumulation in animals (Shaw 2009). However, little is known about the role of this protein kinase family in microalgae stress

response mechanisms and its relation with biotechnological processes, such as the accumulation of high value-added molecules like energetic molecules (sugars and lipids) or pigments (astaxanthin, lutein, and β-carotene).

The Chlorophyceae *Chlamydomonas reinhardtii* is the model organism for microalgae research. The availability of a wide set of molecular tools (Harris 2009) and of its genome sequence (Merchant et al. 2007), the suitability for genetic transformation, and the attractiveness for bioproduction in biotechnology (Neupert et al. 2012), together with its close relation to the economically relevant Chlorella and Dunaliella genera, make this species a strong candidate for microalgal research. Given that it shares common ancestry with vascular plants (Hannon et al. 2010) it is expected that many of their responses to limiting conditions would be similar (Grossman 2000). However, only a few studies suggest the involvement of SnRK in Chlamydomonas stress response, namely under Sulphur (Gonzalez-Ballester et al. 2008; Gonzalez-Ballester et al. 2010) and Nitrogen deprivation (Valledor et al. 2014b) and cold stress (Valledor et al. 2013). Gonzalez-Ballester et al. (2008) reported the existence of eight putative SnRK2 in Chlamydomonas (SnRK2.1-SnRK2.8) while as Valledor et al. (2013) found three homologous sequences to Arabidopsis SnRK (CKIN1, CKIN2, and CKIN3). Both authors suggested that, like in plants, Chlamydomonas stress response is mediated by SnRK. The availability of the complete sequence of the Chlamydomonas genome provides a unique opportunity for identifying SnRK family members in this unicellular microalga. Furthermore, the identification of stress-specific dynamics of SnRK would reveal clear targets for further bioengineering research to accumulate economically relevant biomolecules, since these control entire branches of the metabolism.

In the present study we aim to fully describe the entire SnRK family in Chlamydomonas by using bioinformatic tools and to describe its potential implication in stress response mechanisms by testing the expression levels of each SnRK member under a wide-range of stress conditions by RT-qPCR and data mining of available high-throughput datasets. The results herein present represent a great advance in microalgae and stress biology research since that, although SnRK are a key group of protein kinases for biotechnology, this family was never characterized before in microalgae.

Methods

SnRK sequence identification and classification in Chlamydomonas

An initial set of Chlamydomonas sequences belonging to the SnRK family was obtained by BLAST based homology search of the identified *Arabidopsis thaliana* SnRK protein sequences (Coello et al. 2011) (Supplementary Table 1) (TAIR10, The Arabidopsis Information Resource) (Lamesch et al. 2012) against the Chlamydomonas genome (v5.5, Joint Genome Institute, DOE) (Merchant et al. 2007). All sequences are available at Phytozome (Goodstein et al. 2012). Chlamydomonas protein sequences were considered homologous for e-values lower than 10^{-25} and included in a first uncurated list.

Proteins containing similar domains and showing high homologies to this family (i.e. CDPKs) were filtered out after comparing the domain structure of all candidate sequences using InterProScan (Zdobnov and Apweiler 2001). Furthermore, Arabidopsis domains (Pfam and PANTHER automated annotations detailed in Supplementary Table 2) were used as reference to search for potential SnRK in the Chlamydomonas genome using BIOMART (Smedley et al. 2015). ClustalW alignments led to the identification of conserved regions within each group of proteins.

The SnRK sequences were clustered by UPGMA and neighbour-joining protein sequence trees using the jukes-cantor model to define the SnRK family and its subfamilies and detect misclassified genes (such as the closely related CDPKs). For a better subfamily definition SnRK sequences from *Arabidopsis thaliana* SnRK1, SnRK2 and SnRK3 subfamilies, *Saccharomyces cerevisae* and *Homo sapiens* were used, together with *Chlamydomonas reinhardtii* CDPK sequences (used as outgroup) (Supplementary Table 3).

Chlamydomonas culture and stress response characterization

Chlamydomonas cells were exposed to a wide range of abiotic stress to generate materials for expression quantification of each SnRK under each situation by using quantitative PCR. Chlamydomonas CC-503 cw92, nit1, nit2, agg1+, mt+ was used for all the experiments conducted. Cells were grown on a closed incubator at 25 °C, 120 rpm, a 16:8 h (day:night) photoperiod and a 190-200 µE m^2 s^{-1} light intensity provided by warm white LEDs in liquid HAP culture media (Harris 2009) supplemented with 10mM sodium acetate at an initial cell density of 3-5 x 10^5 cells ml^{-1}. These control conditions were changed to test each one of the stresses studied (Table 1).

Table 1. Stress assays and conditions changed to test each one of them

Stress assay	Changed Condition
Nitrogen deprivation (-N)	Substitution of ammonium chloride for potassium chloride
Carbon deprivation (-C)	Removal of sodium acetate
Sulphur deprivation (-S)	Substitution of sulphate salts for chloride and nitrate salts
Phosphorous limitation (5% P)	5 % of the standard phosphorous content
Heat Stress (40ºC)	Increase incubator temperature to 40 °C
Cold Stress (4ºC)	Decrease incubator temperature to 4 °C
UV radiation Stress (UV)	30 minute UV irradiation each 24 h
Salt stress (0.25M NaCl)	Addition of 0.25 M sodium chloride
Osmotic stress (20% PEG)	Addition of 20 % PEG 4000

Samples were collected at the beginning of the experiment (0 h; control) and 48 h after the start of the assay (stress). Fifty ml of fresh culture were centrifuged (4000 rpm, 6 min at room temperature). Supernatants were discarded, cell pellet masses were estimated gravimetrically, and then immediately frozen in liquid nitrogen. Samples were kept at -80 ºC until RNA extraction. Gravimetrical measures at the stress phase (48 h) were normalized by its corresponding controls (0 h). Furthermore, 2 ml of cell culture were fixed in 3 % (v/v) formaldehyde and stained with Lugol stain and Nile red solutions for starch and lipid observation, respectively. This analysis was performed under an Eclipse E600 fluorescence microscope (Nikon, Japan) following the procedure described by Valledor et al. (2013) and Valledor et al. (2014b), respectively.

RNA extraction, cDNA synthesis, and qPCR

RNA was extracted using the method described by Valledor et al. (2014a). cDNA was synthesized using RevertAid Reverse Transcriptase (Thermo Scientific), random hexamers, and 1.7 µg of RNA in 20 µl final volume reactions following the manufacturer's specifications.

Real time PCR analysis was performed using the CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD). The 20 µL individual reactions prepared contained 1x Maxima Sybr Green qPCR Master Mix (Thermo Scientific), 0.5 µM of each primer, 2 % DMSO, and 0.7-0.8 µg of cDNA. The amplification protocol consisted in an initial denaturation at 95 °C for 10 min followed by 50 cycles of denaturation at 95 °C for 15 s and annealing at 61 °C for 30 s, with fluorescence measurement after this step. The specificity of the PCR amplification was evaluated by a 60 cycle melting curve starting at 60 °C and increasing 0.6 °C/cycle. Relative expression levels were

determined in 24 independent experiments for each primer pair (Supplementary Table 4). Each individual experiment was performed with two analytical replicates for each one of the four individual biological replicates from each condition tested. Ubiquitin and the receptor of activated protein kinase (RCK1) were selected as endogenous controls after testing the expression stability of Actin, Ubiquitin, Tubulin, and RCK1 with geNorm software (Vandesompele et al. 2002). Significant differences between stress conditions were determined for each SnRK after applying ANOVA to the ΔΔCq values (Supplementary Table 5) following Hellemans et al. (2007) recommendations.

Chlamydomonas SnRK family in silico functional characterization

Since SnRK are known to be responsive to abiotic stresses in several plant species, an initial *in silico* functional characterization was performed using the publicly available datasets in the AlgaePath repository (Zheng et al. 2014). Different stress conditions were available: Sulphur depletion (Gonzalez-Ballester et al. 2010), Nitrogen deprivation (Miller et al. 2010), low CO_2 content (Fang et al. 2012), oxidative stress (Urzica et al. 2012), and Iron deprivation (Urzica et al. 2012). SnRK abundance values were obtained by normalizing its abundance under stress conditions against the basal abundance of its corresponding controls.

Bioinformatic and statistical analyses

All the procedures for the identification and classification of Chlamydomonas SnRK were performed locally employing the bioinformatics suite Geneious v7 (Biomatters Inc.), with the exception of InterProScan (Zdobnov and Apweiler 2001) and BIOMART (Smedley et al. 2015) searches that were performed at the European Bioinformatics Institute (Goujon et al. 2010) and Phytozome (Goodstein et al. 2012) websites respectively. R v.2.12 software (R Core Team 2014b) core functions and the gplots2 and pheatmap packages were used under the R Studio Environment (RStudio Team 2014a) to perform the statistical analyses and heatmap plotting. Four biological replicates and two technical replicates were considered for each condition tested. Differences between the control and each one of the stress conditions were tested by Student's t-Test ($p \leq 0.05$). Significant differences between the stress conditions were determined for each SnRK by ANOVA of the ΔΔCq values ($p \leq 0.05$). Clustered heatmaps were constructed using either individual -ΔΔCq values or average of the -ΔΔCq of all samples corresponding to each situation. The *in silico* data heatmap was plotted considering the stress-expression values of each gene normalized by its respective expression in controls.

Results

Identification and classification of the SnRK family in Chlamydomonas

From the initial BLAST search (e-value < 10^{-25}) 112 Chlamydomonas sequences similar to the SnRK Arabidopsis sequences described by Coello et al. (2011) (Supplementary Table 1) were found (data not shown). The use of InterProScan allowed the unequivocal distinction between CDPKs and SnRKs based on its specific domains. By combining the results obtained from BLAST and protein domain validation, 17 SnRK sequences were found in Chlamydomonas (Table 2). Moreover, the use of BIOMART to search for SnRK characteristic domains into the Chlamydomonas genome allowed the identification of 3 more putative SnRK sequences (Table 2). Altogether, a total of 19 putative genes coding for 20 proteins corresponding to the SnRK family were found in *C. reinhardtii* (Table 2). Eleven of the SnRK genes found were previously described by Valledor et al. (2013) and Gonzalez-Ballester et al. (2008), while 9, corresponding to regulatory subunits of the SnRK1 complex and to SnRK2 subfamily proteins, were not previously described in Chlamydomonas.

The construction of sequence trees by UPGMA (Fig. 1) and neighbour-joining (Supplementary Fig. 1) allowed the classification of the putative *C. reinhardtii* SnRK. SnRK1.1 (S1 cluster) grouped with the previously annotated Arabidopsis SnRK1 proteins (*At*AKIN10, *At*AKIN11, and *At*SnRK1.3), Saccharomyces SNF1, and human AMPK. Regarding SnRK1 regulatory subunits (S1 R cluster): Chlamydomonas CKIN3 showed to be closely related to the γ subunit 1 and 2 from *A. thaliana*; Arabidopsis, human and Chlamydomonas β subunits were similar; βγ from *C. reinhardtii* grouped with its Arabidopsis homologous and with *Homo sapiens* AMPK γ subunit. Chlamydomonas SnRK2 (S2 cluster) represents the largest SnRK subfamily (16 elements) and seems to be divided in 2 subgroups, one of them comprising the previously described *C. reinhardtii* SnRK sequences that group with *A. thaliana* SnRK2. However, SnRK2.13 showed different clustering results according to the algorithm used for sequence tree design, not being included in any specific SnRK2 group. Surprisingly, no SnRK3 sequences were found in Chlamydomonas. Moreover, the CDPK sequences used for this analysis group together, as expected. Altogether, we can consider four SnRK clusters in Chlamydomonas: SnRK1 (S1); βγ regulatory subunits of SnRK1 (S1 R); and SnRK2 (S2), which is divided in two subgroups (S2 C and S2 D) (Table 1).

Table 2. Chlamydomonas (*Cre*) SnRK sequences found by BLAST (e-value < 10^{-25}) and protein domain searches. Sequence accessions and corresponding Arabidopsis (*At*) query sequences accession, homology search e-value (or domain if found only by protein search), and Chlamydomonas sequence name. SnRK were grouped according to sequence similarity and protein domains layout (S1: SnRK1; S1R: regulatory subunits of SnRK1; S2: SnRK2; SnRK2 D: SnRK2 containing the Serin/Threonin Kinase SRK2D domain; SnRK2 C: SnRK2 containing the Serin/Threonin Kinase SRK2C domain). *Chlamydomonas sequences previously referred to as SnRK by [1]Valledor et al. (2013) and [2]Gonzalez-Ballester et al. (2008).

***Cre* Accession**	***At* Accession**	**e-value**	**Name**	**Cluster**
Cre04.g211600.t1.1	AT3G01090.1	0	SnRK1.1 *[1]	S1
Cre10.g457500.t1.1	AT4G16360.1	$1.86e^{-69}$	β	S1 R
Cre12.g484350.t1.3	AT1G09020.1	$2.88e^{-52}$	βγ	S1 R
Cre12.g528000.t1.2		Domain	CKIN3 *[1]	S1 R
Cre02.g075850.t1.1	AT4G33950.1	$1.33e^{-57}$	SnRK2.1.t1 *[2]	S2 D
Cre02.g075850.t2.1	AT1G78290.2	$7.08e^{-53}$	SnRK2.1.t2*[2]	S2 D
Cre12.g499500.t1.1	AT1G78290.2	$4.02e^{-117}$	SnRK2.2 *[2]	S2 D
Cre02.g075900.t1.1	AT5G66880.1	$6.60e^{-73}$	SnRK2.3 *[2]	S2 D
Cre11.g477000.t1.2	AT5G08590.1	$7.14e^{-29}$	SnRK2.4 *[2]	S2 D
Cre03.g209505.t1.1	AT1G78290.2	$2.60e^{-63}$	SnRK2.5 *[2]	S2 D
Cre11.g481000.t1.2	AT4G33950.1	$1.91e^{-88}$	SnRK2.6 *[2]	S2 D
Cre06.g292700.t1.2	AT4G33950.1	$8.68e^{-103}$	SnRK2.7 *[2]	S2 D
Cre10.g466350.t1.1	AT4G33950.1	$2.96e^{-152}$	SnRK2.8 *[2]	S2 D
Cre13.g568050.t1.3	AT4G33950.1	$4.77e^{-58}$	SnRK2.9	S2 C
Cre16.g657350.t1.2	AT1G78290.2	$5.77e^{-59}$	SnRK2.10	S2 C
Cre17.g707800.t1.2	AT1G78290.2	$9.81e^{-46}$	SnRK2.11	S2 C
Cre12.g485600.t1.2	AT1G78290.2	$5.64e^{-47}$	SnRK2.12	S2 C
Cre02.g076000.t1.2	AT5G63650.1	$2.10e^{-40}$	SnRK2.13	S2
Cre08.g384250.t1.2		Domain	SnRK2.14	S2 C
Cre17.g707650.t1.1		Domain	SnRK2.15	S2 C

The ClustalW SnRK protein sequences alignment (Fig. 2) supports the CDPK-SnRK sequence trees. The proteins within each group have highly conserved regions that correspond to domains previously described as characteristic from the proteins herein studied. The Serin/Threonin Kinase domain (PTHR24343) is present both in the putative SnRK1 and SnRK2 sequences found in Chlamydomonas and in the corresponding Arabidopsis proteins (Fig. 2; Supplementary Table 2). Conversely, the Serin/Threonin Kinase SRK2C domain (PTHR24343:SF55) seems to be unique to Chlamydomonas SnRK2 (Supplementary Table 2), namely to SnRK2.9-2.12, SnRK2.14, and SnRK2.15, one of the two SnRK2 clusters observed in the sequence tree (Fig. 1 and Supplementary Fig. 1) (named S2 C after this domain). Moreover, we could observe that SnRK2 contain C-ends rich in repetitive amino acids and that SnRK1 contains a highly conserved region that was not previously described (Fig. 2).

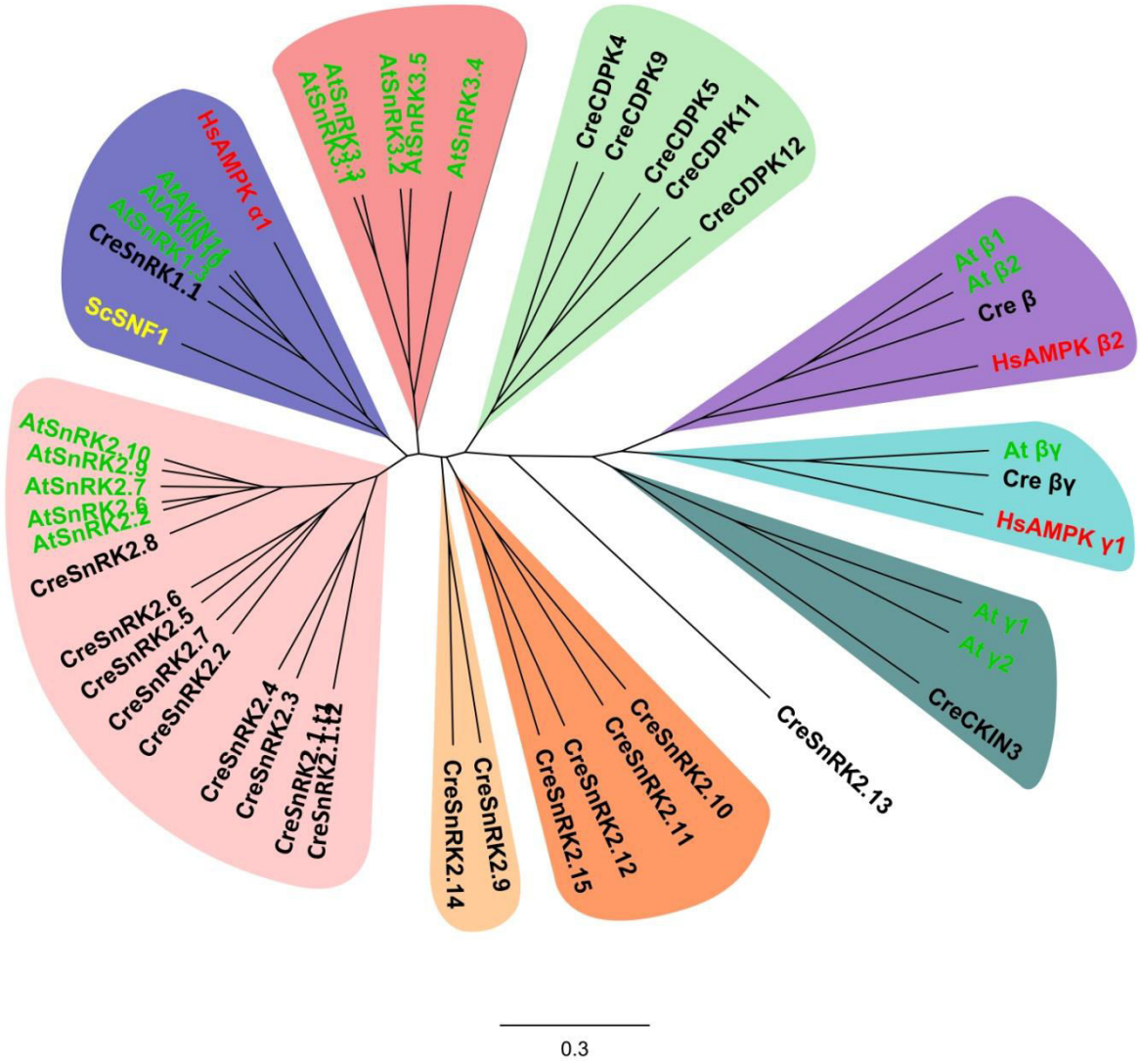

Fig. 1 Sequence tree for the Chlamydomonas SnRK family based on the alignment of protein sequences by UPGMA. Chlamydomonas (Cre; black) CDPK were included as an outgroup. *Arabidopsis thaliana* (At; green), *Homo sapiens* (Hs; red), and *Saccharomyces cerevisae* (Sc; yellow) homologous sequences were also included in the tree.

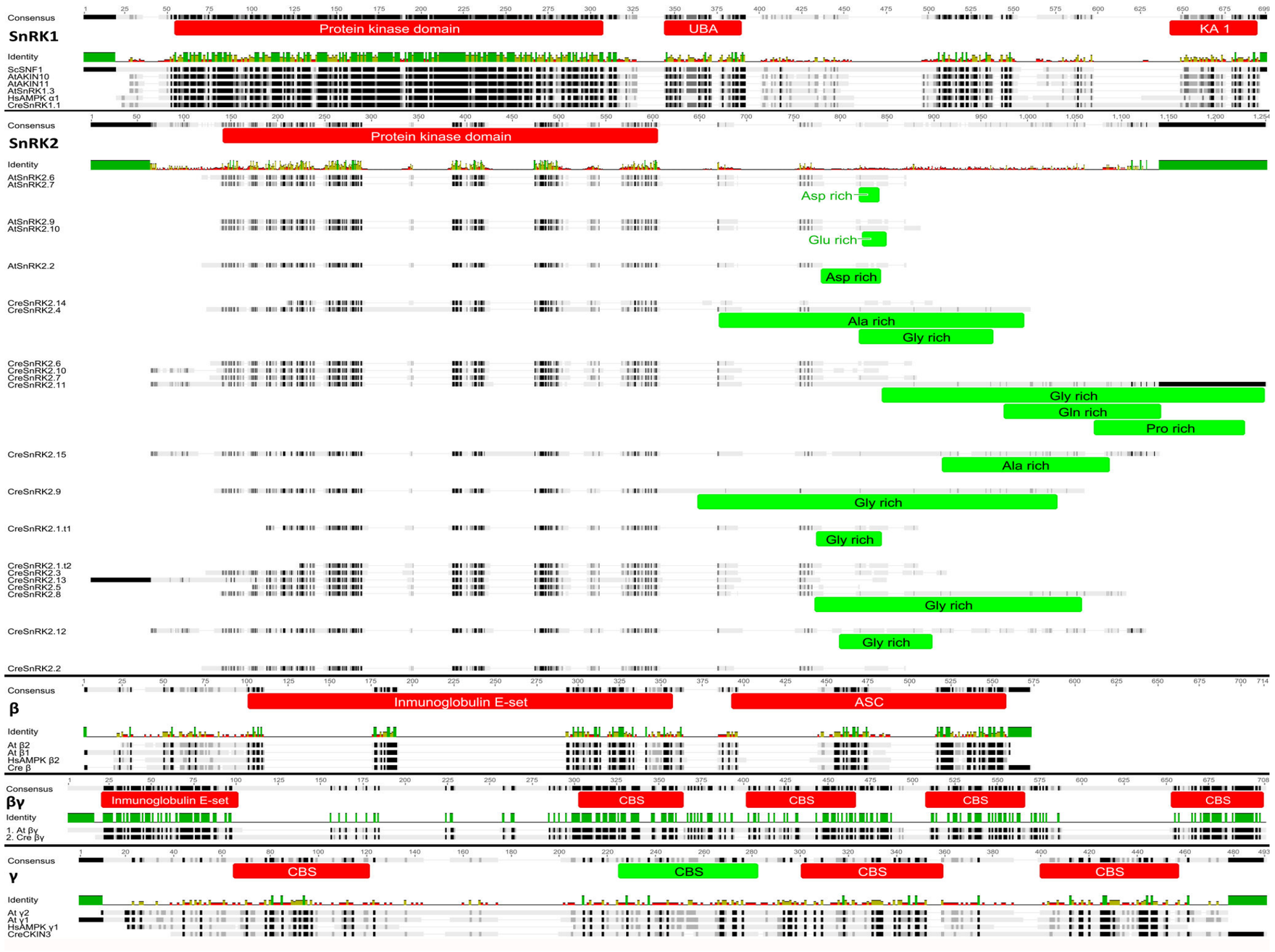

Fig. 2 ClustalW SnRK protein sequences alignment. Consensus sequence for each group of SnRK and identity observed. Red boxes represent conserved domains whereas green boxes correspond to variable domains in each group of proteins. Domains represented: Serin/Threonine Kinase (Protein kinase domain; PTHR24343); Ubiquitin associated domain (UBA; IPR015940); Kinase associated domain (KA1; PF02149); Immunoglobulin E-set (IPR014756); Association with the SNF1 complex domain (ASC, IPR006828); Cystathionine β-synthase (CBS, PF00571); and domains rich in Alanine (Ala), Glutamic acid (Glu), Glutamine (Gln), Aspartic acid (Asp), Glycine (Gly), and Proline (Pro) repetitions.

Physiological responses of Chlamydomonas to the abiotic stresses

Stressful growth conditions reduced Chlamydomonas growth, except for the Phosphorous limitation (5% P) condition (Fig. 3). Lipid bodies and starch accumulation were observed by microscopy (Fig. 4). Nitrogen (-N) and Sulphur (-S) deprivation led to the accumulation of considerable amounts of lipid bodies (Fig. 4A) and starch granules (Fig. 4B) in Chlamydomonas cells. Surprisingly, the irradiation with UV light (UV) also induced the accumulation of lipids, although at a less extent (Fig. 4A). Cold imposition (4°C) increased starch content locally around the pyrenoid (Fig. 4B). Under salt (0.25M NaCl), osmotic (20% PEG) and UV stress cells seem to present a more compact structure (Fig. 4B), probably due to water status changes, that also led to the reduction of cell size after salt and osmotic stress conditions.

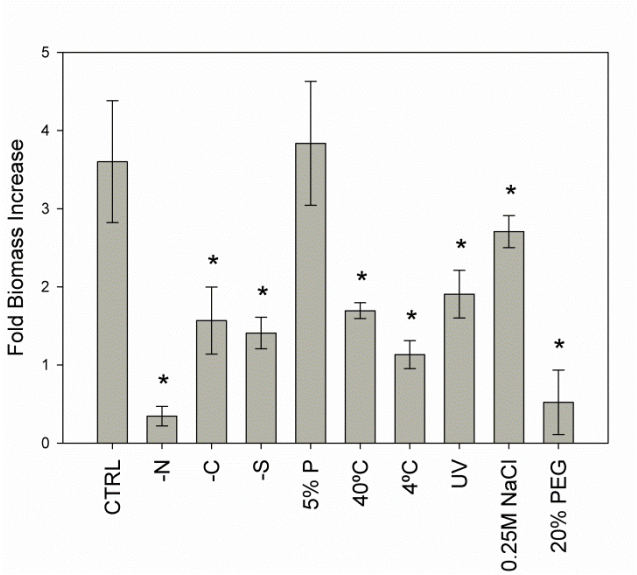

Fig. 3 Fold biomass increase in stress assays. Control (CTRL), Nitrogen (-N), Carbon (-C), and Sulphur (-S) deprivation, Phosphorous limitation (5% P) deprivations, heat and cold stress (40°C and 4°C, respectively), UV radiation stress (UV), salt (0.25M NaCl) and osmotic (20% PEG) stress data are herein represented as mean±SD. Fold biomass increase was obtained by normalizing the stress phase measurements (48 h) with its corresponding controls (0 h). Asterisks indicate significant differences between each stress assay and the control ($p \leq 0.05$).

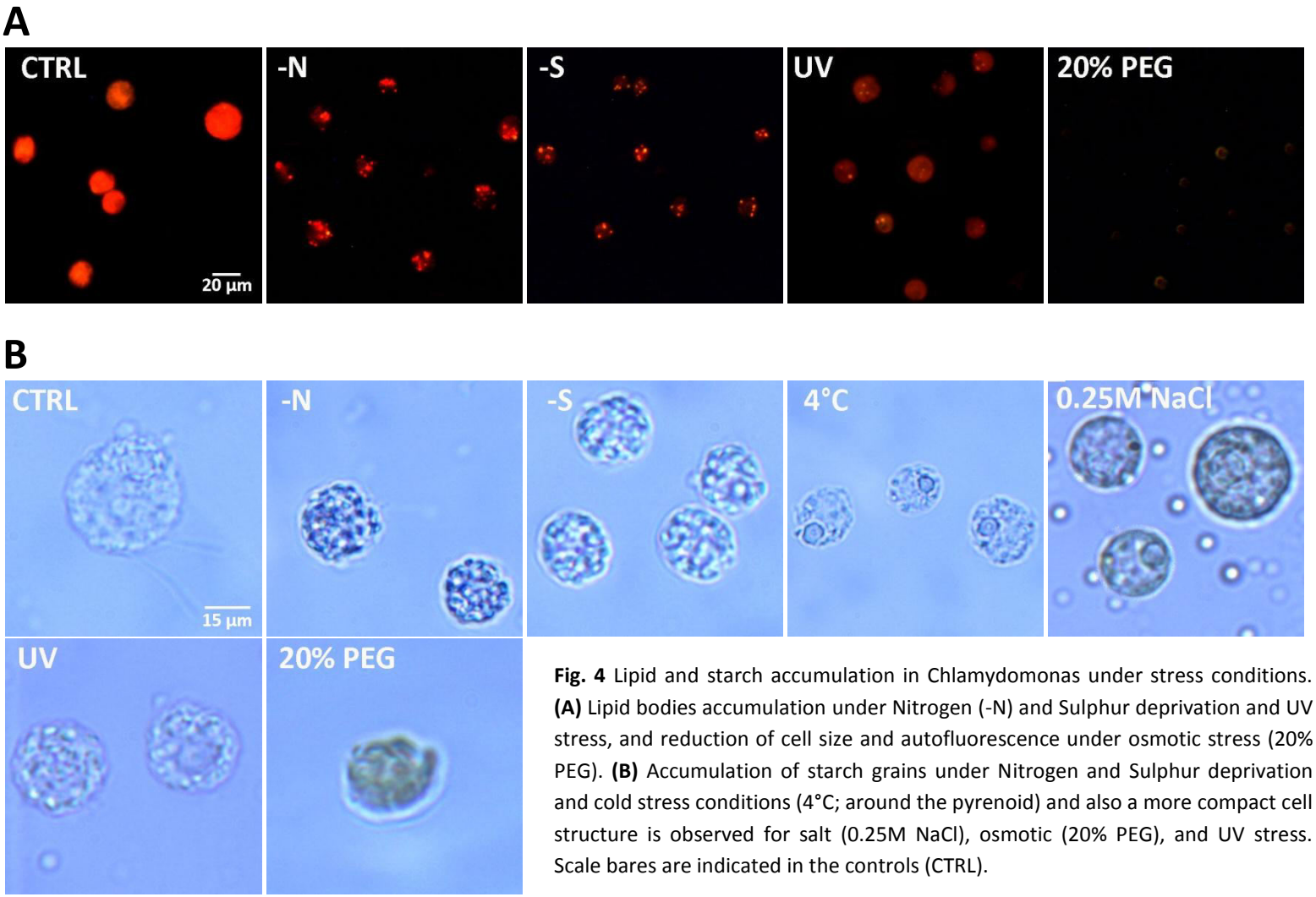

Fig. 4 Lipid and starch accumulation in Chlamydomonas under stress conditions. **(A)** Lipid bodies accumulation under Nitrogen (-N) and Sulphur deprivation and UV stress, and reduction of cell size and autofluorescence under osmotic stress (20% PEG). **(B)** Accumulation of starch grains under Nitrogen and Sulphur deprivation and cold stress conditions (4°C; around the pyrenoid) and also a more compact cell structure is observed for salt (0.25M NaCl), osmotic (20% PEG), and UV stress. Scale bares are indicated in the controls (CTRL).

Expression profiles of Chlamydomonas SnRK genes under the abiotic stresses tested

The expression of the 20 Chlamydomonas SnRK sequences found under several stress scenarios was evaluated by RT-qPCR (Fig. 5, Supplementary Fig. 2, Supplementary Table 5, and Suplemental Table 6). It has been confirmed that the SnRK family widely participates in abiotic stress response. According to their expression patterns under stress the SnRK family was divided into four groups. The first one comprises the SnRK1.1 complex that was overexpressed under all the stress situations, except for Nitrogen and Sulphur deprivation (-N and -S) and the transition from mixotrophic to autotrophic growth (-C). The expression of the different genes encoding the SnRK1.1 complex showed stress-specific abundances. Similarly, a second group of SnRK2 proteins (SnRK2.2, SnRK2.3, and SnRK2.7) also showed to be responsive to all the stresses tested. The remaining SnRK2 genes, with the exception of SnRK2.9 that was down-regulated in all stresses but osmotic (unchanged) and high temperature (up-regulated), were clustered into two differentiated groups according to their specific responses. The first group (SnRK2.1.t1, SnRK2.5, SnRK2.13, SnRK2.14, and SnRK2.15) were induced after osmotic (20% PEG), salt (0.25M NaCl), temperature (40°C and 4°C), and Sulphur (-S) deprivation stresses. This was particularly strong under osmotic stress (20% PEG), once SnRK expressions were significantly increased regarding the other stresses evaluated, except for βγ and γ (Supplementary Table 5; Supplementary Table 6). The last SnRK group (SnRK2.1.t2, SnRK2.4, SnRK2.6, SnRK2.8, SnRK2.10, and SnRK2.12) showed a lower increase of its abundance in the previously described stresses and a higher repression under Nitrogen (-N), Carbon (-C), and Phosphorous (5% P) limitation. Moreover, the two splicing forms of SnRK2.1 showed different expression patterns, being SnRK2.1.t1 highly expressed under Phosphorous limitation (5% P) and osmotic stress (20% PEG), while as SnRK2.1.t2 was greatly induced by Carbon deprivation (-C).

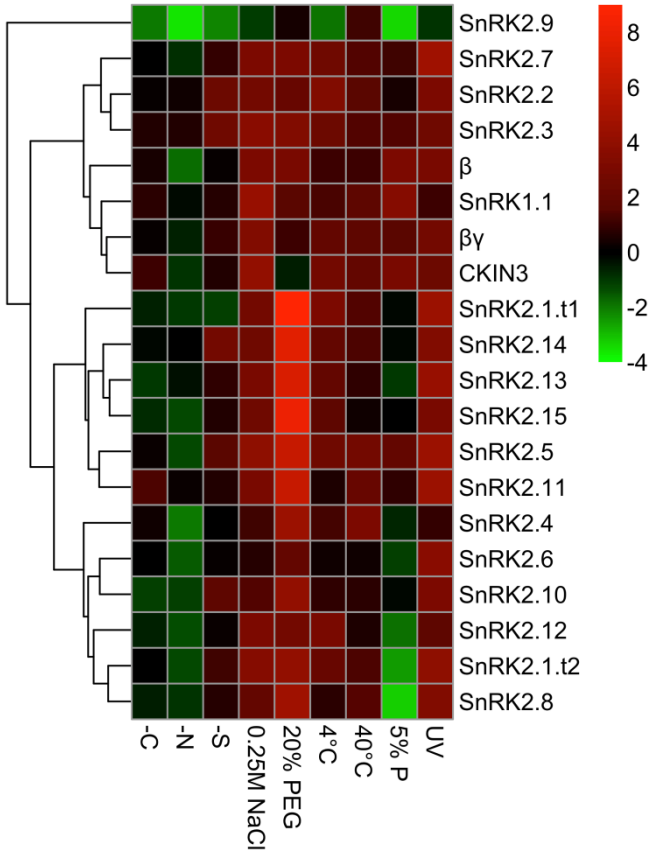

Fig. 5 -ΔΔCt values of each Chlamydomonas SnRK sequence found under the stress conditions tested. The colour scale is shown at the right. Black corresponds to no changes, red to overexpression, and green to down-regulation. Data herein represented correspond to the mean of four biological replicates. Replicates data is available in the Supplementary Fig. 2.

Clustering of the different stress situations based on the similarities of the SnRK expression rates pointed out the similar effects that different stresses caused to the cells. Considering all biological replicates (Supplementary Fig. 2) three major stress responses can be distinguished. The first one could be related to water limiting conditions since it groups osmotic (20% PEG), salt (0.25M NaCl), and UV stresses. These promote the overexpression of all SnRK, except SnRK2.9. The second group can be related to nutrient deficiency (-N, -P, and –C). A last group includes thermal stress (4°C and 40°C) and Sulphur deprivation (-S), which showed a considerable distinct SnRK expression pattern compared to other nutrient limiting stresses.

The available high-throughput RNA-seq data was also mined to intend to investigate the response of the Chlamydomonas SnRK family to stresses not covered by our experiments (oxidative stress, Iron deprivation, and low CO_2 content) and also for validation of the experimental results obtained (Fig. 6). Data showed that, under comparable situations (Carbon, Nitrogen, and Sulphur deficiencies), all genes behave similarly than described by qPCR, although the expression fold changes differ, possibly because of the low resolution of NGS-based methods when analysing sequences with a low number of copies. Also, these results pointed out that the

SnRK family behave in the same way in a wide range of strains, once the results obtained by real time qPCR using CC-503 cw92, nit1, nit2, agg1+, mt+ coincide with the ones resulting from NGS-analysis using CC-4425 cw15 nit2 mt+ (Gonzalez-Ballester et al. 2010), CC-4619 cw15 nit1 mt+ (Miller et al. 2010), CC-125 wild type mt+ (Fang et al. 2012), and CC4532 Mets strain 2137 mt- (Urzica et al. 2012). Considering the stress conditions that were not previously described in Chlamydomonas, Iron deficiency (-Fe) greatly induces the expression of SnRK2.12, while oxidative stress was characterized by an overall down-regulation of this family with the exception of SnRK2.7 that seems to be a marker under such conditions.

Altogether, *in vivo* and *in silico* data confirmed the involvement of the SnRK family in mediating abiotic stress response in Chlamydomonas regardless the strain. Overall specific patterns allowed the clustering of similar stress sources, indicating that different conditions might activate the same stress signalling pathways in which SnRK are probably involved. However, SnRK2.7, SnRK2.9, and SnRK2.12 showed to have a specific stress response under oxidative and heat stress, and iron deficiency, respectively. This makes them interesting targets for future bioengineering studies that confirm their function under such conditions.

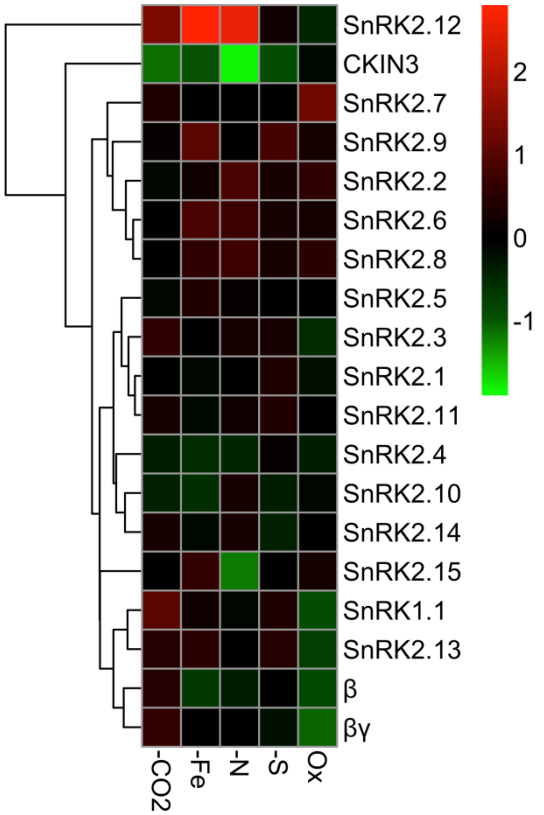

Fig. 6 Fold changes in the transcription of Chlamydomonas SnRK genes relatively to control conditions extracted from the data sets available in AlgaePath (Zheng et al. 2014). Sulphur (-S) and Nitrogen (-N) depletion, low carbon content (-CO_2), Iron deprivation (-Fe), and oxidative damage (Ox) stress scenarios are considered. The colour scale is shown at the right side. Black corresponds to no changes, red to overexpression, and green to down-regulation.

Discussion

Identification and classification of the SnRK family in Chlamydomonas

The SnRK family upsurges in Chlamydomonas as a key group of proteins for understanding abiotic stress response in microalgae and as an interesting target for genetic engineering and consequent valuable molecule accumulation increase, with special focus on the accumulation of energetic (such as TAG or starch) and high value-added (such as pigments, vitamins and bioplastic) biomolecules. It was previously shown that some members of the SnRK family are related to abiotic stress response in *C. reinhardtii* (Gonzalez-Ballester et al. 2008; Gonzalez-Ballester et al. 2010; Valledor et al. 2013; Valledor et al. 2014b). Therefore, the identification of the entire SnRK in Chlamydomonas and of SnRK members that respond specifically to a certain abiotic stress in Chlamydomonas are key steps for future bioengineering-based approaches.

The use of bioinformatics tools allowed the identification of SnRK proteins that are likely to be involved in stress response mechanisms in Chlamydomonas, as they are homologous to the SnRKs previously described in Arabidopsis (Coello et al. 2011) (Supplementary Table 1). The methodology herein applied was effectively used before e.g. for human MAPK (Matsuda et al. 2003) and Arabidopsis bZIP characterization (Jakoby et al. 2002), and more recently to identify a novel human methyltransferase modulating Hsp70 protein, METTL21A (Jakobsson et al. 2013). By combining homologous protein sequence BLAST search and protein domain search a total of 19 SnRK genes coding for 20 proteins were identified in *C. reinhardtii* (Table 2). Almost half of the number of the SnRK members described in Arabidopsis (38 SnRK) (Hrabak et al. 2003). Some of the previously described Chlamydomnas SnRK identified by Gonzalez-Ballester et al. (2008) (SnRK 2.1-2.8) and Valledor et al. (2013) (SnRK1.1 and CKIN3) were confirmed by our results and 9 other novel sequences were found (Table 2). Protein domain search showed to be a mandatory complementary tool to BLAST search for the complete description of this family. This allowed the filtering of SnRK from the great amount of results obtained by BLAST search (112 putative sequences) regarding its domain structure (Supplementary Table 2). In fact, great part of the Chlamydomonas sequences that showed to be similar to Arabidopsis SnRK belonged to the CDPK-SnRK superfamily, which is composed by seven types of serin-threonine kinases (Hrabak et al. 2003). Most of them were CDPK, which were easily distinguished from SnRK thanks to its characteristic calcium-binging EF hands (Hrabak et al. 2003). Moreover, 3 SnRK sequences (CKIN3, SnRK2.14, and SnRK2.15) were only found by searching for specific SnRK domains into the

Chlamydomonas genome using BIOMART. Otherwise these would not be identified and the complete description of the Chlamydomonas SnRK would not be accomplished.

The 20 Chlamydomonas SnRK sequences found were compared to Arabidopsis, Human, and Saccharomyces homologous sequences (Supplementary Table 3) in order to classify them into subfamilies. The results obtained by the design of both UPGMA (Fig. 1) and neighbour joining (Supplementary Fig. 1) sequence trees were strongly supported by the ones resulting from the ClustalW protein sequence alignment (Fig. 2), showing that in fact Chlamydomonas sequences formed different clusters according to its domain structure. ClustalW SnRK protein sequence alignment (Fig. 2) clearly illustrates highly conserved regions for each SnRK cluster that correspond to well-defined protein domains already identified by domain search using InterProScan (Supplementary Table 2). Looking at all these data, the SnRK found in Chlamydomonas were divided into four clusters: SnRK1, the energy sensor of the cell (S1); βγ regulatory subunits of SnRK1 (S1 R), with CKIN3 representing the γ regulatory subunit; and SnRK2 (S2) proteins, which are stress responsive, were divided into two subgroups (S2 D and S2 C) (Table 1). SnRK2 clusters were named as S2 D (SnRK2.1-2.8) and S2 C (SnRK2.9-2.12, SnRK2.14, and SnRK2.15) as it seems that these sequences grouped together (Fig. 1) based on the existence of the Serin/Threonin Protein Kinase SRK2D-Related (PTHR24343:SF99) or the Serin/Threonin Protein Kinase SRK2C (PTHR24343:SF55), respectively (Supplementary Table 2). While S2 D proteins share its characteristic domain with Arabidopsis SnRK2, S2 C proteins specific domain is unique to Chlamydomonas (Supplementary Table 2). Interestingly, the S2 C cluster comprises the SnRK2 that were not previously described by Gonzalez-Ballester et al. (2008), probably because the Chalmydomonas genome information was not complete at that date, which also explains why we considered only two splicing variants of SnRK2.1, while five splicing variants were initially reported. SnRK2.13 did not presented either of these domains (Supplementary Table 2) and its clustering by sequence alignments showed different results according to the algorithm used (Fig. 1 and Supplementary Fig. 1). Therefore, SnRK2.13 could only be assigned to the general SnRK2 subfamily (S2) (Table 1). Also, SnRK2 comprise a C-end rich in repetitive amino acids that vary according to the protein (Fig. 2), which probably correspond to regulatory domains as it was described for the Chlamydomonas SnRK2.1 by Gonzalez-Ballester et al. (2008).

Unlike in Arabidopsis, no SnRK3 proteins were identified in Chlamydomonas. As SnRK2 and SnRK3 are unique to plants (Halford et al. 2000) it would be expected that both of these subfamilies would be present also in Chlamydomonas since it shares common ancestry with

higher plants (Hannon et al. 2010). The fact that in Chlamydomonas no proteins containing the Arabidopsis SnRK3 characteristic NAF/FISL domain (Hrabak et al. 2003) were found can be due to specific variations similar to the ones that occur in some CDPK family proteins (Hamel et al. 2014). Other hypothesis is the presence of a SNF/SKP1/Ubiquitin ligase complex already identified in higher plant, animal and yeast SnRK1 subfamily that allows the action of these proteins in several processes, including hormone, sugar and stress responses (Farrás et al. 2001). The existence of these complexes could compensate the lack of elements from the SnRK3 subfamily. Furthermore, SnRK1 sequences present a highly conserved region that is likely to correspond to a specific protein domain that was not previously described (Fig. 2) which can also compensate the functions of SnRK3. Proteins of these elements were previously defined as cold stress responsive (Valledor et al. 2013), however deeper studies involving functional and protein complex analyses should performed to fully support this hypothesis.

Physiological responses and expression profiles of Chlamydomonas SnRK genes under abiotic stress

The role of SnRK in plant stress response is well studied. However, in Chlamydomonas, although some SnRK were already related to this phenomenon (Gonzalez-Ballester et al. 2008; Gonzalez-Ballester et al. 2010; Valledor et al. 2013; Valledor et al. 2014b), little is known. From the wide-range of stress conditions evaluated, similar effects of both gene expression patterns (Supplementary Fig. 2) and physiological responses (Fig. 4) were observed between some of them. Osmotic, salt and UV stresses grouped together, showing a general great overexpression of the SnRK family (except for SnRK2.9) and a more compact cell structure. These changes are likely to be related to the water status changes sensed by the Chlamydomonas cells, as it is well known that osmotic and salt stress, and, more recently, UV radiation induces water stress in plants (Mao et al. 2012). Although osmotic salt (20% PEG) was the condition that among all showed the most significant SnRK overexpression, cell growth seemed to be considerably reduced (Fig. 4), probably because of the great stress level sensed by the cells. Thus, regarding the aim to increase biomass production to integrate biofuel-based bioproduction into a biorefinery system, the genes related to osmotic stress response do not represent a target of interest. However, UV radiation leads to the increase of lipid bodies accumulation, apparently maintaining cell growth (Fig. 4). Therefore, further studies to unravel the function of the SnRK overexpressed under UV radiation stress might be interesting, especially because applying this stimulus to cultivation ponds or photobioreactors do not represent high costs.

Although Nitrogen and Sulphur deprivation also increased lipid bodies and starch grains accumulation in Chlamydomonas (Fig. 4), applying a nutrient deficit stress involves great costs, since that, after a first phase of cell growth, the media must be substituted. These and Carbon deprivation are the only conditions were the SnRK1.1 complex is not accumulated (Fig. 5). Contrarily, under Phosphorous limitation, this complex was highly expressed. Also, as referred before by Gonzalez-Ballester et al. (2008) SnRK2.1 transcripts clearly showed different behaviours in response to Sulphur deprivation (SnRK2.1.t1: up-regulated; SnRK2.1.t2: down-regulated) and also to Phosphorous limitation (SnRK2.1.t1: no change; SnRK2.1.t2:downregulated) and osmotic stress (SnRK2.1.t1 with a much greater overexpression) (Fig. 5). This suggests that, depending of the limiting conditions sensed by the cell alternative splicing regulates its response to stress. Our data clearly show this because it clusters apart two transcripts from the same gene regarding its expression changes in response to a wide-range of stress conditions (Fig. 5), indicating its possible distinct functionality. In fact, some SnRK2 from Arabidopsis had similarly showed its involvement in Sulphur deprivation (AtSnRK2.3) (Kimura et al. 2006) and water limiting conditions, namely under drought stress (AtSnRK2.8) (Shin et al. 2007) and hyperosmolarity (Kobayashi et al. 2005) (Kobayashi), which supports the idea that the plant-specific SnRK2 subfamily plays a crucial role in stress response signalling both in Arabidopsis and Chlamydomonas.

From all the SnRK family genes tested, only SnRK2.9 showed a stress specific response, being significantly overexpressed exclusively under heat stress. It is clear the importance of this gene as it is displaced in the heatmap as a outgroup (Fig. 5). This makes it a potential target for further bioengineering studies to confirm its function in the stress response mechanisms in Chlamydomonas. Moreover, from the mining of the available high-throughput RNA-seq data, that allowed us to support our data from the coinciding conditions and to observe that stress response is not strain-dependent in Chlamydomonas, other target genes were identified. These were SnRK2.12 that was highly induced by Iron deficiency and SnRK2.7 that appears as a possible specific gene for oxidative stress response in Chlamydomonas (Fig. 6).

Acknowledgments

This research was supported by the EXPL/AAG-TEC /2056/2013 (FCT; Portuguese Foundation for Science and Technology) and AGL2014-54995-P (MINECO; Spain Ministry of Economy and Competiveness). JA, FC, GP, and LV were respectively and generously granted by the BI/UI88/6286/2014 (FCT, Portugal), BP14-138 (Programa Clarín de Ayudas Predoctorales, Autonomous Comunity of Asturias, Spain), SFRH/BPD/101669/2014 (FCT, Portugal) and SFRH/BDP/89765/2012 (FCT, Portugal) fellowships.

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Supplementary Data

Supplementary Table 1. *Arabidopsis thaliana* (*At*) query sequences used for BLAST homology search to identify putative SnRK sequences into the *C. reinhardtii* genome: names and respective identification (ID).

Name	At ID	Name	At ID	Name	At ID
Akin10	AT3g01090	SnRK2.8	AT1g78290	SnRK3.13	AT4g24400
Akin11	AT3g29160	SnRK2.9	AT2g23030	SnRK3.14	AT4g30960
β1	AT5g21170	SnRK2.10	AT1g60940	SnRK3.15	AT5g01820
β2	AT4g16360	SnRK3.1	AT5g01810	SnRK3.16	AT3g17510
β3	AT2g28060	SnRK3.2	AT5g07070	SnRK3.17	AT2g26980
βγ	AT1g09020	SnRK3.3	AT4g14580	SnRK3.18	AT2g25090
γ1	AT3g48530	SnRK3.4	AT5g57630	SnRK3.19	AT2g38490
γ2	AT1g69800	SnRK3.5	AT5g45810	SnRK3.20	AT1g29230
SnRK2.1	AT5g08590	SnRK3.6	AT5g45820	SnRK3.21	AT1g48260
SnRK2.2	AT3g50500	SnRK3.7	AT2g34180	SnRK3.22	AT2g30360
SnRK2.3	AT5g66880	SnRK3.8	AT5g58380	SnRK3.23	AT1g30270
SnRK2.4	AT1g10940	SnRK3.9	AT4g18700	SnRK3.24	AT5g10930
SnRK2.5	AT5g63650	SnRK3.10	AT3g23000	SnRK3.25	AT5g25110
SnRK2.6	AT4g33950	SnRK3.11	AT5g35410		
SnRK2.7	AT4g40010	SnRK3.12	AT1g01140		

Supplementary Table 2. SnRK sequences, protein domain names, and domain identifiers (ID) both in Arabidopsis (*At*) and Chlamydomonas (*Cre*) and corresponding Chlamydomonas sequence names. SnRK clusters according to sequence similarity and protein domain (S1: SnRK1; S1R: regulatory subunits of SnRK1; S2: SnRK2; SnRK2 D: SnRK2 containing the Serin/Threonin Kinase SRK2D domain; SnRK2 C: SnRK2 containing the Serin/Threonin Kinase SRK2C domain).

SnRK	Domain name	*At* Domain ID	*Cre* Domain ID	*Cre* Name	Cluster
SnRK1.1	Kinase associated domain 1 (KA1)	PF02149	PF02149	SnRK1.1	S1
	Serin/Threonin Kinase	PTHR24343	PTHR24343	SnRK1.1	S1
	Ubiquitin associated domain (UBA)	IPR015940	IPR015940	SnRK1.1	S1
β	AMPK , β subunit	PTHR10343	PTHR10343	β	S1 R
	SnRK 1, β-1 regulatory subunit	PTHR10343:SF51	PTHR10343:SF51	β	S1 R
	Immunoglobulin E-set	IPR014756	IPR014756	β	S1 R
	Association with the SNF1 complex domain (ASC)	IPR006828	IPR006828	β	S1 R
γ and βγ	AMPK, γ regulatory subunit	PTHR13780	PTHR13780	βγ	S1 R
				CKIN3	S1 R
	Snrk 1, γ regulatory subunit	PTHR13780:SF36	PTHR13780:SF36	βγ	S1 R
	AMPK , γ regulatory subunit		PTHR13780:SF22	CKIN3	S1 R
	Cystathionine β sinthase (CBS)	PF00571	PF00571	βγ	S1 R
				CKIN3	S1 R
	Immunoglobulin E-set	IPR014756	IPR014756	Bγ	S1 R
SnRK2	Serin/Threonin kinase	PTHR24343	PTHR24343	SnRK2.1.t1	S2
				SnRK2.1.t2	S2
				SnRK2.2	S2
				SnRK2.3	S2
				SnRK2.4	S2
				SnRk2.5	S2
				SnRK2.6	S2
				SnRK2.7	S2
				SnRK2.8	S2
				SnRK2.9	S2
				SnRK2.10	S2
				SnRK2.11	S2
				SnRK2.12	S2
				SnRK2.13	S2
				SnRK2.14	S2
				SnRK2.15	S2
	Serin/Threonin Protein Kinase SRK2D-Related	PTHR24343:SF99	PTHR24343:SF99	SnRK2.1.t1	S2 D
				SnRK2.1.t2	S2 D
				SnRK2.2	S2 D
				SnRK2.3	S2 D
				SnRK2.4	S2 D
				SnRK2.5	S2 D
				SnRK2.6	S2 D
				SnRK2.7	S2 D
				SnRK2.8	S2 D
	Serin/Threonin Protein Kinase SRK2C		PTHR24343:SF55	SnRK2.9	S2 C
				SnRK2.10	S2 C
				SnRK2.11	S2 C
				SnRK2.12	S2 C
				SnRK2.14	S2 C
				SnRK2.15	S2 C
SnRK3	CBL-Interacting Serin/Threonin Protein Kinase 9	PTHR24347:SF140			
	Serin/Threonin Kinase	PTHR24347			
	NAF	PF03822			
	NAF/FISL	PS50816			

Supplementary Table 3. *Arabidopsis thaliana* (*At*), *Saccharomyces cerevisiae* (*Sc*), *Homo sapiens* (*Hs*), and *Chlamydomonas reinhardtii* (*Cre*) sequences used for the design of the sequence trees: organism identification followed by sequence name (Name) and sequence identifier (Seq ID).

Name	Seq ID	Name	Seq ID
*At*AKIN10	AT3g01090	*CreS*nRK2.3	Cre02.g075900.t1.1
*At*AKIN11	AT3g29160	*CreS*nRK2.4	Cre11.g477000.t1.2
*At*SnRK1.3	AT5g39440	*CreS*nRK2.5	Cre03.g209505.t1.1
*Sc*SNF1	NP_010765.3	*CreS*nRK2.6	Cre11.g481000.t1.2
*Hs*AMPK α1	NP_006242.5	*CreS*nRK2.7	Cre06.g292700.t1.2
*Cre*SnRK1.1	Cre04.g211600.t1.1	*CreS*nRK2.8	Cre10.g466350.t1.1
At β1	AT5g21170	*CreSn*RK2.9	Cre13.g568050.t1.3
At β2	AT4g16360	*CreS*nRK2.10	Cre16.g657350.t1.2
*Hs*AMPK β2	NP_005390.1	*CreS*nRK2.11	Cre17.g707800.t1.2
Cre β	Cre10.g457500.t1.1	*CreS*nRK2.12	Cre12.g485600.t1.2
At βγ	AT1g09020	*CreS*nRK2.13	Cre02.g076000.t1.2
*Hs*AMPK γ1	NP_002724.1	*CreS*nRK2.14	Cre08.g384250.t1.2
Cre βγ	Cre12.g484350.t1.3	*CreS*nRK2.15	Cre17.g707650.t1.1
At γ1	AT3g48530	*AtS*nRK3.1	AT5g01810
At γ2	AT1g69800	*AtS*nRK3.2	AT5G07070
*Cre*CKIN3	Cre12.g528000.t1.2	*AtS*nRK3.3	AT4g14580
*At*SnRK2.2	AT3g50500	*AtS*nRK3.4	AT5g57630
*At*SnRK2.6	AT4g33950	*AtS*nRK3.5	AT5g45810
*At*SnRK2.7	AT4g40010	*Cre*CDPK4	Cre06.g296200.t1.2
*At*SnRK2.9	AT2g23030	*Cre*CDPK5	Cre07.g328900.t1.2
*At*SnRK2.10	AT1g60940	*Cre*CDPK9	Cre13.g571700.t1.1
*Cre*SnRK2.1.t1	Cre02.g075850.t1.1	*Cre*CDPK11	Cre19.g750597.t1.1
*Cre*SnRK2.1.t2	Cre02.g075850.t2.1	*Cre*CDPK12	Cre01.g003524.t1.1
*Cre*SnRK2.2	Cre12.g499500.t1.1		

Supplementary Table 4. Primer pairs used for RT-qPCR analyses

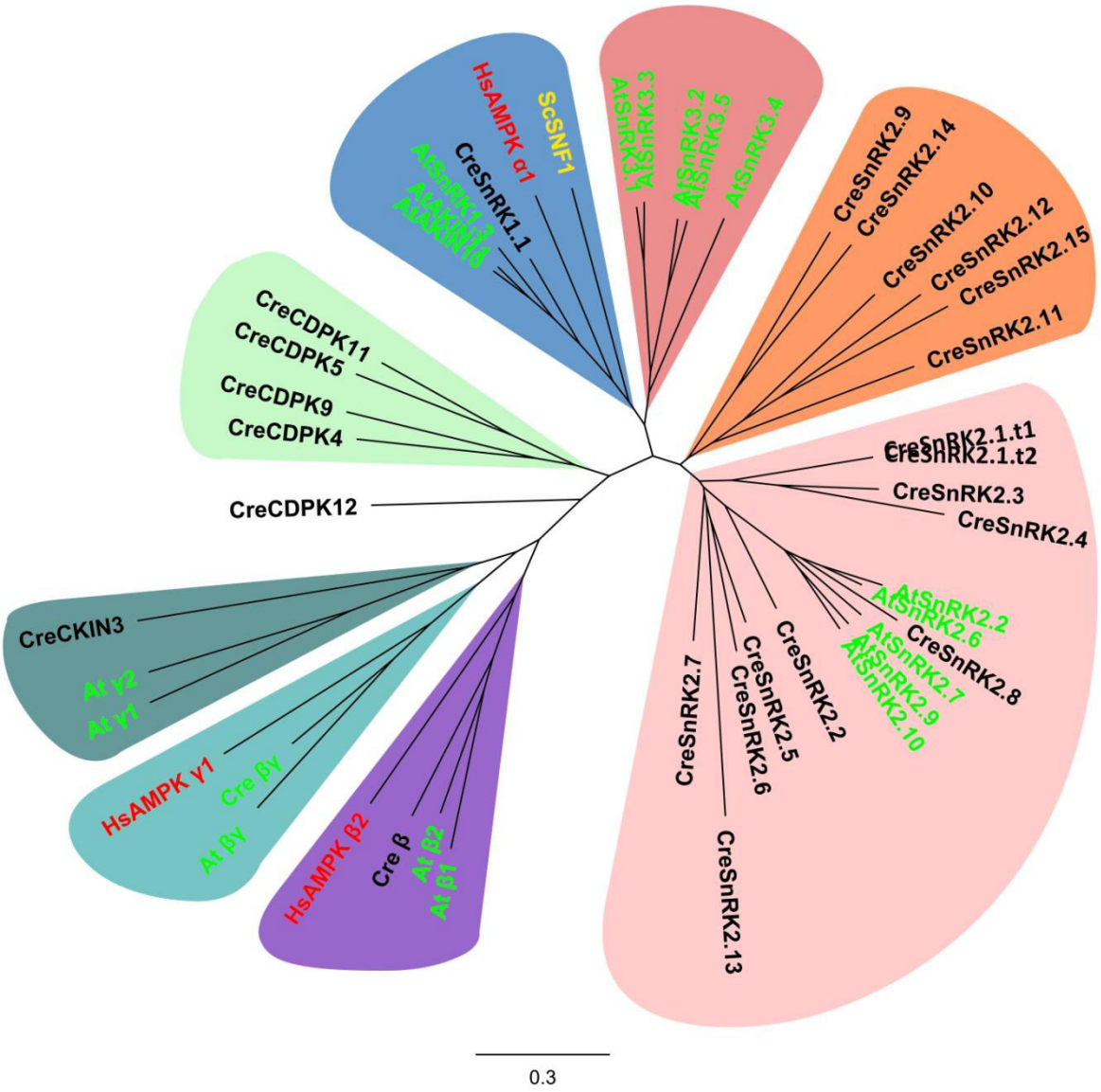
Name	ID	Forward primer sequence	Reverse primer sequence
ACT	Cre13.g603700.t1.2	GCGGCTACTCGTTCACCACCAC	TCTCCTGCTCGAAGTCCAGGGC
UBI	Cre03.g159200.t1.1	CTACCGCCGTTCCTGTTCCTGC	CTGGCGGCAGTAGCACCACATC
TUB	Cre04.g216850.t1.2	GTCCAAGCTGGGCTTCACCGTC	GGCGGCAGATGTCGTAGATGGC
RCK1	Cre06.g278222.t1.1	CGACAAGAGCGTGCTGGTCTGG	GTCAGGCAGAACTGGCCATCGG
SnRK1.1	Cre04.g211600.t1.1	TCATGCACCCGCACATCATCCG	CAGCCGCCCCTTCTCCACAATG
β	Cre10.g457500.t1.1	GATGAGCTGACAGCCGCCAACC	CACCGTGGCTCCACACGATGAC
βγ	Cre12.g484350.t1.3	ACGGCTCTCCTGGGTTGTTTGC	TTGACGTTGCCAAGCGGGTCTG
CKIN3	Cre12.g528000.t1.2	CGTGCTGATGCAGGAGCTGGAG	TCACGGTGTGCACCTTCTTGGC
SnRK2.1.t1	Cre02.g075850.t1.1	GGTCAAGCGTGAAGTGCGAACC	GCAGTCCGCGTACTCCATCACC
SnRK2.1.t2	Cre02.g075850.t2.1	CCGCGTGGGCACACTCTACTAC	GCCGAACGGGTAGGCCTTGAAC
SnRK2.2	Cre12.g499500.t1.1	CATCTGGAGCTGCGGCGTGATG	GCGGCGGGATGTGGTAGTCAAC
SnRK2.3	Cre02.g075900.t1.1	CGTGTTCGGCCTCGACTACTGC	GCAGGTTGAAGGGGTGCTCCAG
SnRK2.4	Cre11.g477000.t1.2	GCGCGAAATTCAGTCCCATCGC	CCCTGGTCAGCGAACTCCATGC
SnRK2.5	Cre03.g209505.t1.1	CGGTGGACTACTGCCACAAGCG	GGACCTGAAGTCGGCCTTGCTG
SnRK2.6	Cre11.g481000.t1.2	GGCGGGAGCTTGTTCCACTACG	TCCAGCTTGATGTCGCGGTTGG
SnRK2.7	Cre06.g292700.t1.2	GCTGCCGCTGCTCAAAATCTGC	GTACTGGTCCGTGGCGCGAATC
SnRK2.8	Cre10.g466350.t1.1	GTGTGCCACCGGGATCTGAAGC	TCTTGGGCTGGCTGTCAAACGC
SnRK2.9	Cre13.g568050.t1.3	CTGGAGCCGCGGGTTCTACAAG	TCCATCACCACCGCCAGGTAGG
SnRK2.10	Cre16.g657350.t1.2	GGACGAGGCGCGGTACTTCTTC	GTCGCACAGCTTCAGCCAGGAG
SnRK2.11	Cre17.g707800.t1.2	GGCCTGCAGCGAGGAGTTCAA	GCGGTTGAAGGTGGGGTAGTGC
SnRK2.12	Cre12.g485600.t1.2	AGGACGAGCACAAGCGCATCA	ACGGGTGGCCAGGTCCTGTATG
SnRK2.13	Cre02.g076000.t1.2	ACCTGAAGCGCGAGGTGGTGTG	GCTCCGCCAGCCACTGCTTGAG
SnRK2.14	Cre08.g384250.t1.2	CATCTGGTCCTGCGGCGTGGTG	TCCTCGCGCGGCACCATGATG
SnRK2.15	Cre17.g707650.t1.1	GGTGATGGAGCTGCTGGGCACAG	GCTTGCAGCTGCAGCTCCATGC

Supplementary Table 5. Expression levels (ΔΔCq values) of Chlamydomonas SnRK under stress conditions. Nitrogen (-N), Carbon (-C), and Sulphur (-S) deprivation, Phosphorous limitation (5% P), heat and cold stress (40°C and 4°C, respectively), UV radiation stress (UV), salt (0.25M NaCl) and osmotic stress (20% PEG) data are herein presented as mean ± SE. Different lowercase letters indicate significant differences between the stress assays tested for each SnRK sequenced analysed (ANOVA; p≤ 0.05).

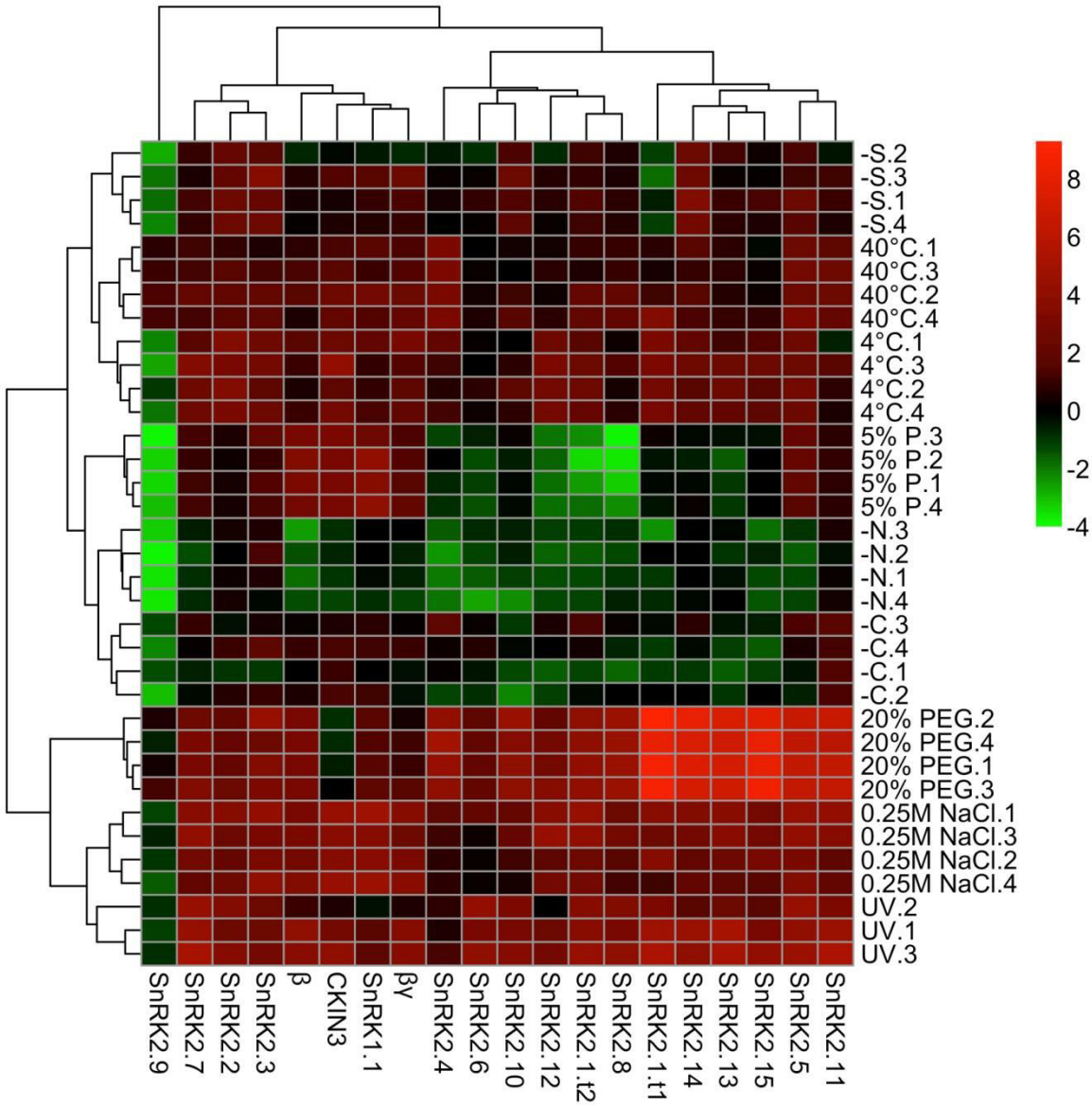
SnRK	Stress Assay								
	-N	-C	-S	5% P	40°C	4°C	20% PEG	0.25M NaCl	UV
SnRK1.1	0.25 ± 0.12a	-0.78 ± 0.43ab	-0.68 ± 0.48bc	-3.52 ± 2.3bc	-1.72 ± 1.18bc	-1.28 ± 0.71c	-1.67 ± 0.52d	-4.22 ± 2.5d	-1.06 ± 0.86e
β	1.78 ± 1a	-0.45 ± 0.22b	-0.14 ± 0.09bc	-3.11 ± 1.26c	-1.1 ± 0.57c	-1.07 ± 0.54d	-3.03 ± 0.49d	-3.12 ± 1.25d	-2.91 ± 2.79e
βγ	0.6 ± 0.32a	-0.16 ± 0.1ab	-0.99 ± 0.87bc	-1.61 ± 0.63cd	-1.84 ± 0.98cd	-2.05 ± 1.26cd	-1.07 ± 0.57de	-3.35 ± 1.89ef	-2.66 ± 2.66f
CKIN3	0.9 ± 0.33a	-1.08 ± 0.47b	-0.59 ± 0.37bc	-3.04 ± 0.95cd	-1.91 ± 1.13de	-2.8 ± 2.14de	0.51 ± 0.26de	-4 ± 2ef	-2.43 ± 2.37f
SnRK2.1.t1	1.03 ± 0.8a	0.58 ± 0.34a	1.16 ± 0.64a	0.15 ± 0.07a	-1.46 ± 1.33b	-3.06 ± 1.04c	-8.82 ± 4.38c	-2.75 ± 2.32d	-4.46 ± 3.58e
SnRK2.1.t2	1.29 ± 0.44a	-0.06 ± 0.05b	-1.16 ± 0.41c	2.5 ± 1.6d	-1.41 ± 0.92de	-2.13 ± 0.92e	-4.11 ± 0.78f	-3.5 ± 2.54f	-3.89 ± 1.93g
SnRK2.2	-0.28 ± 0.11a	-0.13 ± 0.09a	-2.3 ± 0.95a	-0.47 ± 0.14b	-1.5 ± 0.78c	-3.24 ± 1cd	-2.18 ± 0.7d	-2.72 ± 1.73d	-3.18 ± 1.74e
SnRK2.3	-0.59 ± 0.35a	-0.59 ± 0.5a	-2.49 ± 1.74a	-1.42 ± 0.69ab	-1.47 ± 0.95bc	-2.42 ± 1.23c	-3.39 ± 2.33c	-3.67 ± 2.35d	-2.53 ± 1e
SnRK2.4	1.97 ± 0.95a	-0.28 ± 0.24b	-0.08 ± 0.04bc	0.69 ± 0.38c	-3.2 ± 0.54cd	-1.26 ± 0.72d	-4.46 ± 2.33d	-1.14 ± 0.58e	-0.93 ± 0.4f
SnRK2.5	1.27 ± 0.51a	-0.22 ± 0.16b	-1.61 ± 0.96c	-1.99 ± 0.84cd	-2.75 ± 1.26de	-2.63 ± 0.85e	-6.37 ± 2.08f	-3.84 ± 2.32f	-4.41 ± 1.67g
SnRK2.6	1.54 ± 1.05a	0.07 ± 0.05a	-0.12 ± 0.08b	1.17 ± 0.55b	-0.29 ± 0.12b	-0.32 ± 0.15b	-1.93 ± 0.42b	-0.68 ± 0.48c	-3.77 ± 2.32d
SnRK2.7	0.88 ± 0.4a	-0.07 ± 0.05b	-0.9 ± 0.36c	-1.15 ± 0.32c	-1.43 ± 0.77c	-2.55 ± 1.65d	-3.09 ± 1.44d	-3.19 ± 2.42e	-4.7 ± 2.2f
SnRK2.8	0.92 ± 0.36a	0.54 ± 0.39b	-0.67 ± 0.2b	3.26 ± 2.09c	-1.5 ± 0.89cd	-0.73 ± 0.4de	-4.6 ± 0.88e	-2.03 ± 1.36f	-3.42 ± 1.77g
SnRK2.9	3.6 ± 1.35a	1.97 ± 1.37a	2.16 ± 1.09b	3.38 ± 1.55b	-1.11 ± 0.44b	1.92 ± 1.22c	-0.41 ± 0.28c	1.06 ± 0.51d	0.94 ± 0.28e
SnRK2.10	1.14 ± 0.79a	1.13 ± 0.8a	-1.73 ± 0.91b	0.18 ± 0.08c	-0.8 ± 0.52cd	-0.86 ± 0.58cd	-4.05 ± 2.04d	-1.48 ± 1.04e	-3.19 ± 1.36f
SnRK2.11	-0.23 ± 0.1a	-1.38 ± 0.37ab	-0.62 ± 0.4ab	-0.85 ± 0.12ab	-2.27 ± 0.91bc	-0.57 ± 0.42cd	-6.31 ± 2.52d	-2.88 ± 2.46e	-4.46 ± 3.54f
SnRK2.12	1.38 ± 0.48a	0.58 ± 0.44ab	-0.23 ± 0.15bc	1.82 ± 0.46cd	-0.56 ± 0.22d	-2.88 ± 1.08e	-2.78 ± 1.73ef	-3.2 ± 2.67f	-1.75 ± 1.6g
SnRK2.13	0.32 ± 0.16a	1.02 ± 0.56a	-0.83 ± 0.41a	0.96 ± 0.53b	-0.83 ± 0.25b	-1.99 ± 1.19c	-7.15 ± 2.58d	-3 ± 2.29e	-4.28 ± 4.15f
SnRK2.14	0.1 ± 0.02a	0.13 ± 0.09a	-2.79 ± 1.42a	0.18 ± 0.08b	-1.41 ± 0.58bc	-2.02 ± 1.02cd	-7.55 ± 3.75cd	-2.52 ± 1.64d	-3.42 ± 3.29e
SnRK2.15	1.34 ± 0.74a	0.78 ± 0.51ab	-0.6 ± 0.33bc	0.09 ± 0.03c	-0.32 ± 0.17c	-1.82 ± 0.97d	-8.2 ± 3.58de	-2.57 ± 1.58e	-2.96 ± 2.5f

Supplementary Table 6. Expression levels (Rq values) of Chlamydomonas SnRK under stress conditions. Nitrogen (-N), carbon (-C), and sulphur (-S) deprivation, phosphorous limitation (5% P), heat and cold stress (40°C and 4°C, respectively), UV radiation stress (UV), salt (0.25M NaCl) and osmotic stress (20% PEG) data are herein presented as mean ± SE.

SnRK	Stress Assay								
	-N	-C	-S	5% P	40°C	4°C	20% PEG	0.25M NaCl	UV
SnRK1.1	0.75 ± 1.05	1.72 ± 1.1	2.08 ± 1.26	11.98 ± 1.21	3.28 ± 1.24	4.54 ± 1.11	3.38 ± 0.86	18.61 ± 1.14	2.08 ± 1.36
β	0.33 ± 1.05	1.37 ± 0.97	1.42 ± 1.07	8.03 ± 0.89	2.15 ± 1	3.21 ± 0.99	8.33 ± 0.65	8.7 ± 0.88	37.2 ± 1.44
βγ	1.72 ± 1.17	1.12 ± 1.26	5.23 ± 1.51	4.05 ± 1.02	3.57 ± 1.16	8.97 ± 1.25	3.35 ± 1.16	10.18 ± 1.2	51.68 ± 1.63
CKIN3	0.51 ± 0.8	2.11 ± 0.87	1.37 ± 1.06	9.31 ± 0.75	3.75 ± 1.03	6.77 ± 1.2	1.28 ± 0.94	16.03 ± 0.93	119.33 ± 1.41
SnRK2.1.t1	0.76 ± 1.37	0.67 ± 1.19	1 ± 1.14	1.33 ± 1.04	2.75 ± 1.5	13.54 ± 0.93	440 ± 1.09	6.72 ± 1.44	0.79 ± 1.4
SnRK2.1.t2	0.46 ± 1.14	1.04 ± 1.59	2.74 ± 1.15	0.34 ± 1.44	2.65 ± 1.45	5.93 ± 1.23	15.45 ± 0.99	11.29 ± 1.52	22.2 ± 1.29
SnRK2.2	2.72 ± 1.13	1.09 ± 1.46	11.62 ± 1.14	1.98 ± 1.03	2.83 ± 1.25	13.64 ± 1.04	5.15 ± 1.05	6.59 ± 1.37	148.7 ± 1.28
SnRK2.3	1.34 ± 1.45	1.51 ± 1.7	4.73 ± 1.55	3.03 ± 1.33	2.77 ± 1.49	9.95 ± 1.36	8.74 ± 1.54	12.74 ± 1.49	58.56 ± 1.24
SnRK2.4	0.42 ± 1.35	1.22 ± 1.73	1.64 ± 1.36	0.94 ± 1.41	9.16 ± 1.03	2.91 ± 1.44	29.8 ± 1.39	2.21 ± 1.37	12.44 ± 1.29
SnRK2.5	1.5 ± 1.14	1.17 ± 1.48	10.38 ± 1.34	5.16 ± 1.16	6.72 ± 1.2	8.3 ± 1.06	105.61 ± 1.06	14.33 ± 1.34	152.32 ± 1.12
SnRK2.6	1.12 ± 1.31	0.95 ± 1.26	4.44 ± 1.29	0.8 ± 1.1	1.22 ± 1.05	1.86 ± 1.09	8.97 ± 0.84	1.6 ± 1.34	77.24 ± 1.24
SnRK2.7	0.65 ± 1.11	1.05 ± 1.31	2.15 ± 1.06	2.09 ± 0.93	2.69 ± 1.19	6.26 ± 1.3	8.05 ± 1.12	9.1 ± 1.41	124.02 ± 1.12
SnRK2.8	0.62 ± 1.12	0.69 ± 1.44	1.74 ± 1.02	0.19 ± 1.36	2.83 ± 1.31	2.47 ± 1.26	21.2 ± 0.91	4.09 ± 1.39	128.61 ± 1.24
SnRK2.9	0.1 ± 1.07	0.25 ± 1.39	0.28 ± 1.2	0.16 ± 1.15	2.16 ± 1.09	0.38 ± 1.33	1.64 ± 1.37	0.48 ± 1.18	0.97 ± 1
SnRK2.10	1.13 ± 1.4	0.46 ± 1.42	4.99 ± 1.24	1.27 ± 1.13	1.74 ± 1.37	2.12 ± 1.38	16.89 ± 1.21	2.78 ± 1.41	143.14 ± 1.14
SnRK2.11	1.16 ± 0.73	2.61 ± 0.54	1.98 ± 0.91	2.6 ± 0.41	4.82 ± 0.67	2.2 ± 1	71.84 ± 0.67	7.35 ± 1.12	4.03 ± 1.06
SnRK2.12	0.44 ± 1.11	0.67 ± 1.52	1.87 ± 1.4	0.37 ± 1.01	1.47 ± 1.15	9.78 ± 1.14	6.71 ± 1.38	9.19 ± 1.6	8.7 ± 1.67
SnRK2.13	1.17 ± 1.05	0.49 ± 1.1	2.45 ± 1.05	0.72 ± 1.1	1.78 ± 0.85	5.87 ± 1.15	134.08 ± 0.91	7.97 ± 1.31	17.8 ± 1.52
SnRK2.14	2.21 ± 0.83	0.91 ± 1.32	13.77 ± 1.17	1.39 ± 1.09	2.65 ± 1.07	6.67 ± 1.16	230.7 ± 1.16	5.73 ± 1.31	4.36 ± 1.62
SnRK2.15	1.16 ± 1.21	0.58 ± 1.3	4.47 ± 1.2	1.38 ± 0.97	1.25 ± 1.17	6.46 ± 1.19	354.1 ± 1.09	5.94 ± 1.27	34.71 ± 1.5



Supplementary Fig. 1 Sequence tree for the Chlamydomonas SnRK family based on alignment of protein sequences by neighbour-joining. Chlamydomonas (Cre; black) CDPKs were included as an outgroup. *Arabidopsis thaliana* (At; green), *Homo sapiens* (Hs; red), and *Saccharomyces cerevisae* (Sc; yellow) homologous sequences were also included in the tree.



Supplementary Fig. 2 -ΔΔCq values of each Chlamydomonas SnRK sequence found under the stress conditions tested. The colour scale is shown at the right. Black corresponds to no changes, red to over-expression, and green to down-regulation. Data from four biological replicates is herein presented.

Part III

Final considerations

This study arose from the need to identify potential targets for bioengineering the current microalgal strains in order to improve its growth under stress conditions or to simulate a stress conditions with no need to apply it (stress mimicking strategy). This, together with a biorefinery production approach, would greatly reduce the current costs from producing microalgal biofuel, one of the most promising alternative energies to fossil fuels and food-based biofuels. The SnRK family was chosen as it has been proved to be deeply involved in plant stress response networks and, moreover, some studies suggest the same for the model microalgae Chlamydomonas.

Therefore, the aim of this work was to describe SnRK in Chlamydomonas and investigate its involvement in stress response. To do so, the entire SnRK family was genome-wide described by using state-of-the-art bionformatics tools and its potential implication in stress response mechanisms was described testing the expression levels of each SnRK under a wide-range of stress conditions by RT-qPCR and data mining of the available RNA-seq data.

Nineteen SnRK genes coding for 20 SnRK from 4 subfamilies (SnRK1, regulatory subunits of SnRK1, and two groups of SnRK2) were found in Chlamydomonas based on its protein sequence and domain structure similarities to previously described SnRK. Unlike in plants, no SnRK3 were found in Chlamydomonas. From the analyses of the SnRK gene expression under several stress conditions and the mining of the NGS-based data 3 SnRK were identified as likely to be involved in specific stress responses: SnRK2.7 (oxidative stress), SnRK2.9 (heat stress), and SnRK2.12 (Iron deficiency). Moreover, UV radiation showed to induce lipid accumulation, which is highly interesting from the production point of view since it is a stimulus that is easy to apply at reduced costs.

Although the results herein presented represent a great advance for microalgae and stress biology research, further studies are needed in order to confirm the function of the potential target SnRK identified. Once functionally characterized, these can result on a great advantage for the rising microalgal bioproduction industry. Also, studies involving other stress conditions can add some information to our results and alternative target genes can be found.

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