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Regulation of carotene biosynthesis through expression of phytoene desaturase gene in *Chlamydomonas reinhardtii* grown under high light intensity

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External phytoene desaturase genes from *Arabidopsis* (PDS1, AT1G0670.1) and (TP/PDS1 AT4G14210.1) were cloned in PFGC5941 vectors and successfully transformed to *Chlamydomonas renhardtii* independently via *Agrobacterium tumefaciens*. TAP medium and basta sensitivity test as a biomarker were suitable for differentiation between the transformed cells and non-transformed one. The result of transformation manifest that the transformed cells resisted basta up to 30µg/ml (sub lethal dose) whereas the wild able to grow at 10µg/ml only. The transformed colonies were scaled up into liquid TAP media for 8 days and the expression of these colonies confirmed using PCR amplification for PDS1 and TP/PDS1 genes with specific primers. Further the functionality of the inserted desaturase genes was confirmed by restriction enzymes of cloned vectors with *Nco1*and *BamH1*. qRT-PCR of most cell lines resists the basta resulted in that the level of PDS1 expression ranged between (2.28-3.26) and for TP/PDS1 (2.62-3.54) under normal light intensity (80 µmol photon m^{-2} s^{-1}). Further, the functionality of the inserted desaturase genes was confirmed by the metabolic analysis of transformed cultures grown under high light intensity (100-180 µmol photon m^{-2} s^{-1}). The elevating of the biosynthesis and accumulation of carotene as a result of light intensity gave good evidence that phytoene desaturase play a role for protection of the plastids from high intensity of light. Not only the elevating in carotene biosynthesis but also the expressions reached 8 and 19 folds for both PDS1 and TP/PDS1 with respect to wild cell at the high light intensity (180 µmol photon m^{-2} s^{-1}). Such results indicate that the functionality of TP/PDS1 may be light dependent. The positive correlation between the expression of PDS and carotene synthesis (quality and quantity) suggesting differential regulation between the nature of genes and abiotic stress. Also, the functionality of gene expression under high exposure intensity suggested the priority of TP/PDS1 to be over expressed rather than the PDS1 in cytosol and it plays a role as an auto protective against the stress factor.

Keywords: *Chlamydomonas* sp., Phytoene desaturase gene, cloning, PFGC5941 vectors- carotenoids.

INTRODUCTION

Carotenoids are terpenoid compounds synthesized by plants and microalgae. Microalgae have created a wide interest due to its easy to cultivate. So it does not being in competition with food production. It is easy to adapt to environmentally changing conditions by producing a great variety of secondary metabolites, and the

production of these compounds can be triggered by controlling the cultivation conditions (Ibañez & Cifuentes, 2013).

The protective ability of carotenoids against ROS radicals seems to be the reason for their therapeutic applications as anti-diseases and preventative reagents, anti-tumor reagents and stimulators to the immune-system (Knekt et al., 1991; Stahelin et al., 1991) and dietary supplements (Böhm et al., 2012). Carotenoids have been traditionally commercialized as food additives including colorants, antioxidants and vitamins (Metting,1996; Borowitzka and Borowitzka, 1988). The carotenoids biosynthesis pathway has been attracted the attention of several concerns (Barkovich and Liao, 2001). This is due to its commercial values and great market demand, especially for their pharmaceutical and nutritional applications. Moreover it has other biological functions, e.g. regulatory effects on intra-and intercellular signaling and gene expression (Sies and Stahl, 2005).

Carotenoids are generally synthesized via the isoprenoid biosynthetic pathway in plastids (Cunningham and Gantt, 1998; Lenden et al., 1993; Sandmann 2001). For isoprenoid formation the biosyntheses of carotene path through the desaturation step such step is the target point of bleaching herbicide action. It is a key master step to produce different carotenoids isomers, it also modulates and optimized for carotenoids accumulation as well as their structural variability (Pecker et al., 1992; Bartley et al., 1999; Kato et al., 2004).

Phytoene desaturases (PDS) are the key enzymes in the carotenoid biosynthetic pathway and acts as a limiting step in the pathway of most plant species (Chamovitz et al., 1993, Lopez et al., 2007). Many literatures indicate that the inhibition of catalytic activity of PDS by herbicides such as norflurazon resulted in accumulation of phytoene which concomitant with bleaching appearance of plants (Boeger & Sandmann, 1998). Phytoene desaturation process is a complicated process and has an extra variation throughout the plant Kingdome since in algae and higher plant phytoehe converted into ζcarotene through 2-steps of desaturation where as in bacteria and fungi for steps of desaturation process were carried out which lead to maximal saturated lycopene. In the genus *Rhodobacter* 3-steps of desaturation are required to convert phytoene to neurosporene (Sadmann et al., 1992). Pecker et al. (1992) compared different desaturases from various sources, which revealed strong similarities between all the bacterial and fungal enzymes but little conservation in the 2-steps desaturases which is commonly found in organism with oxygenic photosynthesis organisms.PDS genes have been isolated from a wide variety of organisms including higher plants such as tomato ,potato tuber (Ducreux et al., 2005), and carrot (Baranski, 2008) transforming bacteria, yeast and plant with gene cluster encoding carotenoid biosynthetic enzymes (Misawa and Shimada, 1997). *E.coli* which is a non-carotenogenic has widely been used as a host for improved carotenoid production through transformation with appropriate gene cluster. Methods have been developed for increasing biosynthesis of isopentenly pyrophoshate and geranylgeranyl pyrophosphate (GGPP) as precursor in E.coli (Wang et al., 1999; Kristala et al., 2000; Seon-Won et al., 2001) in *Dunaliella* (Sun et al., 2007) and Chlamydomonas (Vila et al., 2007), silencing of the *Chlamydomonas* PSY gene by artificial microRNAs (Molnar et al., 2009), transformation of *H. pluvialis* with a modified phytoene desaturase (Steinbrenner and Sandmann, 2006), the variation of the end product in carotene biosyntheses in *Chlamydomonas* as ketocarotenoid may be explained in the bases of new related enzymes as well as a new iso-enzyme may be expressed to accumulate ketocarotenoid in *Chlamydomonas* through the expression of a foreign β-carotene oxygenase (BKT) gene (León et al., 2007), astaxanthin in the chlorophyta *C. zofingiensis* (Del Campo et al., 2004; Sun, et al., 2008) are some examples. This mean that there are many types of enzymes that play a role in carotene biosynthesis for BKT gene (Huang, et al., 2006), phytoene desaturase (Huang et al., 2008), CHYb (Li et al., 2008), and LCYb (Cordero et al., 2010).

This work aims to evaluate the expected positive contribution of the two transgenes either in plastids (TP/PDS1) or in cytosol (PDS1) in β-carotene production especially under high light intensity as well as pointed out the different kinds of carotene through chromatographic separation.

MATERIALS AND METHODS

2.1. Chemicals.

All chemicals used were of analytical grade and obtained from Sigma-Aldrich (Munich, Germany).

2.2. Strain and culture conditions.

Chlamydomonas reinhardtii strain CC-124 (mt−) was grown in Tris acetate phosphate medium (TAP medium) according to (Gorman and Levine, 1965). The pH of the medium was adjusted to pH7 in liquid TAP medium. For preparation of solid medium, 1.5% (w/v) agar was added in case of growing the algal cells on solid medium and for selection the transformed cell line different concentrations of phosphenotricin were added up to (50µg/ml) medium. The algal cultures were incubated in growth room at 25 °C under long-day photoperiod (16 h light/8 h dark*)* with a light intensity of 80 µ Einstein and mild shaking (80 rpm) in case of liquid cultures.

2.3. Plasmid constract.

Cytosolic Phytoene desaturase gene (PDS1) coding sequence Gene bank: (AT1G06570.1) and chloroplastic TP/PDS1 gene (PDS1 fused to the chloroplast signal sequence (TP)) that targets protein to chloroplasts (TP/PDS1 AT4G14210.1) were amplified by PCR using genomic cDNA isolated from *Arabidopsis thaliana* as template. A forward PDS1 primer with extension for *Nco*I site (5´- CATG CCATGGGCCACCAAAACGCCGCCGTTTC -3´), A forward TP/PDS1 primer with extension for *Nco*I site (5´-CATG CCATGGATGATGACGGCGCTGCGTCGTCG -3´), and for both of them a reverse primer (5´- CGC GGATCCTCATCCCACTAACTGTTTGGCTT -3´) with extensions for BamHI site were used (Fig.1). Amplified PCR PDS fragments were column purified and then ligated into the binary expression vector PFGC5941 (11.406 kb) (Fig. 2) (kindly provided by Dr. Salah Abdel-Ghany Special Assistant Professor department of Biology Colorado State University). This cloning step results in a plant expression construct (PFGC5941-PDS1), the pFgc5941 vector (GenBank Accession No. AY310901) has a size of 11406 bp. It has multiple cloning sites (MCS) 1 and 2 each has different set of restriction enzyme sites. The vector and the specific gene were firstly digested with *Nco*I (MCS1) and *BamH*I (MCS2). Then gene fragment was also digested with the same enzymes, after which were ligated to the vector. Some of the sequences in the MSC was deleted after digestion, therefore the size of the ligated gene will not the exact summation of the vector size and the gene size. And a similar construct But the PDS1 gene was fused to the chloroplast signal sequence (TP) of Arabidopsis that targets protein to chloroplasts (PFGC5941-TP/PDS1) under the control of Cauliflower Mosaic Virus 35S CaMV promoter and OCS, octopine synthase terminator (Fig. 3).

2.4. Transformation of *C. reinhardtii*.

Wild-type *C. reinhardtii* strain CC-124 (mt⁻) was transformed with PFGC9541-PDS1 and another with PFGC9541-TP/PDS1 constructs following the *Agrobacterium tumefaciens* (GV3101) mediated transformation protocol as described by (Kumar, et al., 2004). Single colony of *C. reinhardtii* was inoculated in liquid TAP medium and left to grow until log phase. The cells were then spread on solid TAP medium and incubated under continuous light for 2 days until forming a lawn of cells. An *Agrobacterium* culture (OD600 = 0.6) harboring PFGC9541-PDS1 in one plate and in another plate, PFGC9541-TP/PDS1 plasmids were grown in liquid LB medium supplemented with the appropriate antibiotics (50 mg/l kanamycin and 25 mg/l rifampicin). The cells were spun down and the bacterial pellet was resuspended in 250 µl liquid TAP medium supplemented with acetosyringone (100 µM). The bacterial suspension was then spread onto the layer of *C. reinhardtii* culture grown on the agar plates. Plates were incubated for 2 h at 28 °C then kept overnight at 25 °C (co-cultivation). After that, cells were harvested and washed three times with liquid TAP medium containing 500 mg/l cefotaxime (10 min. each) to remove and kill the *Agrobacterium tumefaciens*. For selection of the transformed cells of *C. reinhardtii*, the washed cells of alga were spread on solid TAP agar plates containing 0.5 mg/ml cefotaxime and 15 µg/ ml Basta (Mohkami, et al., 2015). Such step was repeated with regular increase the concentration of basta up to 50 µg/ ml all the plats incubated at 25 °C in growth chamber for 7 days until the appearance of transformed colonies (Fig. 4). The presence of PDS1 and TP/PDS1 transgene in the selected *C. reinhardtii* colonies were confirmed by colony PCR test using PDS1 and TP/PDS1 genes-specific primers (2.3). After selection of the transformed colonies of *C. reinhardtii*, the selected colonies were inoculated into liquid TAP medium supplemented with phosphynotricine (Basta) (30 µg/ml). Transgenic *C. reinhardtii* cultures were then allowed to grow for 7 days in growth chamber under long-day growth conditions to generate enough algal biomass needed for qRT-PCR analysis and PDS1 and TP/PDS1 proteins isolation and purification.

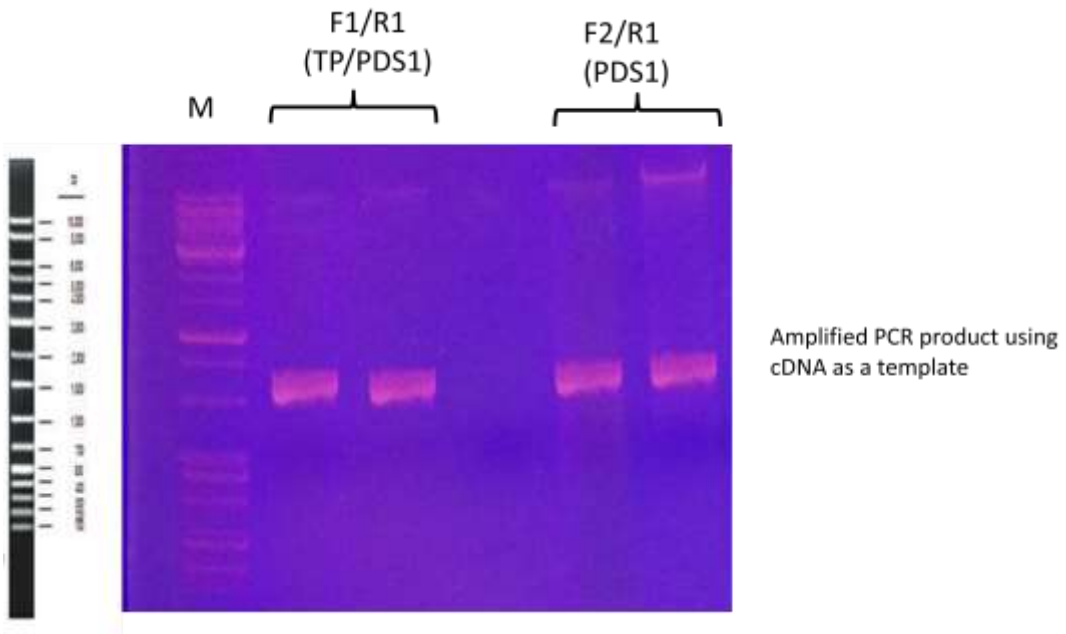

Figure 1; cDNA-PCR product from Arabidopsis TP/PDS1 and PDS1.
Shown is the ethidium bromide mediated fluorescence of DNA fragments (1400bp) after UV excitation. One band in the expected size range appears after a 1 % (w/v) agarose gel in 1x TAE for 50 min at 100 V.

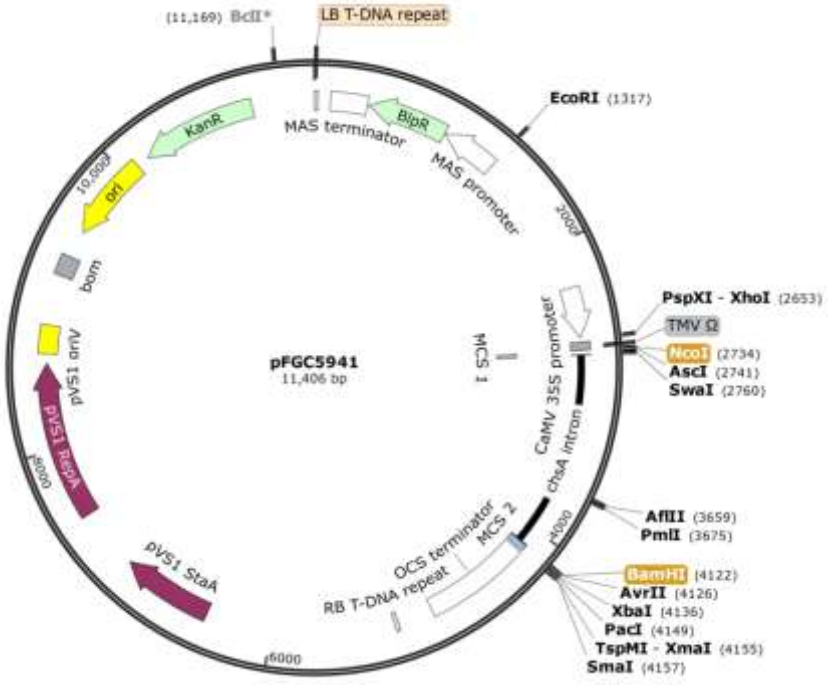

Figure 2; The structure of the pFGC5941 binary vectors (11406 bps).

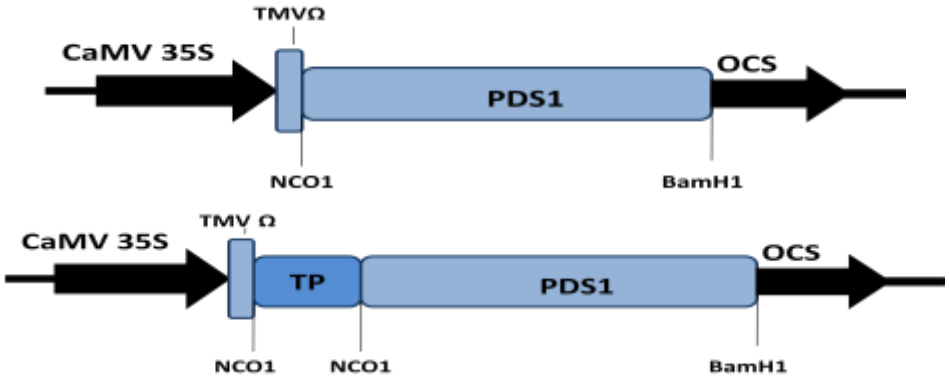

Figure 3; Schematic representation of gene constructs used for plant transformation. (**a**) Binary vector construct containing PDS1gene under the control of Cauliflower Mosaic Virus (35S CaMV) promoter. (**b**) A similar construct like in (**a**) but the PDS1 gene was fused to the chloroplast signal sequence (TP) of Arabidopsis like protein that targets protein to chloroplasts. Restriction sites are explained in the text. OCS, octopine synthase terminator; Ω TMV, 5′ leader sequence of tobacco mosaic virus.

The presence of PDS1 and TP/PDS1 transgene in the growing *C. reinhardii* cultures was confirmed by PCR using PDS1 and TP/PDS1 gene specific primers and genomic DNA isolated from the transgenic lines prior to each assay.

2.5. Quantitative Real-time RT-PCR analysis.

Quantitative real-time RT-PCR was performed as described previously (Kebeish et al., 2015). RNA was extracted from *C. reinhardtii* cells following the 1-bromo-3-chlorpropane (BCP protocol) of (Chomczynski and Mackey,1995). First-strand complementary DNA (cDNA) was synthesized as described by (Niessen et al., 2007). An ABI PRISM1 7300 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) was used following the manufacturer's instructions. PCR amplifications were performed using SYBR Green Reagents (SYBR1 Green ERTM qPCR Super Mixes; Karlsruhe, Germany). Primers were purchased from Intron Biotechnology Inc. (Kyungki-Do, South Korea). For detection of PDS1 transcripts, primers 5′-TGC CTT CTC CTC CGC CTA CTT -3′/5′- TAA ATATCG TCG GCC TGT CAC -3′ for detection of TP- PDS transcripts, primers 5′-GCTCAATGACGATGGCACGGT-3′/5′-TCGACTGGAGCGGCAAACAC-3′ were used. Primers 5′-GCG ATG TGG ACA TCC GCA AG-3′ and 5′-GGG CCG TGA TCT CCT TGC TC-3′ were used for detection of ACTIN transcripts. Primer concentration in the reaction mixture was adjusted to 200 nM. Amplification program used for amplification of different samples and ACTIN fragments was 10 min primary denaturation at 95 ˚C, followed by 40 cycles of 15 s denaturation at 95 °C and 1 min combined annealing and extension at 60 ˚C.

2.6. Response of C.renhardtii either transformed or wild to light intensity.

To manifest the effect of high light intensity on transformed and wild *C. reinhardtii* three groups of 100 ml Erlynmire flasks each containing 50 ml of sterile TAP medium, the first group inoculated with 1ml per each flask of wild algae and acts as control samples the second group inoculated with 1ml of PDS1 transformed alga the third one inoculated with 1ml of TP- PDS transformed alga, all the flasks are incubated under different light intensity (80,100 and 180 µmol photon m^{-2} s^{-1}) for 7 days (mid logarithmic phase) at the end of incubation period algal cells of each sample was separated and washed carefully for further analysis.

2.7.Determination of carotenoids content:

According to Metzner et al. (1965), the O.D. of the clarified extract was measured at 452.5 nm, and then carotenoids content in the extract (mg/l) was calculated from the following equation:

Carotenoids content = 4.2(O.D.452.5) – {0.0264(Chl."a"(mg/l)+0.426 Chl."b" (mg/l)}

2.8. Extraction and Purification of Protein from both PDS1 and PDS-TP from Transgenic *C. reinhardtii* Cells.

Discontinuous SDS-PAGE was used for the separation of protein samples under denaturing conditions according to (Laemmli, 1970). The BIO-RAD MINI PROTEIN II apparatus was used

for all gel electrophoresis. After preparation, each protein sample was mixed with 5 x SDS-PAGE-samples , boiled at 95°C for 5 min, chilled on ice and spun down for 5 seconds. Then the boiled sample was loaded into submerged wells.1 x SDS-PAGE electrophoresis buffer. Protein precipitation and non-specific protein staining with coomassie blue (Wilson, 1993) was used to visualize protein bands.

2.9. Separation of different pigments using thin layer chromatography.

A known weight of each sample was crashed in a mortar with 0.1 g glass beads a mixture of 5 ml hexane and acetone in a ratio 1:1 was added slowly to the fine powder making it a pestle like. The solvent was collected after each addition and the process was repeated for three times of extraction then, the total extracts were collected and the mixture was shaken vigorously and allowed to reduce its volume to 1/10 (Jeyanthi Rebecca et al., 2014). To thin layer chromatography (activated TLC plates) a known volume of each extract was dropped on the line 1.5 cm above the bottom of the plate. The plates was allowed to dry and then placed in glass chamber containing a mixture of hexane and acetone (1:1).This step may be repeated three times up to a well developed separated spots with different colors (Jeyanthi Rebecca et al., 2014). R_f was calculated with respect to standard samples.

2.10. Statistical Analysis.

The data were represented in figures as mean ± standard error (SE) of three independent samples for each genotype.

RESULTS

3.1. Gene cloning and generation of transgenic *C. reinhardtii*.

Phytoene desaturase gene plays a vital role in cartenogenisis processes. Therefore, Phytoene desaturase genes were genetically cloned and transferred into the nuclear genome of the model microalga; *C. reinhardtii.* The binary expression vector, PFGC5941 was used for the transformation of the green microalga, *C. reinhardtii* via *A. tumefaciens* mediated co-cultivation. In order to test the over-expression of PDS genes in alga for enhancing the carotene formation. Transgenic *C. reinhardtii* lines were selected on solid TAP medium supplemented with Phosphenotricin herbicide at 30µg/ml (sublethal dose) (Fig. 4) and re-inoculated in liquid TAP medium. Three independent lines of *C. reinhardtii* transgenic for PDS1 named T1.PDS1, T2.PDS1, and T3.PDS1 and another three independent lines of *C. reinhardtii* transgenic for TP/PDS1 named T1. TP/PDS1, T2.TP/PDS1, and T3.TP/PDS1 were selected and the highest expression level used for the further molecular and biochemical analyses (Fig. 5).

3.2. Effect of different light intensity on carotene quantity.

The biosynthesis of carotene under normal growth conditions of light intensity 80 µmol photon m^{-2} s^{-1} shows slight increase in the biosynthesis of carotenoids by the transformed cells rather than the wild one. The percentage of increase was significantly specially in case of TP/PDS1 transformed cells (57%) and (28%) in case of PDS1 transformed cells more than wild cells.

The previous values (Table 1) shows an obvious elevation as the transformed cells of both genes exposed to 100µmol photon m^{-2} s^{-1} (3.6 folds) in case of PDS1 with respect to wild and (3.2 folds) in case of TP/PDS1. As the intensity elevated to 180µmol photon m^{-2} s^{-1} the level of carotene accumulation reach 4.6 and 6.8 folds of both more than their wild under the same conditions (Fig. 6).This may explain that under the high intensity of light the biosyntheses of pigments starts to depredate and the coloration of algal culture show extraordinary coloration between deep yellow to pale brown (Fig. 7).

3.3. Qualitative analysis of carotene.

Qualitative analysis of acetone extracts of different untreated cell line either transformed or wild *C. reinhardtii* cells. using TLC chromatography evaluate that there are an obvious over-expression of phytoene desaturase specially TP/PDS1 genes with good appearance of β-carotene (R_f 0.923) followed by α-carotene (R_f 0.98) the other spots of leutin another one Methyle lutein (R_f 0.269) and cryptoxanthin (R_f 0.769) in addition to Chl a and Chl b with a moderate appearance (Fig. 7) (Pfander, et al.,1974). Whereas the over expression of PDS1 resulted in a more or less similar appearance of β-carotene and zeaxanthin and leutin with a highly significant response with respect to wild type.

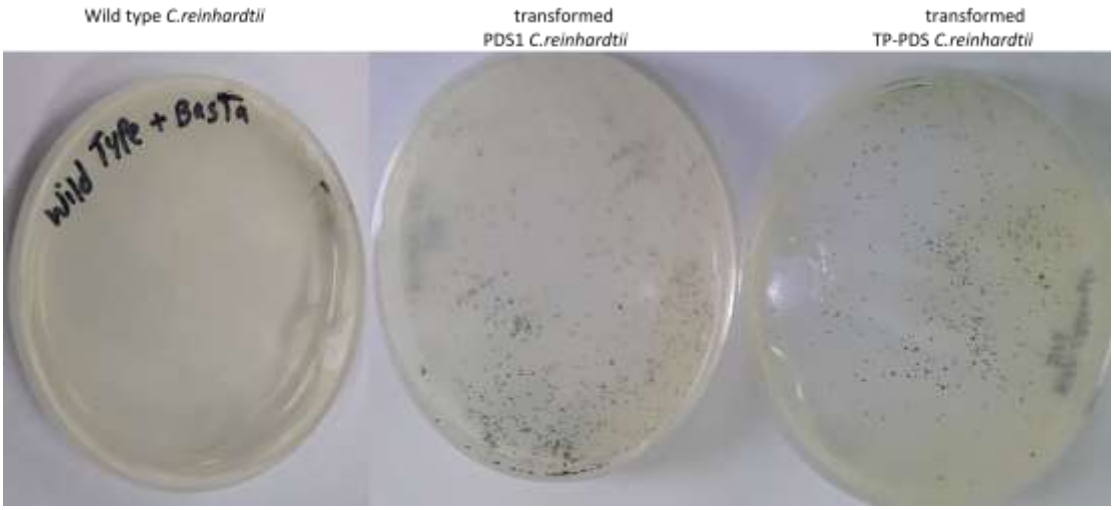

Figure 4; Selection of the transformed cells of *C. reinhardtii* on solid TAP medium after co-cultivation with *Agrobacterium tumefasciens*. **a** Photograph representing the selection of transformed colonies of *C. reinhardtii* and wild-type cells on solid TAP medium supplemented with basta at 30µg/ml.

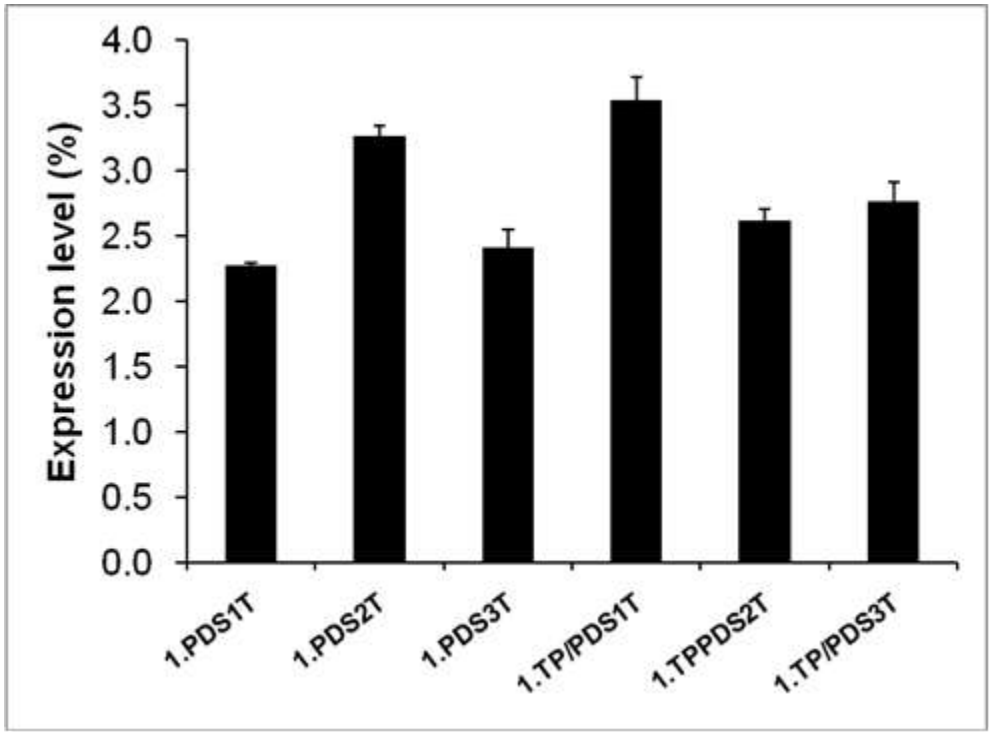

Figure 5; RT-PCR analysis of PDS expression. Amount of PDS mRNA transcripts was measured by real-time RT-PCR and calculated in arbitrary units compared with a standard dilution series.

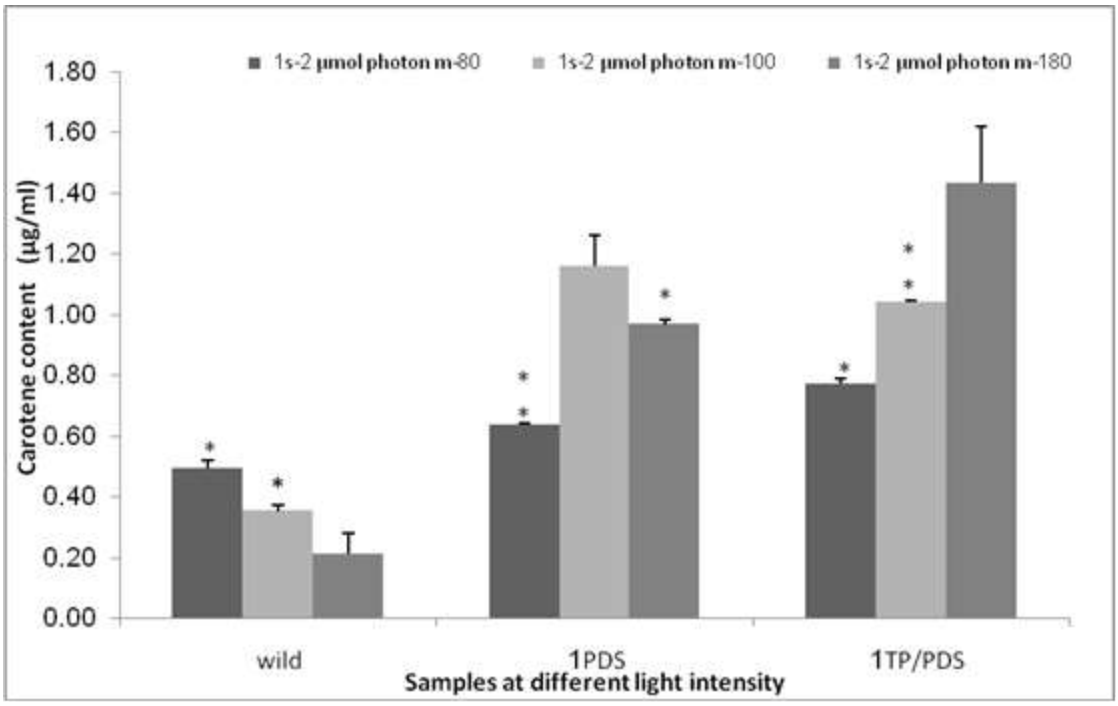

Figure 6; Effect of light intensity on carotene content (µg/ml) both transformed and wild *Chlamydomonas*. Data are means of three independent measurements. Vertical bars represent standard error.

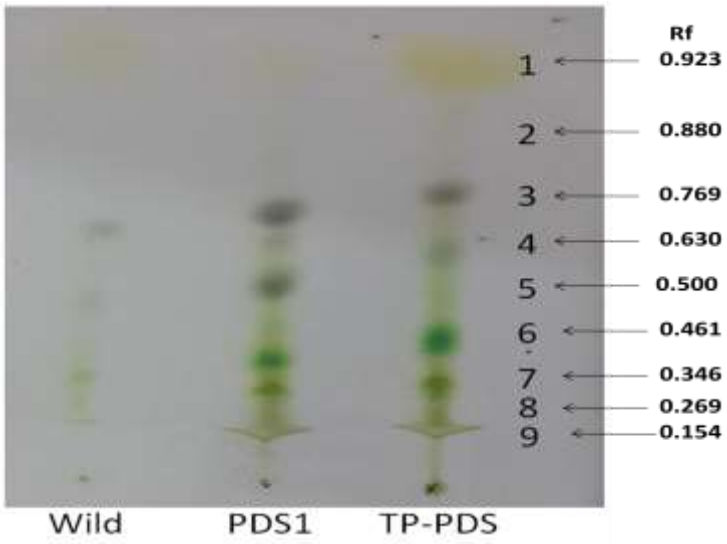

Figure 7; Separation of different pigments using thin layer chromatography at light intensity 180 µmol photon m^{-2} s^{-1}.

Table 1 ;Effect of light intensity on carotene contents (µg/ml) in both transformed and wild *C. reinhardtii.*

Samples	Carotene (µg/ml) at Light intensity (µmol photon $m^{-2} s^{-1}$)		
	80	100	180
wild	0.49±0.025*	0.32±0.020*	0.21±0.06
PDS1	0.63±0.003**	1.16±0.098	0.97±0.017*
TP/PDS1	0.77±0.014*	1.05±0.006**	1.43±0.184

Data are means of three independent measurements ± SE. (*) and (**) represent statistically significant differences when compared to the control samples at $p < 0.05$ and at $p < 0.01$ levels, respectively

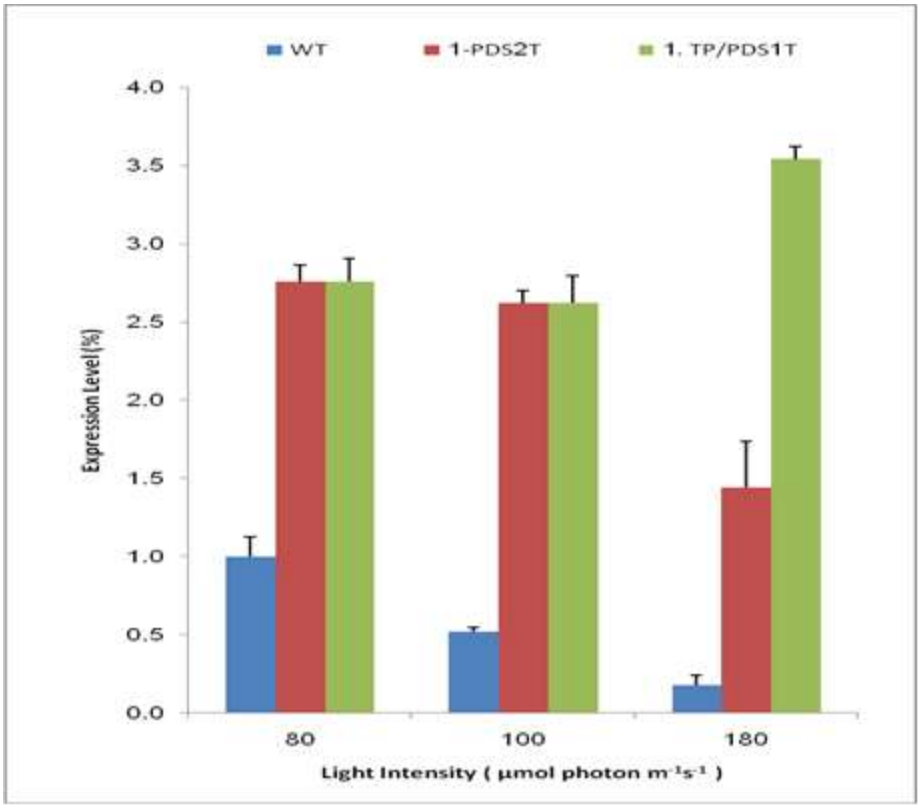

Figure 8; RT-PCR analysis of both wild and PDS expression under different light intensities (80,100 and 180µmol photon $m^{-2} s^{-1}$). Amount of PDS mRNA transcripts was measured by real-time RT-PCR and calculated in arbitrary units compared with a standard dilution series.

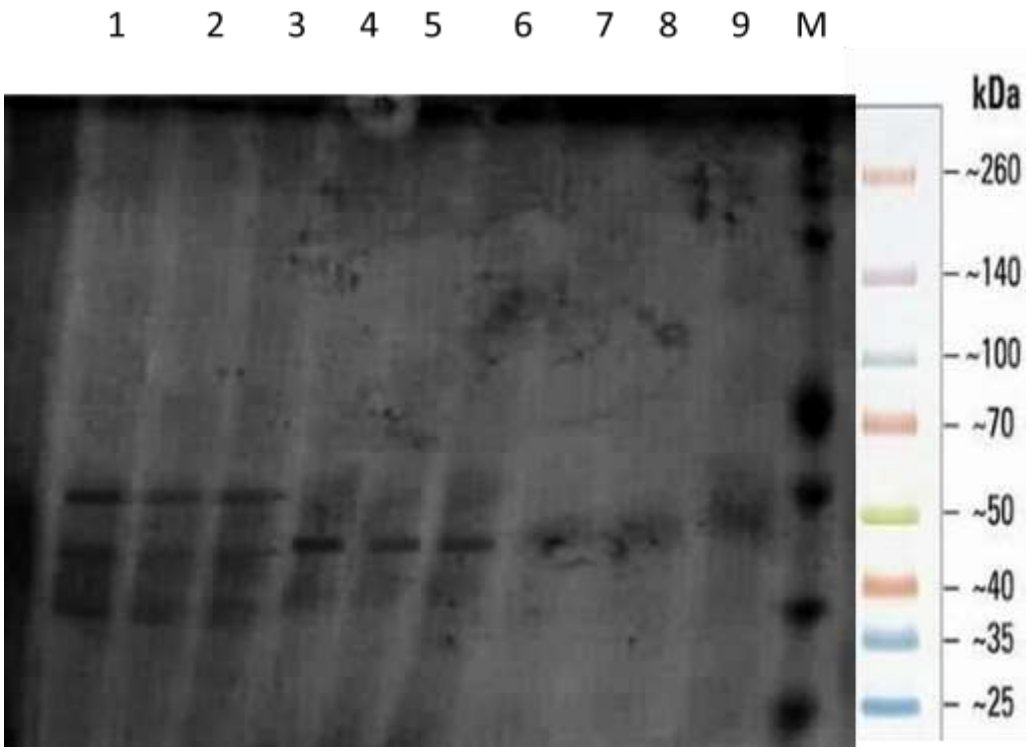

Figure 9; SDS-PAGE analysis of total protein extracts isolated from transgenic *C. reinhardtii*. M: protein ladder, lane 1- 6: total soluble protein extracts isolated from *C. reinhardtii* transgenic. WT control samples (lane7-9).One protein band in the size range of 58 kDa corresponding to TP/PDS1 protein is obvious for protein extracts isolated from transgenic *C. reinhardtii*. (lane1- 3) One protein band in the size range of 48.5 kDa corresponding to PDS1 protein is obvious for protein extracts isolated from transgenic *C. reinhardtii* (lane 4-6). This protein band is completely absent in the control wt. extracts (Lane 7 -9). Based on RT-PCR analysis together with SDS-PAGE analysis, it can be concluded that the TP/PDS1 &PDS1 gene is expressed in *C. reinhardtii*

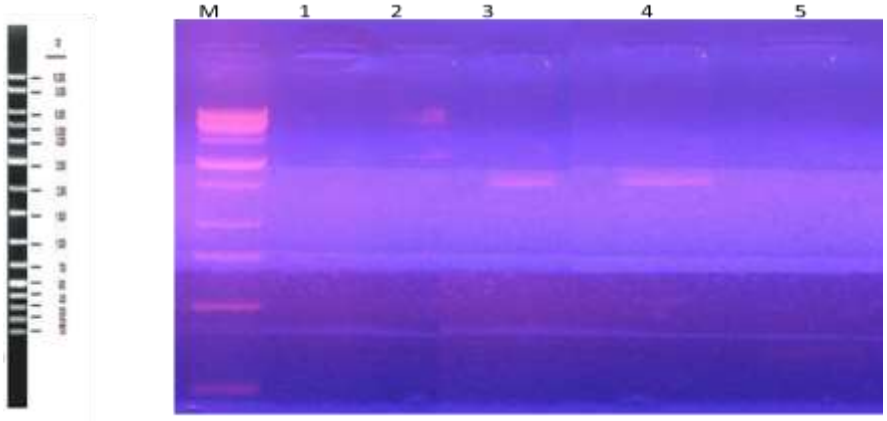

Figure 10; Genomic DNA isolated from PDS1 &TP/PDS1 transgenic *C. reinhardtii*. Shown is the ethidium bromide mediated fluorescence of DNA fragments after UV excitation. The isolated DNA was separated on a 1 % (w/v) agarose gel in 1x TAE buffer containing ethidium bromide for 1h at 12V/cm. M :1kb DNA lader, lane 1-5 : DNA isolated from *C. reinhardtii*

Table 2; R_f values of the different fractions as resolved by acetone:hexane (1:1) (pfander et al.,1974) in high light intensity

R_f and its spot name	*Wild (R_f)*	*PDS1 (R_f)*	*TP-PDS (R_f)*
1-β-carotene R_f (0.923)	+	+++	++++
2-α-carotene R_f (0.880)	--------	++	+++
3-cryptoxanthin R_f (0.769)	+	+++	++
4-unknown R_f (0.630)	+	++	+
5-zeaxanthin R_f (0.500)	----------	+++	++
6- chl b R_f (0.461)	+	+++	+++
7- chl a R_f (0.346)	+	+++	++++
8-Methyle lutein R_f (0.269)	+	+++	+++
9- lutein R_f (0.154)	+	+++	+++

3.4. RT-PCR and PDS1 &TP/PDS1 enzymatic assays.

Figure 8 shows changes in the expression levels of the genes encoding PDS as well as the wild alga (*C. reinhardtii)* following the algal cells grown at different light intensities (80, 100 and 180 µmol photon m^{-2} s^{-1}). Primer systems for PDS1, TP/PDS1, wild and Actin (as a house-keeping gene) were designed and optimized for RT-PCR analysis using serial dilutions of plasmid DNA. Real time polymerase chain reaction (RT-PCR) systems for screening of different gene samples expression levels using TaqMan 7300 series was established. Expression analysis of the different cell line samples of *C.renhardtii* and wild were carried out against Actin 1 and the normalization of transcripts revealed an obvious dropping in the expression of such genes in wild *C.renhartdii* as exposed to high light intensity with special reference to 180 µmol photon m^{-2} s^{-1} where as the expression pattern of PDS 1 and TP/PDS1 show higher expression response specially for TP/PDS1 following the exposure to light intensity of 180 µmol photon m^{-2} s^{-1} it reached 19 folds more than algae growing under 80µmol photon m-2 s-1 and 8 folds for PDS1 as exposed to the previous condition (Fig. 8). These results indicate that the overall expression of PDS genes for primary and secondary metabolism were light intensity dependent (slightly parallel to the dose of exposure).

3.5. SDS-polyacrylamide gel Electrophoresis of phytoene desaturase enzymes.

SDS-polyacrylamide gels manifested that an over expression of PDS1 and TP/PDS1 with approximately size 50 KDa for PDS1 and 60 KDa for TP/PDS1 with regard the protein standard. Separated proteins were visualized by gel staining with Coomassie brilliant blue as shown in (Fig. 9).

DISCUSSION

Plant molecular farming is a globally growing system for the production of recombinant proteins including biopharmaceuticals, industrial proteins, and other secondary metabolites. This technology bases mainly on the genetic transformation of plants and/or plant cell cultures (Barta et al., 1986; Hiatt et al., 1989). Moreover green plants cell cultures, and microalgae-based systems could efficiently be turned into biofactories for production of recombinant proteins on the large scale (Griesbeck, and Kirchmayr, 2012; Obembe et al., 2011). Because microalgae-based systems could potentially combine the advantageous features of plants and microorganisms, they become an effective competitive alternative for gene farming. The unicellular green microalgae *C. reinhardtii* with its preferable growth characteristics such as ease of genetic transformation via *A. tumefaciens* , a short time limit from gene to protein, affordable cultivation, and improved biosafety aspects make this microalgae an ideal candidate for developing a

novel molecular farming system. Moreover its ability to grow auto tropically as well as heterotrophically in TAP medium make the alga is the preferable to be designed for biotechnology (Griesbeck et al., 2006; Harris, 2001).

The reason for why intermediate compounds synthesized and accumulate inside the plastid may be explained that their high lipophilicity.The first committed step of the carotenoid synthesis pathway produces phytoene in a reaction catalysed by phytoene synthase (PSY).Whereas the second step catalyzed by PDS which involving the two-steps dehydrogenation of phytoene to produce zeta-carotene via a phytofluene intermediate (Hugueney et al., 1992; Al-Babili et al., 1996). The second carotenoids protect chloroplasts from excess light energy (Mayer et al., 1990; Norris, et al., 1995) and their absence results in chlorophyll bleaching and eventual cell death (Carol and Kuntz, 2005). In *C. reinhardtii,* PDS is encoded by the single nuclear PDS1 gene (Cre12.g509650) located on chromosome 12 (Bartley,et al., 1999). The *C.reinhardtii* and *A. thaliana* PDS3 (At4G14210.1) proteins are homologous and share 66% amino acid identity. Moreover gel elctrophresss manifest that the total base pair 1400 bp (Fig. 10). Moreover, PDS1 is a polypeptide of 445 aminoacids with a molecular weight of 50KDa, whereas TP/PDS1 is a polypeptide of 566 aminoacids with a molecular weight of 60 KDa (Fig. 9.), this was confirmed with Hugueney et al., 1992 who indicated that PDS was isolated from *Capsicum annuum* chromoplast membranes and exhibited a 56 ± 2 kDa molecular weight and very similar to the corresponding PDS of *Dunaliella* which composed of 420 amino acids. All plant, algal and cyanobacterial PDS sequences are well conserved resemble those of the analogous bacterial PDS enzymes (CRTB). And share an extensive prenyl transferase domain with squalene synthase enzymes. Comparison of *C. reinhardtii* and *D. salina* PDS sequences yields 75% of identity and a similar codon usage. PDSgene from *Dunaliella salina*, has an extraordinary ability to produce carotenoids under certain stress conditions. the central back bone (C40) of 8 isoprene unit forms a polyene chain of conjugated double bonds and establishes an extended p-electron system that account ability to absorb both ultraviolet radiation and visible light (Grossman et al.,2004).Such structure may be oxygenated with cyclic modifications or not to yield classify these colored pigments in to xanthophylls and carotene with variable colures ranged from yellow to red as obvious in (Fig. 7).Carotene compounds has core complexes around photo system PS 1 and PS 11 (Pineau et al., 2001 Ferreira et al., 2004) as well as the different cytochrome complex (Zhang, et al.,1999). Phytoene synthase PSY and phytoene desaturase PDS seem to be under transcriptional control and have an important role in the regulation of carotenoids biosynthesis. Our results were in concomitant with (Steinbrenner and Linden,2001) who found that high light intensity led to a transient increase in carotenoid hydroxylase mRNA followed by moderate astaxanthin accumulation.This means that the light induced expression of genes for carotenoid biosynthesis under photosynthetic control.

The up-regulation of carotenoids biosynthetic genes have been demonstrated in other photosynthetic organism. In the cyanobacterium *Synechococcus* sp. Phytoene synthase and Phytoene desaturase were shown to be under transcriptional control. The promoter activity at the PDS/PSY operon was higher under strong light (Schaeffer et al., 2006). In the unicellular green algae *C. reinhardtii* PSY and PDS showed a fast up-regulation in response to light (Bohne and Linden, 2002). In the green algae *Haematococous pluvialis*, increase in the PDS protein level, was accompanied by increase in PDS mRNA level during the accumulation of ketocarotenoids (Grunewald et al., 2000) It was concluded that Phytoene desaturase is regulated at the mRNA level, most likely by transcriptional control.

Semi quantitative real time PCR showed that the expression of the genes that play a role of carotene biosynthesis namely PDS1 and TP/PDS1 illustrated that the variation in their activities were sosphicated to the controversial changes in the environmental stresses, such as high light intensity but herein , under the normal light intensity (80 µmol photon m^{-2} s^{-1}) the expression levels reached (3.3 and 3.2) in case of PDS1 and TP/PDS1 respectively.Where asthe previous level attained the maximum expression (8 and 19) folds to wild respectively as the light intensity elevated to (180 µmol photon m^{-2} s^{-1}). In this regard (Zamani and Moradshahi, 2016) found that PDS transcript level continued to increase with time reaching a maximum of 2.2-fold, 12 h after shift to higher light intensity and About 4.5-fold increase in PDS mRNA level was observed 48 hr after shift to high light intensity. Possible changes in the mRNA levels of PDS have been studied in other photosynthetic organisms. In sunflower, the steady-state level of PSY was

negatively affected by phytoene accumulation (Salvini et al., 2005). Inhibition of carotenoids synthesis in tomato seedlings resulted in increased PSY and PDS transcript levels (Simkin et al., 2003 a), but this up-regulation was not observed in pepper leaves (Simkin et al., 2003 b).Our results of qRT-PCR analysis indicates that the expression of both PDS1and TP/PDS1 were in parallel with the data obtained from (Zamani and Moradshahi, 2016). (Bohne and Linden, 2002**;** Steinbrenner and Sandmann 2006) who indicated that the increases in PDS transcript expression was correlated with increased carotenoid levels as well as with the Qualitative analysis. (Table 2) revealed that there is an ultra variation in the quantity of different types of carotene between wild and both transformed as exposed to different light intensity in this regard as the exposure.

CONCLUSION

This work reported the increased expression of external phytoene desaturase gene in *Chlamydomonas reinhardtii*. The transformed strain showed increased biosynthesis of carotene especially under high light intensity. Therefore, suggesting differential regulation between the nature of genes and abiotic stress. Eventually, this may increase the protection of the algal cells against stress like high intensity of light.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed in collecting and analyzing data. All authors participated in writing every part of this study. All authors read and approved the final version.

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