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Identification and Functional Characterization of *Arabidopsis icl* Mutant Under Trehalose Feeding in Light and Dark Conditions

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ABSTRACT

Trehalose is a non-reducing sugar that plays an important role in plant growth and development. To study the role of trehalose on lipid metabolism and gluconeogenesis, *Arabidopsis thaliana* wild type (WT) and TreF (a line expressing trehalase) were grown on ½ MS medium with or without 100 mM sucrose and or trehalose in light or continuous darkness. In dark, trehalose leads skotomorphogenesis in WT seedlings and inhibits hypocotyl elongation without altering root growth. Then, a knock out mutant of icl (defective in isocitrate lyase/ICL) was identified in the SALK T-DNA insertion line, yet plants of this line were not altered with regard to growth on MS medium supplemented with or without trehalose in light condition, compared to WT. But the hypocotyl length of icl seedling was shorter than WT when grown on trehalose in darkness. The current data revealed that trehalose feeding altered seedling establishment in both WT and *icl* mutant. ICL enzyme activity measurement showed that the patterns of changes were similar in all treatments. Meanwhile, trehalose feeding reduced *icl* gene expression and enzyme activity. Trehalose fed seedlings demonstrated a high accumulation of total lipids in darkness. Also fatty acids level was higher in seedlings grown in darkness, compared with the light condition. Therefore, trehalose may inhibit lipid utilization by suppressing *icl* gene expression and enzyme activity and thus restrict the supply of carbon sources to the growing seedling. These observations confirm that trehalose regulates plant metabolism in both light and dark.

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Introduction

Trehalose is a non-reducing sugar which consists of two molecule glucose joined by an α, α-1, 1-linkage. This sugar was found in a wide variety of organisms (Elbein *et al.,* 1974). Trehalose-6-phosphate synthase (TPS) catalyzes the conversion of glucose-6-phosphate and UDP-glucose into trehalose-6-phosphate (T6P), the phosphorylated intermediate of trehalose biosynthesis**.** Then, trehalose phosphate phosphatase (TPP) converts T6P to trehalose (Blazquez *et al.* 1998). Trehalose is then cleaved to two molecules of glucose by the enzyme trehalase (Aeschbacher *et al.*, 1999; Muller *et al.*, 2001).

Exogenous trehalose feeding of Arabidopsis increases the trehalose-6-phosphate (T6P) level in 30 minutes (Schluepmann *et al.*, 2004). Increased T6P can change metabolites levels in various higher plants (Schluepmann *et al.*, 2003). T6P has been implicated in control of mechanisms inducing the switch from vegetative to floral development (Van Dijken *et al.,* 2004; Wahl *et al.,* 2013), regulation of photosynthetic capacity (Pellny *et al.,* 2004; Oszvald *et al.,* 2018), resistance to stress (Nounjan *et al.,* 2012) and synthesis of organic acids (Figueroa *et al.,* 2016). T6P is an important signaling molecule that reports the sugar status of the cytosol to the chloroplast (Lunn *et al.,* 2006). In fact, it works

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both as a signal and a negative feedback regulator of sucrose (Yavada *et al.,* 2014).

Exogenously applied trehalose strongly reduces elongation of *Arabidopsis* root length, induces a strong accumulation of starch in cotyledons and an increased activity of ADP-glc pyrophosphorylase (AGPase) (Wingler *et al.,* 2000; Kolbe *et al.,* 2005; Aghdasi *et al.,* 2010). Meanwhile, trehalose feeding inhibits hypocotyls elongation without altering root growth in continuous darkness (Aghdasi 2007; Dellata *et al.,* 2011). Hypocotyl elongation during etiolating has previously been shown to depend on gluconeogenesis from lipids stored in both the embryo and the endosperm of Arabidopsis (Eastmond *et al.,* 2000; Penfield *et al.,* 2006; Pracharoenwattana *et al.,* 2005). Yet some enzymes of the glyoxylate cycle are also repressed by carbon catabolism repression (Graham *et al.,* 1990). The glyoxylate cycle converts acetate (from lipid breakdown) to four carbonate substrates to support seed growth (Cooper and Beevers 1969).

Isocitrate lyase (ICL) and malate synthase (MLS) are two key enzymes in this pathway (Pellicer *et al.,* 1999; McKinney *et al.,* 2000). In the glyoxalate cycle, ICL catalyzes the cleavage of isocitrate to succinate and glyoxalate. Then, MLS converts glyoxalate to malate by using acetyl-COA.

So far mutants lacking the glyoxalate cycle enzymes were identified and characterized. In *Arabidopsis*, *icl* knock-out mutant grows slowly and unable to convert lipids into sugars (Eastmond *et al.,* 2000). In contrast, *mls* mutant produces more sugar in light condition, compared to *icl* mutant (Cornah *et al.,* 2004).

Although the role of trehalose on plant growth under light condition has been reported previously, the effect of trehalose in continuous darkness remained unknown. Therefore, in the present study, the effect of trehalose on plant growth and gluconeogenesis were investigated.

In this way, the *icl* mutant was identified and further characterized in order to allow genetic dissection of the multiple effects of trehalose. In this study *icl* mutant growth and establishment were fully characterized in light and continuous darkness condition.

Materials and Methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* wild type (WT) and TreF 46.2 (a line that expresses *E. coli* trehalase, as a positive control) were used in the current study. Seeds were sterilized with 70% ethanol (5 min) and 20 % commercial bleach. After washing 5 times in sterile milli-Q water, seeds were sown on $^1/_2$ MS medium supplemented with or without 100 mM trehalose and or sucrose. Then, seeds were stratified in darkness at 4ºC for 2 days before transferring to a growth chamber at 25 ºC under a 16 h light /8 h dark photoperiod or continuous dark. Seedlings were grown vertically. After 14 days, pictures were taken and root and hypocotyl length measurements were carried out with the Image J program (Wayne Rasband, NIH Maryland, USA).

Starch staining

For analysis of starch distribution, whole seedlings were taken and destained in 70% and then 90% (v/v) ethanol. Staining was done with KI/I_2 solution and then washed with milli Q water. Pictures were taken using a Nomarski microscope (Jena, Germany).

Sugar measurement

The reducing and non-reducing sugars amounts were determined according to Prado *et al.,* (1998) and Handel (1968) respectively.

ICL enzyme activity assay

In brief, two day old seedlings (0.03 g) were ground in 300 µl cool extraction buffer [0.1 M Tris-HCl pH 7.5; 10 mM, Potassium chloride; 1 mM, Magnesium sulfate; 1 mM, EDTA; 400 mM, Sucrose; 1 mM, PMSF; 0.1 %, Triton X100; 0.6 %, PVPP; and 50 mM, 2-Mercaptoethanol] on ice bath. Crude extracts were incubated for 1 hour at 4 ºC and then centrifuged at 5800 *g* for 15 min. The supernatants were used for ICL activity assay (Sadeghipour and Bhatla, 2002). ICL enzyme activity was recorded spectrophotometrically by phenylhydrazine reagent at 324 nm. The amount of enzyme activity was expressed as nmol glyoxylate hydrolyzed per minute per gram tissue fresh weight (Cooper and Beevers, 1969).

Total lipid and fatty acid measurements

In brief, 0.25 gr of *Arabidopsis* seeds and 0.15 gr of 2 and 4 days old seedlings were homogenized in liquid nitrogen and incubated at 80 °C for 10 min with 5 mL of isopropanol. Then, 10 ml of the hexane-isopropanol mixture (3: 2 ratios) was added and the resulting solution was filtered through filter paper and transferred to pre-washed hexane container. The isopropanol was evaporated and total lipids were quantified by gravimetry after drying the samples (Siloto *et al.,* 2006).

The measurement of fatty acid was performed as described by Chakrabarty *et al.,* (1969) with some modifications. The extracted oil from the previous stage was used to measure the fatty acid content. After adding 300 ml benzene to 300 mg extracted oil, the mixture was vortex at full speed for 2 min. Then, 100 µl of oil and benzene mixture was transferred to a new test tube and resuspended in benzene up to 1000 µl. The amount of fatty acid was recorded spectrophotometrically by Rhodamine 6G reagent at 535 nm.

Isolation and characterization of homozygous T-DNA insertion lines

Seeds were obtained from the insertion collections made available by the Nottingham Arabidopsis Stock Centre (Nottingham UK). Homozygous *icl* mutant plant was isolated from seeds of segregating the T3 line of the T-DNA insertion lines N400723. The genomic DNA was isolated from fresh leaf material using a Quick extraction protocol. In brief, three rosette leaves were ground and homogenized in 200 µl extraction buffer (200 mM Tris-HCL pH 7.5, 25 mM EDTA, 250 mM NaCl). Then potassium acetate was added and centrifuged at 2000 *g* for 2 min. The resulting supernatant was transferred to a new tube. The DNA was precipitated by adding isopropanol and centrifuged at 13000 rpm for 3 min. After washing the pellet with 70% ethanol and air drying for 5 min, the dried pellet was resuspended in 100 µl Tris/EDTA buffer.

PCR screening was used to identify T-DNA insertion in *ICL* (At3g21720) gene. The specific primers used were: *icl*-f (5'-TGTGGTTTCCAAGCTTTCCT) and *icl*-r (5'-AATGGTCCGGTGCTAATTACTATG); *T-DNA*-f (5'-ATGATAACTCGACGGATCGCA) and *T-DNA-r* (5'-CTTGGATGTGGTAGCCGTTTC). The, amplified fragments were sequenced and the T-DNA insertion site was determined. To identify the number of inserted T-DNA into the mutant line, homozygous mutant lines were grown on MS medium with or without 7.5 mg/L sulfadiazine. The sensitive seedlings displayed an obvious yellow phenotype and were unable to produce early rosette leaves (Rosso *et al*., 2003). In other words, the ratio of the resistant to sensitive plant is calculated excluding the yellow phenotype.

RNA Isolation and RT-PCR

Seeds of *Arabidopsis thaliana* accession Columbia-0 and *icl* mutant seeds were grown on ½ MS medium with or without 100 mM sucrose or trehalose for 14 days. Plant material was snap-frozen in liquid nitrogen, pulverized with glass beads for 2 minutes at 2800 rpm in a dismembrator (Braun, Germany).

RNA extraction was performed according to Sambrook and Russell (2001). The extracted RNA was then treated with the DNaseI enzyme to remove the genomic DNA. RNA concentration and purity were determined by measuring absorbance at 260 nm. The cDNA synthesis was performed using the AccuPower CycleScript RT premix (dN6) Kit (BioNeer Korea) according to the manufacturer's instructions. PCR was performed with gene-specific primers (*ICL:* 5'-TGTGGTTTCCAAGCTTTCCT-3' and 5'-AATGGTCCGGTGCTAATTACTATG -3' and for *18S rRNA*: 5'-ATGATAACTCGACGGATCGCA-3' and 5'-CTTGGATGTGGTAGCCGTTTC-3'). After denaturation for 3 min at 94 ºC, DNA amplification was performed with 30 cycles (30 sec 94ºC, 30 sec 56ºC, and 30 sec 72ºC). PCR was completed with a final step at 72ºC for 5 min. Then, the PCR product was run on the agarose gel.

Growth stages analysis

The seeds of *Arabidopsis thaliana* accession Columbia (Col-0) and *icl* homozygote line were sterilized for 5 min with 70% ethanol followed by 10 min in 20% commercial bleach (4% w/v

chlorine) and washed five times in sterile Milli-Q water. Seeds were grown on Murashige and Skoog (MS) medium with or without 100 mM sucrose or trehalose. Seeds were stratified in darkness at 4 °C for 2 days, and then the plates were transferred to a growth chamber at 22°C under a 16-h-light/8-h-dark photoperiod or continuous dark. Daily measurements were performed after removing from the cold room (day 3 after sowing) until the seedlings were harvested. Germination assays were carried out with three replicates of 100 seeds. Seed germination was counted daily for one week after transferring to the growth chamber and determined using Germine-G software. Growth analysis of both WT and *icl* mutant was analyzed according to standards defined for Arabidopsis growth stages (Boyes *et al.,* 2001).

Statistical analysis

All experiments were repeated at least three times with three independent replicates. Data from biochemical measurements were statistically analyzed by Duncan's multiple range tests at $p \leq 0.05$ significant level using SAS software (Version 9.1). Data from developmental stages were analyzed by Student's *t*-test.

Results

Dark development in seedlings growing on 100 mM trehalose

In light condition, trehalose in the medium resulted in decreased root growth length (1.9±0.58) and depletion of starch in columella cells of the root cap in WT seedlings. Root length was very long in *TreF* seedlings (24.9±3.5) and leaf expansion was normal (Fig. 1A, C). There are no significant differences in root length of WT and TreF seedlings when grown on 100 mM trehalose in continuous darkness.

On MS medium with or without 100 mM trehalose, starch did not accumulate in the columella of WT roots grown in darkness, whilst starch did accumulate in the columella of WT roots grown on sucrose and *TreF* expressing roots grown on trehalose as well as on sucrose (Fig. 1B, C). Hypocotyl lengths of WT seedlings were markedly shorter on trehalose compared with MS medium with or without sucrose (Fig. 1D).

Isolation and characterization of T-DNA insertion in *ICL* (At3g21720) gene

To provide evidence on the roles of trehalose on gluconeogenesis, a knockout mutation in the *ICL* gene (At3g21720) was sought. Seed from putative insertion mutants was ordered from NASC (Nottingham, UK) and plants grown from the N400723 were screened for the presence of the insertions by PCR as described in Materials and Methods. We isolated *icl* mutants homozygous with respect to the T-DNA insertion from segregating T3 seeds of GK-008E03-016981line. The knock out phenotype was due to T-DNA insertion in exon 5 of *ICL* gene (Fig. 2A). To ascertain that this line is a truly knockout mutant, expression levels of *ICL* were analyzed by RT-PCR (Fig. 2B). No band was detected in the selected line and confirmed the `true knockout of the gene. Segregation analysis on sulfadiazine showed that the line was homozygous for the T-DNA insertion (Fig. 2C). Meanwhile, ICL enzyme activity was not observed in the T-DNA insertion mutant line (Fig. 2D). The germination of *icl* mutant on MS medium under light condition was identical to those of WT plants (Fig. 3A). The *icl* mutant and WT plants showed different phenotypes when grown in soil. The mutant showed a shorter height, compared to WT plants (Fig. 3B).

To analyze Arabidopsis growth stages, Boyes *et al.,* (2001) model was applied. In this model, they used a BASIC scale which provides a generic nomenclature to analysis Arabidopsis phenotypes (Lancashire *et al.*, 1991). According to this model, *Arabidopsis* growth stages consist of different steps, from seed imbibition through the completion of flowering and seed maturation. The number of rosette leaves was recorded every 2 days from stage 1.02 (when the first two rosette leaves appeared) to stage 5.01 (when the first flower bud appeared). Meanwhile, stage 6.00 (when 10% of flowers to be produced have opened) and stage 6.90 (when flowering is completed) were recorded in both WT and *icl* mutant plants, when grown on soil under 16 h daylight. The current results showed *icl* mutant delayed the vegetative growth stages, including the emergence of rosette leaves (stage 1.02) and

the emergence of the first flower bud (stage 5.10). However, the step between stage 5.10 and 6.00 (called bolting) was shorter and the first flower was opened (step 6.00) earlier in the mutant line. Furthermore, the *icl* mutant was different with respect to the flowering time when compared with the WT ones. Flowering time (stage 6.90) was also longer in the *icl*, compared to WT plants.

Overall, the current data revealed that the life cycle in the *icl* mutant is approximately 12 days longer than WT, which could be due to delayed entry into the reproductive phase (Fig. 3C). The *icl* mutant had lower seed weight than that of WT plants. The results showed a 38% decrease in the seed weight of *icl* mutant (Fig. 3D).

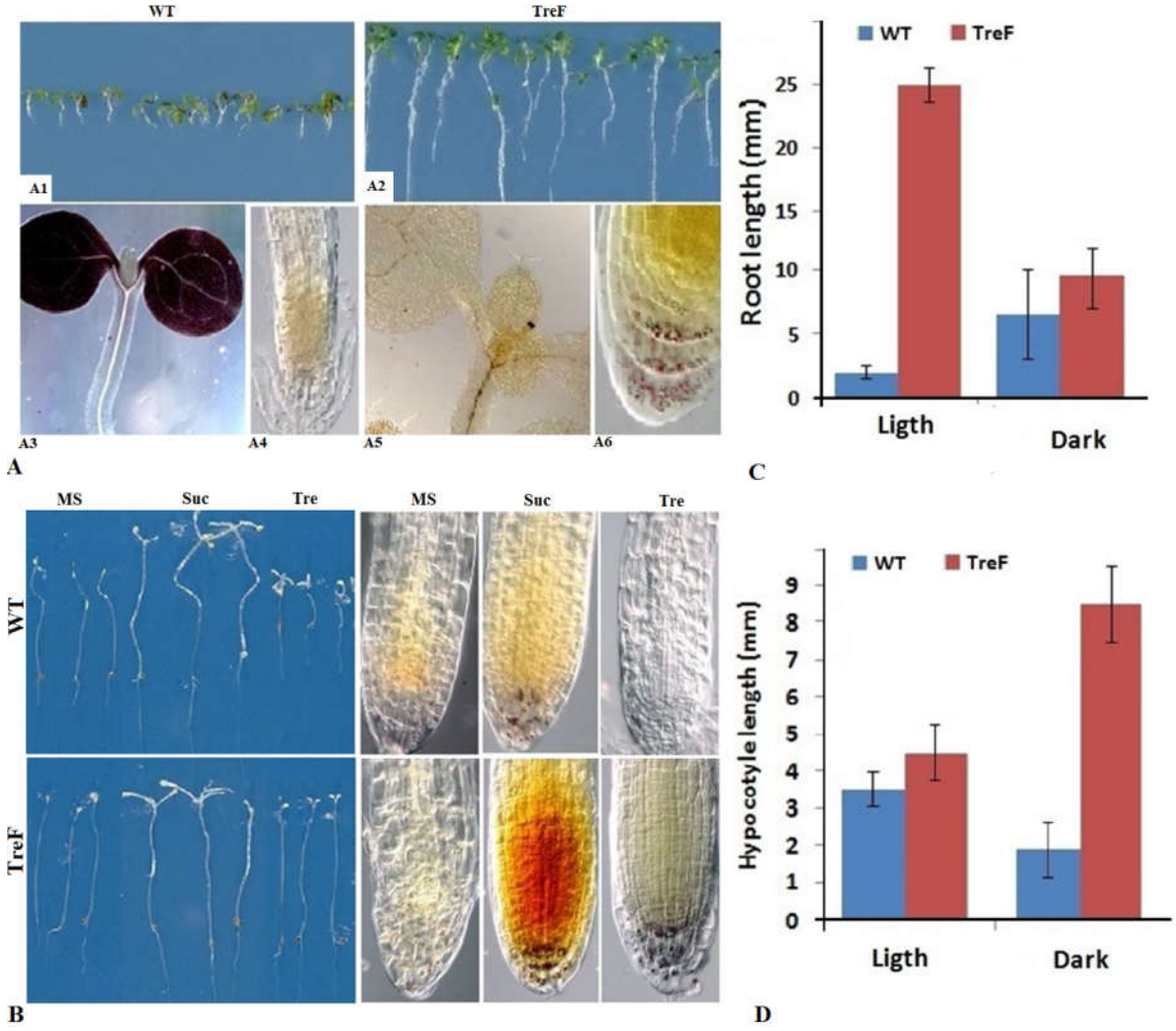

Fig. 1. The effect of 100 mM trehalose on the growth and starch accumulation of WT and TreF seedlings in light and dark conditions: A1-A2) Growth and A3-A6) Starch staining in 14-day-old seedlings from WT and TreF grown on MS medium supplemented with 100 mM trehalose in the 16 h-light/8 h-dark photoperiod; B) Growth and starch accumulation in 14-day-old seedlings from WT and TreF on MS medium with or without 100 mM sucrose and or trehalose in continuous dark; C & D) Root and hypocotyl length in WT and TreF seedlings on MS medium supplemented with 100 mM trehalose in light and continuous dark, respectively; Data are presented as the means ± SD. Bars with different letters are significantly different at $p \leq 0.05$, according to Duncan's test; WT= Wild-type; TreF= Trehalase over-expressing line

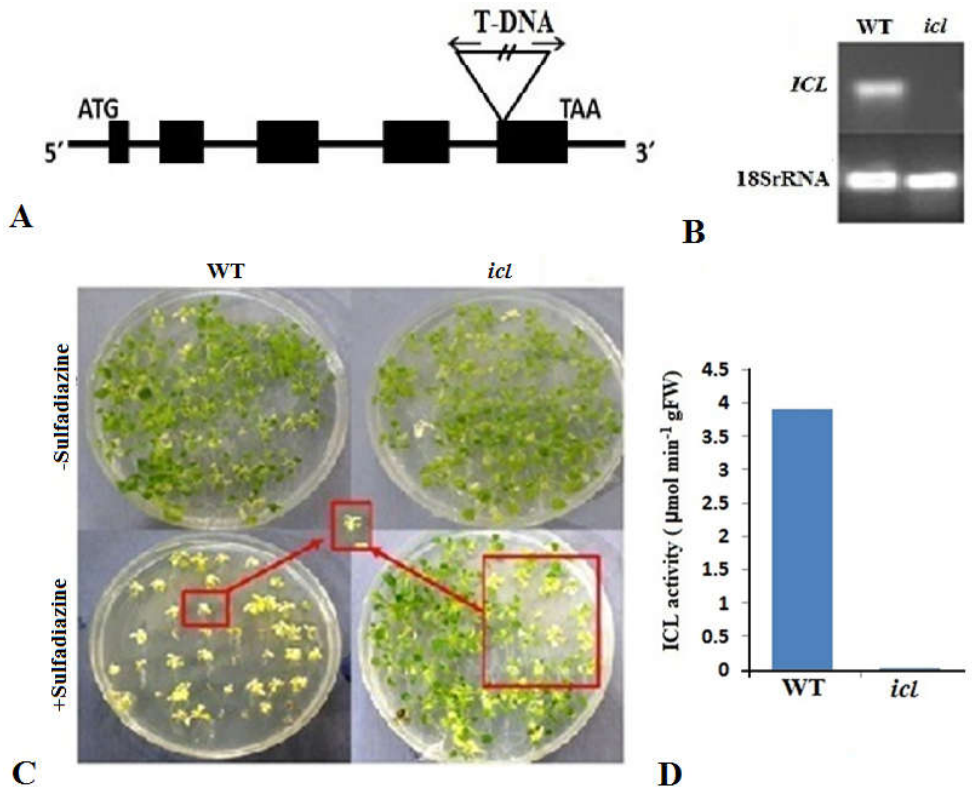

Fig. 2. Confirmation of *icl* knock out mutant: A) Schematic representation of T-DNA insertion site in *icl* mutant; B) Expression of *ICL* in wild-type (WT) and *icl* knock out mutant; C) Phenotype of WT and *icl* mutant on MS medium with or without Sulfadiazine; D) ICL activity of WT and *icl* mutant on MS medium; Data are presented as the means ± SD. Bars with different letters are significantly different at $p \leq 0.05$, according to Duncan's test.

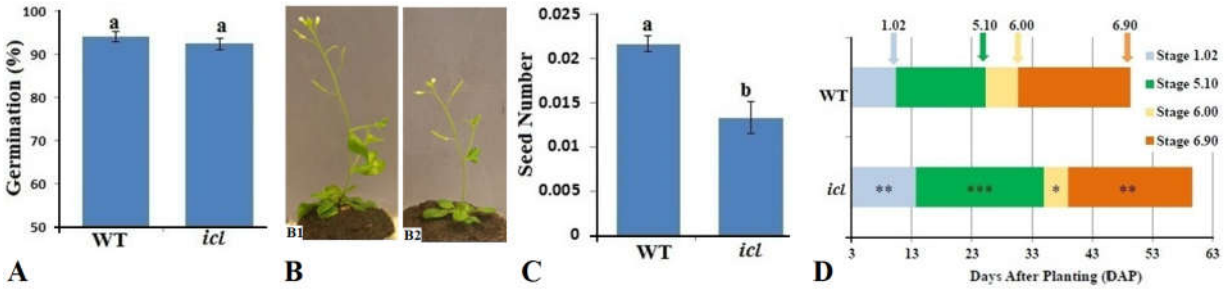

Fig. 3. Characterization of *icl* mutant: A) Germination rate; B) Growth phenotype, B1=WT, B2= *icl* mutant; C) Seed number of wild-type (WT) and *icl* knock out mutant on soil; D) Growth stage progression; Data are presented as the means ± SD. Bars with different letters are significantly different at $p \leq 0.05$, according to Duncan's test. Stage 1.02: Appearance of the first two rosette leaves; Stage 5.01: the appearance of the first flower bud; Stage 6.00: the opening of 10% of produced flowers; Stage 6.90; completed flowering.

icl knock out mutant is sensitive to trehalose

Seed germination was not different between *icl* and WT on MS medium with or without 100 mM sucrose and or trehalose under 16 h light or continuous dark conditions (Fig. 4A, B). While trehalose feeding suppresses root length in both WT and *icl* mutant in light condition, it was identical in seedlings grown on MS medium with or without sugars in the continuous dark (Fig. 4A). The hypocotyl length of the mutant seedlings was shorter than that of WT ones when grown on the MS medium supplemented with or without trehalose in darkness (Fig. 4C).

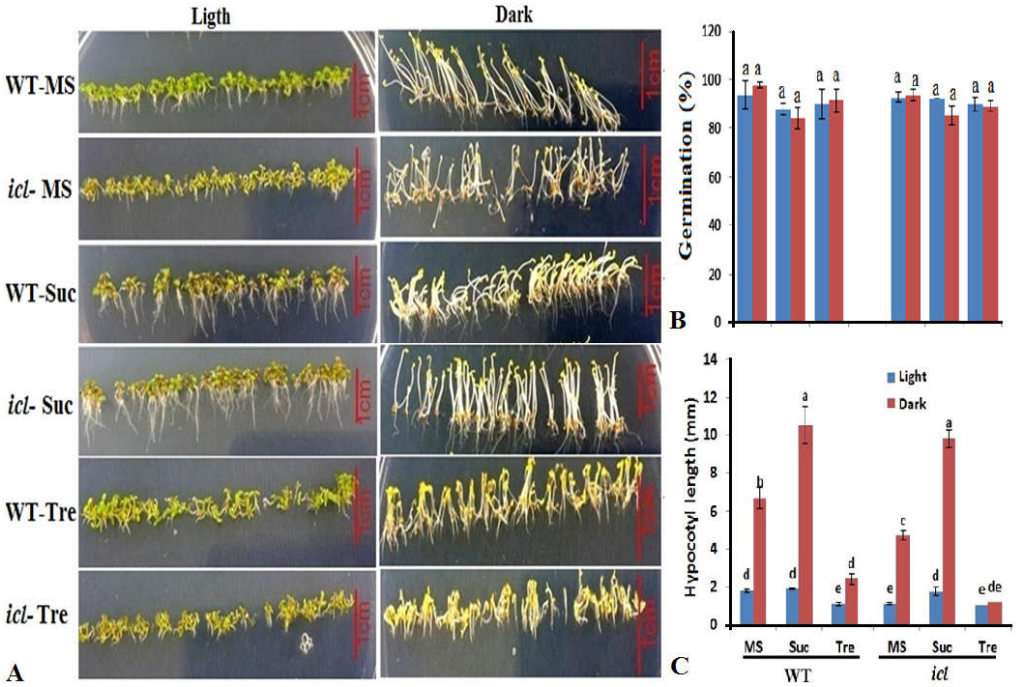

Fig. 4. Light and dark development phenotypes of WT and *icl* mutant: A) Phenotype, B) Germination, C) Hypocotyl length of wild-type (WT) and *icl* knock out mutant on MS medium with or without 100 mM sucrose (Suc) and or trehalose (Tre) in the 16h light/8h dark photoperiod or continuous dark condition. Data are presented as means ± SD. Bars with different letters are significantly different at $p \leq 0.05$, according to Duncan's test.

Trehalose feeding changes ICL enzyme activity during seed germination

To further characterize the effect of trehalose on plant metabolism, ICL enzyme activity and gene expression were assayed in both WT and *icl* mutant on investigated medium. The expression of *ICL* was low in WT on MS medium with or without 100 mM trehalose. Hence, sucrose feeding enhanced *ICL* gene expression in WT plants (Fig. 5).

ICL enzyme activity was evaluated on 0, 2, 3 and 4 days after imbibition. The obtained data revealed that ICL activity was initially very low and gradually increased in WT plants on MS medium with or without sugars. Interestingly, the patterns of changes in enzyme activity were similar in all treatments. The highest enzyme activity was observed in WT plants after 2 days imbibition when grown on MS medium supplemented with sucrose. But the lowest ICL activity was obtained in WT plants grown on trehalose, after 4 days imbibition (Fig. 5). *ICL* gene expression and enzyme activity were not recorded in *icl* mutant on all investigated medium.

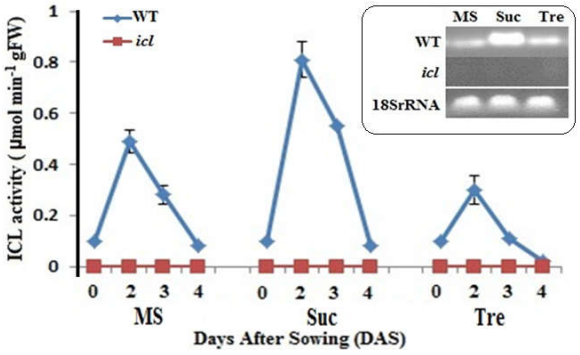

Fig. 5. ICL enzyme activity and gene expression: ICL enzyme activity in wild-type (WT) and *icl* knock out mutant. Seeds were sown on MS medium with or without 100 mM sucrose (Suc) or trehalose (Tre) and then enzyme activity was evaluated on 0, 2, 3 and 4 days after imbibition. Data are presented as the means ± SD (n=3); *ICL* Gene expression pattern were showed in the rounded rectangle box, Seeds were grown on MS medium with or without 100 mM sucrose (Suc) and or trehalose (Tre) for 14 days. Levels of gene expression were determined with reference to *18S rRNA (n=3).*

Trehalose feeding changes growth stages progression

Both WT and *icl* plants reached growth stage up to 1.04 (appearance 4 rosette leaves) when grown on MS medium with or without sucrose in light condition. However, growth stage 1.00 was longer in the *icl* mutant when grown on MS medium, compared to WT. The growth stage pattern was similar in WT and *icl* plants when treated with 100 mM sucrose. By trehalose feeding, both WT and *icl* only reached to growth stage up to 1.02 (Fig. 6A).

The progression of growth stages was almost similar in both wt and *icl have* grown on MS medium with or without sucrose in the continuous dark. But seedlings grown on sucrose triggered up to growth stage 1.02 (appearance 2 rosette leaves >1 mm). Whereas seedlings grown on MS medium with trehalose triggered growth stages up to 1.0 (Cotyledons fully opened). Meanwhile, hypocotyl and cotyledon emergence (stage 0.7) and full cotyledons opening (stage 1.0) have delayed in *icl* seedlings when grown on trehalose in the continuous dark (Fig. 6B).

Trehalose feeding affect plant metabolism during light and dark condition

To determine whether trehalose control over lipid metabolism, the amount of total lipid and fatty acids were assayed in WT and *icl* seedlings at days 0, 2 and 4 after sowing in light and continuous dark conditions. There was no significant difference in total lipid between WT and *icl* seedlings when grown on MS medium in light condition. The amount of total lipid of both WT and *icl* mutant was high at day 0 but gradually decreased at days 2 and 4, when grown on MS medium with or without sucrose. Under trehalose feeding, the seedling total lipid was higher than those grown on MS medium with or without sucrose at days 2 and 4 after sowing (Fig. 7A). In both WT and *icl* seedlings, the number of total lipids was high on day 0, which then decreased at day 2 and 4 after sowing under continuous dark. Trehalose feeding of both WT and *icl* was associated with higher levels of total lipids, compared with seedlings grown on MS medium with or without sucrose (Fig. 7B).

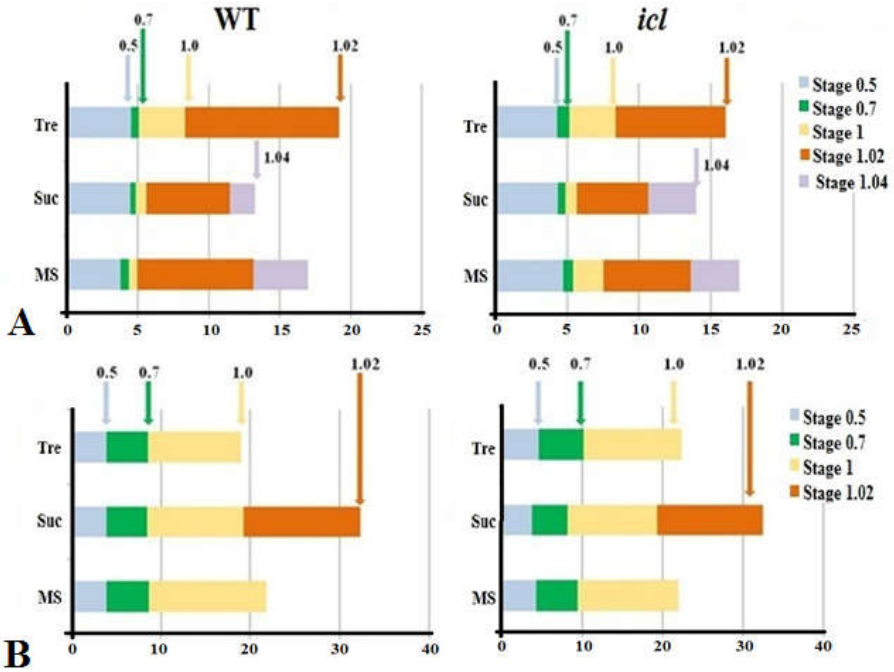

Fig. 6. The *icl* growth stages: A) Growth Stage Progression of wild-type (WT) and *icl* knock out mutant grown on MS medium with or without 100 mM sucrose (Suc) and or trehalose (Tre) in the 16h light/8h dark photoperiod or continuous dark condition; B). Stage 0.5: Radicle emergence; Stage 0.7: Hypocotyl and cotyledon emergence; Stage 1.0: Cotyledons fully opened; Stage 1.02: appearance 2 rosette leaves >1 mm; Stage 1.04: appearance 4 rosette leaves >1 mm.

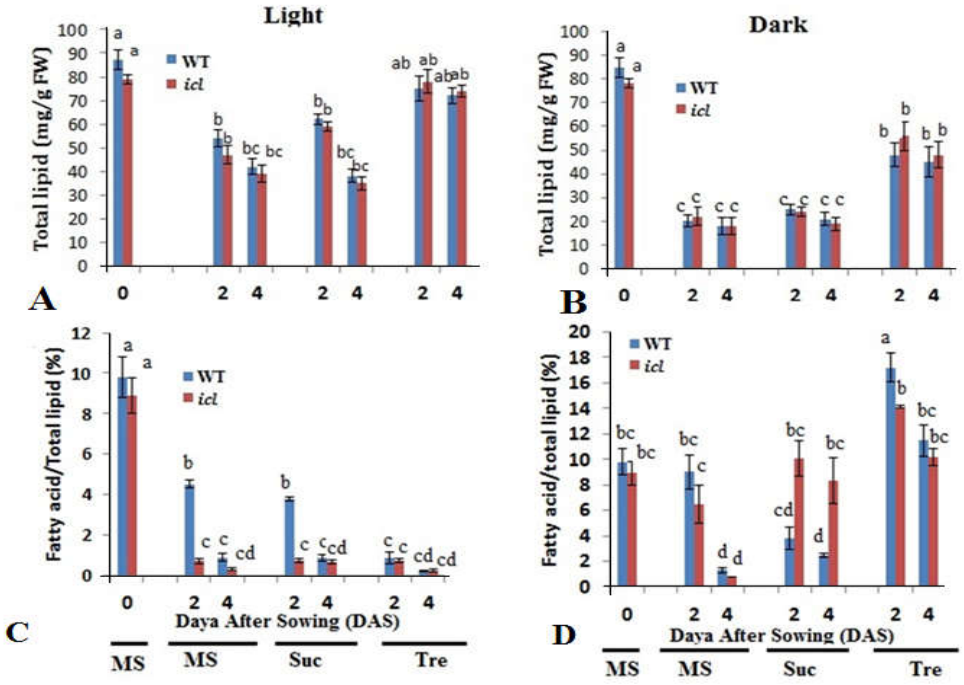

Fig. 7. Lipids and fatty acid levels in *icl*: A, B) Total lipid and C, D) Fatty acid ratio/total lipid in wild-type (WT) and *icl* knock out mutant grown on MS medium with or without 100 mM sucrose (Suc) and or trehalose (Tre) in the 16h light/8h dark photoperiod or continuous dark condition. Data are presented as means ± SD. Bars with different letters are significantly different at $p \leq 0.05$, according to Duncan's test.

The fatty acid amount of seedlings was high on day 0, which reduced at day 2 and 4 after sowing in light condition. However, WT seedlings on MS medium with or without sucrose showed the higher fatty acid amount on day 2 after sowing, compared with *icl* mutant (Fig. 7C). The level of fatty acids was higher in the continuous dark compared with the light condition. Furthermore, the relative abundance of fatty acids in *icl* was significantly higher than WT when grown on MS medium supplemented with sucrose. But by trehalose feeding, the abundance of fatty acids of WT seedlings at day 2 after sowing was higher than other investigated seedlings (Fig. 7D)

When cultivated on MS medium, the amount of reducing sugar was decreased in WT and *icl* mutant at day 4 after sowing in light condition (Fig. 8A). The addition of sucrose increased the amount of reducing sugar in both WT and *icl*, in light and dark conditions (Fig. 8B).

The amount of non-reducing sugar of WT plants was significantly higher than those of the *icl* when grown on MS medium supplemented with trehalose in light condition (Fig. 8C). In light and continuous dark, 2 days old seedlings of WT and *icl* showed the highest amount of non-reducing sugar, when subjected to sucrose treatment (Fig. 8C-D).

Discussion

The most striking example of altered allocation control is observed when Arabidopsis seedlings are grown on trehalose. When this sugar is supplied to the medium it spurs massive accumulation of starch in source tissue and traditional sinks such as meristems and roots to lack carbon and are growth inhibited (Wingler *et al.,* 2000; Aghdasi *et al.,* 2010). In the light, growth arrest on 100 mM trehalose is due to T6P accumulation and can be rescued by exogenous supply of metabolizable sugar (Schluepmann *et al.,* 2004). In the dark, 100 mM trehalose leads to sugar-induced skotomorphogenesis in seedlings yet it inhibits hypocotyl elongation without altering root growth. Seedling establishment and hypocotyl elongation in the dark are dependent on gluconeogenesis from lipids (Graham *et al.,* 1990). Trehalose feeding inhibits gluconeogenesis which can be because of catabolism repression in the source tissue, the cotyledon.

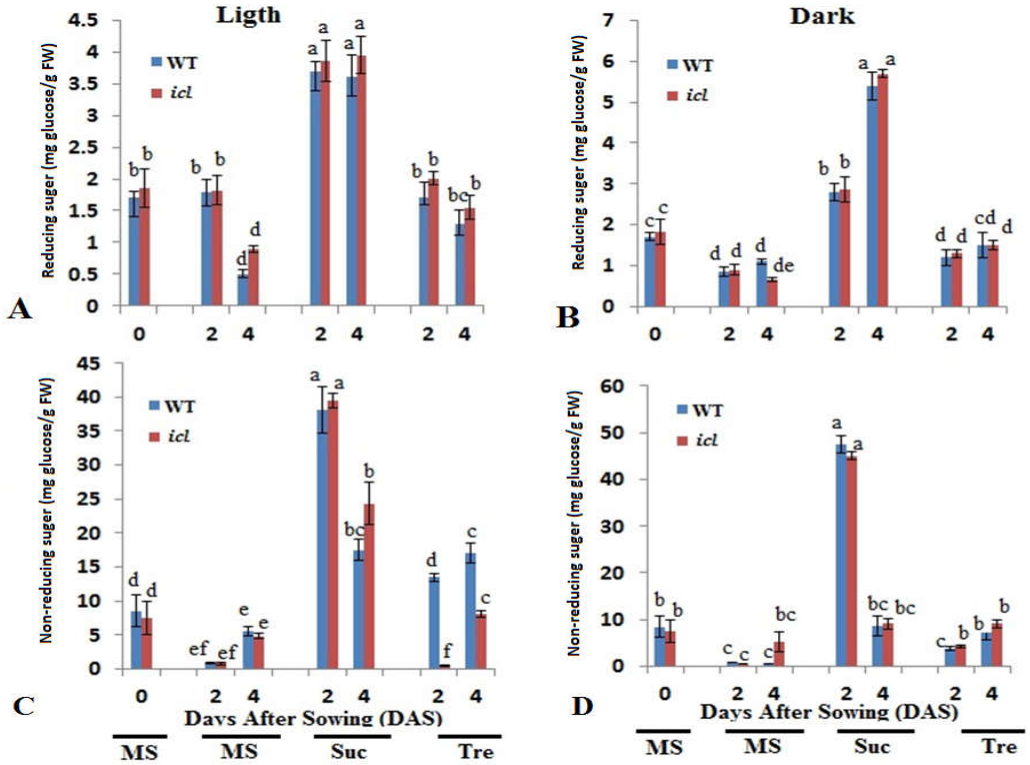

Fig. 8. Sugar levels in *icl* mutant: A, B) Reducing sugar and C, D) Non-reducing sugar in wild-type (WT) and *icl* knock out mutant grown on MS medium with or without 100 mM sucrose (Suc) and or trehalose (Tre) in the 16h light/8h dark photoperiod or continuous dark condition. Data are presented as means ± SD. Bars with different letters are significantly different at $p \leq 0.05$, according to Duncan's test.

Further lipids are stored in the endosperm and have been shown to contribute to hypocotyls extension in the dark (Penfield *et al.,* 2006), and so it is likely that T6P accumulation controls lipid remobilization from the endosperm as well. Trehalose, therefore, triggers skotomorphogenesis as sucrose does, but trehalose does not support or inhibits hypocotyl elongation like sucrose. The data is consistent with the starvation of the elongating hypocotyls on trehalose. These observations showed that growth arrest by T6P accumulation is found both in the light and in the darkness condition. It means trehalose feeding not only alters carbon allocation in the light but also stopped the use of carbon from lipid utilization needed for hypocotyl elongation in darkness condition (Dellata *et al.,* 2011).

In the current study, the effect of trehalose feeding on germination and seedling establishment was investigated in WT and *icl* knock out mutant under both light and continuous darkness. The obtained results showed that the germination rate was not different between WT and *icl*. In Arabidopsis, germination and root emergence are dependent on the limited sources of stored sugar in the seed. But Seedling growth from the embryo is primarily driven by the enzymatic breakdown of the seed reserved lipids (Cornah *et al.,* 2004). ICL and glyoxalate cycle are not necessary for seed germination (Eastmond *et al.,* 2000). The hypocotyl elongation was strongly inhibited by trehalose feeding in WT and *icl* seedlings. Removing the endosperm from Arabidopsis seed reduces hypocotyl elongation in the darkness which shows gluconeogenesis in the endosperm is necessary for skotomorphogenesis. However, this phenotype can be rescued by the exogenous supply of sucrose (Eastmond *et al.,* 2000; Penfield *et al.,* 2006). In continuous darkness, *icl* mutant showed a short hypocotyl phenotype (Cornah *et al.,* 2004). On the other hand, WT Arabidopsis seedlings showed a short hypocotyl phenotype after trehalose feeding in continuous darkness. Therefore, it seems trehalose somehow

affects the key enzymes of the lipid break down and gluconeogenesis pathway, which affects hypocotyl elongation in darkness.

ICL enzyme activity pattern was similar in all treatments. Meanwhile, the *icl* gene expression pattern shows consistency with trends of ICL activity during the investigated period (Eastmond *et al.,* 2000; Cornah *et al.,* 2004). Several lines of evidence have demonstrated that genes encoding *ICL* and *MLS* are regulated at the transcriptional level (Comai *et al.,* 1989; Graham *et al.,* 1990; Sarah *et al.,* 1996) and both enzymes are expressed synchronously during the early stages of post-germination and senesces (Gut and Matile, 1988; Comai *et al.,* 1989). Other studies have shown that the mRNA level, enzyme activity and gene expression of the glyoxalate cycle are not detectable until 4 days after imbibitions (Rylott *et al.,* 2001). The current results also revealed that trehalose feeding suppresses ICL enzyme activity and gene expression. These observations indicate that trehalose regulates ICL activity on gene expression levels. Several documents confirmed that T6P regulates many metabolic processes in plants (Zhang *et al.,* 2009; Paul *et al.,* 2010; Zhan *et al.,* 2018).

The current data showed that the number of total lipids in WT decreased during the time which indicates the utilization of stored lipids. Therefore, it shows WT seedlings have not any restriction to break down stored lipids in both light and dark conditions. But the *icl* mutant utilized stored lipids by providing alternative carbon sources, such as sucrose. It was shown that *icl1-1* and *icl1-2* Arabidopsis mutant needs an additional source of carbon to utilize lipids and seedling establishment in the darkness (Eastmond *et al.,* 2000). These mutants showed a short hypocotyl phenotype when grown on sugar-free medium in the darkness. When sucrose was added to the medium, hypocotyl elongation was induced (Cornah *et al.,* 2004). Results from the biochemical analysis revealed that trehalose has a profound effect on the total lipid level. Most interestingly, the amount of total lipid in both WT and *icl* seedlings grown on trehalose was higher than those which grown on MS medium with or without sucrose. This can be due to impaired lipid utilization in trehalose feed seedlings. Trehalose can also induce *ABI4* gene expression (Ramon *et al.,* 2007). On the other hand, the expression of genes involved in lipid utilization, *ACX3* (Acyle CoA oxidase) and *MLS*, was severely suppressed by ABA. ACX3 and MLS are two key enzymes in the β-oxidation pathway and the glyoxalate cycle (Pritchard *et al.,* 2002). Meanwhile, it was shown that ABA can inhibit ICL activity in the endosperm of *Ricinus communis* and *Nicotiana tabacum* (Finkelstein and Lynch, 2000; Zhan *et al.,* 2018).

The current data suggest that the trehalose pathway regulates lipid utilization. Trehalose/ T6P control gene expression or enzyme activity involving in lipid breakdown. But whether it controls gluconeogenesis is not well understood. Gomez et al (2006) reported that *TPS1* is essential for seedling establishment. Arabidopsis *tps1* mutant embryos exhibit high accumulation of proteins, lipids and sugars. Meanwhile, transcript analysis showed the induction of storage lipid mobilization pathway in the *tps1* mutant. In *tps1* mutant transcript level of *ICL* and *MLS* was higher than WT.

Conclusion

The present experiment outlines new aspects of the trehalose effect on plant growth and gluconeogenesis. In conclusion, our study revealed that the trehalose pathway regulates carbon partitioning both in light and dark. It can be suggested that trehalose regulates key genes expression and enzyme activity involving fatty acid metabolism. Further confirmation of the above results requires more experiments.

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Conflicts of interest

The authors declare that they have no conflicts of interest

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