OC kappa increases in the levels of glucose, trehalose, TOR-P and transcripts encoding proteins involved in photosynthesis, and basal and secondary metabolisms in *Eucalyptus globulus*

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Oligo-carrageenan (OC) kappa increases net photosynthesis, and basal and secondary metabolism enzyme activities in *Eucalyptus globulus* trees. Here, trees were sprayed on leaves with water (control) or with OC kappa 1 mg ml^{-1}, once a week, four times in total, and cultivated for 17 additional weeks (21 weeks in total). Height, level of glucose, trehalose, TOR phosphorylated in Ser2448 (TOR-P) and transcripts encoding TOR and S6 kinase (S6K) as well as the level of transcripts encoding proteins and enzymes involved in glucose accumulation, photosynthesis, C, N and S assimilation, and synthesis of phenylpropanoid compounds (PPCs) and terpenes were determined. Treated trees showed an increase in height of 105% compared to controls at week 21. Treated trees showed an increase in glucose and trehalose level having an oscillatory pattern with maximal levels for glucose at week 1, 9-11 and 17-19, and for trehalose at weeks 1-3, 5, 8-9, 12, 15-16 and 18-21. TOR-P showed increases from week 1 until the end of the experiment with peaks at weeks 2, 6, 12 and 16. The level of *tor* transcripts showed peaks at weeks 3, 6, 10-11 and 13 whereas the level of *s6k* transcripts remained unchanged. In addition, transcripts encoding proteins involved in photosynthesis, and enzymes involved in glucose accumulation, C, N and S assimilation, and synthesis of secondary metabolites showed an oscillatory pattern with increases mainly at weeks 3-4, 5-6, 10-11, and in some cases at weeks 13-14 and 16-18. Thus, the increases in trehalose levels better correlate with increases in TOR-P and transcript levels. Therefore, OC kappa induced an increase in the levels of glucose, trehalose, TOR-P and expression of genes involved in photosynthesis, and basal and secondary metabolism which may explain, at least in part, the increase in growth and defense responses in *E. globulus* trees.

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Keywords basal metabolism, *Eucayptus globulus*, glucose, growth, OC kappa, secondary metabolism, TOR kinase, trehalose.

Abstract

Oligo-carrageenan (OC) kappa increases net photosynthesis, and basal and secondary metabolism enzyme activities in *Eucalyptus globulus* trees. Here, trees were sprayed on leaves with water (control) or with OC kappa 1 mg ml^{-1}, once a week, four times in total, and cultivated for 17 additional weeks (21 weeks in total). Height, level of glucose, trehalose, TOR phosphorylated in Ser2448 (TOR-P) and transcripts encoding TOR and S6 kinase (S6K) as well as the level of transcripts encoding proteins and enzymes involved in glucose accumulation, photosynthesis, C, N and S assimilation, and synthesis of phenylpropanoid compounds (PPCs) and terpenes were determined. Treated trees showed an increase in height of 105% compared to controls at week 21. Treated trees showed an increase in glucose and trehalose level having an oscillatory pattern with maximal levels for glucose at week 1, 9-11 and 17-19, and for trehalose at weeks 1-3, 5, 8-9, 12, 15-16 and 18-21. TOR-P showed increases from week 1 until the end of the experiment with peaks at weeks 2, 6, 12 and 16. The level of *tor* transcripts showed peaks at weeks 3, 6, 10-11 and 13 whereas the level of *s6k* transcripts remained unchanged. In addition, transcripts encoding proteins involved in photosynthesis, and enzymes involved in glucose accumulation, C, N and S assimilation, and synthesis of secondary metabolites showed an oscillatory pattern with increases mainly at weeks 3-4, 5-6, 10-11, and in some cases at weeks 13-14 and 16-18. Thus, the increases in trehalose levels better correlate with increases in TOR-P and transcript levels. Therefore, OC kappa induced an increase in the levels of glucose, trehalose, TOR-P and expression of genes involved in photosynthesis, and basal and secondary metabolism which may explain, at least in part, the increase in growth and defense responses in *E. globulus* trees.

Introduction

It is now well known that growth and development in mammals, nematodes yeast, plants and algae is controlled by the kinase Target of Rapamycin (TOR) (Xiong and Sheen, 2015; Rexin et al., 2015; Dobrenel et al., 2016). TOR is a phosphoinositol-related kinase (PIK) having serine/treonine protein kinase activity and is a key regulatory kinase of the TOR pathway (Xiong and Sheen, 2015; Rexin et al., 2015; Dobrenel et al., 2016). TOR kinase is large protein constituted by several domains: a N-terminal Huntingtin, Elongation Factor 3, Regulatory Subunit A of PPA2, TOR1 (HEAT) domain containing several HEAT repeats which are constituted by 37-47 amino acids forming two α -helices and a solenoid structure that is involved in protein-protein interactions and interaction with Regulatory-Associated Protein of mTOR (RAPTOR) (Kim et al., 2002; Mahfouz et al., 2006). Contiguous to HEAT domain, is FRAP, ATM; TTRAP (FAT) domain that is present in most PIK and is involved in protein-protein interactions (Bosotti et al., 2000). Contiguous to FAT domain is FRB FKBP-Rapamycin-Binding (FRB) domain that binds to FKBP12-rapamycin complex (Banaszynski et al., 2005; Rodriguez-Camarzo et al., 2012). Contiguous to FRB domain is the catalytic domain (CD) that interacts with Lethal with SEC13 protein 8 (LST8) regulatory protein (Schalm et al., 2003) and two TOR-LST8 complexes form a dimer mediated by TOR-TOR interactions (Baretic et al., 2016). Contiguous to CD, is the C-terminal FAT domain (FATC) that is redox-sensitive and binds to membranes (Takahashi et al., 2000; Dames, 2010). In mammals, TOR kinase is inhibited by nanomolar concentrations of the macrolide rapamycin produced by the bacteria *Streptomyces hygroscopicus* (Crespo et al., 2005). In contrast, plant TOR kinases are only moderately sensitive to rapamycin (Ren et al., 2011; Ren et al., 2012). In this respect, it has been shown that when FKBP12, a prolyl isomerase, is overexpressed or replaced by human or yeast FKBP12 in *Arabidopsis thaliana*, TOR kinase becomes sensitive to rapamycin (Sormani et al., 2017; Xiong and Sheen, 2012).

In mammals, TOR is a large protein of around 280 kDa that is activated by phosphorylation in Treo 2446, Ser 2448, Ser 2481 and Ser1261 (Chiang and Abraham, 2005; Acosta et al., 2009). TOR pathway is activated by growth factors, pro-inflammatory cytokines, insulin, glucose, amino acids as glutamine and leucine, and lipids; the latter leads to an increase in anabolic reactions, cell division and growth (Jewell et al., 2015). In mammals, there is single gene encoding TOR, although TOR kinase can interact with proteins RAPTOR, LST8 and FKBP12 to form complex TORC1, which is sensitive to rapamycin, and it can also interact with RICTOR, LST8 and SIN1, to form TORC2, which is insensitive to rapamycin (Sarbassov et al., 2004; Sarbassov et al., 2005;

Wullschelger and Loewith, 2006). TORC1 regulates the equilibrium among anabolism and catabolism, cell proliferation and temporal growth whereas TORC2 modulates cytoskeleton structure, spatial cell growth, cell polarity and apoptosis (Wullschelger and Loewith, 2006; Martin and Hall, 2005).

In plants, TOR is a protein of around 250 kDa, 39% identical in its amino acid sequence to human TOR (Dobrenel et al., 2016) and TOR is phosphorylated in Ser2448 since antibodies anti-human TOR-P Ser2448 recognize phosphorylated TOR (TOR-P) in cells of maize callus (Garrocho-Villegas et al., 2013). In addition, it has been shown that insulin-like growth factors (IGF) and bovine insulin promote growth in maize cells which correlates with the increase in the level of TOR-P. On the other hand, rapamycin inhibits IGF-induced growth stimulation as well as TOR phosphorylation in Ser 2448 (Garrocho-Villegas et al., 2013). In plants, TOR kinase interacts with RAPTOR, LST8 and rapamycin-FKBP12 (Dobrenel et al., 2016; Mahfouz et al., 2006; Garrocho-Villegas et al., 2013). TOR pathway in plants is activated by glucose, sucrose and amino acids, among others (Dobrenel et al., 2016; Ren et al., 2012). On the other hand, TOR is inhibited by the kinase SnRK1 that phosphorylates RAPTOR leading to inhibition of TOR kinase activity (Nukarinen et l., 2016). In turn, snRK1 is directly inhibited by glucose-6-P (G6P) and trehalose-6-P (T6P) (Toroser et al., 2000; Zhang et al., 2009). Thus, the increase in glucose and trehalose may lead to an increase in G6P and T6P and the inhibition of snRK1 resulting in the activation TOR kinase, and the stimulation of growth and development in plants.

It has been shown that activation of TOR pathway increases growth mainly by activating cell division through phosphorylation of E2Fa and E2Fb transcription factors (Xiong and Sheen, 2013), and by activating protein translation through phosphorylation of S6 kinase (S6K) that, in turn, phosphorylates the ribosomal small subunit 6 (Xiong and Sheen, 2013). More recently, it has been demonstrated that activation of TOR pathway in *A. thaliana* results in an increased expression of genes coding for enzymes involved in anabolic reactions, such as those related to the syntheses of proteins, amino acids, RNA, DNA and cell wall, as well as synthesis of enzymes involved in glycolysis, TCA cycle and proteins of mitochondrial electron transport chain (Ren et al., 2012; Xiong and Sheen, 2013; Caldana et al., 2013). Moreover, the inhibition of TOR pathway using the inhibitor of TOR kinase AZD8055 leads to a decrease in transcripts encoding proteins involved in photosynthesis, chlorophyll synthesis and C assimilation (Montane and Menand, 2013; Dong et al., 2015) indicating that the activation of TOR pathway leads to the increase photosynthesis and basal metabolism. Furthermore, the activation of TOR pathway down-regulates expression of genes coding for catabolic enzymes involved in protein, amino

acids, lipid syntheses, and related to starch degradation, autophagy and glyoxylate cycle (Xiong at al., 2013). In addition, activation of TOR pathway also increased the expression of genes coding for enzymes involved in secondary metabolism and defense responses, such as those that synthesize glucosinolates (Xiong et al., 2013). Similarly, TOR pathway mediates the increase in the level of phenylpropanoid compounds (PPCs) and glucosinolates in *A. thaliana* (Caldana et al., 2013). It has been recently shown that inhibition of TOR pathway with AZD8055 down-regulates the synthesis and signaling of phytohormones such as auxin, gibberelins, cytokinines and brassinosteroids (Dong et al., 2015; Deng et al., 2016), indicating that activation of TOR pathway also increase the synthesis of growth-promoting hormones.

On the other hand, marine algae oligo-carrageenans (OCs) enhance growth and defense responses in terrestrial plants (Moenne et al., 2016). OCs kappa, lambda and iota are obtained by acid hydrolysis of pure carrageenans kappa, lambda and iota, respectively, and displayed a DP=20-25 (Vera et al., 2011). It was initially determined that OCs kappa, lambda and iota applied on plant leaves at a concentration of 1 mg mL^{-1}, once a week, four times in total, mediated an increase in height and plant biomass in tobacco plants (var. Xhanti) cultivated in control conditions as well as tobacco plants (var. Burley) cultivated in the field for four months (Castro et al., 2012). In addition, OCs kappa, lambda and iota applied at a concentration of 1 mg mL^{-1}, once a week, four times in total, induced and increase in height, trunk diameter, net photosynthesis, and in the levels of PPCs and essential oils in *Eucalyptus globulus* cultivated in the field for three years (González et al., 2013b). On the other hand, it was shown that OC kappa mediated an increase in the synthesis of reducing compounds such as NADPH, ascorbate (ASC), and glutathione (GSH), as well as increased activities of thioredoxin reductase and thioredoxin in *E. globulus* trees cultivated for four months outdoors. In addition, OC kappa increases the activity of enzymes involved basal metabolism, C, N and S assimilation, purine and pyrimidine syntheses, and in the activities of Krebs cycle enzymes (González et al., 2014a). Furthermore, in *E. globulus,* OC kappa increased the level of growth-promoting hormones such as auxin, gibberellin and cytokinines in *E. globulus* trees (González et al., 2014b) as well as in pine trees (Saucedo et al., 2015). In addition, OC kappa increases the amount of volatile terpenes, and new terpenes having potential anti-pathogenic activities in *E. globulus* trees (González et al., 2014c). Thus, OC kappa mediated an increase in net photosynthesis, basal metabolism and secondary metabolism in *E. globulus* trees.

Considering that OC kappa increases net photosynthesis, the level of growth-promoting hormones, and basal and secondary metabolisms in plants, and that the activation of TOR

pathway lead to an increase in plant growth, photosynthesis, growth hormone level and basal an secondary metabolisms in plants (see above), we hypothesize that OC kappa may induce an increase in glucose and trehalose levels which may lead to an increase in G6P and T6P which may inhibit SnRK1 which may increase the level of TOR-P which may lead to increase in expression of genes encoding proteins of photosystems and enzymes involved in chlorophyll synthesis, C, N and S assimilation, and PPCs and terpenes synthesis in *E. globulus* trees.

Materials and Methods

Plant culture, treatment with OC kappa and measurement of height

E. globulus trees were obtained from seeds produced by Semillas Imperial S.A. (Los Angeles, Chile). Plants having an initial height of approximately 30 cm (n=10 for each control and treated groups) were sprayed on leaves with water (control group) or with 5 mL of an aqueous solution containing OC kappa at a concentration of 1 mg mL^{-1} once at the beginning of each week, four times in total, and cultivated outdoors in plastic bags containing composted soil for 17 additional weeks during spring and summer of 2015. Leaves (10 g) were obtained from the middle height part of control and treated trees, one day after each treatment, at the same time in the day (11 h in the morning), divided into three samples (n=3) and frozen in liquid nitrogen for further analyses. The height of *E. globulus* trees were determined using measuring tape.

Quantification of total chlorophyll

Quantification of chlorophylls *a* and *b* was performed as described in Lichtenthaler and Wellburn (1983). Fresh leaves (0.1 g) were frozen in liquid nitrogen and homogenized in a mortar with a pestle. One mL of acetone was added and the mixture was incubated at 4°C for 90 min. The mixture was centrifuged at 14.000 rpm for 5 min using a micro-centrifuge. The supernatant was recovered and the absorbance determined at 665 and 649 nm using a Hewlett Packard/Agilent spectrophotometer model 8453 (Santa Clara, CA, USA). Total chlorophyll was calculated by addition of chlorophylls *a* and *b* and the concentration of chlorophylls was calculated using the following formula:

$$\text{Chlorophyll } a \text{ } (\mu g\ mL^{-1}) = 13.96\ A_{665} - 6.88\ A_{649}$$

$$\text{Chlorophyll } b \text{ } (\mu g\ mL^{-1}) = 24.96\ A_{665} - 7.32\ A_{649}$$

Quantification of total reducing sugars

Quantification of total reducing sugars was performed as described in Hansen and Möller (1975). Fresh leaves (0.1 g) were frozen in liquid nitrogen and homogenized in a mortar. One mL of ethanol was added and the mixture centrifuged at 14.000 rpm for 5 min; the supernatant was then recovered. An aliquot of the supernatant (25 µL) was added to 475 µL of sulfuric anthrone solution prepared by mixing 150 mg of anthrone in 100 mL of sulfuric acid; the mixture was incubated at 60°C for 15 min. Absorbance was determined at 620 nm and the concentration

calculated using a calibration curve prepared with glucose at concentrations ranging from 0.065 to 0.5 mg mL^{-1}.

Quantification of glucose

Fresh leaves (0.1 g) were frozen in liquid nitrogen and homogenized in a mortar. Five hundred µL of distilled water were added and the mixture centrifuged at 14.000 rpm for 5 min. The supernatant was recovered and an aliquot of 30 µL was added to 500 µL of glucose oxidase/peroxidase kit reaction mixture (Valtek Diagnostics, Santiago, Chile). The absorbance was determined at 505 nm and the concentration was calculated using a calibration curve prepared using glucose at concentrations of 0.2 to 2 mg mL^{-1}.

Quantification of trehalose

Quantification of trehalose was performed as described in Ahmed et al. (2013). Fresh leaves (0.1 g) were frozen in liquid nitrogen and homogenized in a mortar. Two mL of ethanol were added; mixture was boiled for 1 h and ethanol was left to evaporate at 60° in an oven. Five mL of 5 mM sulfuric acid were added and the mixture was centrifuged at 3.200 rpm for 10 min. The supernatant was filtered through 0.2 µm pore PDVF filters and boiled in water for 1 h to hydrolyze sucrose. Once cold, the pH was neutralized with sodium hydroxide, the solution evaporated and the residue was dissolved in distilled water. The calibration curve was prepared using trehalose at concentrations ranging 0 to 5 mg mL^{-1}.

Preparation of protein extracts

Protein extracts were prepared as described in Faurobert et al. (2007). Fresh leaves (1 g) were frozen with liquid nitrogen and homogenized in a mortar. Three mL of extraction buffer (0.5 M Tris-HCl, 0.7 M sucrose, 1 mM PMSF, 50 mM EDTA, 0.1 M KCl and 0.2% β-mercaptoethanol pH 8.0) were added and the homogenate was shaken on ice for 10 min. One mL of phenol at pH 6.6-8.0 was added, the mixture was shaken on ice for 10 min and centrifuged at 3.200 rpm for 10 min at 4°C. The organic phase was recovered and mixed with 4 volumes of 0.1 M ammonium acetate solubilized in methanol. The mixture was shaken using a vortex and incubated overnight at -20ºC for protein precipitation. The mixture was centrifuged at 3.200 rpm for 15 min at 4ºC, and the protein pellet was washed twice with ammonium acetate at 0.1 M in methanol, and then once at the same concentration in acetone; the pellet was dried at room temperature and

196 solubilized in 50 mM Tris-HCl pH 8.0. Proteins were quantified using Bradford (1976) reagent
197 and the calibration curve was prepared using bovine serum albumin.

198 **Quantification of phosphorylated TOR (TOR-P)**
199 Proteins (5 µg) were separated using a biphasic denaturant polyacrylamide gel (6% stacking
200 phase and 12% resolving phase), and electrophoresis was performed at 110 V for 1.5 h. Proteins
201 were electro-transferred to a nitrocellulose membrane using a TransBlot system (Bio-Rad) and
202 400 mA, at 4 ºC for 1 h. The transfer of protein was verified by staining the membrane with
203 Ponceau Red dye. The membrane was blocked with 5% skim milk solubilized in TTBS buffer (20
204 mM tris-HCl pH 7.5, 0.1 mM NaCl and 0.1% Tween 20), and washed three times with TTBS at
205 room temperature for 10 min. The membrane was incubated with the monoclonal antibody anti-
206 TOR-P Ser2448 (1:1000, Abcam ab109268) or anti-RbcL (1:2500, Agrisera AS03037) at room
207 temperature for 1 h. The membrane was washed three times with TTBS at room temperature for
208 10 min, incubated with the secondary antibody anti-Rabbit IgG conjugated with HRP (Agrisera,
209 AS09602) at room temperature for 1 h, and washed three times with TTBS at room temperature
210 for 10 min. The membrane was incubated with a chemo-luminiscent substrate (SuperSignal West
211 Femto, Thermo Scientific, Rockford, IL, USA) for 5 min and was exposed to an X-ray film
212 (Thermo Scientific, Rockford, IL, USA) for 3 min to detect TOR-P ser2448, or for 30 s to detect
213 RbcL. Bands in the film were scanned and then quantified using Image Studio software (Li-Cor,
214 USA).

215 **RNA extraction**
216 Total RNA was extracted from *Eucalyptus* leaves as described in Morante-Carriel et al. (2014).
217 Fresh leaves (1 g) were frozen in liquid nitrogen and homogenized in a mortar with a pestle. Ten
218 mL of solution A containing 100 mM Tris-HCl pH 8.0, 0.35 M sorbitol, 10% (w/v)
219 polyethylenglycol 6000 and 2% (w/v) of β-mercaptoethanol were added and the mixture was
220 shaken for 1 min. The mixture was centifuged 3.500 rpm, at 4°C, for 15 min, and the supernatant
221 was discarded. The pellet was solubilized in 10 mL of solution B containing 300 mM Tris-HCl
222 pH 8.0, 25 mM EDTA, 2 M NaCl, 2% (w/v) CTAB, 0.05% (w/v) spermidine, 2% PVPP and 2%
223 (w/v) β-mercaptoethanol; the mixture was heated at 65ºC and incubated at 65ºC for 10 min and
224 shaken every 2 min using a vortex. A similar volume of a solution of chlorophorm/isoamyl
225 alcohol (24:1) was added and the mixture was centrifuged at 3.200 rpm at 4ºC for 10 min. The
226 aqueous phase was extracted once more with a similar volume of chlorophorm/isoamyl alcohol

and centrifuged at 3.200 rpm at 4ºC for 10 min. The aqueous phase was recovered and total RNA precipitated by addition of 0.1 volume of 0.3 M sodium acetate, pH 5.2, and 0.6 volumes of isopropanol; the mixture was incubated at -80°C for 30 min. The mixture was centrifuged at 14.000 rpm, at 4ºC, for 20 min, and the supernatant discarded. The pellet was solubilized in 1 mL of nuclease free (DEPC-treated) water and total RNA was precipitated adding 0.3 volumes of 10 M lithium chloride; the mixture was incubated at 4°C overnight. The mixture was centrifuged at 13.000 rpm, at 4ºC, for 30 min, and the supernatant was discarded. The pellet was solubilized in 0.1 mL of DEPC-treated water and total RNA precipitated adding 0.1 volume of 3 M sodium acetate pH 5.2, and 2 volumes of 70% cold ethanol; the mixture was centrifuged at 13.000 rpm at 4ºC for 20 min, and the supernatant discarded. The pellet was washed with 200 µL of 70% cold ethanol and centrifuged at 13.000 rpm at 4ºC for 10 min, and the supernatant discarded. The pellet was dried at room temperature and solubilized in 50 µL of DEPC-treated water. The concentration and purity of total RNA was determined measuring the absorbance at 260 and 280 nm, and in an agarose gel; RNA was stored at -80ºC for further gene expression analyses.

Quantification of transcript levels by qRT-PCR

The relative level of transcripts was quantified by qRT-PCR using a real-time thermocycler Rotorgene 6000 (Corbett, Australia). Transcripts involved in glucose accumulation and consumption: were those encoding fructose-1,6-bisphosphatase 1 (f*bp1*), a key enzyme in glucose synthesis; a-amylase 3 (amy3), an important enzyme in starch degradation and glucose production; ADP-glucose pyrophophorylase 1 (*apl1*), determinant enzyme in starch synthesis. Transcripts of proteins involved in TOR pathway were: TOR Kinase (*tor*), the regulatory kinase of TOR pathway; S6K (*s6k*), a kinase that is activated by TOR. Transcripts encoding photosystem proteins were: the subunit A of photosystem II (*psbA*), Rieske subunit of cytochrome b6f (*petC*), plastocyanin (*petE*), subunit A of photosystem I (*psaF*). Transcripts encoding enzymes were magnesium chelatase (*chlH*), a key enzyme of chlorophyll synthesis; the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) (*rbcL*), a key enzyme in C assimilation; glutamine synthase (*gs1*), an enzyme involved in N assimilation; glutamate dehydrogenase (*gdh2*), an enzyme involved in N assimilation; O-acetylserine thiol-lyase (*cysK*), an enzyme involved in S assimilation; 5'-adenilylsulfate reductase (*apr2*), an enzyme involved in S assimilation; phenylalanine ammonia-lyase 1 (*pal1*), a key enzyme of phenylpropanoid pathway; and terpene synthase 1 (*ts1*), an enzyme involved in terpenes synthesis. RNA 18S was used as housekeeping gene. PCR primers are listed in Supplementary Table 1. qRT-PCR reactions

were performed using Sensimix One-step kit (Quantace, UK), 75 ng of total RNA, 200 nM primer solution and 3 mM magnesium chloride. Relative transcript level from three independent replicates was expressed as described by Livak and Schmittgen (2001) using the $2^{-\Delta\Delta CT}$ method. To this end, mean values of control samples were subtracted to mean values of treated samples to determine fold-change in expression.

Statistical analyses

Data were subject to one-way analysis of variance (ANOVA) and *post hoc* Tukey Test, previous to the evaluation of the requirements of normality and homogeneity of variance. Significant differences were estimated over 3 independent replicates at a 95% confidence interval.

Results

OC kappa-induced increases in levels of glucose and trehalose

Eucalyptus trees treated with OC kappa showed significant higher increase in height compared to controls, which started at week 9 and became more evident with time until week 21 (Fig. 1A). Treated trees showed an average height of 72 cm at week 21, whereas control trees showed an average height of 35 cm, indicating 105% higher increase in height in OC kappa-treated trees with respect to controls. In addition, trees treated with OC kappa showed a higher level of total chlorophylls starting at week 3 and remaining until week 11; the following week, the levels of total chlorophylls decreased to reach control levels in a continuing pattern until the end of the experiments (Fig. 1B). OC kappa showed no clear effects on the levels of total sugars if compared with controls; although there were a clear higher significant peaks in total sugar with respect to controls at week 12 and 20 (Fig. 1C). Furthermore, treated trees showed in general higher levels of glucose compared with controls; these differences were significant at weeks 1, from weeks 9-11, and from weeks 17-19 (Fig. 1D). Treated trees showed a trend of higher levels of trehalose if compared with controls with maximal level at weeks 1-3, 5, 8-9, 12, 15-16 and 18-21 (Fig. 1E).

OC kappa-induced levels of transcripts encoding enzymes involved in glucose accumulation

In order to analyze the reasons explaining the increases in the level of glucose induced by OC kappa, the level of transcripts encoding the enzymes fructose-1,6-bisphosphatase involved in glucose synthesis, fbp1; α-amylase 3 involved in starch degradation and production of glucose, *amy3*; and ADP-glucose pyrophosphorylase involved in starch synthesis, *apl1*, were detected. Trees treated with OC kappa showed an increase in the level of *fbp1* transcripts at weeks 3, 6, 8, 10, 13 and 15 with respect to controls (Fig. 2A). Transcript levels of *amy3* peaked at weeks 1, 3, 7-8, 10-12 and 16, compared to controls; peaks were the highest at weeks 7 and 11 (Fig. 2B). *Apl1* transcripts increased only at week 18 (Fig. 2C).

OC kappa-induced increase in the level of TOR-P and *tor* transcripts but not *s6k* transcripts

In order to analyze whether the increase in glucose and/or trehalose may induce the activation of TOR kinase, the level of TOR phosphorylated in ser2448 (active TOR-P) as well as that of the large subunit (RbcL) of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) were detected using specific antibodies (Fig. 3A). The level of active TOR was normalized using

the level of RbcL (Fig. 3B). Trees treated with OC kappa showed a higher relative level of TOR-P from week 1 to the end of the experiment (week 21) and increases at weeks 1,2, 6, 12 and 16 (Fig. 3A-C). In order to detect whether the increase in active TOR-P is due to the increase in *tor* transcripts, the relative level of tor was detected. Trees treated with OC kappa showed peaks of increase in transcript levels encoding TOR kinase (*tor*) at weeks 3, 6, 10-11 and 13, compared to controls (Fig. 3C). In contrast, no significant changes were observed for transcripts encoding S6 kinase (*s6k*) throughout the experiments (Fig. 3D).

OC kappa-induced increase in the levels of transcripts encoding proteins involved in photosystems and chlorophyll synthesis

In order to analyze the reasons explaining the increase in net photosynthesis observed in previous works, the levels of transcripts of a subunit of photosystem (PS) II, *psbA*; the Rieske subunit of cytochrome b6f, *petC*; plastocyanin, *petE*; a subunit of PSI, *psaF*; and the enzyme magnesium chelatase involved in chlorophyll synthesis, *chlH*, were analyzed. Trees treated with OC kappa showed significant increase in the level of *psbA* transcripts at weeks 3-4, 8, 10-14 and 18 (Fig. 4A). Peaks of *petC* expression were observed to be significant at weeks 3-6, 10 and 17-18 (Fig. 4C). The increase of *petE* transcripts were significant at weeks 3, 5, 9-10, 14 and 18 (Fig. 4C). Expression of *psaF* was significantly higher at weeks 3, 10-11, 14 and 18 (Fig 4D). Finally, *chlH* transcripts increased significantly from weeks 5 to 15 (Fig. 4E).

OC kappa-induced increases in the level of transcripts encoding enzymes involved in C, N and S assimilation

In order to analyze the reasons explaining the increase in activities of enzymes involved in C, N and S assimilation observed in previous works, the level of transcripts encoding the large subunit of the enzyme rubisco involved in C assimilation, *rbcL*; the enzyme glutamine synthase (GlnS) involved in N assimilation, gs1; the enzyme glutamate dehydrogenase (GDH) involved in N assimilation, *gdh2*; the enzyme 5'-adenilylsulfate reductase (APR) involved in S assimilation, *apr2*, and the enzyme O-acetylserine thiol-lyase (O-ASTL) involved in S assimilation, *cysK*. Treated trees showed a significant increase in *rbcL* transcripts at weeks 4, 6, 11 and 14 (Fig. 5A), in *gs1* at weeks 3-4, 7, 9-11, 13-14 and 18-19 (Fig. 5B), in *gdh2* at weeks 3-4, 6, 10 and 14 (Fig. 5C), in *apr2* at weeks 1, 3-4, 7 and 17 (Fig. 5D) and in *cysK* at weeks 3-6, 10, 14 and 17-18 (Fig. 5E).

OC kappa-induced increase in the level of transcripts encoding enzymes involved in secondary metabolism

In order to analyze the reasons explaining the increase in PPCs and terpenes induced by OC kappa and reported in previous works, the level of transcripts encoding enzyme phenyalanine ammonia-lyase (PAL) involved in PPCs synthesis, *pal*, and the enzyme terpene synthase involved in terpenes synthesis, *ts1*, were analyzed. Trees treated with OC kappa showed an increase in *pal1* transcripts at weeks 3, 6-7, 10 and 18 (Fig. 6A), and in *ts1* transcripts at weeks 3, 6 and 10-14 (Fig. 6B).

Discussion

OC kappa induced increases in the level of glucose and trehalose that are coincident with increases in TOR-P increases but precedes the increase in transcript levels

In this work, we showed that treatment with OC kappa in *Eucalyptus* trees induced an initial increase in glucose level at week 1 and in trehalose level at weeks 1-3. In addition, the increases in glucose and trehalose are coincident with the initial increase in TOR-P observed at week 1-2 suggesting that the increases in these sugars could be related with phosphorylation and activation of TOR kinase. In this respect, it has been shown that the increase in G6P and T6P inhibit SnrK1, a kinase that inhibits TOR by phosphorylation, which results in TOR activation (Toroser et al., 2000; Zhang et al., 2009). In addition, it has been shown that trehalose applied on leaves of wheat induced an increase in growth suggesting that this increase results in the increase T6P levels (Ibrahim et al., 2016). Thus, the increases in glucose and trehalose levels induced by OC kappa in *E. globulus* trees may result in the increase in G6P and T6P levels that may inhibit snRK1 that, in turn, may trigger the increase in active TOR-P leading to the stimulation of growth. Furthermore, OC kappa induced additional increases in glucose level at weeks 9-11 and 17-19. Moreover, the level of transcripts encoding enzymes leading to glucose accumulation, fructose-1,6-bisphosphatase and α-amylase, increased weeks 1, 3, 7-8, 10-11, 13, 15 and 16. The latter suggests that the increase in glucose level may be due, at least in part, to the increase in the level of transcripts of enzymes that produce glucose.

On the other hand, the increases in trehalose level showed an oscillatory pattern with peaks at weeks 1-3, 5, 8-9, 12, 15-16 and 18-21 which correlates with the increase in the level of transcripts encoding proteins of PS and enzymes of basal and secondary metabolisms observed at weeks 3-4, 5-6, 10-11, and in some cases at weeks 13-14 and 16-18. In this sense, it has been shown that activation of different isoforms of trehalose 6-P synthase (TPS), the enzyme that produces trehalose, can be activated by snRK1 and/or by calcium-dependent protein kinases, CDPKs (Glinski and Weckwert, 2005). Thus, the oscillatory pattern observed in T6P level, TOR-P and transcripts encoding proteins involved in photosynthesis, and basal and secondary metabolisms, may be explained by the activation of CDPKs that increases trehalose and T6P levels inhibiting SnRK1and activating TOR kinase. In this sense, it is important to mention that *Eucalyptus* plants treated with 50 μM rapamycin or 250 μM AZ8055 did not show growth inhibition (data not shown) but they displayed greater height compared to the controls.

Consequently, *Eucalyptus* trees might have alternative pathways different from snRK1/TOR pathway, probably involving CDPKs.

OC kappa-induced increase in the level of TOR-P and *tor* transcripts but not *s6k* transcripts

OC kappa increases in the level of TOR-P from week 1 until the end of the experiment (week 21) with peaks at weeks 1, 2, 6, 12 and 16 compare to control plants. In this sense, it has been shown that an insulin-like growth factor (zmIGF) a well as bovine insulin induced an increase in cell division and growth in maize callus, phosphorylation of TOR in ser2448 and S6K in thr389 (Garrocho-Villegas et al., 2013). In addition, it was shown that rapamycin inhibited TOR and S6K phosphorylations. Thus, growth stimulation induced by zmIGF and insulin in maize is due to the activation of TOR pathway involving phosphorylation of TOR and S6K. In addition, it was shown that zmIGF interact with a receptor located in the plasma membrane of maize cells (Garrocho-Villegas et al., 2013). Therefore, it is possible that OC kappa may interact with a receptor located in the plasma membrane leading to the production inositol 1,4,5 triphosphate (IP_3) which induces realease of calcium from ER activating CDPKs na the latter may may activate enzymes that produced G6P and T6P which inhibit SnRK1 leading to TOR activation explaining, at least in part, the stimulation of growth observed in *E. globulus* trees (see model in Fig. 7). On the other hand, it was determined that the level of *tor* transcripts showed increases at weeks 3, 6, 10-11 and 13 whereas *s6k* transcripts levels did not change. Thus, OC kappa induce an increase expression of *tor* gene expression indicating that TOR level is transcriptionally-regulated and the increase in TOR could be realated to the increase in TOR-P level.

OC kappa-induced increases in the level of transcripts encoding proteins of photosystems and chlorophyll synthesis

OC kappa induced an increase in the levels of transcripts encoding proteins of PS II and I which may explain, at least in part, the increase in net photosynthesis previously observed in *E. globulus* trees (González et al., 2014a; González et al., 2013a). In this sense, it has been shown that the inhibition of TOR with AZD8055 leads to a decrease in transcripts encoding proteins involved in photosynthesis, chlorophyll synthesis (Dong et al. 2015) indicating that the activation of TOR pathway leads to the increase photosynthesis. In addition, the levels of transcripts encoding PS proteins showed an oscillatory pattern. As mentioned before, OC kappa may activate CDPKs that may phosphorylate enzymes that synthesize G6P and T6P synthase leading to an increase in G6P and T6P levels which may inhibit SnRK1 leading to TOR activation, resulting in

the increase in genes encoding proteins involved in photosynthesis and chlorophyll synthesis, thus, increasing growth in *E. globulus* trees (see model in Fig. 7). On the other hand, OC kappa induced and increase in total chlorophyll observed at weeks 3-11 and this increase partially overlaps with the higher expression of magnesium chelatase, a key enzyme in chlorophyll synthesis, which increases at weeks 5-15. Thus, the increase in chlorophyll level may occur due to an increased expression of enzymes involved in chlorophyll synthesis.

OC kappa-induced increase in the level of transcripts encoding enzymes of basal and secondary metabolism

Treatment of *Eucalyptus* with OC kappa induced higher expression of genes involved in C, N and S assimilation which is in accord with previous results showing an increase in activities of enzymes involved in C assimilation, rubisco; in N assimilation, GlnS and GDH; and in S assimilation, APR and O-ASTL in *E. globulus* trees (González et al., 2014a). In addition, transcripts encoding enzymes involved in secondary metabolism, PAL and TPS, also increased in response to OC kappa. The latter explain previous results obtained *E. gobulus* trees treated with OC kappa showing an increase in PPCs levels (González et al., 2013b) as well as in volatile terpenes (González et al., 2014b). These results may indicate that the increased activities of enzymes involved in C, N and S assimilation as well as the increase in the level of PPCs and terpenes is due to the increase in expression of the genes encoding enzymes involved in basal and secondary metabolism in *Eucalyptus* trees treated with OC kappa. Moreover, the increase in expression of enzymes involved in basal and secondary metabolisms also showed an oscillatory pattern. As mentioned before, OC kappa may induce the release of calcium from ER which may activate CDPKs that may activate enzymes that synthesize G6P and T6P which may inhibit snRK1 and activate TOR which may lead to activation of transcription regulatory factors resulting in the increase in expression of genes encoding enzymes involved in basal and secondary metabolism resulting in an enhancement of growth and defense responses in *E. globulus* trees (see model in Fig. 7).

Conclusions

E. globulus trees treated with OC kappa displayed an initial increase in glucose and trehalose levels at week 1-3, an increase in the level of TOR-P at week 1-2 and an increase in the level of transcripts encoding proteins involved in photosynthesis and enzymes related to chlorophyll synthesis, and C, N and S assimilation, and associated with the syntheses of PPCs and terpenes,

beginning at weeks 3-4 and displaying an oscillatory pattern. Thus, OC kappa may induce an increase in glucose (G6P) and trehalose (T6P) levels which may inhibit SnRK1 leading to the activation of TOR pathway which may induce the increase in expression of genes involved in photosynthesis, basal metabolism and secondary metabolisms, leading to the enhanced in growth and defense responses in *E. globulus* trees (see model in Fig. 7).

Acknowledgments

This work was funded by Sirius Natura S.A. and VRIDEI-USACH. Silvia Saucedo was financed by SENECYT-Ecuador, Convocatoria 2011.

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Figure legends

Figure 1. Increase in height (A), level of total chlorophyll (B), total reducing sugars (C), glucose (D) and trehalose (E) in control (open circles) and in *E. globulus* trees treated with OC kappa at 1 mg mL^{-1} (black circles). The increase in height is expressed in centimeters, the level of total chlorophyll is expressed in micrograms per gram of fresh tissue and the level of total reducing sugars, glucose and trehalose are expressed in milligram per gram of fresh tissue. Numbers over circles highlight weeks when the most important peaks were observed. Within each experimental week, asterisks (*) indicate when there are significant differences ($p < 0.05$) between OC kappa-treated and control trees. Circles represent the mean value of three independent triplicates ±SD.

Figure 2. Relative level of transcripts encoding enzymes fructose-1,6-bisphosphatase (f*bp1*, A), amylase (*amy3*, B), and ADP-glucose pyrophosphorylase (*apl1,* C) in control and in *E. globulus* trees treated with OC kappa at 1 mg mL^{-1}. Asterisks (*) represent significant differences ($p < 0.05$) between the level of transcripts at a certain week compared with the expression at the beginning of the experiments (week 0). Relative level of transcripts is expressed as $2^{-\Delta\Delta Ct}$. Circles represent the mean value of three independent triplicates ±SD.

Figure 3. Level of active TOR kinase (TOR-P ser4448), and large subunit of ribulose-1,5-bisphosphate carboxylase/oxigenase (RbcL) in control (A) and in *E. globulus* trees treated with OC kappa at 1 mg mL^{-1} (B). Levels of active TOR (C) are expressed in relative units of band intensity corresponding to the ratio TOR/RbcL. Relative level of transcripts encoding TOR kinase (*tor*, D) and S6 kinase (*s6k*, E) in control and *E. globulus* trees treated with OC kappa at 1 mg mL^{-1}. Numbers over circles highlight weeks when the most important parameter peaks were observed. For Fig. 3C, within each experimental week, asterisks (*) indicate when there are significant differences ($p < 0.05$) between OC kappa-treated and control trees. For Fig. 3D-F, asterisks (*) represent significant differences ($p < 0.05$) between the level of transcripts at a certain week compared with the expression at the beginning of the experiments (week 0). The level of transcripts is expressed as $2^{-\Delta\Delta Ct}$. Circles represent mean values of three independent experiments ±SD.

Figure 4. Relative level of transcripts encoding subunit A of photosystem II (*psbA*, A), subunit Rieske of cytochrome b6f *(petC*, B), plastocyanin (*petE*, C), subunit F pf photosystem I (*psaF*, D)

and magensium chelatase (*chlH*, E), in control (open circles) and *E. globulus* trees trated with OC kappa at 1 mg mL^{-1} (black circles) Asterisks (*) represent significant differences ($p < 0.05$) between the level of transcripts at a certain week compared with the expression at the beginning of the experiments (week 0). The level of transcripts is expressed as $2^{-\Delta\Delta Ct}$. Circles represent mean values of three independent experiments ±SD.

Figure 5. Relative level of transcripts encoding the large subunit of ribulose-1,5-carboxylase/oxigenase (r*bcL*, A), glutamine synthase (*gs1*, B), glutamate dehydrogenase (*gdh2*, C), 5'-adenilylsulfate reductase (*apr2*, D) and O-acetylserine thiol-lyase (*cysK*, D) in control (open circles) and *E. globulus* trees treated with OC kappa at 1 mg mL^{-1} (black circles). Asterisks (*) represent significant differences ($p < 0.05$) between the level of transcripts at a certain week compared with the expression at the beginning of the experiments (week 0). The level of transcripts is expressed as $2^{-\Delta\Delta Ct}$. Circles represent mean values of three independent experiments ±SD.

Figure 6. Relative level of transcripts encoding phenylalanine ammonia-lyase (*pal1*, A) and terpene synthase (*ts1*, B), in control and in *E. globulus* trees treated with OC kappa at 1 mg mL^{-1} (black circles). Asterisks (*) represent significant differences ($p < 0.05$) between the level of transcripts at a certain week compared with the expression at the beginning of the experiments (week 0). The level of transcripts is expressed as $2^{-\Delta\Delta Ct}$. Circles represent mean values of three independent experiments ±SD.

Figure 7. Proposed model of OC kappa-induced signaling in *E. globulus*. OC kappa binds to a membrane-associated receptor (Receptor), potentially coupled to a G protein (G); this may activate phospholipase C (PLC) leading to the release of inositol 1,4,5 triphosphate (IP_3) which may activate an IP_3-dependent channel in the endoplasmic reticulum (ER) leading to the calcium release; calcium may activate calcium dependent protein kinases (CDPKs) which, in turn, may activate enzymes that synthesize G6P and T6P. The increases in G6P and T6P levels may inhibit SnRK1 leading to the activation of TOR kinase. Then TOR may mediate the activation of transcription factors (TF) leading to the increase in expression of genes encoding proteins of photosystems and enzymes of basal metabolism, enhancing plant growth, as well as enzymes of secondary metabolism increasing defenses responses in *E. globulus* trees.

Figure 1

Increase in growth and status of photosynthesis and sugars

Increase in height (A), level of total chlorophyll (B), total reducing sugars (C), glucose (D) and trehalose (E) in control (open circles) and in *E. globulus* trees treated with OC kappa at 1 mg mL^{-1} (black circles). The increase in height is expressed in centimeters, the level of total chlorophyll is expressed in micrograms per gram of fresh tissue and the level of total reducing sugars, glucose and trehalose are expressed in milligram per gram of fresh tissue. Numbers over circles highlight weeks when the most important peaks were observed. Within each experimental week, asterisks (*) indicate when there are significant differences ($p < 0.05$) between OC kappa-treated and control trees. Circles represent the mean value of three independent triplicates ±SD.

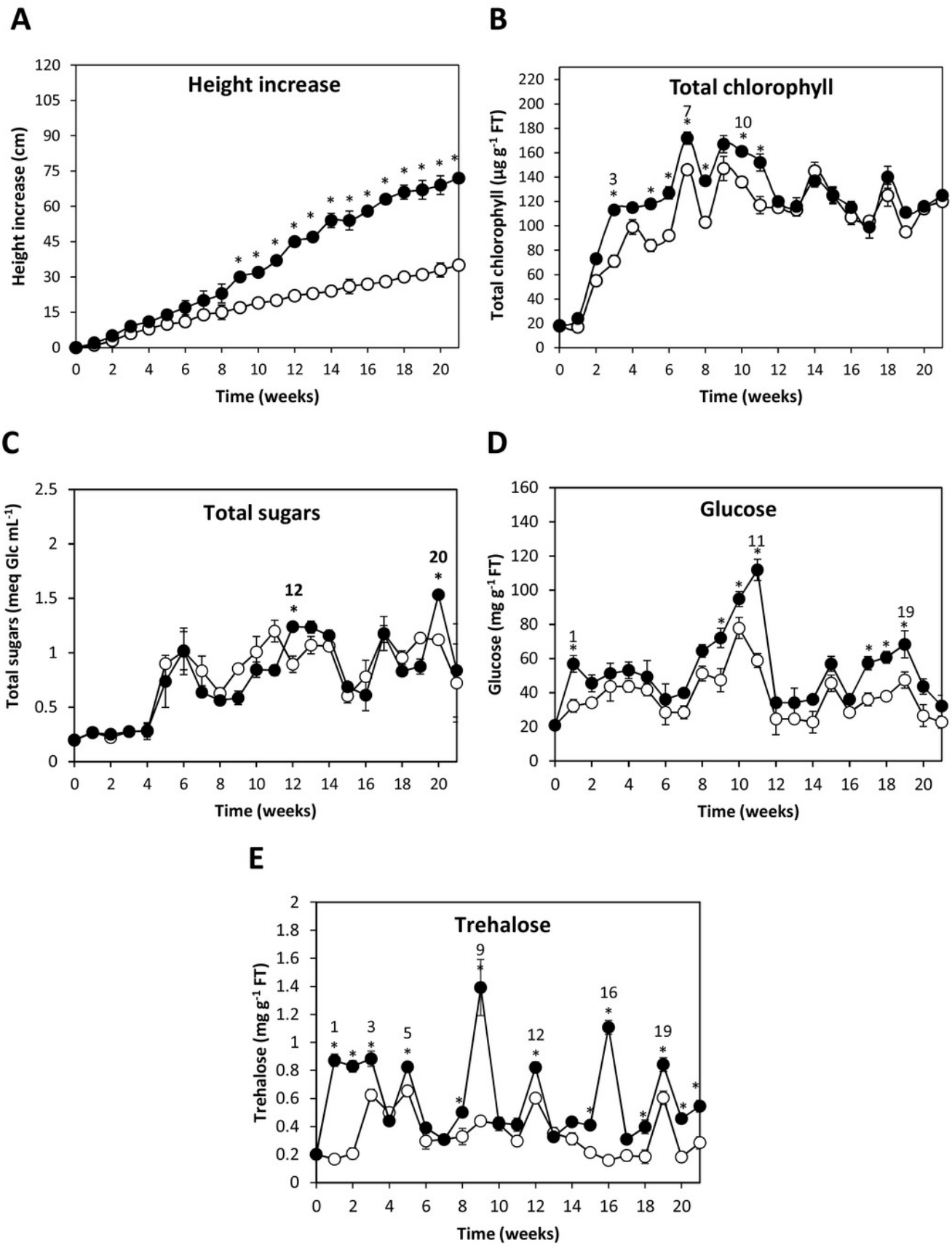

Figure 2

Transcript levels of sugar metabolism genes

Relative level of transcripts encoding enzymes fructose-1,6-bisphosphatase (*fbp1*, A), amylase (*amy3*, B), and ADP-glucose pyrophosphorylase (*apl1,* C) in control and in *E. globulus* trees treated with OC kappa at 1 mg mL^{-1}. Asterisks (*) represent significant differences ($p < 0.05$) between the level of transcripts at a certain week compared with the expression at the beginning of the experiments (week 0). Relative level of transcripts is expressed as $2^{-\Delta\Delta Ct}$. Circles represent the mean value of three independent triplicates ±SD.

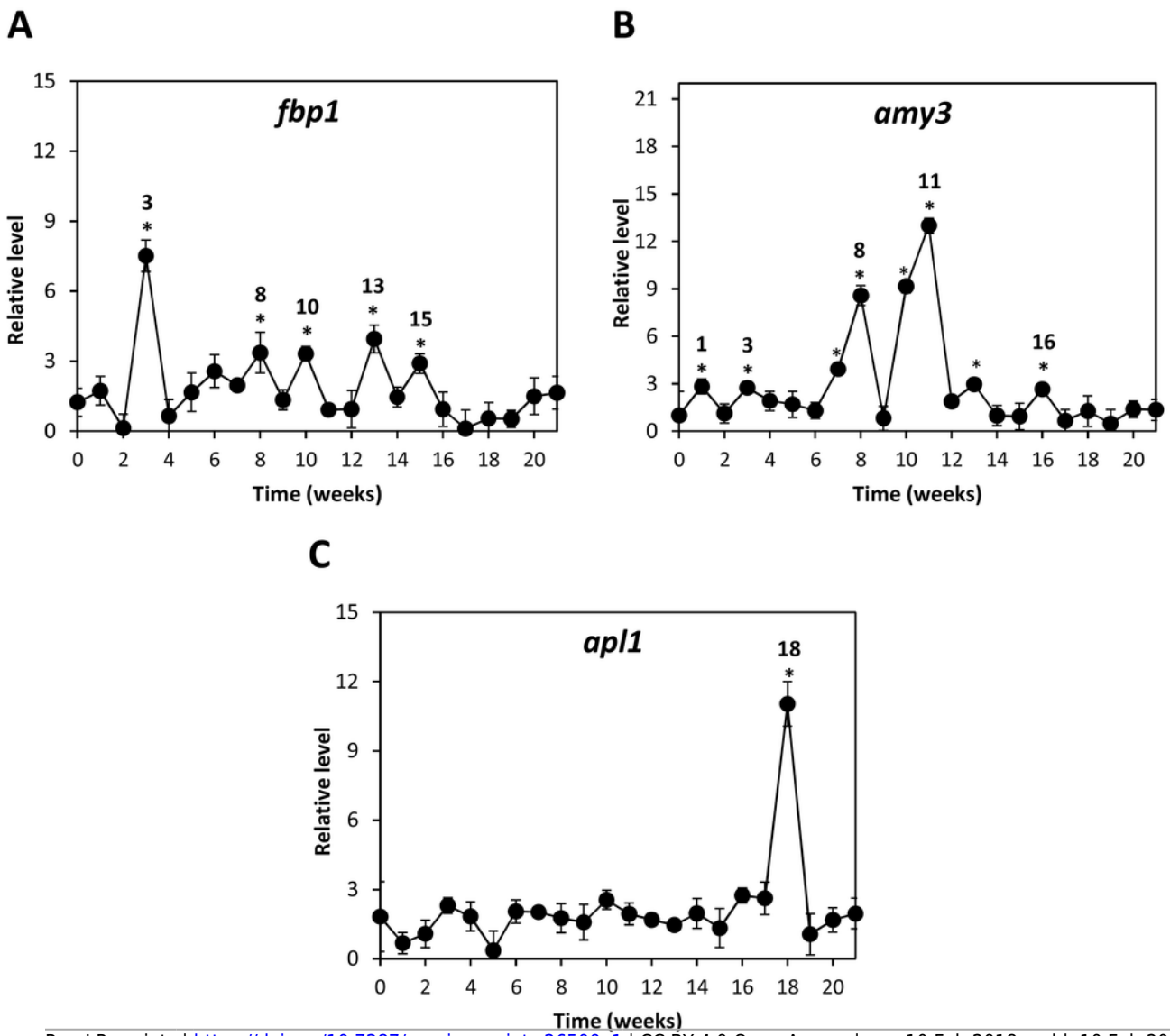

Figure 3

Activation of TOR kinase induced by OC kappa

Level of active TOR kinase (TOR-P ser4448), and large subunit of ribulose-1,5-bisphosphate carboxylase/oxigenase (RbcL) in control (A) and in *E. globulus* trees treated with OC kappa at 1 mg mL^{-1} (B). Levels of active TOR (C) are expressed in relative units of band intensity corresponding to the ratio TOR/RbcL. Relative level of transcripts encoding TOR kinase (*tor*, D) and S6 kinase (*s6k*, E) in control and *E. globulus* trees treated with OC kappa at 1 mg mL^{-1}. Numbers over circles highlight weeks when the most important parameter peaks were observed. For Fig. 3C, within each experimental week, asterisks (*) indicate when there are significant differences ($p < 0.05$) between OC kappa-treated and control trees. For Fig. 3D-F, asterisks (*) represent significant differences ($p < 0.05$) between the level of transcripts at a certain week compared with the expression at the beginning of the experiments (week 0). The level of transcripts is expressed as $2^{-\Delta\Delta Ct}$. Circles represent mean values of three independent experiments ±SD.

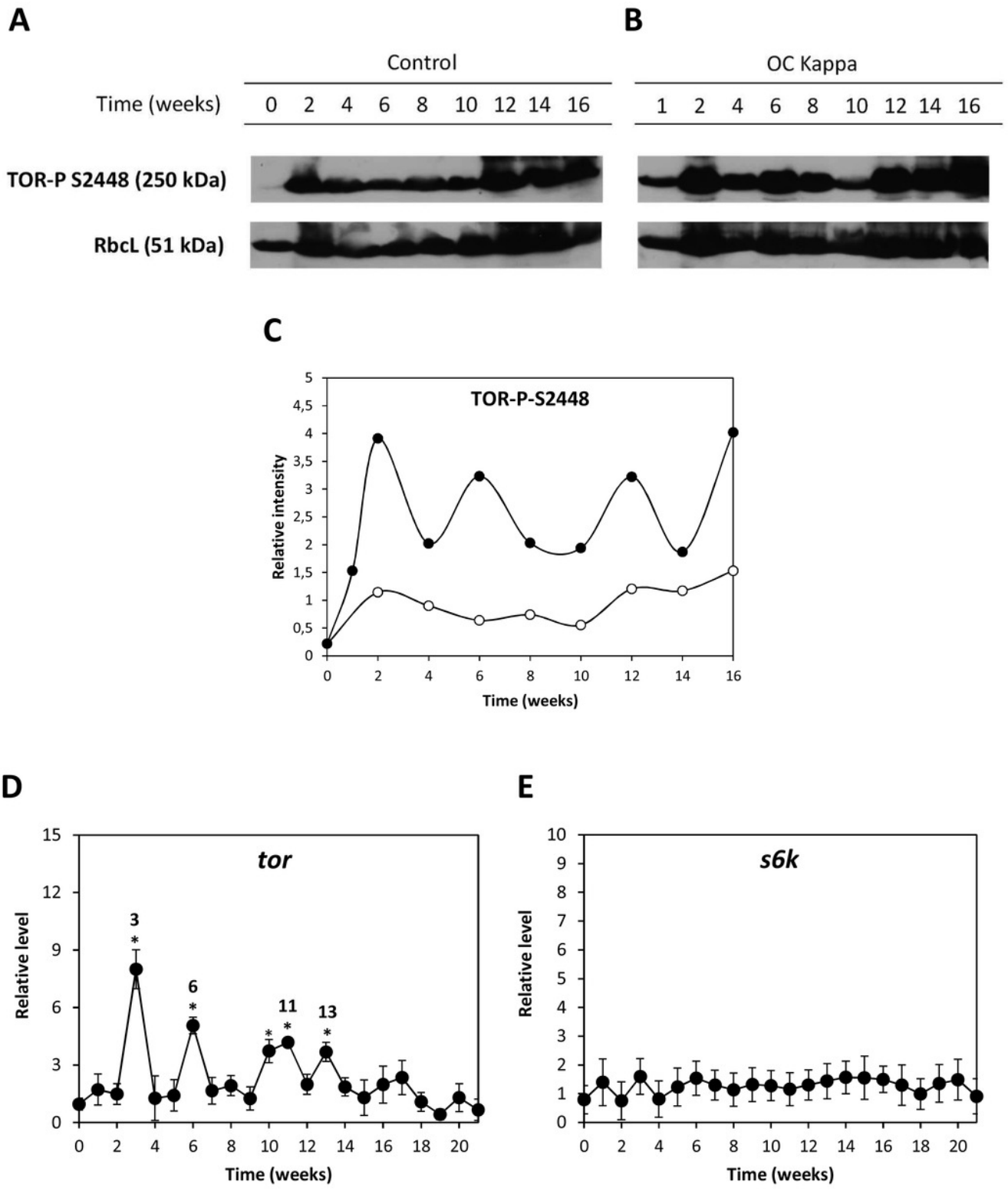

Figure 4

Transcript levels of photosyntesis related genes

Relative level of transcripts encoding subunit A of photosystem II (*psbA*, A), subunit Rieske of cytochrome b6f *(petC*, B), plastocyanin (*petE*, C), subunit F pf photosystem I (*psaF*, D) and magensium chelatase (*chlH*, E), in control (open circles) and *E. globulus* trees trated with OC kappa at 1 mg mL^{-1} (black circles) Asterisks (*) represent significant differences ($p < 0.05$) between the level of transcripts at a certain week compared with the expression at the beginning of the experiments (week 0). The level of transcripts is expressed as $2^{-\Delta\Delta Ct}$. Circles represent mean values of three independent experiments ±SD.

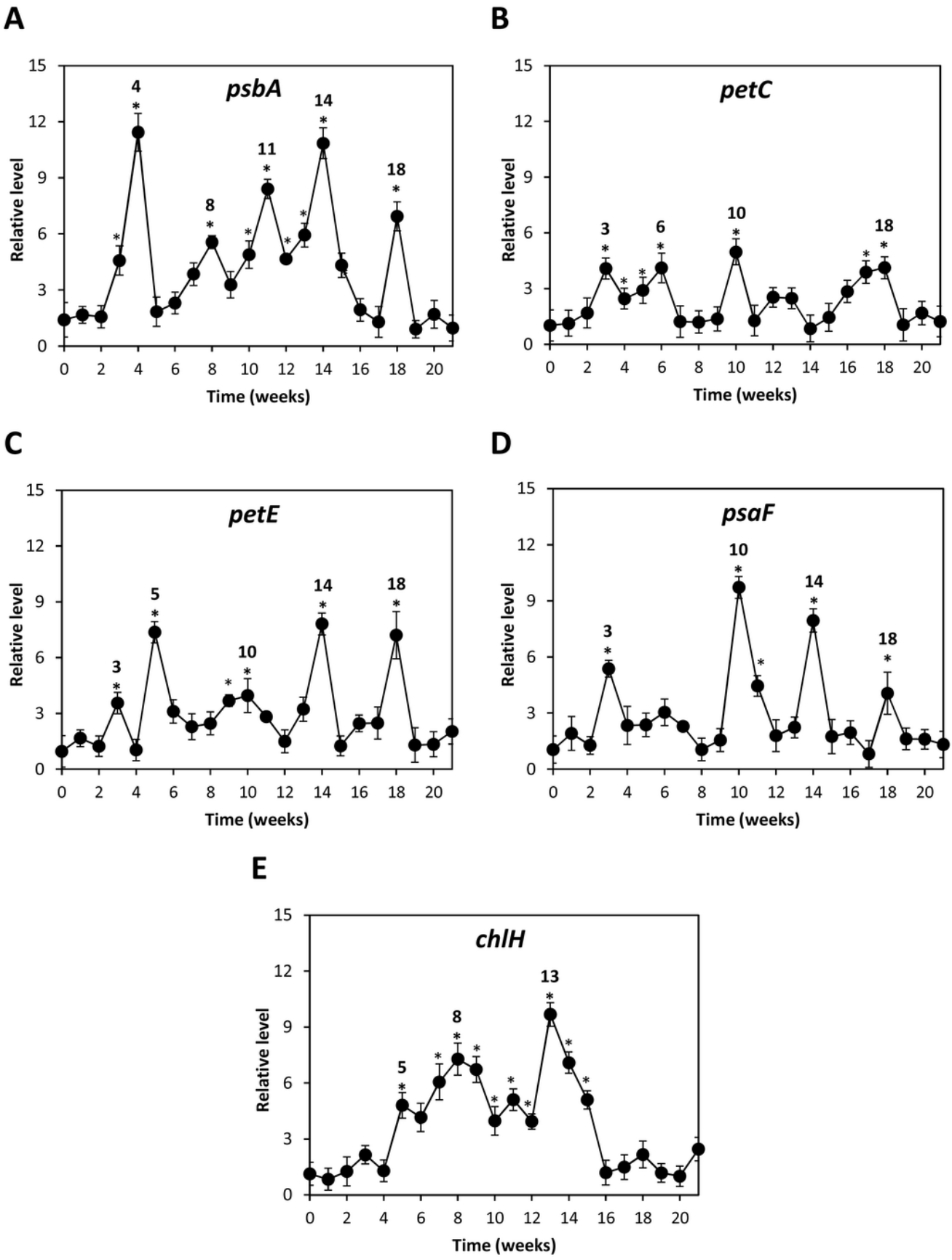
A
psbA
Relative level
Time (weeks)
B
petC
Relative level
Time (weeks)
C
petE
Relative level
Time (weeks)
D
psaF
Relative level
Time (weeks)
E
chlH
Relative level
Time (weeks)

Figure 5

Transcript levels of carbon, nitrogen and sulfur assimilation related genes

Relative level of transcripts encoding the large subunit of ribulose-1,5-carboxylase/oxigenase (r*bcL*, A), glutamine synthase (*gs1*, B), glutamate dehydrogenase (*gdh2*, C), 5'-adenilylsulfate reductase (*apr2*, D) and O-acetylserine thiol-lyase (*cysK*, D) in control (open circles) and *E. globulus* trees treated with OC kappa at 1 mg mL^{-1} (black circles). Asterisks (*) represent significant differences ($p < 0.05$) between the level of transcripts at a certain week compared with the expression at the beginning of the experiments (week 0). The level of transcripts is expressed as $2^{-\Delta\Delta Ct}$. Circles represent mean values of three independent experiments ±SD.

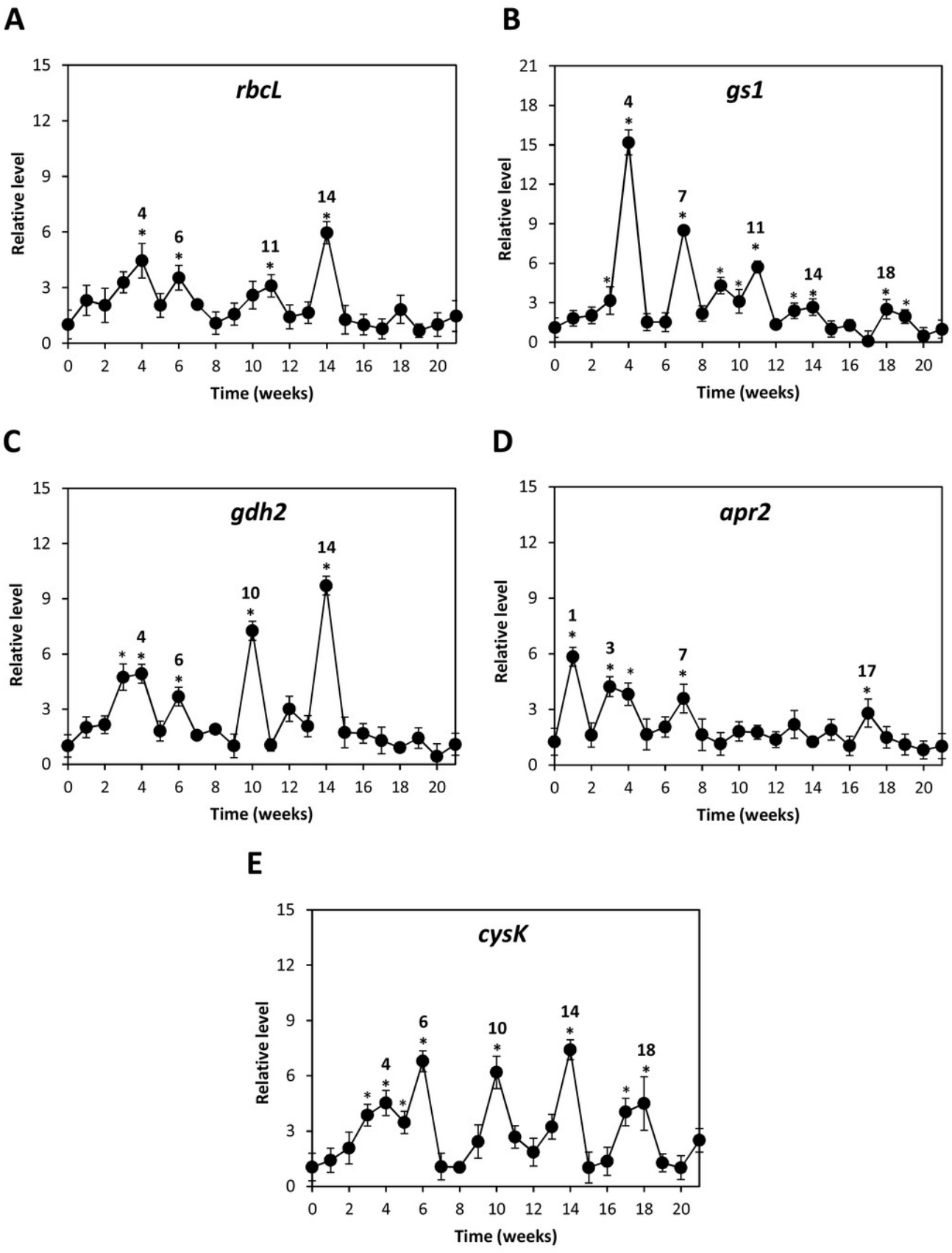

Figure 6

Transcript levels of secondary metabolism related genes

Relative level of transcripts encoding phenylalanine ammonia-lyase (*pal1*, A) and terpene synthase (*ts1*, B), in control and in *E. globulus* trees treated with OC kappa at 1 mg mL^{-1} (black circles). Asterisks (*) represent significant differences ($p < 0.05$) between the level of transcripts at a certain week compared with the expression at the beginning of the experiments (week 0). The level of transcripts is expressed as $2^{-\Delta\Delta Ct}$. Circles represent mean values of three independent experiments ±SD.

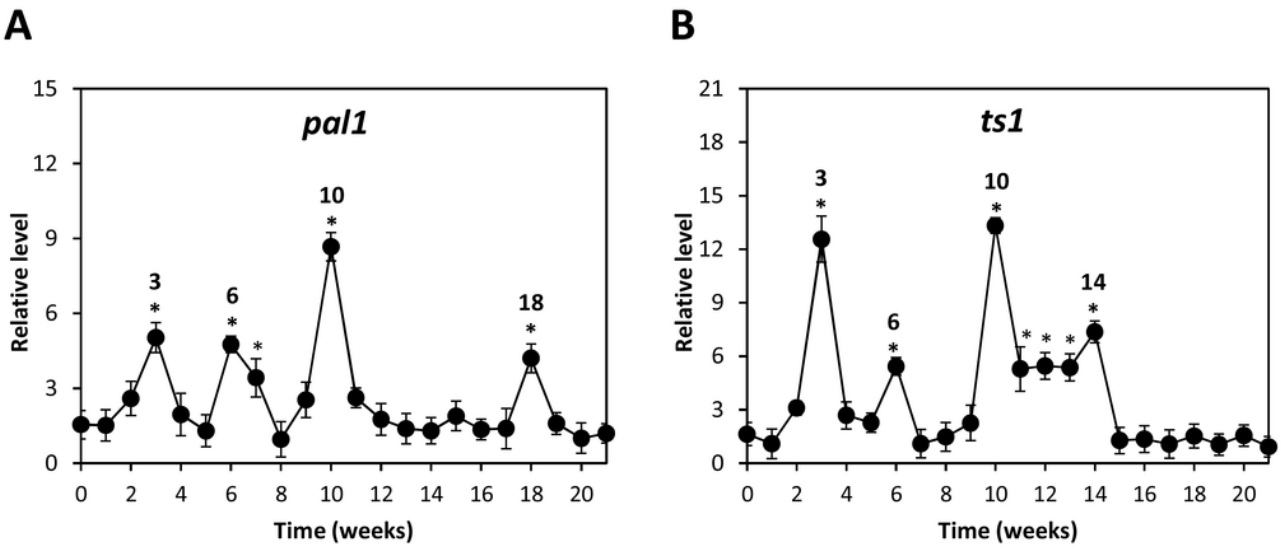

Figure 7

Proposed model of OC kappa-induced signaling in *E. globulus*

OC kappa binds to a membrane-associated receptor (Receptor), potentially coupled to a G protein (G); this may activate phospholipase C (PLC) leading to the release of inositol 1,4,5 triphosphate (IP_3) which may activate an IP_3-dependent channel in the endoplasmic reticulum (ER) leading to the calcium release; calcium may activate calcium dependent protein kinases (CDPKs) which, in turn, may activate enzymes that synthesize G6P and T6P. The increases in G6P and T6P levels may inhibit SnRK1 leading to the activation of TOR kinase. Then TOR may mediate the activation of transcription factors (TF) leading to the increase in expression of genes encoding proteins of photosystems and enzymes of basal metabolism, enhancing plant growth, as well as enzymes of secondary metabolism increasing defenses responses in *E. globulus* trees.

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