J Plant Growth Regul (2012) 31:173–185
DOI 10.1007/s00344-011-9229-5

Oligo-Carrageenans Stimulate Growth by Enhancing Photosynthesis, Basal Metabolism, and Cell Cycle in Tobacco Plants (var. Burley)

Jorge Castro · Jeannette Vera · Alberto González · Alejandra Moenne

Received: 2 March 2011 / Accepted: 11 July 2011 / Published online: 18 August 2011

Abstract Leaves of tobacco plants var. Burley were sprayed with water (control) or with oligo-carrageenans kappa2 (K), lambda (L), and iota (I) at 1 mg ml^{-1}, once a week, four times in total, and cultivated for 4 months without treatment. Plants treated with K, L, and I showed an increase in leaf biomass of 1.9, 1.8, and 2.5 times, respectively, despite that cellular size was similar in control and treated plants. In addition, net photosynthesis, efficiency of photosystem II and the level of chlorophyll *a* and *b* increased in plants treated with oligo-carrageenans. Moreover, the activity of ribulose 1,5-biphosphate carboxylase/oxygenase (rubisco) increased in treated plants, mainly with oligo-carrageenan I, whereas its content did not change compared to control plants. The activity of several NAD(P)H-synthesizing enzymes involved in basal metabolism increased in treated plants, mainly with oligo-carrageenan I. Furthermore, the relative level of transcripts encoding the cell cycle regulatory cyclins A and D and CDKs A and B increased in treated plants, mainly with oligo-carrageenan I. In addition, the amount of ascorbate (ASC) and the activity of ascorbate peroxidase (AP) increased in treated plants, mainly with oligo-carrageenan I, whereas the level of glutathione remained unchanged. Interestingly, the increases in total ascorbate and AP activity linearly correlated with the increase in leaf biomass. Thus, oligo-carrageenans enhanced photosynthesis, basal metabolism, and cell cycle as well as ASC levels and AP activity which may explain, at least in part, the stimulation of plant growth.

J. Castro · J. Vera · A. González · A. Moenne (✉)
Department of Biology, Faculty of Chemistry and Biology, University of Santiago, casilla 40, correo 33, Santiago, Chile
e-mail: alejandra.moenne@usach.cl

Keywords Oligo-carrageenans · Growth · Photosynthesis · Basal metabolism · Cell cycle · Tobacco plants

Introduction

Plant growth and development are determined by various endogenous and environmental factors such as hormones, light, temperature, and nutrient availability (Lau and Deng 2010). In addition, it has been shown that plant oligosaccharides can stimulate or inhibit growth and development in plants (Tran Thanh Van and others 1985; Eberhard and others 1989; McDougall and Fry 1990; Zhou and others 2003; Bilisics and others 2004; Nge and others 2006). Moreover, marine algae oligo-saccharides can also stimulate plant growth (Laporte and others 2007). In this sense, it has been reported that a depolymerized fraction of mannuronic acid obtained from alginates of a brown alga (Poly-Ma) and a depolymerized fraction of sulfated galactan from a red alga (Poly-Ga) at a concentration of 0.5 mg ml^{-1} increased fresh weight biomass of tobacco plants var. Xanthi by 43 and 27%, respectively (Laporte and others 2007). In addition, Poly-Ga and Poly-Ma increased total glutathione (GSH), whereas Poly-Ga increased only total ascorbate (ASC) level (Laporte and others 2007). Moreover, Poly-Ga and Poly-Ma enhanced ascorbate peroxidase (AP) activity, and its enhancement linearly correlated with the increase in leaf biomass (Laporte and others 2007). Interestingly, it was shown that Poly-Ga induced long-term protection against tobacco mosaic virus (TMV) in tobacco plants by increasing the activity of the defense enzyme phenylalanine ammonia lyase and the accumulation of phenylpropanoid compounds (Vera and others 2011). Recently, we prepared oligo-carrageenans kappa2 (K), lambda (L), and iota (I) by acid

hydrolysis of commercial pure carrageenans (Moenne 2009). These oligo-saccharides, which are structurally related to Poly-Ga, are constituted by around 20 units of sulfated galactose linked by β-1,3, and α-1,4 alternate bonds and, in some cases, units of anhydrogalactose. It was shown that oligo-carrageenans K, L, and I sprayed on leaves of tobacco plants var. Xanthi, at a concentration of 1 mg ml^{-1}, increased fresh leaf biomass by 108, 31, and 121%, respectively (Moenne 2009). In addition, oligo-carrageenans enhanced protection against TMV infection in tobacco plants (Moenne 2009). Thus, it is possible that oligo-carrageenans K, L, and I may increase growth of commercial tobacco plants as well as that of other plants of commercial interest.

On the other hand, plant growth is determined mainly by the increase in cell number rather than in cell size (Mizukami 2001). Regarding plant cell division, it has been shown that ASC is required for cell cycle differentiation and division (De Pinto and others 1999, 2004). In addition, ASC level increased during seed germination (Arrigoni and others 1992; Tomassi and others 2001), and levels were higher in meristematic tissue than in adjacent tissues (Córdoba-Pedregosa and others 2005). Moreover, ASC is also a cofactor of several dioxygenases required for cell division (De Tullio and others 1999; Arrigoni and De Tullio 2002). Furthermore, the total ASC level increased in tobacco plants treated with Poly-Ga oligo-saccharide as well as leaf biomass, suggesting that an increase in ASC may favor plant growth (Laporte and others 2007).

To analyze the potential stimulation of growth induced by oligo-carrageenans K, L, and I in commercial tobacco plants (var. Burley), we determined height and weight of leaf biomass in control and treated plants. To determine the physiological and biochemical mechanisms involved in the stimulation of growth, the level of CO_2 incorporation, stomatal conductance, net photosynthesis, and the efficiency of photosystem II (PSII) were measured as well as the activity and amount of ribulose 1,5-biphosphate carboxylase/oxygenase (rubisco). In addition, the activities of several NAD(P)H-synthesizing enzymes involved in basal metabolism, the level of transcripts encoding cell cycle regulatory proteins, the level of the antioxidant compounds ASC and GSH, and the activity of the antioxidant enzyme AP were also determined in control and treated plants.

Materials and Methods

Preparation and Analysis of Oligo-Carrageenans

Carrageenans are constituted by alternate β-1,3- and α-1,4-linked sulfate galactose units containing sulfate groups in positions 2 and 4 of the galactose ring and, in some cases, units of anhydrogalactose. Pure (free of proteins and secondary metabolites) commercial K, L, and I carrageenans (20 g) were purchased from Gelymar S.A. and were solubilized in 2 l of water at 60°C. Concentrated HCl (36.2 N) was added to reach a final concentration of 0.1 N. The solution was incubated for 45 min at 60°C, and 1 M NaOH was added to obtain a neutral pH. A sample of 10 µl of each depolymerized carrageenan (oligo-carrageenan) was analyzed by electrophoresis in an agarose gel (1.5% w/v) at 100 V for 1 h and dextran sulfate of 8 and 10 kDa as standards (Sigma, St. Louis, MO, USA). The gel was stained with 15% w/v Alcian blue dye in 30% v/v acetic acid for 1 h at room temperature and washed with 50% v/v acetic acid for 1 h. Oligo-carrageenans K, L, and I were visualized as a relative discrete band of around 10 kDa.

Plant Culture, Treatments, and Analysis of Leaf Biomass

Tobacco plants of var. Burley ($n = 10$) were cultivated outdoors in plastic bags containing compost during spring and summer 2009 and watered every 3 days. Tobacco plants ($n = 10$, initial height = 25 cm) were sprayed in the upper and lower parts of the leaves with 2 ml of water per plant (control group) or with 2 ml of an aqueous solution of oligo-carrageenan K, L, or I at a concentration of 1 mg ml^{-1}, once a week, 4 times in total, and cultivated without treatment for four additional months. Tobacco plants were cut at the bottom, hanged at the roof of the greenhouse, and maintained 45 days at room temperature. The leaves of each control and treated tobacco plant ($n = 10$) were pooled and weighed using a balance (no dried leaves fell).

Visualization of Cell Size

Leaves located in the middle part of control tobacco plants ($n = 3$) and in plants treated with oligo-carrageenan I were cut in slices of around 40 µm in the middle part of the leaf using a razor blade. Leaf cells were visualized using a confocal microscope (Axiovert 100, Carl Zeiss, Jena, Germany) with an emission wavelength of 543 nm (20% light intensity) from an argon laser, and a filter of 560–615 nm (pinhole = 119 µm) to detect red autofluorescence of chloroplasts. Cell sizes were measured in 10 cells per slice using the software LSM Image Browser belonging to the confocal microscope.

Detection of CO_2 Incorporation, Stomatal Conductance, and Net Photosynthesis

CO_2 incorporation, stomatal conductance, and net photosynthesis were detected in five leaves located in the middle

part of each control and treated tobacco plant ($n = 10$) using a portable infrared gas analyzer (Ciras-1, PP systems, Hitchin, UK), a leaf cuvette of 12.5 cm^2, a red/white LED light source, a photon irradiance of 1,000 µmol quanta m^{-2} s^{-1} photosynthetic active radiation (PAR), a CO_2 concentration of 500 ppm, and relative humidity of 70% at 24°C for 1 min.

Detection of PSII Efficiency

Maximal quantum efficiency of PSII was detected in five leaves located in the middle part of each control and treated plant ($n = 10$) using a portable photosystem efficiency analyzer (Hansatech Instruments, Norfolk, UK). Leaves were adapted to darkness for 30 min using a leaf clip. The minimal fluorescence (F_0) was detected at 650 nm with a photon irradiance of 3,000 µmol m^{-2} s^{-1} PAR for 50 µs using an ultrabright red LED emission source and a long-pass photodiode detector RG9. The maximal fluorescence (F_m) was detected at 650 nm for 30 ms. The maximal quantum efficiency of PSII (F_v/F_m) was calculated according to Roháček (2002).

Detection of Chlorophyll *a* and *b* Content

Tobacco leaves (6 g) were frozen in liquid nitrogen and homogenized in a mortar with a pestle. Twelve milliliters of hexane-acetone (3:1) was added and the mixture was incubated overnight at room temperature. The mixture was filtered on Miracloth paper (Calbiochem, Darmstadt, Germany), and the absorbance of chlorophyll *a* and *b* was detected at 663 and 646 nm, respectively, using a Genesys 5 spectrophotometer (Spectronic, Milton Roy Co., Waltham, MA, USA). The amounts of chlorophyll *a* and *b* were calculated according to Lichtenthaler and Wellburn (1983) using the following formulae:

$$\text{Chlorophyll } a\left(\mu g ml^{-1}\right) = 12.5A_{663} - 2.79A_{646}$$

$$\text{Chlorophyll } b\left(\mu g ml^{-1}\right) = 20.5A_{646} - 5.1A_{663}$$

Preparation of Protein Extracts

Tobacco leaves of each plant (20 g) were frozen in liquid nitrogen and pulverized in a mortar with a pestle. Sixty milliliters of 0.1 M phosphate buffer (pH 7.0) containing 5 mM β-mercaptoethanol and 20% (v/v) glycerol was added and the homogenization was pursued. After thawing, the homogenate was filtered through Miracloth (Calbiochem, San Diego, CA). The filtrate was centrifuged at 7,400 *g* for 15 min and the supernatant was recovered and stored at −80°C. Protein concentration was determined according to Bradford (1976) and was normally 1 mg ml^{-1}.

Detection of Rubisco Activity

Rubisco activity was detected as described by Lilley and Walker (1974) in 1 ml of reaction mixture containing 100 mM Tris–HCl (pH 8.0), 1 mM ribulose 1,5-biphosphate, 10 mM $KHCO_3$, 20 mM $MgCl_2$, 5 mM creatine phosphate, 3 mM ATP, 10 U phosphoglycerate kinase, 10 U glyceraldehyde 3-phosphate dehydrogenase, 10 U creatine kinase, 0.15 mM NADH, and 10 µg protein extract. One enzyme unit (U) corresponds to the amount of enzyme that converts 1 µmol of substrate at pH 7.0 and 25°C .The decrease in absorbance at 340 nm due to NADH consumption was detected for 3 min. Rubisco activity was calculated using the extinction coefficient of NADH ($\varepsilon = 6.2$ mM^{-1} cm^{-1}).

Detection of Rubisco Level

The amount of rubisco was detected by Western blot analysis using a polyclonal antibody prepared against the large subunit of rubisco enzyme (Agrisera, Vännäs, Sweden). Proteins from tobacco leaves (3 µg) were separated by electrophoresis in a 10% denaturing polyacrylamide gel for 2 h at 100 V using a prestained protein standard (BioRad, Hercules, CA, USA). Proteins were electrotransferred to a nitrocellulose membrane (BioRad) at 150 mA for 1 h. The membrane was incubated in TTBS [20 mM Tris–HCl (pH 7.5), 0.1 mM NaCl, 0.1% Tween-20] supplemented with 5% w/v skim milk for 1 h at room temperature, with TTBS supplemented with anti-rubisco antibody diluted 50,000 times for 1 h and washed 3 times with TTBS supplemented with 3% w/v of skim milk for 10 min. The membrane was incubated in TTBS supplemented with a polyclonal antibody prepared against rabbit IgG coupled to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 5,000 times for 1 h, and washed 3 times with TBS [20 mM Tris–HCl (pH 7.5), 0.1 mM NaCl] for 10 min. The membrane was incubated with luminol and hydrogen peroxide (Biological Industries, Beit Haemek, Israel) for 2 min and exposed to a clear blue X-ray film (Thermo Scientific, Rockford, IL, USA) for 1 min.

Detection Enzyme Activities Involved in Basal Metabolism

Pyruvate dehydrogenase (PDH) activity was detected as described by Reid and others (1977) in 1 ml of reaction mixture containing 50 mM Tris–HCl (pH 7.5), 1 mM sodium pyruvate, 0.5 mM thiamine pyrophosphate, 1 mM cysteine, 0.1 mM coenzyme A, 20 mM $MgCl_2$, 1 mM NAD, and 70 µg of protein extract. The increase in absorbance was detected at 340 nm due to the synthesis of NADH for 1 min.

The activity of PDH was calculated using the extinction coefficient of NADH ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

Isocitrate dehydrogenase (IDH) activity was detected as described by Lemaitre and others (2007) in 1 ml of reaction mixture containing 50 mM HEPES (pH 7.0), 10 mM sodium isocitrate, 1 mM NAD, 20% glycerol, and 30 μg of protein extract. The increase in absorbance due to NADH synthesis was detected at 340 nm for 1 min. The activity of IDH was calculated using the extinction coefficient of NADH ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

Inosine monophosphate dehydrogenase (IMPDH) activity was detected as described by Shelp and Atkins (1983) in 1 ml of reaction mixture containing 100 mM Tris–HCl (pH 7.2), 100 mM KCl, 2 mM inosine monophosphate, 1 mM NAD, 3 mM EDTA, 1 mM DTT, and 10 μg of protein extract. The increase in absorbance due to NADH synthesis was detected at 340 nm for 3 min. The activity of IMPDH was calculated using the extinction coefficient of NADH ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

Dehydroorotate dehydrogenase (DHODH) activity was detected as described by Ullrich and others (2002) in 1 ml of reaction mixture containing 50 mM Tris–HCl (pH 7.2), 150 mM KCl, 5 mM magnesium chloride, 1 mM L-dehydroorotate, 1 mM decylubiquinone, 0.1 mM 2,6-dichlorophenolindophenol, 1 mM NAD, 0.1% Triton X-100, and 30 μg of protein extract. The increase in absorbance due to NADH synthesis was detected at 340 nm for 3 min. The activity of DHODH was calculated using the extinction coefficient of NADH ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

Glucose 6-phosphate dehydrogenase (G6PDH) activity was detected as described by González and others (2010) in 1 ml of reaction mixture containing 0.1 M phosphate buffer (pH 7.4), 4 mM glucose 6-phosphate, 0.6 mM NADP, 5 mM $MgCl_2$, and 40 μg of protein extract. The increase in absorbance due to NADPH synthesis was detected at 340 nm for 8 min. The activity of G6PDH was calculated using the extinction coefficient of NADPH ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

Fatty acid synthase (FAS) activity was detected as described by Lynen (1969) in 1 ml of reaction mixture containing 100 mM phosphate buffer (pH 7.4), 3 mM acetyl-CoA, 10 mM malonyl-CoA, 0.3 mM NADPH, 1 mM EDTA, 1 mM DTT, and 20 μg of protein extract. The decrease in absorbance due to NADPH consumption was detected at 340 nm for 2 min. The activity of FAS was calculated using the extinction coefficient of NADPH ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

Glutamate dehydrogenase (GDH) activity was detected as described by Turano and others (1996) in 1 ml of reaction mixture containing 100 mM phosphate buffer (pH 7.4), 50 mM ammonium sulfate, 2 mM 2-oxoglutarate, 0.3 mM NADH, and 60 μg of protein extract. The decrease in absorbance was detected at 340 nm due to the consumption of NADH for 1 min. The activity of GDH was calculated using the extinction coefficient of NADH ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$).

Detection of Transcripts Encoding Cyclins and CDKs

Total RNA was extracted from tobacco leaves (0.1 g) using the FavorPrep Plant Total RNA kit (Favorgene, Ping-Tung, Taiwan) and quantified with Quanti-iT Ribogreen RNA assay kit (Invitrogen, Carlsbad, CA, USA).

The relative levels of cyclin A, cyclin D, cyclin-dependent kinase (CDK) A, and CDK B were detected using actin (ACT) transcripts as internal control, PCR primers listed in Table 1, and a real-time thermocycler Rotor Gene 6000 (Corbett Research, Sidney, Australia). To detect cyclins, RT-PCR reactions were performed using a Sensimix One-Step kit (Quantace, London, UK), 10 μg of total RNA, 8 μM of each primer, and 2 mM $MgCl_2$. The reverse transcription step was done for 20 min at 45°C; the inactivation step was 5 min at 95°C; and the amplification step was performed using 40 cycles of 20 s at 95°C for denaturation, 30 s at 58°C for primer annealing, and 15 s at 72°C for elongation. To detect CDKs, RT-PCR reactions were performed with 10 μg of total RNA, 15 μM each primer, and 2 mM $MgCl_2$. The reverse transcription was done for 20 min at 42°C; the inactivation step for 10 min at 92°C; and the amplification step was performed using 40 cycles of 20 s at 95°C for denaturation, 30 s at 62°C for primer annealing, and 25 s at 72°C for elongation.

Fragments amplified by RT-PCR were detected by fluorescence using SYBR® Green I included in the amplification kit. RT-PCR reactions were done in triplicate with total RNA extracted from three independent replicates. Samples were averaged and normalized using the ΔΔCT method and mean value control was subtracted from mean treated to determine the fold of change in treated samples. The relative transcript level was expressed as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001).

Table 1 PCR primers to quantify cell cycle regulatory proteins

Gene	Sense	Sequence
Actin	F	ACATTGTGCTCAGTGGTGGTACT
	R	CCACCTTAATCTTCATGCTGCT
Cyclin A	F	AATCACACGAGCACGAGCAAG
	R	CTCCCAATGCCTGCTTCTTATC
Cyclin D	F	AAGATAAGCCTTGGATGATTCAAC
	R	GAGTTTCTTCCACTTTAGCAGC
CDKA	F	ATTCCTGTCAGAACTTTCAC
	R	CAAACATCAACAGGAGTAGAG
CDKB	F	CACCCATGAGATTGTTAC
	R	CCTTCTAACCATCTCGGC

Detection of Ascorbate and Glutathione Levels

The levels of ASC, dehydroascorbate (DHA), reduced glutathione (GSH), and oxidized glutathione (GSSG) were determined as described by Laporte and others (2007).

Detection of Ascorbate Peroxidase Activity

AP activity was detected as described by Laporte and others (2007) in 1 ml of reaction mixture containing 0.1 M phosphate buffer (pH 7.0), 0.5 mM ASC, 0.5 mM hydrogen peroxide, and 100 µg of protein extract. The decrease in absorbance due to ASC consumption was determined at 290 nm for 1 min. AP activity was calculated using the extinction coefficient of ASC ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$).

Statistical Analysis

Significant differences were determined by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests (T). Differences between mean values were considered to be significant at a probability of 5% ($P < 0.05$) (Zar 1999).

Results

Oligo-Carrageenans Increased Tobacco Height and Leaf Biomass

Tobacco plants treated with oligo-carrageenans K, L, and I showed an increase in plant height and weight of leaf biomass compared to control plants (Fig. 1). The height of control plants was 116 cm and that of plants treated with K, L, and I was 184, 179, and 172 cm, respectively, which corresponds to increases of 1.6, 1,5, and 1.5 times, respectively (Fig. 1a). These increases were not significantly different between treatments. In addition, the weight of leaf biomass from control plants was 0.68 kg of dry tissue (DT) and that of leaf biomass from plants treated with K, L, and I was 1.31, 1.26, and 1.73 kg DT, respectively, which correspond to increases of 1.9, 1.8, and 2.5 times, respectively (Fig. 1b). The highest increase of leaf biomass was observed with oligo-carrageenan I. On the other hand, control plants and plants treated with oligo-carrageenan I showed similar cell sizes with mean values of 585 and 583 μm^2, respectively (Fig. 1c, d). Thus, oligo-carrageenans induced an increase in plant height and weight of leaf biomass that is determined, at least in part, by the increase in cell number.

Oligo-Carrageenans Increased Photosynthesis and Efficiency of PSII

Plants treated with oligo-carrageenans showed an increase in net photosynthesis and quantum efficiency of PSII (Fig. 2). CO_2 incorporation increased in plants treated with oligo-carrageenans K, L, and I by 2.3, 1.8, and 1.5 times, respectively, and these increases were not significantly different between treatments (Fig. 2a). Mean values of stomatal conductance increased in plants treated with oligo-carrageenans K, L, and I by 3.8, 2.4, and 5.1 times, respectively (Fig. 2b). The highest increase in stomatal conductance was observed with oligo-carrageenan I. Net photosynthesis increased in plants treated with oligo-carrageenans K, L, and I by 4, 3.6, and 4.3 times, respectively, and these increases were not significantly different between treatments (Fig. 2c). In addition, quantum efficiency of PSII (F_v/F_m) increased from 0.78 in control plants to 0.85, 0.83, and 0.85 in plants treated with oligo-carrageenans K, L, and I, respectively, and these increases were not significantly different between treatments (Fig. 2d).

Oligo-Carrageenans Increased Chlorophyll *a* and *b* Content and Rubisco Activity

Tobacco plants treated with oligo-carrageenans showed an increase in chlorophyll *a* and *b* content and in rubisco activity (Fig. 3). The level of chlorophyll *a* was 1.3 mg g^{-1} fresh tissue (FT) in control plants and 1.6 mg g^{-1} FT in plants treated with oligo-carrageenans K, L, and I (Fig. 3a), which corresponds to increases of 1.2 times. These increases were not significantly different between treatments. The level of chlorophyll *b* was 0.8 mg g^{-1} FT in control plants and 1 mg g^{-1} FT in plants treated with oligo-carrageenans K, L, and I (Fig. 3b), which corresponds to increases of 1.3 times. These increases were not significantly different between treatments.

In addition, rubisco activity increased from 35 µmol min^{-1} mg^{-1} protein in control plants to 82, 92, and 149 µmol min^{-1} mg^{-1} protein in plants treated with oligo-carrageenans K, L, and I, respectively (Fig. 3c), which correspond to increases of 2.3, 2.6, and 4.3 times. These increases were significantly different between treatments and the highest increase was detected with oligo-carrageenan I. In contrast, the amount of rubisco enzyme was similar in control and treated plants (Fig. 3d). Thus, the increase in rubisco activity detected in treated plants was not due to an increase in enzyme content.

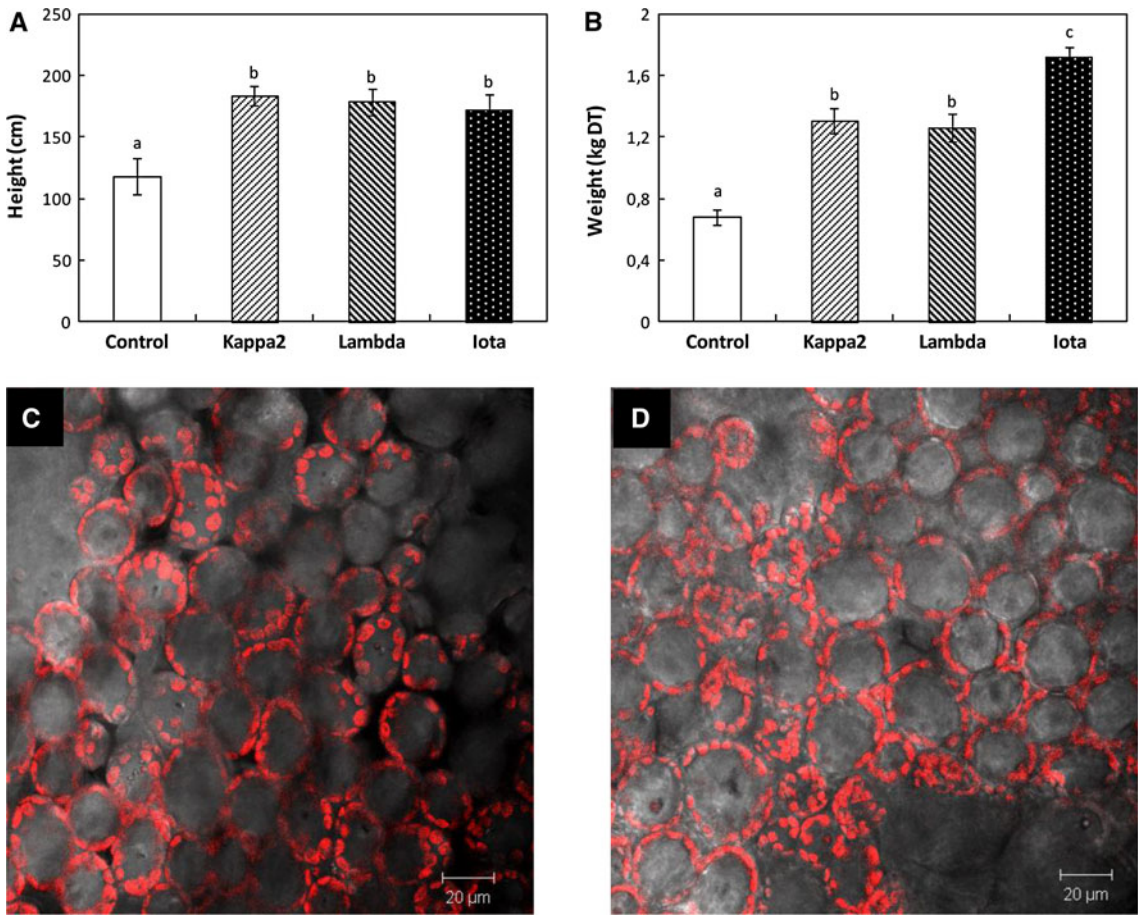


Fig. 1 Height (**a**) and weight of leaf biomass (**b**) of control tobacco plants and plants treated with oligo-carrageenans kappa2, lambda, and iota. Plant height is expressed in cm and leaf biomass is expressed in kg of dry tissue (DT). *Bars* represent mean values of ten independent replicates ± standard deviation. *Different letters* indicate significant differences ($P < 0.05$). Visualization of cell size in leaves of control tobacco plants (**c**) and in plants treated with oligo-carrageenan iota (**d**). The *white bar* represents 20 µm

Oligo-Carrageenans Increased Activities of Enzymes Involved in Basal Metabolism

Tobacco plants treated with oligo-carrageenans showed an increase in activity of enzymes involved in basal metabolism (Fig. 4). First, the NADH-synthesizing enzymes PDH and IDH, belonging to the Krebs cycle, increased in plants treated with oligo-carrageenans. PDH activity increased by 2.4, 2.3, and 3 times in plants treated with oligo-carrageenans K, L, and I, respectively, (Fig. 4a), and IDH activity increased by 2, 1.3, and 3 times, respectively (Fig. 4b). The highest increases in PDH and IDH activities were observed with oligo-carrageenan I.

In addition, the activities of NADH-synthesizing enzymes IMPDH and DHODH, involved in purine and pyrimidine synthesis, increased in plants treated with oligo-carrageenans. IMPDH activity increased by 2, 1.3, and 2.5 times (Fig. 4c) and DHODH activity increased by 1.5, 1.6, and 1.9 times in plants treated with oligo-carrageenans K, L, and I, respectively (Fig. 4d). The highest increases in IMPDH and DHODH activities were observed with oligo-carrageenan I.

Moreover, the activity of NADPH-synthesizing enzyme G6PDH, involved in ribose 5-phosphate synthesis, increased in plants treated with oligo-carrageenans. G6PDH activity increased by 2.5, 2.3, and 2.8 times in plants treated with oligo-carrageenans K, L, and I, respectively (Fig. 4e). The highest increase in G6PDH activity was observed with oligo-carrageenan I.

The activity of FAS, a key enzyme of fatty acid synthesis, increased in plants treated with oligo-carrageenans K, L, and I by 1.6, 1.8, and 1.9 times, respectively (Fig. 4f). The higher increases in FAS activity were detected with oligo-carrageenan L and I.

Finally, the activity of GDH, a key enzyme for amino acid synthesis and nitrogen fixation, increased by 2.3, 2.2, and 2.9 times in plants treated with oligo-carrageenans K, L, and I, respectively (Fig. 4g). The highest increase in GDH activity was detected with oligo-carrageenan I. Thus, oligo-carrageenans, mainly oligo-carrageenan I, induced the

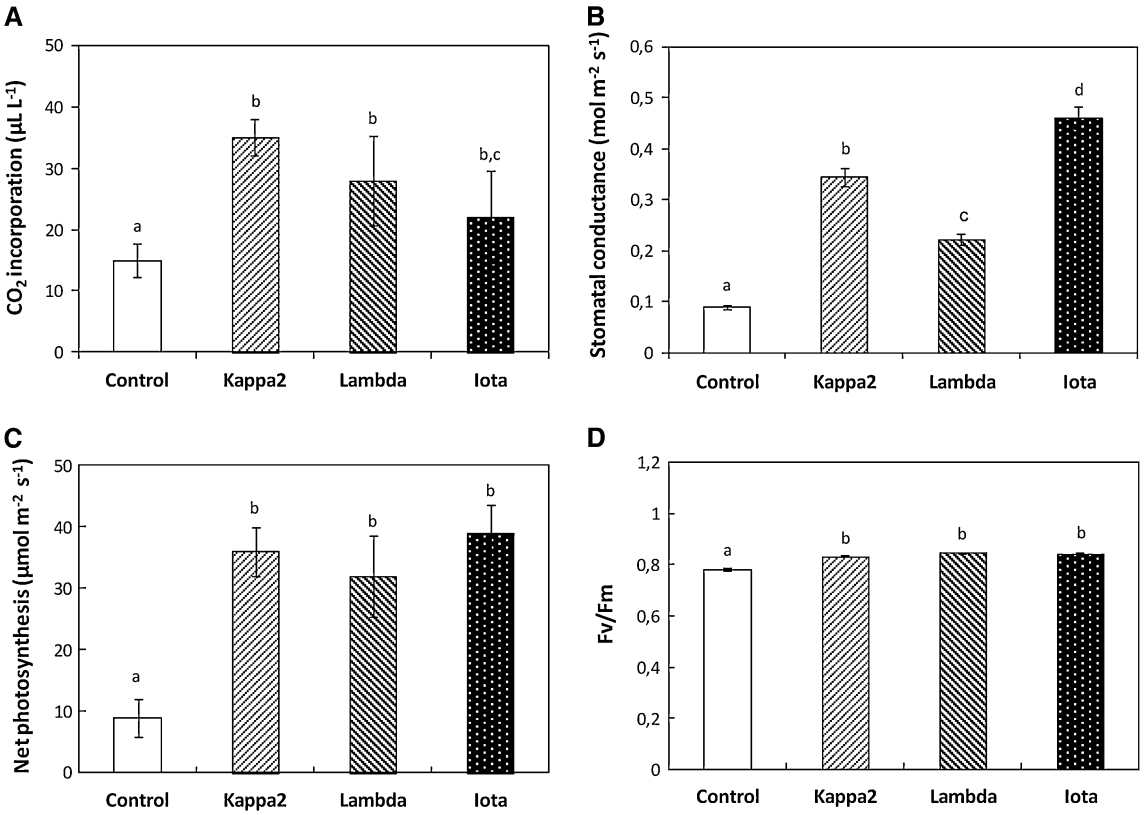


Fig. 2 CO_2 incorporation (**a**), stomatal conductance (**b**), net photosynthesis (**c**), and efficiency of PSII (**d**) in control tobacco plants and in plants treated with oligo-carrageenans kappa2, lambda, and iota. CO_2 incorporation is expressed in µl l^{-1}, stomatal conductance is expressed in mol m^{-2} s^{-1}, net photosynthesis is expressed in µmol m^{-2} s^{-1}, and efficiency of PSII is expressed as the ratio of variable fluorescence and maximal fluorescence. Bars represent mean values of ten independent replicates ± standard deviation. *Different letters* indicate significant differences ($P < 0.05$)

activation of enzymes involved in basal metabolism and nitrogen fixation.

Oligo-Carrageenans Increased Transcript Levels of Cell Cycle Regulatory Proteins

Tobacco plants treated with oligo-carrageenans showed an increase in the level of transcripts coding for cell cycle regulatory proteins (Fig. 5). The relative level of transcripts encoding cyclin A increased by 2.2, 1.5, and 3.5 times (Fig. 5a) and that of cyclin D by 3.6, 2.2, and 5.1 times (Fig. 5b) in plants treated with oligo-carrageenans K, L, and I, respectively. These increases were significantly different between treatments and the highest increases in cyclin A and D were detected with oligo-carrageenan I. In addition, the relative level of transcripts encoding CDK A increased by 2.2, 1.8, and 4.2 times (Fig. 5c) and the level of CDK B transcripts increased by 1.9, 1.6, and 3.8 times (Fig. 5d) in plants treated with oligo-carrageenans K, L, and I, respectively. These increases were significantly different between treatments and the highest increases in CDKs A and B were detected with oligo-carrageenan I. Thus, oligo-carrageenans, mainly oligo-carrageenan I, may induce a stimulation of cell cycle and cell division in tobacco plants.

Oligo-Carrageenans Increased Ascorbate Level and AP Activity

Tobacco plants treated with oligo-carrageenans showed an increase in ASC level, a decrease in DHA, and an increase in AP activity, whereas the levels of reduced GSH and GSSG remained unchanged (Fig. 6). The level of ASC was 0.5 µg g^{-1} of dry tissue (DT) in control plants and 1.1, 1.1, and 1.8 µg g^{-1} DT in plants treated with oligo-carrageenans K, L, and I which correspond to increases of 2.2, 2.2, and 3.6 times, respectively (Fig. 6a). These increases were significantly different between treatments and the highest increase in ASC level was detected with oligo-carrageenan I. In addition, the DHA level decreased in treated plants (Fig. 6b). The ratio ASC/DHA in control plants was 1.1 and increased to 6.6, 3.3, and 10.8 in plants treated with oligo-carrageenans L, K, and I, respectively (data not shown). In contrast, the levels of GSH (Fig. 6c) and GSSG (Fig. 6d) were similar in control and treated plants and the ratio GSH/GSSG was 2.5. On the other hand,

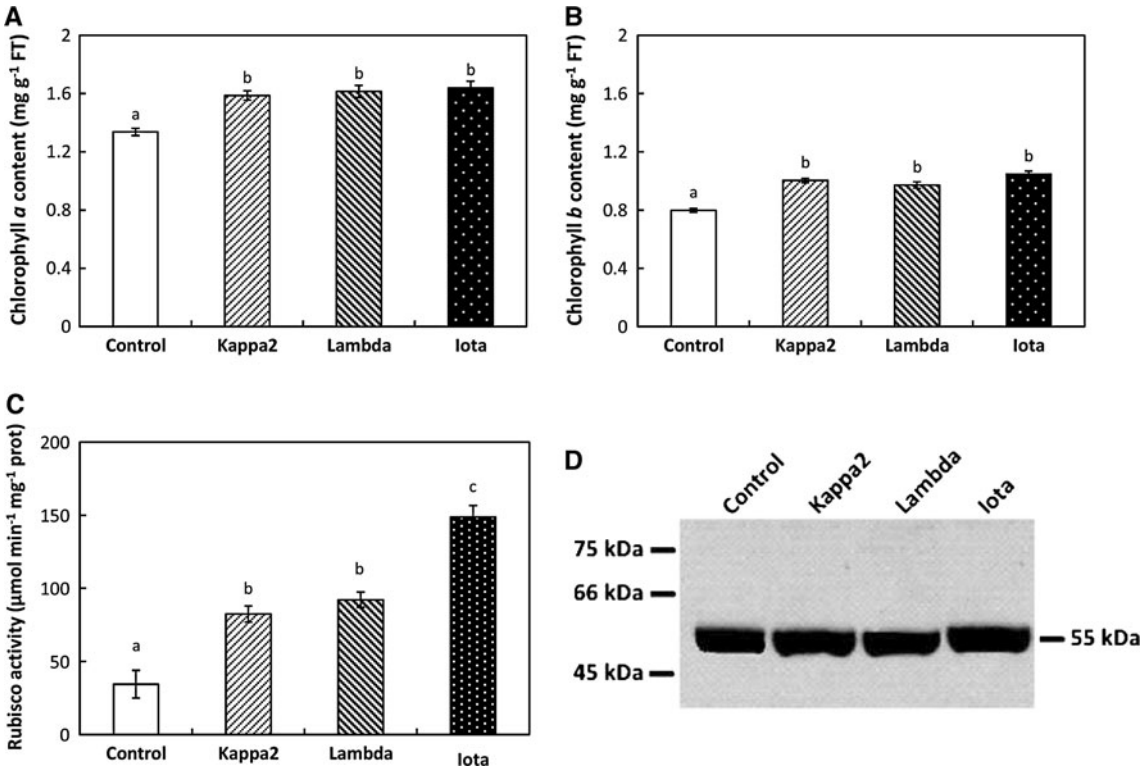


Fig. 3 Level of chlorophyll *a* (**a**) and chlorophyll *b* (**b**), rubisco activity (**c**), and rubisco content (**d**) in control tobacco plants and in plants treated with oligo-carrageenans kappa2, lambda, and iota. The levels of chlorophyll *a* and *b* are expressed in mg g^{-1} fresh tissue (FT) and rubisco activity is expressed in µmol min^{-1} mg^{-1} protein. The large subunit of rubisco (55 kDa) is indicated by a hyphen as are molecular weights of protein standards. *Bars* represent mean values of ten independent replicates ± standard deviation. *Different letters* indicate significant differences ($P < 0.05$)

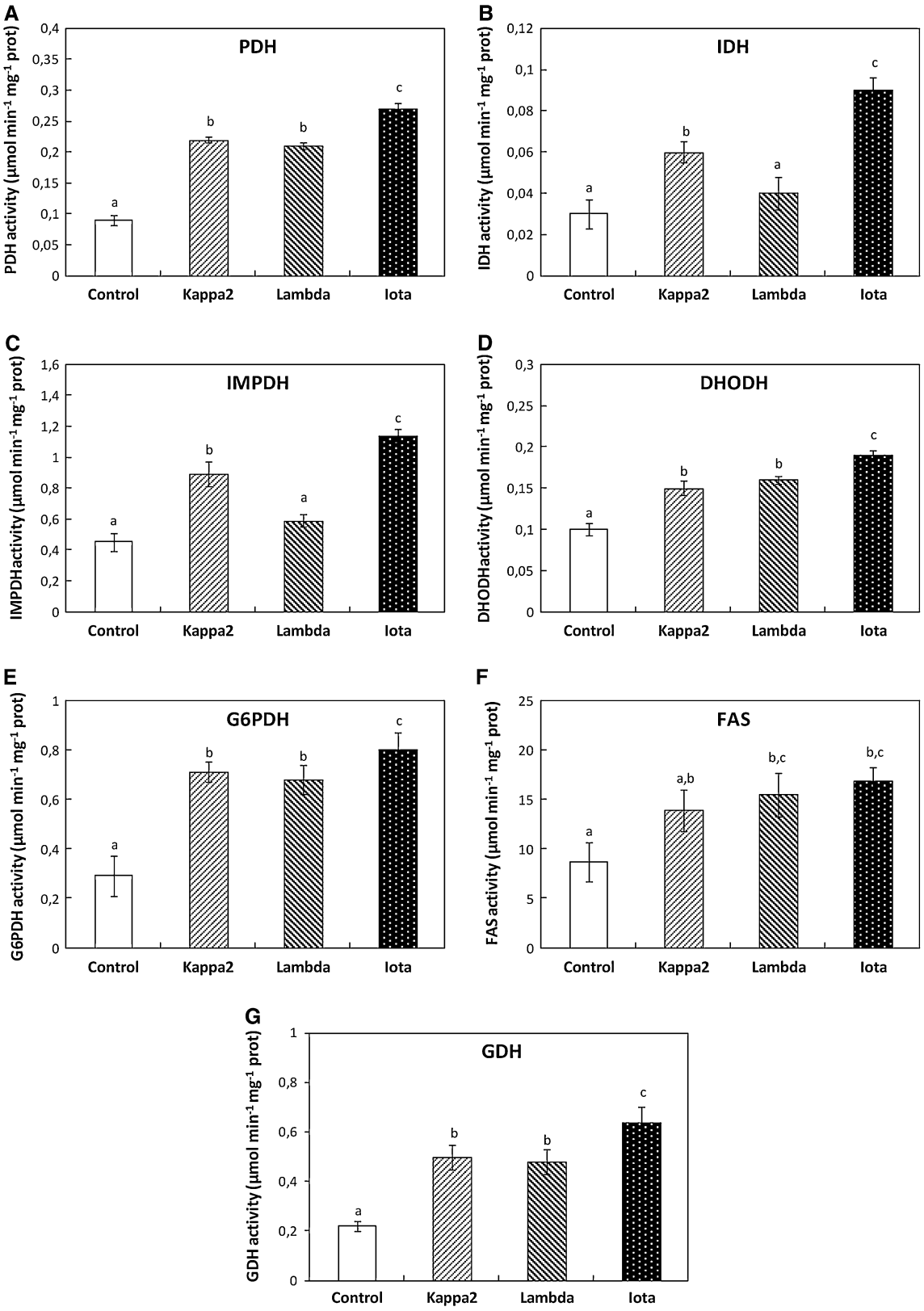
AP activity increased from 0.6 µmol min^{-1} mg^{-1} of protein in control plants to 1.5, 1.1, and 2.6 min^{-1} mg^{-1} of protein in plants treated with oligo-carrageenans K, L, and I which correspond to increases of 2.5, 1.8, and 4.3 times, respectively (Fig. 6e). These increases were significantly different between treatments and the highest increase in AP activity was detected with oligo-carrageenan I. Interestingly, the increase in total ascorbate (ASC + DHA) showed a linear correlation ($r^2 = 0.89$) with the increase in plant biomass (Fig. 6f) and the increase in AP activity ($r^2 = 0.99$, data not shown). Thus, oligo-carrageenans induce an increase in ASC level and AP activity that linearly correlate with the increase in leaf biomass indicating that both parameters may contribute to stimulation of plant growth.

Discussion

Oligo-Carrageenans Increased Tobacco Biomass and Stimulated Photosynthesis

In this study we showed that oligo-carrageenans induced an increase in leaf biomass and net photosynthesis in tobacco plants (var. Burley). Interestingly, these oligo-saccharides also induced an increase in net photosynthesis and rubisco activity in sugar beet plants (Moenne and others 2010). It is important to mention that oligo-carrageenans induced a greater increase in plant biomass than that obtained in Free-Air CO_2 Enrichment (FACE) field experiments performed with a high concentration of CO_2, that is, 580 ppm (Ainsworth and Rogers 2007; Leaky and others 2009). FACE experiments showed an increase in crop and legume biomass of 13 and 19%, respectively, which were lower than the theoretical values expected in an atmosphere containing 580 ppm of CO_2, that is, 38%. The enhancement in net photosynthesis determined in plants treated with oligo-carrageenans resides in a concomitant increase in CO_2 incorporation and stomatal conductance. This

Fig. 4 Activities of basal metabolism enzymes in control tobacco ▶ plants and in plants treated with oligo-carrageenans kappa2, lambda, and iota. Activities of pyruvate dehydrogenase (PDH, **a**), isocitrate dehydrogenase (IDH, **b**), inosine monophosphate dehydrogenase (IMPDH, **c**), dehydroorotate dehydrogenase (DHODH, **d**), glucose 6-phosphate dehydrogenase (G6PDH, **e**), fatty acid synthase (FAS, **f**), and glutamate dehydrogenase (GDH, **g**) are expressed as µmol min^{-1} mg^{-1} protein. Bars represent mean values of 10 independent replicates ± standard deviation. *Different letters* indicate significant differences ($P < 0.05$)


A
PDH
PDH activity (µmol min-1 mg-1 prot)
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
a
b
b
c
Control
Kappa2
Lambda
Iota
B
IDH
IDH activity (µmol min-1 mg-1 prot)
0,12
0,1
0,08
0,06
0,04
0,02
0
a
b
a
c
Control
Kappa2
Lambda
Iota
C
IMPDH
IMPDHactivity (µmol min-1 mg-1 prot)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
a
b
a
c
Control
Kappa2
Lambda
Iota
D
DHODH
DHODHactivity (µmol min-1 mg-1 prot)
0,3
0,25
0,2
0,15
0,1
0,05
0
a
b
b
c
Control
Kappa2
Lambda
Iota
E
G6PDH
G6PDH activity (µmol min-1 mg-1 prot)
1
0,8
0,6
0,4
0,2
0
a
b
b
c
Control
Kappa2
Lambda
Iota
F
FAS
FAS activity (µmol min-1 mg-1 prot)
25
20
15
10
5
0
a
a,b
b,c
b,c
Control
Kappa2
Lambda
Iota
G
GDH
GDH activity (µmol min-1 mg-1 prot)
1
0,8
0,6
0,4
0,2
0
a
b
b
c
Control
Kappa2
Lambda
Iota

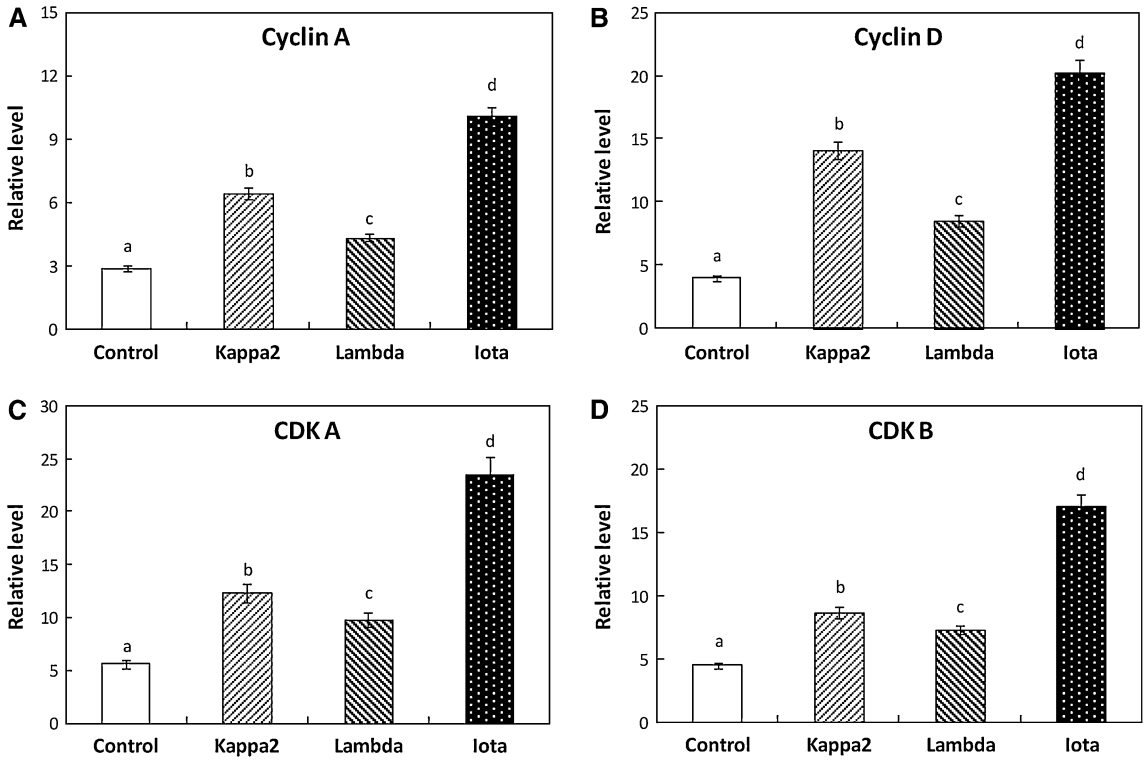


Fig. 5 Relative transcript levels of cyclin A (**a**), cyclin D (**b**), CDK A (**c**), and CDK B (**d**) in control tobacco plants and in plants treated with oligo-carrageenans kappa2, lambda, and iota. *Bars* represent mean values of three independent replicates ± standard deviation. *Different letters* indicate significant differences ($P < 0.05$)

contrasts with results obtained from FACE experiments where the increase in atmospheric CO_2 leads to an increase in CO_2 incorporation but to a decrease in stomatal conductance (Leaky and others 2009). Mechanisms determining the coordinated increase of internal CO_2 concentration and stomatal conductance induced by oligo-carrageenans in tobacco plants remain to be determined.

Oligo-Carrageenans Increased Chlorophyll Content, PSII Efficiency, and Rubisco Activity

Oligo-carrageenans induced the increase of chlorophyll *a* and *b* content in tobacco plants and the efficiency of PSII. In this sense, the increase in the level of total chlorophyll may determine the enhancement of quantum efficiency of PSII induced by oligo-carrageenans. In addition, treated plants showed an increase in rubisco activity which was not accompanied by a higher enzyme content, indicating that the increase in rubisco activity may be due to enzyme regulation. It has been shown that rubisco activity is mainly regulated by the ATP-dependent chaperone rubisco activase. This protein interacts with the large subunit of the enzyme inducing a conformational change that removes inhibitory sugar phosphates bound to the active site (Portis and others 2008). It has been shown that one of the two isoforms of rubisco activase is regulated by the redox state in *Arabidopsis thaliana* and spinach chloroplast and that its reduction involves thioredoxins which are in turn reduced by NADPH-dependent thioredoxin reductases (Zhang and Portis 1999; Motohashi and others 2001). Thus, the activation of rubisco activase is dependent on the content of NADPH in chloroplasts which is a by-product of photosynthesis. Interestingly, tobacco chloroplasts contain only the small form of rubisco activase which is also activated by thioredoxins (Ruuska and others 2000). In this study, it was shown that oligo-carrageenans increased net photosynthesis which may lead to an enhancement in NADPH content, which may in turn induce a shift in the intracellular redox state to a more reducing condition favoring the activation of thioredoxins, rubisco activase, and rubisco enzyme. On the other hand, oligo-carrageenans induced an increase in total ASC level and in AP activity (see below). In this sense, it has been shown that the increases in ASC level and AP activity protect chloroplasts from photooxidative stress (Chen and Gallie 2005; Talla and others 2011). Thus, the increase in photosynthesis, NADPH content, rubisco activity, ASC level and AP activity may favor carbon fixation in chloroplasts leading to the stimulation of plant growth.

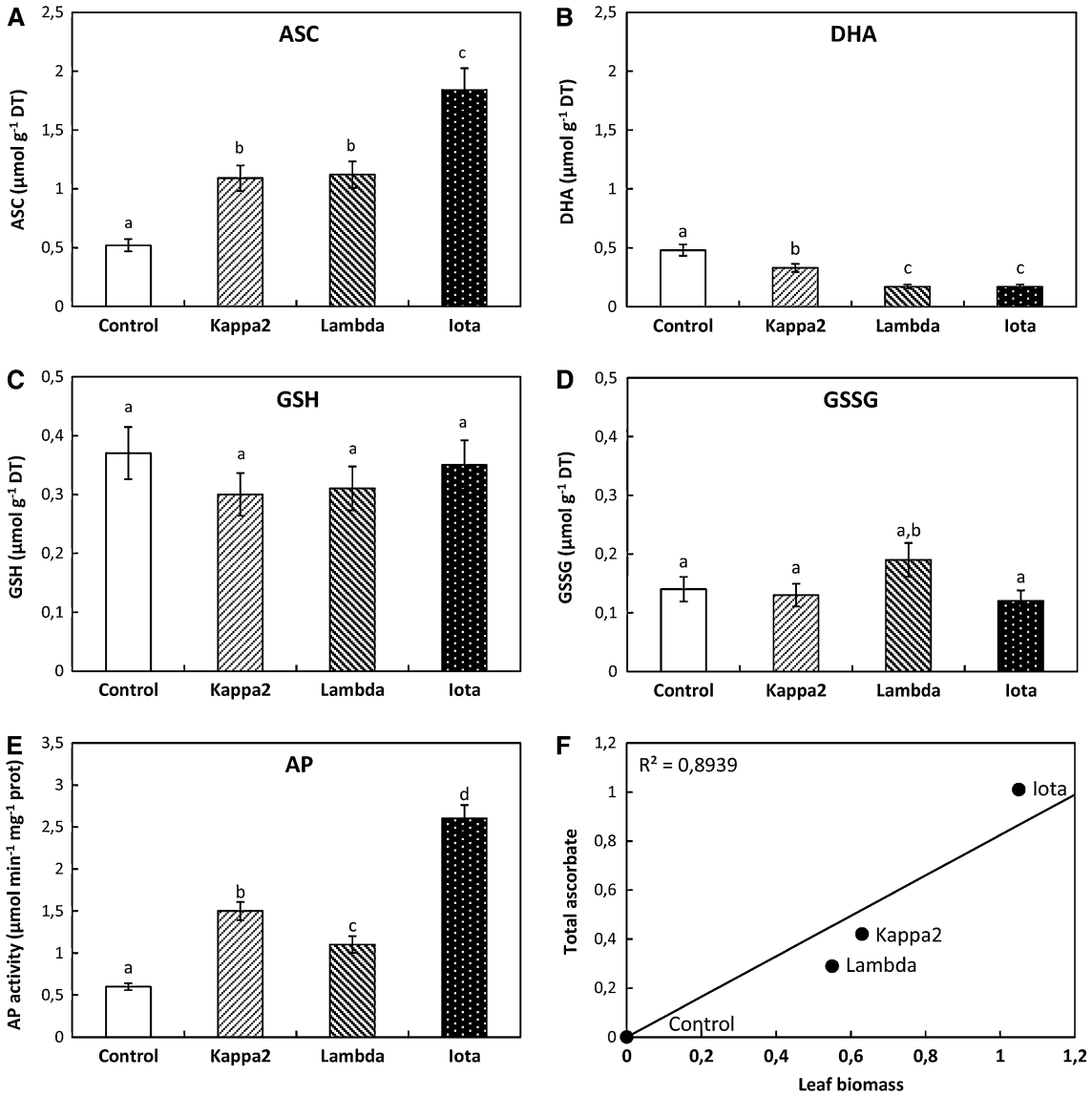


Fig. 6 Level of ascorbate (ASC, **a**), dehydroascorbate (DHA, **b**), reduced glutathione (GSH, **c**), oxidized glutathione (GSSG, **d**), and ascorbate peroxidase activity (AP, **e**) in control tobacco plants and in plants treated with oligo-carrageenans kappa2, lambda, and iota. The levels of ASC, GSH, DHA, and GSSG are expressed in µmol g^{-1} dry tissue (DT) and AP activity is expressed in µmol min^{-1} mg^{-1} protein. Bars represent mean values of 10 independent replicates ± standard deviation. *Different letters* indicate significant differences ($P < 0.05$). Correlation between the increase in ASC content (µmol g^{-1} DT) and the increase in leaf biomass (kg DT) (**f**)

Oligo-Carrageenans Increased Activities of Enzymes Involved in Basal Metabolism

Oligo-carrageenans induced the increase in activities of several enzymes of basal metabolism located in different cellular compartments. In particular, oligo-carrageenans induced the activation of PDH and IDH, which are NADH-synthesizing enzymes of the Krebs cycle, located in mitochondria (Malhotra and Spencer 1970). In addition, they induced the activation of the NADH-synthesizing enzyme glyceraldehyde 3-phosphate dehydrogenase (data not shown) located in chloroplasts and in the cytoplasm (Winter and others 1982). Furthermore, oligo-carrageenans induced the activation of the NADPH-synthesizing enzyme G6PDH, involved in ribose synthesis, which is located in chloroplasts and cytoplasm in plants (Esposito and others 2001), and that of the NADH-synthesizing enzymes IMPDH and DHODH, involved in purine and pyrimidine synthesis, which are located in the cytoplasm (Christensen and Jochimsen 1983). Thus, the activation of several NAD(P)H-synthesizing enzymes may determine an increase in NAD(P)H content in different cellular compartments, changing the intracellular redox state to a more reducing condition which may favor photosynthesis (see above) and

cell division (see below). Furthermore, oligo-carrageenans induced an increase in the activity of GDH, a key enzyme of nitrogen assimilation and amino acid synthesis (Forde and Lea 2007), suggesting that nitrogen fixation is also increased in plants treated with oligo-carrageenans, which may also contribute to the stimulation of plant growth.

Oligo-Carrageenans Increased the Expression of Cell Cycle Regulatory Proteins

Oligo-carrageenans did not induce an increase in tobacco cell size, indicating that the increase in leaf biomass is determined by an increase in cell number. In this sense, it was determined that the level of transcripts encoding cyclins A and D and CDKs A and B is increased in plants treated with oligo-carrageenans, indicating a promitotic effect of oligo-carrageenans in tobacco plants. In addition, it has been shown that ASC can stimulate cell cycle progression and mitosis (De Pinto and others 1999, 2004) and that its level increases during seed germination (Arrigoni and others 1992; Tomassi and others 2001) and in meristematic tissues (Córdoba-Pedregosa and others 2005). Therefore, the increase in ASC levels detected in plants treated with oligo-carrageenans may stimulate cell cycle progression and cell division leading to an increase in plant growth.

Oligo-Carrageenans Increased Ascorbate Level and AP Activity

Oligo-carrageenans, mainly oligo-carrageenan I, induced an increase in total ASC level, a decrease in DHA level, and an increase in AP activity. It has been shown that tobacco plants treated with Poly-Ga, an oligosaccharide structurally related to oligo-carrageenans, exhibited an increase in total ASC level and AP activity (Laporte and others 2007). In addition, the increase in AP activity in plants treated with Poly-Ga linearly correlated with the increase in plant biomass, suggesting that the stimulation of AP activity may contribute to plant growth (Laporte and others 2007). In this work we showed that oligo-carrageenans also induced an increase in total ASC level and AP activity and that the increase in both parameters linearly correlated with the increase in plant biomass. Thus, the increase in ASC level and in AP activity may be a key factor in the regulation of plant growth. On the other hand, the level of GSH and GSSG did not change in plants treated with oligo-carrageenans, which contrasts with the increase in GSH level observed in plants treated with Poly-Ga (Laporte and others 2007). Interestingly, the ratio GSH/GSSG in control tobacco plants var. Burley was similar to that detected in control tobacco plants var. Xanthi. It is important to mention that Poly-Ga and oligo-carrageenans may trigger different signal transduction pathways because oligo-carrageenans did not increase the GSH level in tobacco plants, as did Poly-Ga.

It is interesting to point out that oligo-carrageenan I is the oligosaccharide that induced the greatest increase in tobacco plant biomass, in rubisco and GDH activities (carbon and nitrogen fixation), in NAD(P)H-synthesizing enzyme activities, in cyclins and CDKs transcript levels, in ASC level, and in AP activity, suggesting that these parameters are key factors for plant growth regulation.

In summary, oligo-carrageenans, mainly oligo-carrageenan I, stimulate photosynthesis, basal metabolism, and cell cycle, inducing an increase in growth and biomass in tobacco plants (var. Burley). These oligosaccharides may induce a shift in the cellular redox state to a more reducing condition which may stimulate cell division leading to an increase in plant growth.

Acknowledgments This study was financed by Sirius Natura S.A.

References

Ainsworth EA, Rogers A (2007) The response of photosynthesis and stomatal conductance to rising [CO_2]: mechanisms and environmental interactions. Plant Cell Environ 30:258–270

Arrigoni O, De Tullio MC (2002) Ascorbic acid: much more than just an antioxidant. Biochim Biophys Acta 1569:1–9

Arrigoni O, De Gara L, Tomassi F, Liso R (1992) Changes in the ascorbate system during seed development of *Vicia faba* L. Plant Physiol 99:235–238

Bilisics L, Vojtassák J, Capek P, Kollárová K, Lisková D (2004) Change in glycosidase activities during galactoglucomannan oligosaccharide inhibition of auxin induced growth. Phytochemistry 65:1903–1909

Bradford MM (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chen Z, Gallie DR (2005) Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. Plant Physiol 138:1673–1689

Christensen TM, Jochimsen TU (1983) Enzymes of ureide synthesis in pea and soybean. Plant Physiol 72:56–59

Córdoba-Pedregosa MC, Villalba JM, Córdoba F, González-Reyes JA (2005) Changes in intracellular and apoplastic peroxidase activity, ascorbate redox status, and root elongation induced by enhanced ascorbate content in *Allium cepa* L. J Exp Bot 56:685–694

De Pinto M, De Gara L (2004) Changes in the ascorbate metabolism of apoplastic and symplastic spaces are associate with cell differentiation. J Exp Bot 55:2559–2569

De Pinto MC, Francis D, De Gara L (1999) The redox state of the ascorbate-dehydroascorbate pair as a specific sensor of cell division in tobacco BY-2 cells. Protoplasma 209:90–97

De Tullio M, Paciolla C, Dalla Vecchia F, Rascio N, D' Emerico S, De Gara L, Liso R, Arrigoni O (1999) Changes in onion root development induced by the inhibition of peptidyl-prolyl hydroxylase and influence of the ascorbate system on cell division and elongation. Planta 209:424–434

Eberhard S, Doubrava N, Marfa V, Mohnen D, Southwick A, Darvill A, Albersheim P (1989) Pectic cell wall fragments regulate tobacco thin-cell-layer explants morphogenesis. Plant Cell 1: 747–755

Esposito S, Carfagna S, Massaro G, Vona B, Di Martino Rigano B (2001) Glucose-6-phosphate dehydrogenase in barley roots: kinetic properties and localization of the isoforms. Planta 212: 627–634

Forde BG, Lea PJ (2007) Glutamate in plants: metabolism, regulation and signalling. J Exp Bot 58:2339–2358

González A, Vera J, Castro J, Dennett G, Mellado M, Morales B, Correa JA, Moenne A (2010) Co-occurring increases of calcium and organellar reactive oxygen species determine differential activation of antioxidant and defense enzymes in *Ulva compressa* (Chlorophyta) exposed to copper excess. Plant Cell Environ 33:1627–1640

Laporte D, Vera J, Chandía NP, Zúñiga E, Matsuhiro B, Moenne A (2007) Structurally unrelated algal oligosaccharides differentially stimulate growth and defense against tobacco mosaic virus in tobacco plants. J Appl Phycol 19:79–88

Lau OS, Deng XW (2010) Plant hormone signaling lightens up: integrators of light and hormones. Curr Opin Plant Biol 13: 571–577

Leaky ADB, Ainsworth EA, Bernacchi CJ, Rogers A, Long SP, Ort DR (2009) Elevated CO_2 effects on plant carbon, nitrogen, and water relations: six important lessons from FACE. J Exp Bot 60:2859–2876

Lemaitre T, Urbanczyk-Wochniak E, Flesh V, Bismuth E, Fernie AR, Hodges M (2007) NAD-dependent isocitrate dehydrogenase mutants of *Arabidopsis* suggest the enzyme is not limiting for nitrogen assimilation. Plant Physiol 144:1546–1558

Lichtenthaler HK, Wellburn AR (1983) Determinations of total carotenoids and chlorophylls *a* and *b* in leaf extracts in different solvents. Biochem Soc Trans 11:591–592

Lilley RM, Walker DA (1974) An improved spectrophotometric assay for ribulosebiphosphate carboxylase. Biochim Biophys Acta 358:226–229

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408

Lynen F (1969) Yeast fatty acid synthase. Methods Enzymol 14:17–33

Malhotra SS, Spencer M (1970) Changes in the respiratory, enzymatic, and swelling and contraction properties of mitochondria from cotyledons of *Phaseolus vulgaris* L. during germination. Plant Physiol 46:40–44

McDougall G, Fry S (1990) Xyloglucan oligosaccharides promote growth and activate cellulase: evidence for cellulase in cell expansion. Plant Physiol 93:1042–1048

Mizukami Y (2001) A matter of size: developmental control of organ size in plants. Curr Opin Plant Biol 4:533–539

Moenne A (2009) Composition and method to stimulate plant growth and defense against pathogens in plants. U.S. Patent Office Application No. 12/666,700

Moenne A, Castro J, Vera J (2010) Method to stimulate carbon fixation with an aqueous solution of oligo-carrageenans selected from kappa1, kappa2, lambda or iota. U.S. Patent Office Application No. 12/911,790

Motohashi K, Kondo A, Stumpp M, Hisabori T (2001) Comprehensive survey of proteins targeted by chloroplast thioredoxins. Proc Natl Acad Sci USA 98:11224–11229

Nge KL, Nwe N, Chandrkrachang S, Stevens W (2006) Chitosan as a growth stimulator in orchid tissue culture. Plant Sci 170: 1185–1190

Portis AR, Li CS, Wang DF, Salvucci ME (2008) Regulation of rubisco activase and its interaction with rubisco. J Exp Bot 59: 1597–1604

Reid EE, Thompson P, Lyttle R, Dennis DT (1977) Pyruvate dehydrogenase complex from higher plant mitochondria and proplastids. Plant Physiol 59:842–848

Roháček K (2002) Chlorophyll fluorescence parameters: the definitions, photosynthetic meaning, and mutual relationships. Photosynthetica 40:13–29

Ruuska SA, Andrews J, Badger MR, Price D, Von Caemmerer S (2000) The role of chloroplast electron transport and metabolites in modulating rubisco activity in tobacco. Insights from transgenic plants with reduced amounts of cytochrome b/f complex or glyceraldehyde 3-phosphate dehydrogenase. Plant Physiol 122: 491–504

Shelp BJ, Atkins CA (1983) Role of inosine monophosphate oxidoreductase in the formation of ureides in nitrogen-fixing nodules of cowpea (*Vigna unguiculata* L. Walp.). Plant Physiol 72:1029–1034

Talla S, Riazunnisa K, Padmavathi L, Sunil B, Rajsheel P, Raghavendra AS (2011) Ascorbic acid is a key participant during the interaction between chloroplast and mitochondria to optimize photosynthesis and protect against photoinhibition. J Biosci 36:163–173

Tomassi F, Paciolla C, De Pinto MC, De Gara L (2001) A comparative study of glutathione and ascorbate metabolism during germination of *Pinus pinea* L. seeds. J Exp Bot 52:1647–1654

Tran Thanh Van K, Toubart P, Cousson A (1985) Manipulation of the morphogenetic pathways of tobacco by oligosaccharins. Nature 314:615–661

Turano FJ, Dashner R, Upadhyaya A, Caldwell CR (1996) Purification of mitochondrial glutamate dehydrogenase from dark-grown soybean seedlings. Plant Physiol 112:1357–1364

Ullrich A, Knecht W, Piskur J, Löffler M (2002) Plant dehydroorotate dehydrogenase differs significantly in substrate specificity and inhibition from the animal enzymes. FEBS Lett 529:346–350

Vera J, Castro J, González A, Barrientos H, Matsuhiro B, Arce P, Zúñiga G, Moenne A (2011) Long-term protection against TMV infection is induced by the marine alga oligo-sulphated-galactan Poly-Ga in tobacco plants. Mol Plant Pathol 12:437–447

Winter K, Foster JG, Edwards GE, Holtum JA (1982) Intracellular localization of enzymes of carbon metabolism in *Mesembrianthemum cristallinum* exhibiting C(3) photosynthetic characteristics or performing crassulacean acid metabolism. Plant Physiol 69:300–307

Zar J (1999) Biostatistical analysis, 4th edn. Prentice-Hall, Englewood Cliffs

Zhang N, Portis AR (1999) Mechanism of light regulation of rubisco: a specific role for the largest rubisco activase isoform involving reductive activation by thioredoxin-*f*. Proc Natl Acad Sci USA 96:9438–9443

Zhou LA, Yang CZ, Li JQ, Wang SL, Wu JY (2003) Heptasaccharide and octasaccharide isolated from *Paris polyphylla* var. yunnanensis and their plant-growth regulatory activity. Plant Sci 165: 571–575