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PLANT PHYSIOLOGY

Glyceryl Tricaprate in Thylakoids of *Stevia rebaudiana* and Its Physiological Role

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Abstract—The short-day plant stevia (*Stevia rebaudiana*) was earlier reported to produce electron-dense thylakoids in the chloroplasts under long-day conditions. In the present work, such thylakoids were analyzed by transmission electron microscopy and gas chromatography–mass spectrometry. It was found that they contain glyceryl tricaprate, which belongs to triacylglycerols. A hypothesis is advanced that glyceryl tricaprate acts as a photoprotector for short-day plants against redundant solar radiation.

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INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni, Asteraceae) is an endemic Paraguayan plant; its critical day length for flowering is 12–14 h. Its leaves contain diterpene steviol glycosides (SGs) (Chalapathi, 1997; Brandle et al., 1998; Soejarto, 2002). Under a long day, the plant intensively vegetates without a transfer to flowering. It was earlier shown by us that electron-dense thylakoids are present in the stevia leaves under these conditions (Sukhanova et al., 2007; Ladygin et al., 2008; Bondarev et al., 2010). Such thylakoids were earlier observed in such species as the perilla and potato (Kashina et al., 1981; Semenova, 1985), i.e., in the introduced plants. It should be noted that perilla is also a short-day plant, similar to stevia in this regard. It was found that the electron-dense thylakoids appear in perilla under constant illumination, while typical electron-transparent thylakoids were observed under a short day (Kashina et al., 1981). Danilova and Kashina (1999) have hypothesized that the revealed thylakoid dimorphism in perilla chloroplasts (the electron-transparent thylakoids as a regular type and the electron-dense ones as a modified type) is the structural basis for the contribution of chloroplasts to photoperiodical light reactions. However, repeated experimental attempts have failed both to identify the electron-dense stuff inside thylakoids and to propose its nature so far.

The objective of this study was to elucidate the nature and physiological role of the electron-dense compounds in the chloroplast thylakoids of *Stevia rebaudiana* that emerge upon its growing under a long day.

MATERIALS AND METHODS

Plants of *S. rebaudiana* Bertoni were grown in a glass greenhouse under natural illumination on a soil mixture of equal parts of turf soil, sand, and peat at 30 ± 5°C. The material for transmission electron microscopy was prepared as previously described (Bondarev et al., 2010).

The intact chloroplasts and thylakoids were isolated from stevia leaves to examine the content of the electron-dense thylakoids. Leaves (100 g) were homogenized in A buffer (0.33 M sorbitol, 2 mM EDTA, 5 mM β-mercaptoethanol, and 50 mM tricine, pH 8). The homogenate was filtered through one layer of gauze and two layers of Miracloth (Calbiochem-Behring, United States) followed by centrifugation. The pellet of organelles was resuspended in A buffer and fractionated in a percoll step gradient 40 to 70% upon 30-min centrifugation at 4000 *g*. The intact chloroplasts were harvested from the border between 40 and 70% percoll, washed with A buffer, sedimented, and resuspended in the same buffer. Afterward, they were lysed by hypoosmotic shock and the thylakoids were sedimented by centrifugation. After disruption of the thylakoid membranes, their content was isolated and its chemical composition was analyzed by gas-liquid chromatography–mass spectrometry. For this purpose, an Agilent Technologies (United States) device was used. It consisted of a 7890 gas chromatographer (HP-5 column 50 m × 320 μm × 1.05 μm) and a 5975C mass-selective detector with a quadrupole mass analyzer.

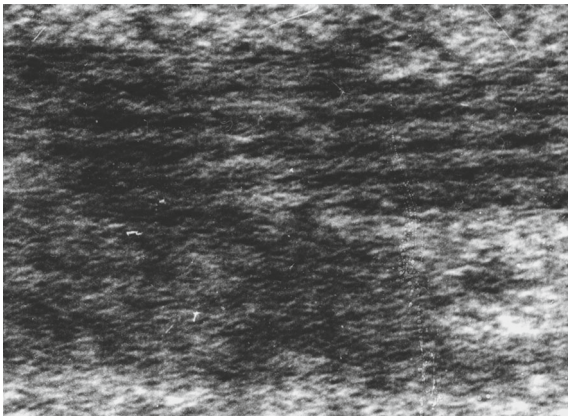

Fig. 1. Thylakoids of the chloroplasts of stevia leaf parenchyma filled with an electron-dense material.

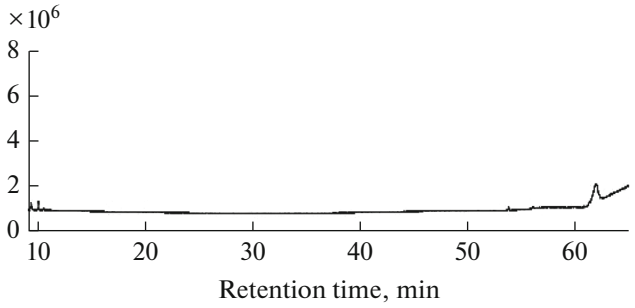

Fig. 2. Chromatogram of the sample isolated from the content of electron-dense thylakoids of stevia.

Conditions of chromatography. The temperature program was a succession of an isotherm at 40°C for 2 min, a programmed heating up to 250°C at 5°C/min, an isotherm at 250°C for 15 min, a programmed heating to 320°C at 25°C/min, and an isotherm at 320°C for 5 min. The sample volume was 1 μL. The injector separated the flow 1 : 50, the injector temperature was 250°C, the interface temperature was 280°C, and the carrier gas was helium at a flow rate of 1 mL/min. Sample chromatograms were drawn by the full ionic current. The ChemStation E 02.00 software was used.

Conditions of mass spectrometry. Electron ionization proceeded at the energy of ionizing electrons 70 eV, and registration of mass-spectra in positive ions was at $m/z = 20–450$, where m is the mass of a particle and z is its charge. The scan rate was 2.5 scans/s. Identification of the component content (qualitative analysis) was performed with the help of the NIST-05 library of complete mass-spectra.

RESULTS AND DISCUSSION

Transmission microscopy showed that the entire intrathylakoid space of the chloroplasts of stevia leaves was filled with electron-dense matter (Fig. 1). In this regard, the electron-dense thylakoids were isolated by the technique described above and their content was analyzed by chromatography–mass spectrometry.

Figure 2 represents the resulting chromatogram. The presence of glyceryl tricaprate (GTC) or glyceryl tridecanoate, which belongs to triacylglycerols (TAGs), was found in this sample. The mass-spectrum obtained by detection of the compound corresponding to the peak (with a retention time of 61.88 min) is given in Fig. 3. Comparison of the obtained mass-spectrum with that of GTC taken from the NIST-05 database found a fairly good coincidence of both sets of the fragment ions and the relative intensities of the corresponding peaks.

It should be highlighted that GTC, as a component of essential oils, may fulfill adaptive functions in the plant organism. It comprises residues of a saturated fatty acid (FA)—*n*-capric acid—possessing ten carbon atoms in the molecule. It was shown that the cell is prone to accumulation of the TAGs bearing residues of saturated FAs because their transformation into the necessary polyunsaturated FAs requires less energy, whereas on the contrary, oxidation of these compounds liberates more energy (Solovchenko, 2012).

TAGs are known to be essential storage compounds—sources of energy and carbon—in higher plants. There are two biosynthetic pathways yielding TAGs in plants. One path is situated in plastids (Banaš et al., 2000; Lung and Weselake, 2006; Durrett et al., 2008), and glycero-3-phosphate is a source of the glycerol residue in this case (Weselake et al., 2009).

The specificity of acyl transferases—the enzymes participating in TAG biosynthesis—may be a key factor determining biosynthesis of TAGs involving unusual FAs with an intermediate chain length (10 to 14 carbon atoms) (Frentzen, 1993). In particular, the specificity of glycerol-3-phosphate acyl transferase may be employed by plants in their responses to the stress caused, for example, by a sharp decrease in the ambient temperature (Turnbull et al., 2001). In addition, different plant species may have different isoforms of lysophosphatidic acid acyl transferases, which may be specific to acyls with an intermediate chain length. For instance, the microsomes of embryos of the cigar flower (*Cuphea lanceolata*) synthesize dicaprinoil-diacylglycerols in a culture in vitro (Bafor et al., 1990). A similar incorporation of the above-mentioned FAs into TAGs was also found in different plant species in vitro (Wiberg, 1994). These data suggest that such a biosynthetic specificity is a stress response to conditions in vitro.

Importantly, higher plants deposit TAGs in the cytoplasmic oleosomes or lipid globules (Athenstaedt and Daum, 2006). Microalgae also accumulate TAGs in similar structures (Solovchenko, 2012), but sometimes TAG-containing lipid globules appear in the interthylakoid space (Hu et al., 2008). In stevia, in

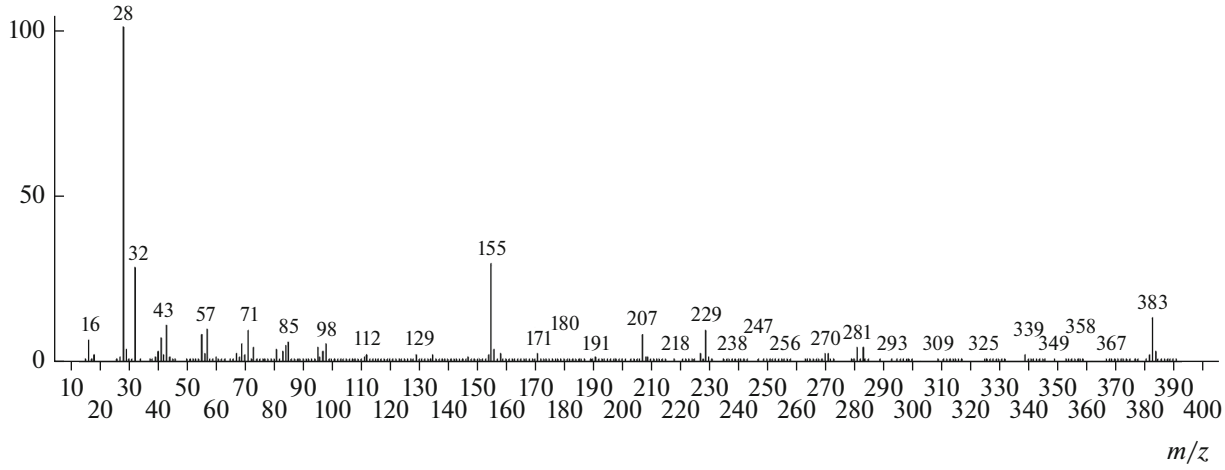

Fig. 3. Mass-spectrum of glyceryl tricaprate in the sample isolated from the content of electron-dense thylakoids of stevia.

addition to the GTK in thylakoids identified here, we earlier observed roundish electron-dense lipid globules in the cytoplasm (Sukhanova et al., 2007).

Oleosomes have been discovered in many types of plant cells. Details of TAG biosynthesis in tissues other than seeds have not been satisfactory disclosed. It is most likely that they are synthesized in response to stresses or attacks of pathogens (Chapman et al., 2012). It was reported that the oil content in seeds of soybean, rapeseed, and *Arabidopsis* increases under excessive illumination (Ruuska et al., 2004; Goffman et al., 2005).

It is important to say that eukaryotic microalgae also broadly use TAGs in the processes of adaptations to the environment. Thus, in cells of many species of microalgae, stress factors, such as intense photosynthetically active radiation (PAR), entail TAG accumulation (Merzlyak et al., 2007). Biosynthesis of TAGs creates a sink for photoassimilates. This prevents photooxidative damage conferred by those stresses, which reduce the cell potential of utilization of photosynthesis products (Hu et al., 2008; Lemoine and Schoefs, 2010). Carotenoids are well-known potent antioxidants functioning as free radical scavengers and singlet oxygen quenchers (Ladygin and Shirshikova, 2006). In this regard, TAGs create a depot of secondary carotenoids that act as an optical shield against excessive PAR and protect against photodamage as a consequence (Solovchenko, 2012). A tight relation is established between syntheses of secondary carotenoids and TAGs (Solovchenko, 2013).

It is interesting that accumulation of SGs is also significantly increased in leaves of stevia under redundant light (Bondarev et al., 2008, 2012). This may be interpreted to mean that surplus PAR often elevates levels of reactive oxygen species (Ort, 2001), while diterpene glycosides of stevia have a strong antioxidant capacity as revealed recently (Stoyanova et al., 2011).

Therefore, the intense biosyntheses of GTK and SGs in leaves of stevia, presumably, hinder development of photooxidative damage. The latter may be associated with an increased level of reactive oxygen in the short-day plants when they are exposed to a long-day regimen. In addition, accumulation of GTK, as well as different TAGs, in plant cells entails depot formation of secondary carotenoids that screen an intense PAR and, hence, protect against photodamage.

REFERENCES

Athenstaedt, K. and Daum, G., The life cycle of neutral lipids: synthesis, storage and degradation, *Cell Mol. Life Sci.*, 2006, vol. 63, pp. 1355–1369.

Bafor, M., Jonsson, L., Stobart, A.K., and Stymne, S., Regulation of triacylglycerol biosynthesis in embryos and microsomal preparations from the developing seeds of *Cuphea lanceolata, Biochem. J.*, 1990, vol. 272, pp. 31–38.

Banaš, A., Dahlqvist, A., Ståhl, U., Lenman, M., and Stymne, S., The involvement of phospholipid: diacylglycerol acyltransferases in triacylglycerol production, *Biochem. Soc. Trans.*, 2000, vol. 28, pp. 703–705.

Bondarev, N.I., Reshetnyak, O.V., and Nosov, A.M., Effect of photoperiod and irradiation intensity on the development of *Stevia rebaudiana* shoots in vitro and the synthesis of steviol glycosides in them, *Izv. Timiryaz. S.-Kh. Akad.*, 2008, no. 4, pp. 102–107.

Bondarev, N.I., Sukhanova, M.A., Semenova, G.A., Goryaeva, O.V., Andreeva, S.E., and Nosov, A.M., Morphology and ultrastructure of trichomes of intact and in vitro plants of *Stevia rebaudiana* Bertoni with reference to biosynthesis and accumulation of steviol glycosides, *Moscow Univ. Biol. Sci. Bull.*, 2010, vol. 65, no. 1, pp. 12–16.

Bondarev, N.I., Reshetnyak, O.V., Bondareva, T.A., and Nosov, A.M., Distribution and dynamics of the content of diterpene glycosides in intact *Stevia rebaudiana* Bertoni plants, *Biotekhnologiya*, 2012, no. 5, pp. 50–54.

Brandle, J.E., Starratt, A.N., and Gijzen, M., *Stevia rebaudiana*: its agricultural, biological and chemical properties, *Can. J. Plant. Sci.*, 1998, vol. 78, pp. 527–536.

Chalapathi, M.K., Natural non-calorie sweetener stevia (*Stevia rebaudiana* Bertoni), *Future Crop India. Crop Res.*, 1997, vol. 14, pp. 347–350.

Chapman, K.D., Dyer, J.M., and Mullen, R.T., Biogenesis and functions of lipid droplets in plants, *J. Lipid Res.*, 2012, vol. 53, pp. 215–226.

Danilova, M.F. and Kashina, T.K., *Strukturnye osnovy aktinoritmicheskoi regulyatsii tsveteniya* (Structural Basis of Actinorhythmic Flowering Regulation), St. Petersburg: Nauka, 1999.

Durrett, T.P., Benning, C., and Ohlrogge, J., Plant triacylglycerols as feedstocks for the production of biofuels, *Plant J.*, 2008, vol. 54, pp. 593–607.

Frentzen, M., Acyltransferases and triacylglycerols, in *Lipid Metabolism in Plants*, Moore, T.S., Jr., Ed., Boca Raton, FL: CRC Press, 1993, pp. 195–230.

Goffman, F.D., Alonso, A.P., Schwender, J., Shachar-Hill, Y., and Ohlrogge, J.B., Light enables a very high efficiency of carbon storage in developing embryos of rapeseed, *Plant Physiol.*, 2005, vol. 138, pp. 2269–2279.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., and Darzins, A., Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances, *Plant J.*, 2008, vol. 54, pp. 621–639.

Kashina, T.K., Danilova, M.F., and Moshkov, B.S., Ultrastructure of chloroplasts in the leaves of *Perilla ocymoides* (Lamiaceae) and photoperiodic control of flowering, *Bot. Zh.*, 1981, vol. 66, no. 12, pp. 1685–1694.

Ladygin, V.G. and Shirshikova, G., Modern ideas about the functional role of carotenoids in eukaryotic chloroplasts, *Zh. Obshch. Biol.*, 2006, vol. 67, pp. 163–190.

Ladygin, V.G., Bondarev, N.I., Semenova, G.A., Smolov, A.A., Reshetnyak, O.V., and Nosov, A.M., Chloroplast ultrastructure, photosynthetic apparatus activities and production of steviol glycosides in *Stevia rebaudiana* in vivo and in vitro, *Biol. Plant.*, 2008, vol. 52, no. 1, pp. 9–16.

Lemoine, Y. and Schoefs, B., Secondary ketocarotenoid astaxanthin biosynthesis in algae: a multifunctional response to stress, *Photosynth. Res.*, 2010, vol. 106, pp. 155–177.

Lung, S.C. and Weselake, R.J., Diacylglycerol acyltransferase: a key mediator of plant triacylglycerol synthesis, *Lipids*, 2006, vol. 41, pp. 1073–1088.

Merzlyak, M., Chivkunova, O., Gorelova, O., Reshetnikova, I., Solovchenko, A., Khozin-Goldberg, I., and Cohen, Z., Effect of nitrogen starvation on optical properties, pigments, and arachidonic acid content of the unicellular green alga *Parietochloris incisa* (Trebouxiophyceae, Chlorophyta), *J. Phycol.*, 2007, vol. 43, pp. 833–843.

Ort, D., When there is too much light, *Plant Physiol.*, 2001, vol. 125, pp. 29–32.

Ruuska, S.A., Schwender, J., and Ohlrogge, J., The capacity of green oilseeds to utilize photosynthesis to drive biosynthetic processes, *Plant Physiol.*, 2004, vol. 136, pp. 2700–2709.

Semenova, G.A., Electron-dense material in the mesophyll cells of potato leaves, 1985, vol. 32, no. 3, pp. 461–464.

Soejarto, D.D., Ethnobotany of *Stevia* and *Stevia rebaudiana*, in *Stevia: The Genus Stevia*, Kinghorn, A.D., Ed., London: Taylor Francis, 2002, pp. 40–67.

Solovchenko, A.E., Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses, *Russ. J. Plant Physiol.*, 2012, vol. 59, no. 2, pp. 167–176.

Solovchenko, A.E., Physiology and adaptive significance of secondary carotenogenesis in green microalgae, *Russ. J. Plant Physiol.*, 2013, vol. 60, no. 1, pp. 1–13.

Stoyanova, S., Geuns, J., Hideg, E., and Ende, W., The food additives inulin and stevioside conteract oxidative stress, *Int. J. Food Sci. Nutr.*, 2011, vol. 62, no. 3, pp. 207–214.

Sukhanova, M.A., Bondarev, N.I., Goryaeva, O.V., Andreeva, S.E., and Nosov, A.M., Ultrastructural characterization of plant cells and callus cultures of *Stevia rebaudiana* in relation to the synthesis of steviol glycosides, *Biotekhnologiya*, 2007, no. 5, pp. 51–59.

Turnbull, A.P., Rafferty, J.B., Sedelnikova, S.E., Slabas, A.R., Schierer, T.P., Kroon, J.T., Simon, J.W., Fawcett, T., Nishida, I., Murata, N., and Rice, D.W., Analysis of the structure, substrate specificity, and mechanism of squash glycerol-3-phosphate (1)-acyltransferase, *Structure*, 2001, vol. 9, pp. 347–353.

Weselake, R.J., Taylor, D.C., Rahman, M.H., Shah, S., Laroche, A., McVetty, P., and Harwood, J., Increasing the flow of carbon into seed oil, *Biotechnol. Adv.*, 2009, vol. 27, pp. 866–878.

Wiberg, E., Tillberg, E., and Stymne, S., Substrates of diacylglycerol acyltransferase in microsomes from developing oil seeds, *Phytochemistry*, 1994, vol. 36, pp. 573–577.

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