Agronomy Research **9** (Special Issue II), 501–507, 2011

Differences in Rubisco and Chlorophyll Content among Tissues and Growth Stages in Two tomato (*Lycopersicon esculentum Mill.*) Varieties

R. Vicente[1,2], R. Morcuende[1] and J. Babiano[2]

[1]Institute of Natural Resources and Agrobiology of Salamanca, IRNASA–CSIC, Apartado 257, 37071 Salamanca, Spain; e-mails: ruben.vicente@irnasa.csic.es; rosa.morcuende@irnasa.csic.es

[2]University of Salamanca, Department of Plant Physiology, Campus Miguel de Unamuno, 37008 Salamanca, Spain; e-mail: babiano@usal.es

Abstract. Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) is a key enzyme in the photosynthetic assimilation of CO_2 and the most abundant leaf protein. The amounts of chlorophyll (chl) and Rubisco have often been considered, respectively, as indices of light harvesting and Calvin cycle capacities of leaves. The purpose of this study was to analyze the changes in chlorophyll content and the level of Rubisco protein in various plant tissues at different growth stages in two tomato (*Lycopersicon esculentum Mill.*) varieties. The results show an increase of the amount of both chlorophyll and Rubisco protein at vegetative growth stages (leaf expansion), which was followed by a gradual decline during anthesis, probably as a consequence of changes in the balance of their synthesis and degradation reported previously – Rubisco could be remobilized and reused in the production of reproductive structures. However, the increase in the amount of Rubisco and chlorophyll at ripening stage (more in Tres Cantos variety) contrasts with the decrease reported in other studies when degradation is becoming predominant during senescence.

Key words: chlorophyll, growth stages, leaf nitrogen, protein, Rubisco, tissues, tomato

INTRODUCTION

The rate of photosynthesis and biomass accumulation depends largely on the quantity and activity of Rubisco (Lorimer, 1981). Rubisco is the first and key enzyme in the Calvin cycle of photosynthetic assimilation of CO_2 in C_3 plants. It catalyzes the fixation of atmospheric CO_2 to ribulosa-1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglycerate (3PGA), which is subsequently used to build organic molecules. The enzyme is extremely inefficient and its carboxylation activity is compromised by competing side–reactions, the most notable being with another atmospheric gas, O_2. Both CO_2 and O_2 are mutually competitive at the same large-subunit active site. Whereas carboxylation accounts for net CO_2 fixation, oxygenation leads to the loss of CO_2 in the photorespiratory pathway. In order to catalyse photosynthetic CO_2 fixation at high rates, large amounts of Rubisco are needed to compensate the slow catalytic rate of the enzyme. It has been estimated that Rubisco

accounts for a quarter of leaf nitrogen and up to half of the soluble protein in leaves of C_3 plants (Ellis, 1979), and it is probably the most abundant protein of the world (Portis & Parry, 2007). In higher plants Rubisco is composed of large and small subunits in a hexadecameric structure. The catalytic large subunit is encoded by a single gene in the chloroplast genome and the small subunit is coded by a family of closely related nuclear genes (Portis & Parry, 2007; Andersson & Backlund, 2008).

The amount of Rubisco in a leaf is determined by the balance between its synthesis and degradation (Imai et al., 2008). Rubisco synthesis is most active during leaf expansion or during the greening of etiolated leaf tissue (Ishizuka et al., 2004; Imai et al., 2005; 2008; Suzuki et al., 2010) but very limited after full leaf expansion (Miller & Huffaker, 1982), and it is actively degraded during leaf senescence (Miller & Huffaker, 1982; Suzuki et al., 2010). Thus, in many higher plants Rubisco also appears to serve as a leaf storage protein that can be hydrolyzed and the nitrogen derived from the degraded Rubisco is remobilized and reused in developing new tissues (Evans, 1989; Imai et al., 2005) at the expense of leaf photosynthetic activity, which declines in parallel with Rubisco content (Makino et al., 1992). Changes in Rubisco synthesis have been primarily explained by changes in transcript abundance of the encoding genes of the enzyme. The levels of these mRNAs are high in expanding leaves and decline in senescing leaves. Different studies suggest a highly positive correlation between Rubisco content and mRNA levels in the leaves of plants grown under different nitrogen supplies (Evans, 1989; Makino et al., 1992; Imai et al., 2005; 2008; Suzuki et al., 2010). In addition, nitrogen influx into the leaf seems to be closely related to Rubisco synthesis, and N influx declines in the senescent leaf.

Chlorophylls are the most important group of photosynthetic pigments responsible for light absorption and are found in the thylakoids of the chloroplasts. They contain a porphyrin ring with a magnesium ion in the center of the molecule and a long hydrophobic tail that anchors them in the membrane. It has also been shown that the level of chlorophyll is increased in young expanding leaves and decreased substantially during senescence (Imai et al., 2005; 2008).

Tomato is one of the most important vegetable crops cultivated for its fleshy fruit, which is a rich source of minerals, vitamins, organic acids and dietary fibers and is considered as a protective food. The dual role of Rubisco as a key photosynthetic enzyme and a major nitrogen-containing compound in leaves predetermines its importance for plant productivity and its use as a selection criterion for high yield can be hypothesized. With this objective the aim of the present study was to compare the developmental changes in the chlorophyll content and the level of Rubisco protein in different plant tissues in two tomato varieties –Tres Cantos and Cherry, which were grown hydroponically under controlled environmental conditions.

MATERIALS AND METHODS

Plant material and growth conditions

Tomato seeds (*Lycopersicon esculentum Mill*.) of two varieties, Tres Cantos and Cherry, were sown in rockwool in darkness at 24°C for germination. Afterwards, seedlings were grown hydroponically in pots containing vermiculite. The pots were placed in a growth chamber with 140 ± 14 μmol m^{-2} s^{-1} photon flux density, 19–

20.5°C temperature, 510 ± 15 μmol mol^{-1} CO_2 and 60% relative humidity with an 18 h photoperiod for the first four weeks and 12 h for the rest of the growth period. Plants were fertilized with nutrient solution depending of the growth stage according to the manufacturer's specifications (Flora Series, General Hydroponics Europe) and were watered daily to maintain field capacity. Different plant organs (upper-sunlit leaf, stem and tomato fruit –exocarp and mesocarp–) were harvested and immediately plunged into liquid nitrogen at five growth stages in Tres Cantos variety: vegetative growth stage, anthesis, appearance of the first very small green tomato fruit and fruit ripeness at two different time points (with fully green tomatoes and fully ripe red tomatoes); and at three growth stages in Cherry variety: vegetative growth stage, anthesis and fruit ripeness (fully ripe red tomatoes). The samples were kept at −80°C until analysis.

Protein extraction, chlorophyll and protein determination

Aliquots of frozen plant material were ground in a mortar with liquid nitrogen and extracted with 1x PEB (0.105 M Tris-HCl pH 8.5, 0.28 M Tris Base, 2% LDS v/v, 10% glycerol v/v and 0.5 mM EDTA); the extracts were frozen in liquid nitrogen and subjected to cycles of sonication and freezing three times. Afterwards, the samples were centrifugated (3 min, 4 °C, 10 000 g) and the supernatants were used for determination of chlorophyll in acetone 80% (Arnon, 1949) using for quantification the method suggested by Wathley & Arnon (1963); total soluble protein was measured according to Bradford (1976).

Western blotting and quantification of Rubisco

10 μg of protein were loaded in the slots of a 12% SDS–polyacrylamide gel. Electrophoretic protein separation (Laemmli, 1970) was done in a Midi Format 1–D Electrophoresis gel system (Bio Rad, Spain) at room temperature and a constant voltage of 180 V and the proteins blotted on a PVDF membrane using a semi-dry transfer unit (Mini Trans–Blot, Bio Rad, Spain) at 4°C for 2 h at 80 V. In parallel other gels with the same samples were fixed and stained in methanol-acetic acid-Coomassie Brilliant Blue (Sigma) mixture for 1 h, and subsequently rinsed in water-methanol-acetic acid mixture to remove excess stain. The protein blots were immunostained using a polyclonal antibody specific for the large Rubisco subunit (Agrisera, Sweden) in a 1:10 000 dilution. Later, the blots were incubated with a secondary antibody (goat anti-biotin IgG peroxidase conjugate, Agrisera, Sweden) in a 1:5 000 dilution. The immunodetection was performed with an Immun-Blot Opti-4CN Colorimetric kit (Bio Rad, Spain) following the manufacturer's instructions. The relative amount of Rubisco large subunit was calculated by densitometric scanning of the PVDF membranes by image analysis using the Scion ImagePC software (Scion, MD, USA) and expressed in arbitrary units.

Statistical analyses

All statistical analyses were carried out using Microsoft Excel 2010 (Microsoft, USA). Results are given as the mean of three replicates for chlorophyll content and two replicates for Rubisco protein content determinations from each tissue and growth stage studied, with its corresponding standard deviation (SD).

RESULTS AND DISCUSSION

For the two studied tomato varieties the Rubisco and chlorophyll content was determined in several plant tissues at different growth stages. Leaves and stems of both tomato varieties showed the same pattern of changes in chlorophyll content during development, with maximal accumulation at vegetative growth stages and a reduction during the anthesis and the initial phase of fruit development (Fig. 1) followed by a slight increase at fruit ripeness stages. The described changes were more dramatic in Cherry than Tres Cantos leaves, while the reverse was true for stems. This contrasts with the results shown for other plant species in which the chlorophyll content increases during leaf expansion, then gradually declines, and more drastically, during leaf senescence (Imai et al., 2005; 2008; Simova-Stoilova, 2001). The accumulation of chlorophyll was higher in leaves than stems (3.2–1.7 mg g FW^{-1}; 0.36–0.05 mg g FW^{-1}, respectively). Negligible amounts of chlorophyll were detected in fruit tissues as compared to the other analyzed tomato organs. In Tres Cantos variety the level of the mentioned pigment was slightly higher in fully green tomato tissues than in fully ripe red tomato.

In our experiment the soluble protein content showed a similar pattern of changes from the total chlorophyll amount (data not shown) which are initially consistent with previous results showing an increase of protein in young leaf during expansion and decrease in senescent leaf (Simova-Stoilova, 2001; Imai et al., 2008) and contrast with the increase of soluble protein we have found during the fruit ripeness phase.

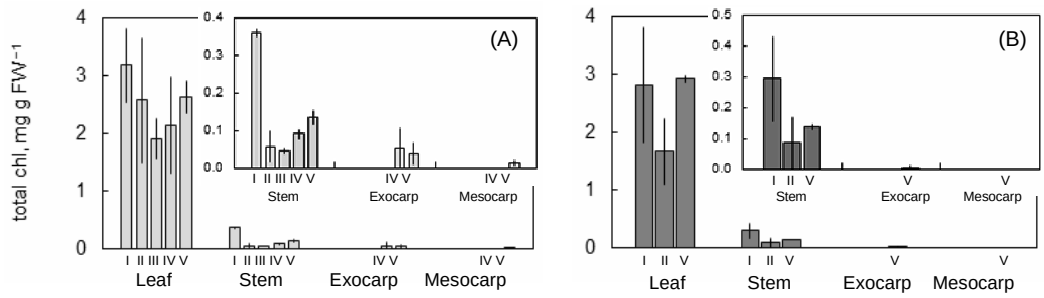

Figure 1. Changes in chlorophyll content of leaves, stems, exocarp and mesocarp in Tres Cantos (A) and Cherry (B) tomato plants at different growth stages: vegetative growth (I), anthesis (II), appearance of the first very small green tomato fruit (III) and fruit ripeness at two different time points, with fully green (IV) and fully ripe red tomatoes (V). The results are the mean ± SE of three separate tissues.

Rubisco content during early stages of development (Figs. 2 A, B) was high in both tomato varieties in agreement with previous studies (Ishizuka et al., 2004; Imai et al., 2005; 2008; Suzuki et al., 2010). During leaf expansion or the greening of etiolated leaf tissue Rubisco content reaches a maximum, because its synthesis predominates over degradation leading to an accumulation of the protein (Suzuki et al., 2010); these changes have been correlated with a higher level of transcripts of the encoding genes of

the enzyme. During anthesis, the increasing demand of nutrients from developing organs (reproductive structures) could lead to the decline in Rubisco observed in the analyzed tissues. Rubisco plays a role as a photosynthetic enzyme but it is also considered a nitrogen storage protein (Eichelmann & Laisk, 1999), a function which has been often discussed but not yet elucidated. It could happen that at this growth stage the degradation of Rubisco is induced and the nitrogen derived from the degraded Rubisco used to supply new developing organs (Simova–Stoilova, 2001; Suzuki et al., 2009; 2010). In Tres Cantos variety we have found an increase of Rubisco protein during ripeness fruit stages, which were correlated with an increase in total leaf nitrogen (data not shown), when the level of protein should remain constant or gradually decrease as has been shown by Suzuki et al. (2010). Imai et al. (2008) suggested that although Rubisco synthesis and transcript encoding for the enzyme subunits decrease in senescing leaves, in this stage, leaves could have the potential to activate *de novo* synthesis of Rubisco by increasing the nitrogen availability. It may be that during fruit ripeness phases in our experiment, the demand of assimilates decreases as compared to early stages of fruit development. This could help to recover the amount of Rubisco or could also be correlated with the optimum supply of nitrogen and its further effect on the growth rate, as suggested by the longitudinal growth analysis (data not shown).

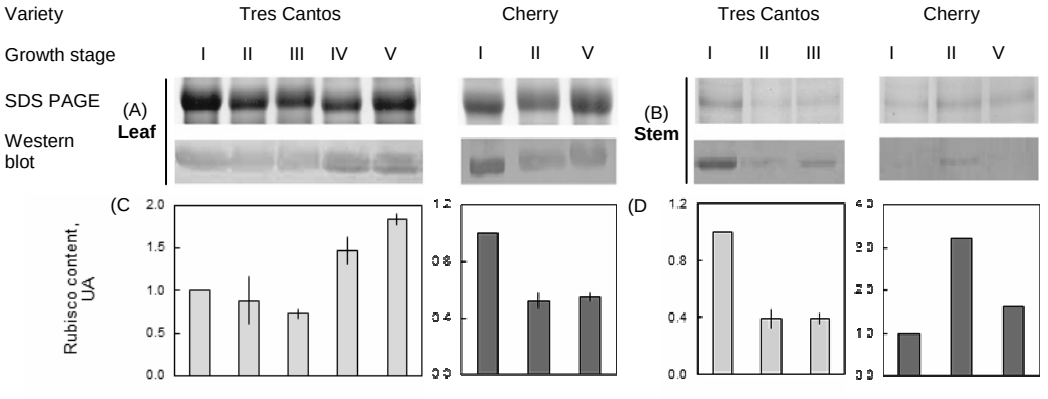

Figure 2. Changes in Rubisco content of leaves (A) and stems (B) in Tres Cantos and Cherry tomato varieties at different growth stages. For details see the legend of Figure 1. (C, D) Quantified signals (UA, arbitrary units) of Rubisco content in PVDF membranes. The results are the mean ± SE of two separate tissues and in Cherry stem is shown a single tissue.

It should be highlighted that we have also found an important amount of Rubisco in tomato stems, and its accumulation pattern was different in the two tomato varieties analyzed. While in Tres Cantos it reached a maximum in vegetative growth stages and decreased gradually during development, in Cherry it increased slightly until anthesis

and decreased afterwards. However, we have not detected the presence of Rubisco in fruit tissues, mesocarp and exocarp, independently of the ripening stage in both varieties, in contrast to other studies with pericarp of tomato (Barsan et al., 2010). As should be expected, the highest amount of Rubisco was found in leaves in agreement with the suggestion that Rubisco represents up to 50% of the soluble protein in leaves of C_3 plants (Ellis, 1979).

CONCLUSIONS

Our study shows that the pattern of changes of Rubisco and chlorophyll content was similar for the two tomato varieties at different growth stages, which differs from other plant species in late growth stages.

ACKNOWLEDGEMENTS: This work is associated with the Agrobiotechnology master of the University of Salamanca and it has been carried out in the Department of Plant Physiology. R. Vicente had an FPI fellowship funded by the Spanish Ministry of Sciences and Innovation. We thank Wilson Acosta for his technical cooperation.

REFERENCES

Andersson, I. & Backlund, A. 2008. Structure and function of Rubisco. *Plant Physiol. Biochem.* **46**, 275–291.

Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol*. **24**, 1–15.

Barsan, C., Sanchez-Bel, P., Rombaldi, C., Egea, I., Rossignol, M., Kuntz, M., Zouine, M., Latché, A., Bouzayen, M. & Pech, J. C. 2010. Characteristics of the tomato chromoplast revealed by proteomic analysis. *J. Exp. Bot.* **61**(9), 2413–2431.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Eichelmann, H. & Laisk, A. 1999. Ribulose-1,5-bisphosphate carboxylase/oxygenase content, assimilatory charge, and mesophyll conductance in leaves. *Plant Physiol*. **119**, 179–189.

Ellis, J. R. 1979. The most abundant protein in the world. *TIBS* **4**, 241–244.

Evans, J. R. 1989. Photosynthesis and nitrogen relationships in leaves of C_3 plants. *Oecologia* **78**, 9–19.

Imai, K., Suzuki, Y., Makino, A. & Mae, T. 2005. Effects of nitrogen nutrition on the relationships between the levels of *rbcS* and *rbcL* mRNAs and the amount of ribulose-1,5-bisphosphate carboxylase/oxygenase synthesized in the eighth leaves of rice from emergence through senescence. *Plant Cell Environ.* **28**, 1589–1600.

Imai, K., Suzuki, Y., Mae, T. & Makino, A. 2008. Changes in the synthesis of Rubisco in rice leaves in relation to senescence and N influx. *Ann. Botany* **101**, 135–144.

Ishizuka, M., Makino, A., Suzuki, Y. & Mae, T. 2004. Amount of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein and levels of mRNAs of *rbcS* and *rbcL* in the leaves at different positions in transgenic rice plants with decreased content of Rubisco. *Soil Sci. Plant Nutr*. **50**(2), 233–239.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lorimer, G. H. 1981. The carboxylation and the oxygenation of ribulose-1,5-bisphosphate: the primary event in photosynthesis and photorespiration. *Ann. Rev. Plant Physiol*. **32**, 349–383.

Makino, A., Sakashita, H., Hidema, J., Mae, T., Ojima, K. & Osmond, B. 1992. Distinctive responses of ribulose-1,5-bisphosphate carboxylase and carbonic anhydrase in wheat leaves to nitrogen nutrition and their possible relationships to CO_2-transfer resistance. *Plant Physiol.* **100**, 1737–1743.

Miller, B. L. & Huffaker, R. C. 1982. Hydrolysis of ribulose-1,5-bisphosphate carboxylase by endoproteinases from senescing barley leaves. *Plant Physiol.* **69**, 58–62.

Portis, A. R. & Parry, M. A. J. 2007. Discoveries in Rubisco (ribulose-1,5-biphosphate carboxylase/oxygenase): a historical perspective. *Photosynth. Res.* **94**, 121–143.

Simova–Stoilova, L., Stoyanova, Z. & Demirevska-Kepova, K. 2001. Ontogenic changes in leaf pigments, total soluble protein and Rubisco in two barley varieties in relation to yield. *Bulg. J. Plant Physiol.* **27**(1–2), 15–24.

Suzuki, Y., Nakabayashi, K., Yoshizawa, R., Tadahiko, M. & Makino, A. 2009. Differences in expression of the RBCS multigene family and Rubisco content in various rice plant tissues at different growth stages. *Plant Cell Physiol.* **50**, 1851–1855.

Suzuki, Y., Kihara-Doi, T., Kawazu, T., Miyake, C. & Makino, A. 2010. Differences in Rubisco content and its synthesis in leaves at different positions in *Eucalyptus globulus* seedlings. *Plant Cell Environ.* **33**, 1314–1323.

Whatley, F. R. & Arnon, D. I. 1963. Photosynthetic phosphorylation in plants. In Colowick, S. P. & Kaplan, N. O. (eds), *Methods in Enzymology VI*. Academic Press, NY, pp. 308–313.