Importance of Rubisco activase in maize productivity based on mass selection procedure

A. Morales¹, M.L. Ortega-Delgado¹, J. Molina-Galán¹ and E. Sánchez de Jiménez²,³

¹ Institutos de Recursos Naturales and Recursos Genéticos y Productividad, Colegio de Postgraduados, Montecillo, Edo. de México, México
² Departamento de Bioquímica, Facultad de Química, Ciudad Universitaria, UNAM México, D.F. 04510, México

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Abstract
The original maize (Zea mays L.) var. Zacatecas 58 (Z₅₈) and five composites of cycles 5, 10, 15, 20, and 23 of stratified mass selection (Z₅₈, Z₁₀₅, Z₁₅₅, Z₂₀₅, and Z₂₃₅) for improved productivity applied to the original variety were used as the model system. Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) (Rubisco) activity and the rates of net photosynthesis in the leaves above the ear were compared in all the composites during the grain filling period. Leaves were sampled weekly from anthesis to physiological maturity. Results showed a significant and gradual increase in Rubisco activity for the improved populations. In vivo photosynthesis measured by IRGA during the same period also showed increased levels associated with increases of grain yield. The highest Rubisco activity, photosynthetic rate and grain yield were found in the Z₂₃₅ population. Western blot analysis for Rubisco protein did not show significant differences either during the filling period or between populations. The same analysis, however, for Rubisco activase protein showed increasing contents in the improved populations. These data confirm and complement previous findings, indicating that the stratified visual mass selection procedure applied to maize plants is associated with leaf content of Rubisco activase protein. The possible regulatory role of Rubisco activase during grain filling is discussed.

Key words: Plant productivity, photosynthetic rate, Rubisco, Rubisco activase, sink–source relationship, Zea mays L.

Introduction
In higher plants, sink/source relationships are established between photosynthetic and the developing organs (Rogers et al., 1996; Fischer et al., 1997). In these plants, enzymes responsible for sucrose uptake and assimilation on one side, and photosynthetic rates, sucrose synthesis and transport on the other, have been described as participants in the mechanisms determining plant yield (Gifford and Evans, 1983; Daie, 1985; Hay and Walker, 1989; Jones et al., 1996). Indeed, it has been demonstrated that in higher plants, carbohydrate depletion up-regulates genes for photosynthesis, remobilization and export, indicating a mechanism for controlling resource distribution among tissues and organs (Koch, 1996).

Several studies in C₃ plants have pointed to ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity and photosynthetic capacity of the source leaves as possible limiting factors for plant growth and fruit development (Jang and Sheen, 1994; Rogers et al., 1996). On the other hand, scarce information is available about these mechanisms in C₄ plants, as maize, suggest there is not a relationship between photosynthetic rate and grain yield (Crobie et al., 1981; Hageman and Lambert, 1988). Moreover, to determine which enzymatic activities control carbohydrate flux is further complicated by the additional complexity of cell specialization (Furbank and Taylor, 1995; Dai et al., 1995).

The role of Rubisco activase (R-A) in this process might be relevant since this protein is known to activate in vivo Rubisco (for reviews see Portis, 1990, 1992) from an inactive complex (Rubisco: Pentose-P) by restoring the active Rubisco structure (Andrews et al., 1995; Sánchez de Jiménez et al., 1995; Salvucci and Ogren, 1996). Previous reports from this laboratory have indicated that in yield-improved maize (Zea mays L.) plants a positive correlation between increased Rubisco activity and gain in grain production exists (Loza-Tavera et al., 1987; Martínez-Barajas et al., 1992, 1997). On the other
hand, the leaf Rubisco protein content in these plants was indistinguishable from the original and a yield improved maize population, whereas R-A significantly increased in the latter as compared with the control (Martinez-Barajas et al., 1997), suggesting that Rubisco activation might account for this phenomenon.

Based on these antecedents, the present work focuses on elucidating whether C₄ selected plants for larger sink strength might have up-regulated photosynthesis and the CO₂-fixation process. The aim of this research was to gain knowledge on the biochemical and molecular pathways underlying maize productivity.

Materials and methods

Biological materials

The biological material chosen for this study was a series of maize plant populations generated by stratified mass selection for yield improvement, based on visual selection of the top 5% plants bearing the best aspect of ear characteristics. Whole population size was 6000 plants, and the selected sample comprised 300 plants per cycle (Molina-Galán, 1983). Plants of Zea mays L. cv. Zacatecas 58 were used in this work. Six populations were tested: Zₒ, Z₅, Z₁₀, Z₁₅, Z₂₀, and Z₃₃ (subindices refer to the number of selection cycles). Plants from all these populations were grown simultaneously in the same field to avoid differences introduced by changes in environmental conditions. The plants were cultivated under irrigation in a completely randomized block design at Montecillo, State of México, located at 19°53’ N, 98°53’ W and 2245 m altitude. The plots comprised two rows 80 cm apart of 12 hills each or R-A, respectively) were subjected to SDS electrophoresis following the established protocol of Robinson et al. (1988), modified by Loza-Tavera et al. (1990). The assay mixture contained: assay buffer (100 mM Tricine-NaOH, pH 8.0, 10 mM MgCl₂, 4.16 mM DTT), 1.66 mM ribulose-1,5-bisphosphate tetrasodium salt (RuBP), 10 mM NaH¹³CO₃ (100 μCi μmol⁻¹, Amershams), and 5 μl of leaf extract in a final volume of 65 μl. Initial velocity was calculated from four determinations at 0, 20, 40, and 60 s in each sample. The radioactivity incorporated in the acid stable products was quantified with a Packard Liquid Scintillation Counter (Minaxi β serie S400). One enzyme unit is equal to 1 μmol of ¹³CO₂ fixed min⁻¹ under the assay conditions. Soluble protein was determined by the method of Bradford (1976), using bovine serum albumin (Sigma) as standard.

Western blot analysis

Leaf extracts (either 5 μg or 50 μg of soluble protein for Rubisco or R-A, respectively) were subjected to SDS electrophoresis by the Laemmli method (Laemmli, 1970). Thereafter, proteins were blotted onto nitrocellulose membranes in a Hoefer Scientific electroblotting apparatus. The nitrocellulose membranes were processed following the method described by Robinson et al. (1979). Anti-Rubisco large subunit (LS) antibody (1:1500) (Martinez-Barajas et al., 1997) or anti-R-A antibody (1:700) were used for the assay coupled with second-stage antibody-peroxidase conjugate (Sigma). The blots were developed by the 4-chloro-1-naphthol method. Relative densitometric quantification in films was performed using a densitometer Lasser × Ray Ultrascan XL (Pharmacia, LKB), and taking Zₒ band intensity as 1 in each case. The values were normalized by the total protein loaded per slot.

Statistical analysis

All determinations are averages of the number of samples indicated for each parameter. Variance analysis and mean value comparisons were performed by the statistical SAS computer package (SAS Institute Inc., Cary, NC, USA). Tukey’s procedure was used for mean comparisons.

Results

The original variety Zac 58 (Zₒ) and its five selection composites (Z₅, Z₁₀, Z₁₅, Z₂₀, and Z₃₃) were cultivated in the same field under the same enviromental conditions to assess their agronomical, physiological and biochemical characters.

Data collected at the end of the agronomical cycle
showed that some of the main agronomical and physiological parameters differed among plants from different improved selection cycles. Plant height and leaf area (measured in the ear leaf plus one) increased along with the number of selection cycles from 155 to 220 cm for the former, and 290 to 543 cm² for the latter, in populations $Z_0$ and $Z_{23}$, respectively (Table 1). However, the period to male flowering (DTF) changed only slightly, from 64 to 71 d from $Z_0$ to $Z_{23}$ (Table 1). On the other hand, yield-related parameters were significantly improved. Ear length and diameter increased in the selected populations from 10.3 to 14.2 cm and 4.0 to 4.5 cm in $Z_0$ and $Z_{23}$, respectively. Grain yield increased from 3.2 to 7.0 t ha⁻¹ from $Z_0$ to $Z_{23}$ populations, an increase of more than 100% throughout 23 cycles of mass selection ($P \leq 0.05$) (Table 2).

Previous work from this laboratory on some of these maize populations (Loza-Tavera et al., 1987; Martínez-Barajas et al., 1992, 1997) showed a transient increase of Rubisco activity on the ear leaf plus one after anthesis, which was not accompanied by increments in Rubisco protein. To reproduce and further confirm this phenomenon, total protein and Rubisco protein were measured in extracts of the ear leaf plus one taken at different periods from 0 to 60 DAA in all the populations indicated above. Results showed steady values for total protein during most of the period followed by a slight decrease toward the end, with no significant differences between populations (Table 3). Rubisco protein was determined in the same extracts by Western blots. Equal samples (5 μg) of the leaf extracts, were applied for the SDS-PAGE analysis. The Western blots revealed similar amounts of Rubisco LS protein for all the populations (Fig. 1A). Indeed, the densitometric analysis performed in the Western blot films showed that Rubisco protein remained approximately constant for all population, even within the lapse of maximum change of Rubisco activity (around 24 DAA) (Fig. 1B). These results were reproducible and very similar for samples taken at either 0, 24, or 40 DAA of the filling period.

Photosynthesis was determined in situ in the ear leaf plus one of plants from early, middle and advanced mass selection cycles, at different stages of the grain filling period. Results showed a transient increase for this parameter around the middle of the grain filling period in all populations (Fig. 2A). The increment in photosynthetic rate, however, was larger in populations of advanced selection cycle. These differences between populations were not due to changes in light intensity registered during the experimental period, since increased photosynthetic rates were observed in populations of advanced cycles of yield improvement even during days with low light intensity (Fig. 2A). Moreover, $Z_{23}$ showed significantly higher photosynthetic rate than $Z_0$ at all sampled periods ($P \leq 0.05$), as shown by the mean comparison test performed among populations, regardless of variations in light intensity (Fig. 2A). This pattern became even more pronounced when photosynthetic rates were calculated per whole leaf area (Fig. 2B).

Following the same experimental design, Rubisco activity was measured in the ear leaves plus one of all plant populations at closer intervals within the middle part of the period than the ones used for photosynthetic rate measurement.
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Fig. 1. Western blot analysis of Rubisco protein. (A) SDS-PAGE and Western blot analysis of Rubisco protein were performed. Leaf extracts (5 μg protein per slot) of each population from Z₀ to Z₂₃ (1 to 6) were compared. Antibodies versus spinach Rubisco LS, dil 1:1500, were used to reveal the corresponding band. The picture corresponds to data from samples at 24 DAA. Molecular mass markers are indicated at the left. (B) Densitometric analysis of the Western blot film was performed by Lasser X-Ray Ultra scan XL. The values were normalized by total protein content per slot. No significant differences were found among populations.

determinations (Fig. 3). Rubisco activity showed similar patterns to those for photosynthetic rates, i.e. transient increases during the filling period, increasing as the selection cycle of the maize population increased. Statistical analysis of Rubisco activity, expressed as enzyme units per milligram of protein, revealed significant differences between Z₀, Z₅ and Z₁₀ compared with Z₂₃ (P<0.05) (Fig. 3). Z₁₅ was also larger than Z₂₃ in four out of six periods tested and Z₀ showed significantly lower values than those in populations Z₁₅ or Z₂₀ in all but one of the periods (see letters on Fig. 3). These differences became even more pronounced when Rubisco activity was expressed in terms of activity per whole leaf, as leaf area also increased with the number of selection cycles (Table 1). The above data indicate an association between photosynthetic rate and Rubisco activity in the yield-improved maize populations.

To investigate whether the increments in Rubisco activity observed during grain filling and among improved populations were associated with leaf R-A, the content of this protein was determined by Western blot analysis. This was performed using extracts of ear leaves plus one for all maize populations at three DAA stages within the experimental period (0 DAA, 24 DAA and 48 DAA). Results for the 24 DAA stage are presented (Fig. 4A) as a representative set of data from these experiments. As can be observed, the band corresponding to R-A shows increased intensities for the improved populations during the selection process. The densitometric analysis of these data show more clearly this phenomenon (Fig. 4B). The other two periods tested, 0 DAA and 48 DAA, gave similar patterns for both proteins, i.e. Rubisco protein, photosynthetic rate and Rubisco activity in the yield-improved maize populations.

Fig. 2. Rate of net photosynthesis during the grain filling period. Photosynthesis was measured in situ by IRGA in the ear leaf plus one of three maize populations (Z₀, Z₁₀, Z₂₃), at different stages during the filling period: 8 (□), 16 (□), 40 (□), 48 (□), 56 (□) DAA. Photosynthetic rates are expressed either per unit area (A) or per whole leaf (B). Light intensity at each time of photosynthesis measurements is shown in the upper part of (A). Average values with same letters at a given time within populations are not statistically different. Vertical lines in the bars stands for ± standard deviation.

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Fig. 3. Rubisco activity in leaves of six maize populations during the grain filling period. Rubisco activity was measured in extracts of the ear leaf plus one of six maize populations from Z₀ to Z₂₃, at 0 (○), 16 (●), 24 (▲), 32 (▲), 40 (▲), and 48 (▲) DAA. Values are expressed as average of CO₂ nmol fixed per mg of protein at each stated period. Average values at a given time with same letter(s) within populations are not significantly different. Vertical lines in the bars stand for ± standard deviations.

Fig. 4. Western blots of Rubisco activase (A) SDS-PAGE and Western blot analysis of leaf extracts from all populations were determined at 24 DAA. Same amount (50 μg of protein) of leaf extract was applied to each slot. Antibodies against spinach R-A (dil 1:700) were used (C). The picture corresponds to data from samples at 24 DAA. Molecular mass markers are indicated at the left. (B) Denatimetric analysis of the Western blot film was performed with a Laser X-Ray Densitometer Ultra scan XL. The values were normalized by total protein content per slot.

Fig. 5. Relative values of densitometric R-A/LS ratios at three stages (DAA) during the filling period. Densitometric measurements of the Western blot analysis of Rubisco activase and Rubisco LS were performed in leaves of Z₀ to Z₂₃ populations at 0 (○), 24 (●), and 40 (▲) DAA during the filling period. R-A/LS ratios of the Z₀ population were arbitrarily made equal to 1.0 and the relative values for the ratios of the other populations were calculated.

data, including those from 0 and 48 DAA, allowed the relative R-A/LS protein ratios to be calculated at the three stages. The R-A/LS ratios for Z₀ were 0.189 at 0 DAA, 0.161 at 24 DAA and 0.164 at 40 DAA. Taking each of these values arbitrarily as 1.0, the relative values for R-A/LS ratios for the other populations were calculated (Fig. 5) and the ratios were always higher. Since the content of LS was fairly constant for all populations (Fig. 1B) these data indicate an association between the number of mass selection cycles for yield improvement and the content of R-A in the ear leaf plus one for the three DAA stages tested.

Discussion

A series of visual stratified mass selected maize populations converging to yield improvement, were used to test whether up-regulation of photosynthesis and CO₂-fixation activity might be involved in the process leading to improved plant productivity.

According to previous reports, high CO₂ exchange rate (CER) has not been found associated to increased yield in corn (Crosbie et al., 1981). However, genetic variability among corn has made it difficult to evaluate the actual contribution of specific physiological traits to yield improvement (Hageman and Lambert, 1988).

The aim of the maize populations tested in the present research is the genetic constancy of the corn populations analysed. They derived from the same original genetic background after cycles of visual stratified mass selection converging to increasing yield. This condition facilitated the recognition of traits associated with the increase in grain yield through the series of improved selection cycles.

Indeed, the changes in photosynthetic rates were found associated with the progressive increase in grain yield in...
the populations (Table 2; Fig. 2). To account for this improvement, some basic biochemical characters were modified throughout the selection procedure, since Rubisco activity was also progressively improved in the high yielding selected populations (Fig. 3).

Analysis of this phenomenon revealed that Rubisco activity increments during the filling period, and among plant populations, were not due to increases in Rubisco protein content in the leaves, as judged by the densitometric analysis of the Western blot results (Fig. 1B), but to Rubisco activation (Fig. 3). The mechanism behind this enzymatic activation seems to rely on the accumulation of R-A, both within the filling period and progressively during the selection process (Figs 4, 5), that follows a similar pattern to that shown by Rubisco activity in the same leaves.

The molecular mechanism by which R-A elicits Rubisco activation is not completely understood (He et al., 1997). However, the role of R-A in vivo has been demonstrated in C₃ plants by analysing mutants lacking the rca gene (Salvucci et al., 1985) or reducing R-A levels in antisense transgenic plants (Mate et al., 1993; Jiang et al., 1994). These authors have shown that Rubisco cannot achieve and maintain a satisfactory level of CO₂-fixation for growth with reduced levels of R-A, particularly at high light intensities (Andrews et al., 1995). Recent data (Martinez-Barajas et al., 1997) have also demonstrated that the addition of purified R-A and the ATP generating system to Z₀ leaf extracts increases Rubisco activity to similar levels of Z₂₀ leaf extracts, suggesting that leaf R-A concentration might be a limiting factor for full and sustained Rubisco activity expression in these leaves.

In conclusion, the data presented here suggest that the mass selection procedure applied to maize plants for improved productivity might be selecting for increased source strength in terms of Rubisco activation during the filling period, this is basically due to the enrichment of R-A leaf content. Based on these findings, research on the mechanism regulating R-A expression in maize leaves becomes more relevant for further designing strategies on plant productivity improvement. Research in this direction is currently being performed in this laboratory.

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