ISOLATION, CLONING AND OVEREXPRESSION OF THE COTTON RUBISCO ACTIVASE GENE

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Abstract

Activation of Rubisco, the primary carboxylating enzyme in photosynthesis, is promoted *in vivo* by the enzyme Rubisco activase. As a first step in investigating Rubisco activation in cotton, we screened a cotton (*Gossypium hirsutum* L.) cDNA library to isolate the cotton activase (*rca*) gene. Restriction and sequence analysis of positive clones indicated that there were three classes of cotton *rca* cDNAs. One of these classes encodes for a mature protein of 380 amino acids (42357 Da), while the other two encode for mature proteins of 419 amino acids (46590 Da). These results were consistent with Southern analysis which indicated that there are multiple cotton activase genes and with Western blot analysis which showed the presence of two activase polypeptides in cotton leaf tissue. The deduced amino acid sequences of the cotton *rca* clones shared 80 to 89% identity with activases from other higher plants. One of the cotton activase clones was overexpressed in *E. coli*, enabling the production of large amounts of soluble cotton activase protein for functional analysis.

Introduction

The primary enzyme of photosynthetic carbon fixation, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), is regulated by **Rubisco activase,** a regulatory enzyme that controls the amount of Rubisco that is active in the light (Salvucci et al., 1985). Activase is required for "activation" of Rubisco and the activation process is essential for photosynthesis (Salvucci et al., 1986; Mate et al., 1994). Rubisco activation appears to be very sensitive to heat inactivation (Weiss, 1981), probably because of the heat lability of activase (Robinson and Portis, 1989; Holbrook et al., 1991). However, little is known about Rubisco activation in cotton and other tropical C_3 plants and the effect of temperature on the process.

As a first step in investigating Rubisco activation in cotton, we isolated and cloned the cotton activase gene. Sequence comparisons showed that the primary structure of the cotton enzyme is similar to activases from other sources. The cotton enzyme appears to be comprised of two related polypeptide types, similar to activases from several other plant species. One of the cotton activase clones was overexpressed in *E. coli*, enabling the production of large amounts of soluble cotton activase protein.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 2:1178-1181 (1996)
National Cotton Council, Memphis TN

Materials and Methods

Isolation of Cotton Activase cDNA Clones

Cotton activase cDNA clones were isolated from a lambda-zap cDNA library made from young expanding leaves of upland cotton (*Gossypium hirsutum* L. cv Acala SJ-2). The library was screened with a tobacco activase cDNA probe (Salvucci and Klein, 1994) and positive clones were sequenced at the University of Kentucky Macromolecular Facility.

Western Analysis

Cotton leaves (glandless Coker 100A) were extracted in cold 7% (v/v) perchloric acid. Following centrifugation, the protein pellet was resuspend in buffer containing 1% (w/v) SDS and immediately boiled. Alternatively, leaves were extracted in 100 mM MOPS-KOH, pH 7, 2 mM EDTA, 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, 10 mM leupeptin and 2% (w/v) PVPP. The homogenate was centrifuged for 3 min and the supernatant was supplemented with SDS and boiled. Polypeptides were electrophoresed and electroblotted to PVDF membrane and the membrane probed with anti-tobacco activase IgG followed by alkaline phosphatase-conjugated anti-mouse IgG (Klein and Salvucci, 1995).

Southern Analysis

Genomic DNA was isolated from glandless Coker 100A cotton by the cetyltrimethylammonium bromide method. Genomic DNA was screened at high stringency with a mixed probe made from the three cotton cDNAs (Sambrook et al., 1989)

Overexpression of Cotton Activase in *E. coli* and Partial Purification

A full-length cotton activase cDNA was cloned into a pET23d vector and expressed in *E. coli* strain BLR(DE3)pLysS (Salvucci and Klein, 1994; van de Loo and Salvucci, unpublished). Activase was purified from IPTG-induced *E. coli* cells by ammonium sulfate precipitation and rate zonal centrifugation (Salvucci and Klein, 1994).

Results and Discussion

Cotton Activase cDNA Clones

Several positive clones were isolated when a cDNA library made from young expanding leaves of upland cotton (*Gossypium hirsutum* L. cv Acala SJ-2) was screened with a tobacco Rubisco activase cDNA probe. Based on restriction analysis and preliminary sequence data, the positive clones obtained were assigned to three classes. Complete sequences were determined for a full length clone (GhRca2O) of one class, and near-full length clones (GhRca27, GhRca28) of the other two classes (Fig. 1).

Clone GhRca2O contains a 1559 bp insert including a 1314 bp open reading frame that encodes a 438 amino acid predicted protein with strong homology to activase of other species. Comparison to activase sequences from spinach and tobacco, for which the chloroplast transit peptide cleavage site has been determined by sequencing of the mature leaf protein (Wang et al., 1992; Salvucci and Klein, 1994), predicted that GhRca2O encodes a mature protein of 380 amino acids (42357 Da). Clones GhRca27 and GhRca28 encode similar proteins. These proteins have a carboxy-terminal extension that is identical in length to long forms of the protein generated by alternative splicing in spinach, *Arabidopsis* and barley (cf. Werneke et al., 1989). Clone GhRca27 contains a 1462 bp insert, extending into the region encoding the predicted transit peptide, and encodes a predicted mature protein of 419 amino acids (46590 Da).

Comparisons between the three sequences indicated that clones GhRca27 and GhRca28 were most alike, with 97% and 98% identity at the nucleotide and amino acid levels, respectively. These clones had about 87% (nucleotide) and 92% (amino acid) identity with clone GhRca2O. Sequence differences clearly indicated that the three clones each represent separate cotton genes. In addition, five activase bands were discerned by Southern analysis (data not shown), consistent with the presence of multiple activase genes in cotton. For each of the three classes of cDNA clones, partial sequences were obtained for two shorter clones, and in all cases were collinear with the clones described above in the region where alternative splicing is observed in other species.

The deduced amino acid sequences of the three cotton clones share 80% - 89% identity with Rubisco activases from other higher plants. A multiple alignment of all available sequences identified a small number of residues that are perfectly conserved among other species but divergent in cotton. These were conservative substitutions with the exception of Ala to Ser at position 374.

Western Blot Analysis

Antibodies directed against tobacco activase, recognized two polypeptides of 46 and 42 kDa on Western blots of cotton leaf proteins (Fig. 2). Interestingly, the activase polypeptides were extensively proteolyzed when extracts were not immediately quenched by extraction in perchloric acid. The presence of two immunologically-related activase polypeptides is consistent with the presence of both long and short form *rca* cDNAs in cotton and indicates that cotton activase is heteromeric, comprised of two types of polypeptides that differ primarily in length. The presence of two types of activase polypeptide has been observed in several other species (Salvucci et al., 1987). The functional significance of an activase comprised of two related activase polypeptides in unknown, particularly since activase is comprised of only a single polypeptide type in some species.

Overexpression and Purification of Recombinant Cotton Activase

To facilitate study of the enzymatic properties of cotton activase, the full-length GhRca2O clone was engineered for expression of the mature protein by replacement of the sequence encoding the transit peptide with an NcoI site encoding for an initiating Met. When *E. coli* cells harboring the activase clone in a pET23d vector were incubated with IPTG, the cells synthesized copious amounts of the 42 kDa form of activase (Fig. 3). After partial purification by ammonium sulfate precipitation and rate zonal centrifugation on sucrose gradients, recombinant cotton activase represented >80% of the protein in the preparation. Enzyme assays showed that recombinant cotton activase had ATPase activity, however, the activity was relatively low compared to the recombinant tobacco enzyme.

Acknowledgments

We thank Drs. Thea Wilkins (Dept. of Agronomy and Range Science, University of California at Davis) and Barbara Triplett (USDA, ARS, SRRC, New Orleans) for providing cotton cDNA libraries. We thank Dr. Robert Houtz and the Dept. of Horticulture and Landscape Architecture (University of Kentucky) for providing laboratory space and facilities to F. J. L.

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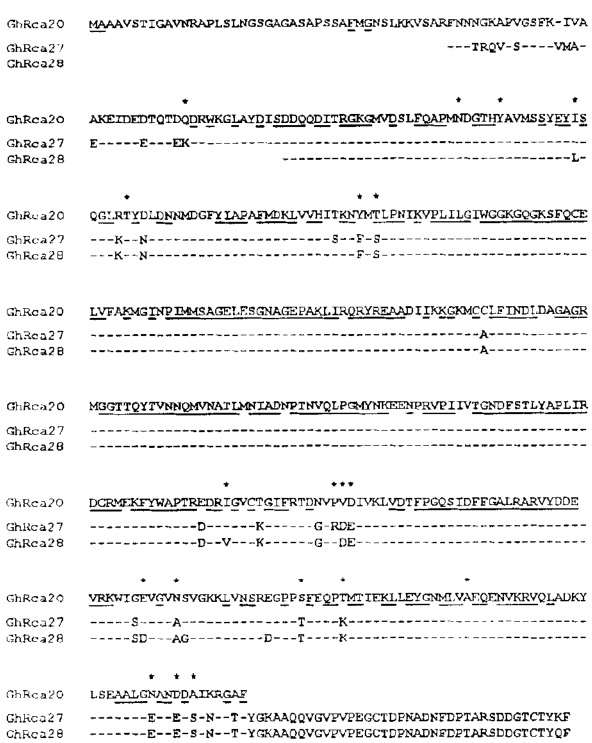

Figure 1. Deduced amino acid sequences of cotton activase cDNA clones GHRca20, GHRca 27 and GHRca 28. Dashes indicate residues in GHRca 27 and GHRca 28 that are identical to GHRca20. Underlined residues are totally conserved in all activase higher plant sequences. The asterisks mark positions where one or more of the cotton activase sequences differ from other higher plant activase sequences.

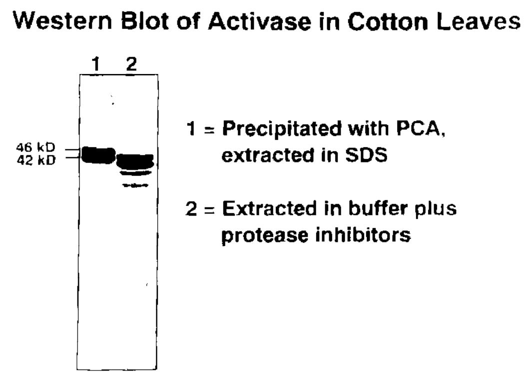

Figure 2. Western blot of activase in cotton leaves. Leaves were extracted in either 7% perchloric acid (PCA, lane 1) or buffer plus protease inhibitors (lane 2).

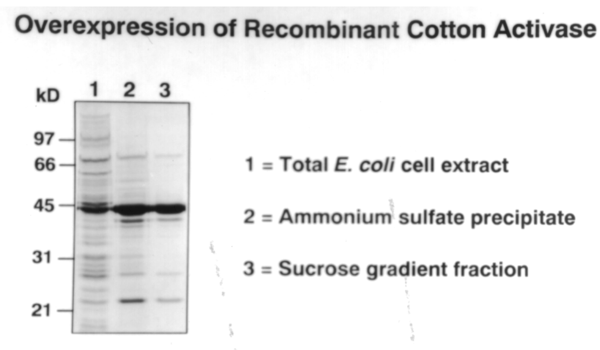

Figure 3. Overexpression and partial purification of recombinant cotton activase. SDS-PAGE gel showing expression of the 42 kD cotton activase polypeptide in extracts of *E. coli* cells (lane 1) and after partial purification by ammonium sulfate precipitation (lane 2) and rate zonal centrifugation in sucrose gradients (lane 3)