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Research article

Changes in Rubisco activase gene expression and polypeptide content in *Brachypodium distachyon*

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ABSTRACT

Regulation of Rubisco (D-ribulose-1,5-bisphosphate carboxylase/oxygenase activase (RCA) gene expression and polypeptide content were determined in *Brachypodium distachyon* leaves, stems and ear elements at different developmental stages under optimal growth conditions as well as under drought and salt stress conditions. *B. distachyon* leaf contains a much greater amount of Rubisco activase small (RCAS) isoform than the large one (RCAL) under optimal growth conditions. Increased levels of the RCAL isoform compared with the RCAS isoform were found in leaves and in green stems under salt and drought stress, respectively. Transcriptional levels of RCA are almost identical in different leaf positions. Short-term drought and salt stresses did not cause the impairment of RCA gene expression in early seedlings. But gradually increasing drought stress significantly decreased gene expression in early seedling samples. Amounts of the RCAS isoform were found to be more in different leaves of the plant compared with the RCAL isoform and their ratio was constant under normal condition. In green stems gene expression of RCA decreased under salt and drought stresses, although as it was in green leaves protein amounts of RCAL isoform increased compared with the RCAS isoform. All of the above described results clearly indicate that the accumulation of each RCA isoform is differentially regulated by developmental and environmental cues.

1. Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the key enzyme catalyzing carbon assimilation in plants, exhibits maximal activity when all its active sites are in the proper conformation and are available for catalysis. In plant Rubisco, the lysine residue at position 201 (numbering based on residue positions in tobacco Rubisco) in the active site pocket must be carbamylated and bind a Mg^{2+} ion as cofactor for the enzyme to become catalytically active. Binding of ribulose-1,5-bisphosphate to uncarbamylated Rubisco, or of 2-carboxy-D-arabinitol 1-phosphate to the carbamylated enzyme at night, results in the trapping of sugar phosphate and in the inhibition of the enzyme. However, Rubisco sites can be deactivated by various mechanisms, and the rate of deactivation increases with temperature (Spreitzer and Salvucci, 2002). Rubisco activity during photosynthesis is regulated by the Rubisco activase (RCA), which facilitates the dissociation of ribulose-1,5-bisphosphate and other inhibitory sugar phosphates from the active site of Rubisco in an ATP-dependent reaction. Activase is a relatively abundant nuclear-encoded AAA^+ protein located in the chloroplasts of higher plants and algae. Like other AAA^+ proteins, activase functions as a mechanical motor, remodeling the conformation of its target protein, Rubisco. RCA rescues Rubisco sites from deadend inhibition by promoting ATP-dependent conformational changes that open closed sites, making them more accessible to solvent and facilitating the dissociation of inhibitory sugar phosphates (Portis et al., 2008). In this way, RCA is a molecular chaperone, controlling the switching of Rubisco conformation from inactive to active (Spreitzer and Salvucci, 2002). In several plant species, RCA consists of two polypeptides, which differ in length by the presence of an extra 30 amino acids at the C-terminus of one of the forms (Salvucci et al., 1987; Werneke et al., 1988). Even under non-stressed conditions, control of RCA expression is complicated and is regulated at various levels in different species. Most species studied contain two isoforms of RCA, an α-isoform of 46–48 kDa and a β-isoform of 41–43 kDa.

The Rubisco activase large isoform (RCAL) differs from the Rubisco activase small isoform (RCAS) by the presence of a carboxy-terminus extension, which contains the redox-sensitive disulfides.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonylfluoride; PVP, polyvinylpolypyrrolidone; Rubisco, Ribulose-1,5-bisphosphate carboxylase/oxygenase; Rubisco LS, Rubisco large subunit; RCA, Rubisco activase; RCAL, Rubisco activase large isoform; RCAS, Rubisco activase small isoform; RT-PCR, reverse transcription polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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The activity of longer isoform was regulated by redox state *via* thioredoxin f and this redox regulation was due to an interaction between carboxyl extension and nucleotide-binding pocket in RCAL. The C-terminal extension of the α-isoform contains two redox-regulated Cys residues that are modulated by thioredoxin f. When these residues are oxidized to a disulfide, the affinity for ATP decreases and enzyme activity is more sensitive to inhibition by ADP. Physiological ratios of ADP to ATP significantly inhibit the activity of the Arabidopsis α-isoform when in the oxidized state, but inhibition is much less when this isoform has been reduced by thioredoxin. In contrast, the shorter Arabidopsis β-isoform is not redox regulated and is less sensitive to inhibition by ADP (Zhang and Portis, 1999).

In most of the species that have been examined, the two isoforms are products of a single, alternatively spliced pre-mRNA. In plants such as Arabidopsis, spinach, and rice, which have two RCA isoforms, the two forms are encoded by mRNAs produced from alternative splicing of the transcribed pre-mRNA from a single RCA gene (Zielinski et al., 1989; Zhang and Komatsu, 2000). However, in cotton, the two isoforms are encoded by separate genes (Salvucci et al., 2003). In barley there are two genes, one that is alternatively spliced and a second that encodes only the smaller isoform. Two RCA polypeptides are encoded by two separate genes and the transcription rate of the two RCA genes differed during leaf development in barley leaves (Rundle and Zielinski, 1991). Ayala-Ochoa et al. (2004) reported that the accumulation of two maize RCA polypeptides, encoded by two separate genes, was regulated during leaf development. RCA gene expression seems to be tissue specific in all higher plants examined. It occurs almost only in green parts of the plant and is developmentally regulated by leaf age and light. The number of RCA -encoding genes varies depending on the plant species. Certain plant species like Arabidopsis, camelina, and spinach express equal amounts of α-isoform and β-isoform, while others like rice (*Oryza sativa*) and wheat (*Triticum aestivum*) accumulate much more β-isoform than α-isoform (Fukayama et al., 2012).

RCA is also considered to be a heat-labile protein and a key regulation point for photosynthesis, especially under moderately high temperature stress (Portis, 2003). Previously, a temperature-dependent dual function has been proposed for RCA at optimal temperatures. It works in releasing inhibitory sugar phosphates from the Rubisco active site, but during heat stress, RCA might function as a chaperone, protecting the protein synthesis machinery against heat inactivation (Rokka et al., 2001). Over-expression of sedoheptulose-1,7-bisphosphatase in rice suggested that yields were also improved under drought and heat stress by protecting the RCA (Feng et al., 2007). Research with the model plant, Arabidopsis, has already demonstrated that the thermotolerance of photosynthesis can be improved by increasing the thermal stability of RCA (Kumar et al., 2009).

Currently available X-ray crystallographic data of RCA provide for a spiral subunit packing arrangement within the crystal with unknown physiological relevance (Stotz et al., 2011).

RCA structure, activity and protein expression have been the focus of studies examining the effect of heat stress on this enzyme, but less is known about the response of RCA gene expression and protein content under salt and drought stress conditions. The genome of *B. distachyon*, a wild annual grass endemic to the Mediterranean and Middle East, has been sequenced. *B. distachyon* represents a wild, undomesticated grass species, in contrast to cultured crops such as rice, wheat, barley, sorghum, and maize. Comparison of the Brachypodium, rice and sorghum genomes shows a precise history of genome evolution across a broad diversity of the grasses, and establishes a template for analysis of the large genomes of economically important pooid grasses such as wheat. The high-quality genome sequence, coupled with ease of cultivation and transformation, small size and rapid life cycle, will help Brachypodium reach its potential as an important model system for developing new energy and food crops (Vogel et al., 2010).

The aim our research is to study RCA gene expression and protein content in *B. distachyon* grown under normal and also salt and drought stress conditions.

2. Results

2.1. Western blot analysis of Rubisco activase

Western blot analysis indicated that there are two RCA proteins in *B. distachyon*. The molecular mass of these polypeptides were approximately 46 and 42 kDa. Expression of RCA was detected in leaves, green stems and ear elements. RCA protein amount changed differently in leaves and stems of the early seedlings of plants subjected to drought and watered with 50 mM NaCl solution (Fig. 1). RCAL amount increased in initial leaves of seedlings exposed to drought as well as to salt stress, but this increase was more pronounced under salt stress. In stems of the early seedlings amount of the RCAL was sharply induced under drought, whereas under salt stress only the expression of the RCAS occurred. The results showed that content of RCAS was 10-fold more than that of RCAL content in different mature leaf positions and the RCAL/RCAS ratio reached maximum in the second and third leaf under optimal growth conditions (Fig. 2). RCAS and RCAL protein abundance levels decreased, in *B. distachyon* mature leaves in response to drought stress. Significant decrease of RCAL abundant was observed after 5 days of drought treatment (Fig. 2). But RCAL content increased after 24 h of rewatering. RCAL content increased in stem after 7 days of drought stress. RCAL/RCAS ratio increased under salt stress and this increase became more pronounced with increasing duration of the stress (Fig. 3). Contrary to the plants grown under normal conditions at the stages of ear forming and flowering, protein contents of the RCAS and RCAL isoforms were close and their total amount was less compared with that in leaves. At the beginning of the grain filling stage amount of the RCAL was more than that of the RCAS in awns (Fig. 4). Amounts of both isoforms appeared to be less in glumes compared with awns. Amounts of both isoforms increased parallely in awns under drought stress compared with watered plants, while in glumes more pronounced increase was observed for the RCAS. Immunoblot analysis did not reveal RCA protein expression in developing grains (Fig. 4). In contrast, expression of Rubisco large subunit (Rubisco LS) observed at the initial stages of grain development sharply decreased at the final stages and disappeared in fully mature grains. Rubisco protein content was found to be higher in awns and glumes compared with developing grains. Both RCA isoforms and Rubisco LS protein contents appeared to be high in glumes. However, unlike to RCA, Rubisco LS quantity decreased in glumes and awns in response to drought stress (Fig. 5).

2.2. Expression patterns of B. distachyon RCA

RCA transcript level was assessed by reverse transcription-polymerase chain reaction (RT-PCR). The expression of RCA gene

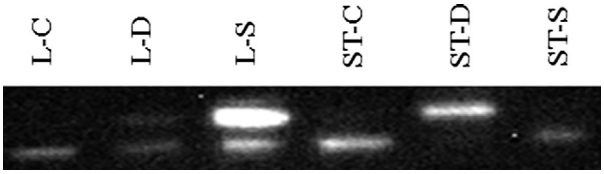

Fig. 1. Western-blot analysis of Rubisco activase in *Brachypodium distachyon* early leaf (L) and stem (ST) samples under control (C), salt stress (S) and drought stress (D) conditions.