Pak. J. Bot., 46(4): 1381-1388, 2014.

TWO RUBISCO ACTIVASE GENES FROM *IPOMOEA BATATAS* HAVE DIFFERENT ROLES IN PHOTOSYNTHESIS OF ARABIDOPSIS

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Abstract

Rubisco activase (RCA) that functions as a molecular chaperone regulates the activity of the Calvin-Benson cycle via regulation of the Rubisco activity. In plants such as *Arabidopsis thaliana*, *Spinacia oleracea*, and *Oryza sativa*, there are two RCA isoforms from two mRNAs that are produced from alternative splicing of the transcribed pre-mRNA of a single RCA gene. However, this research reported that the transcripts of the two IbRCA isoforms in sweet potato (*Ipomoea batatas*) were transcribed from two different genes. To study the roles of these two IbRCA isoforms in photosynthesis, we inserted these two *IbRCA* genes into the genome of Arabidopsis with deletion of RCA gene (ΔRCA), resulting in *IbRCAs*- and *IbRCAl*-expressing plants, respectively. Analysis of these transgenic Arabidopsis indicated that the *IbRCAs*-expressing plants were similar to wild-type plants under ambient CO_2 concentration and 22°C conditions, suggesting that expression of *IbRCAs* gene was sufficient for functional complementation of ΔRCA plants under normal conditions. However, *IbRCAs*-expressing plants were more susceptible to moderate heat stress (26°C) compared to wild-type plants. In contrast, although the *IbRCAl*-expressing plants had to grow normally in high CO_2 concentration conditions, there were almost no differences in growth and photosynthesis between normally grown and heat-treated plants, implying that *IbRCAl*-expressing plants had a better heat-resistance than *IbRCAs*-expressing plants.

Introduction

Photosynthesis is highly susceptible to inhibition by moderate heat stress. Of the components of photosynthesis that are impaired as temperature increases, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) activation has long been recognized as one of the most sensitive process (Kobza & Edwards, 1987; Weis, 1981a; b). Rubisco exists in three different statuses in photosynthetic cells: 1) non-carbamylated Rubisco (Type E); 2) Rubisco uncarbamylated and containing bound RuBP (type ER); 3) carbamylated enzyme with bound Mg^{2+} (Type ECM). The proportion of these Rubisco forms directly influences photosynthesis in plants (Crafts-Brandner & Salvucci 2000; Kubien & Sage, 2008; Wang *et al*., 2009). Rubisco activase (RCA, EC 4.1.1.36), a chloroplast protein encoded by the nuclear genome, regulates the activated status of Rubisco and improves the photosynthetic rate. In most plants, two forms of RCA (short isoform, RCAs; long isoform, RCAl) are present, and they differ only at the C-terminus. Unlike the RCAs, the RCAl holds a C-terminal extension that contains the redox-sensitive Cys residues (Portis *et al*., 2008; Salvucci *et al*., 2003). Two *RCA* isoforms can be produced by alternative splicing at the 3' end of a transcript, giving rise to two transcripts with about 100 nt difference or may be produced from two separate genes (Salvucci *et al*., 2003; Werneke *et al*., 1989). The role of RCA as Rubisco's chaperone (Portis, 2003; Zheng *et al.,* 2012) becomes most apparent during periods of stress, when Rubisco deactivation accelerates and photosynthesis is inhibited. Much attention has focused on the function of two RCA isoforms since its discovery. However, in previous studies, few researchers separately investigated the function of each RCA isoform on photosynthesis and/or plant growth. So, it is not clear whether the two RCA isoforms have different physiological functions or whether they both play important roles in photosynthesis. *In vitro*, it was shown that both RCAl and RCAs from spinach were capable of promoting Rubisco activation but they had marked difference in enzyme activity (Shen *et al*., 1991). In maize, Ayala-Ochoa (2004) and Vargas-Suarez (2004) observed that heat stress increased the accumulation of RCAs and induced the appearance of a novel RCAl (Ayala-Ochoa *et al.,* 2004; Vargas-Suarez *et al.,* 2004). Similarly, in cotton, Law (2001) detected a new heat-induced form of RCA at 46 kDa. It is considered that the expression pattern of activase was changed by undergoing heat-stress (Law *et al.,* 2001). Crafts-Brandner (1997) found that RCAl was more thermostable than RCAs and its optimum temperature for ATP hydrolysis was much higher (Crafts-Brandner *et al*., 1997). As we know, sweet potatoes exhibit the characteristics of efficient and longstanding photosynthesis even though they are grown under stress conditions. Meanwhile, in previous work, we found that the amount of IbRCA in sweet potato was greater than that of many other plants, and the accumulation of IbRCAs was much higher than IbRCAl (Xu *et al*., 2010). Are these superordinary characteristics of sweet potato related to these two isoforms of RCA? What is the relationship between these two RCA isoforms, and how do they play important roles in photosynthesis? In order to better understand the roles of the two IbRCA isoforms of sweet potato, we firstly cloned these two *IbRCA* isoform genes and over-expressed these genes in RCA-deletion mutant, respectively. The temperature tolerance of growth and photosynthetic efficiency were determined in the *IbRCA*-transgenic and wild-type Arabidopsis. The results indicated that these two RCA isoforms played different roles in regulating photosynthesis.

Materials and Methods

Plant material and growth conditions: The *Arabidopsis thaliana* (ecotype Columbia) was used as the wild-type control in this experiment. The Arabidopsis of RCA-deletion mutant (salk_118831) was isolated from the T-DNA-tagged pools at the Salk Institute T-DNA Express

(http://signal.salk.edu/cgi-bin/tdnaexpress). For screening of homozygote and transformation, the plants were grown in 22°C under an irradiance of 150 μ mol photons m^{-2} s^{-1} with a 10 h light/14 h dark photoperiod with a high CO_2 concentration (about 600 ppm). The relative humidity was maintained at 80–90% and plants were watered as necessary with 1/2 MS nutrient solution. Wild-type and T3 homozygous seeds of three independent *IbRCAs*-expressing lines (S1, S8, and S13) were seeded on pots in a climate box under ambient CO_2 concentration and 22°C or 26°C conditions. Wild-type and T3 homozygous seeds of three independent *IbRCAl*-expressing lines (L1, L5, and L7) were seeded on pots in a climate box under high CO_2 concentration and 22°C or 26°C conditions. Three-week old plants were carried out measuring photosynthetic rate. The temperatures of heat stress treatment were 26°C during the light cycle and the temperature during the 10 h dark cycle remained at 22°C.

Isolation of RNA, DNA and cloning of *IbRCA*: Total RNAs were isolated from leaves using Trizol reagent (Invitrogen, California, USA) according to the instruction manual and were treated with RNase-free DNase I (TaKaRa, Dalian, China). RNA concentration and RNA quality were determined using a spectrophotometer (GeneQuant pro, Biochrom, England). 1 μg of total RNAs were subjected to reverse transcription using 200 units of M-MLV reverse transcriptase (TaKaRa), 40 units of RNase inhibitor (TaKaRa), 100 ng of oligo(dT)12-18 primer, 10 mM (each) dNTPs, 4 μL reverse transcriptase buffer, in a total volume of 20 μL at 42°C for 1 h. The reaction was terminated by heating at 70°C for 15 min. Genomic DNA was extracted from mature leaves of sweet potato using the cetyl-trimethyl-ammonium bromide protocol as described by Weising *et al*., (1995) .

According to the results of MALDI-TOF-TOF/MS and the local transcriptomic database of sweet potato (Tao *et al*., 2012), primers were designed to amplify the cDNA and genomic DNA fragments of *IbRCAs* (for the sequence of the short *IbRCA* ORF) and *IbRCAl* (for the sequence of the long *IbRCA* ORF) using KOD FX DNA polymerase (TOYOBO, Shanghai, China). Then, these fragments were cloned into pMD19 vectors and sequenced. The primers used in PCR are: for *IbRCAs*, csF (5'- A T G G C T A C C T C A G T G T C C A C A A T T - 3') and csR (5'-T T A G C T A G C A A A A A A G G T T C C A T T G T T - 3'); for *IbRCAl*, clF (5'-A T G G C T G C C A C T G T C T C C A C - 3') and clR (5'-T T A A A C CT G G T A C G T G C A G G T T C - 3').

Expression plasmid construction: For expression plasmid construction, fragments of *IbRCAS* and *IbRCAl* were amplified using the PMD-*IbRCAs* and PMD-*IbRCAl* plasmids as templates, respectively. The primers used for PCR are: for *IbRCAs*, esF (5'- C G A C C A T G G C T A C C T C A G T G T C C A C A A T T - 3') and esR (5' – C G A G G T C A C C T T A G C T A G C A A A A A A G G T T C C A T T G T T - 3'); for *IbRCAl*, elF (5' – C G A C C A T G G C T G C C A C T G T C T C C A C - 3') and elR (5' – C G A G G T C A C C T T A A A C CT G G T A C G T G C A G G T T C - 3') (Restriction sites are underlined). The product of *IbRCAs* (or *IbRCAl*) was digested with *Nco*I and *Bst*EII, and inserted into binary vector pCAMBIA3302 (derived from pCAMBIA1302) with a 35S promoter and a Bar selection gene to generate a constitutive expression of *IbRCAs* (or *IbRCAl*) and the expression plasmid was designated p35S-*IbRCAs* (or p35S-*IbRCAl*).

Arabidopsis transformation and screening: Expression plasmids p35S-*IbRCAs* and p35S-*IbRCAl* were transformed into Agrobacterium tumefaciens strain GV3101 by freeze-thaw, respectively. The transformed Agrobacterium was grown overnight on a rotary shaker at 30°C in the dark in YEB medium (pH 7.0) containing the antibiotics rifampicin (50 mg/L), gentamicin (50 mg/L) and kanamycin (50 mg/L). The cultures were centrifuged and resuspended in fresh MS medium (Murashige and Skoog 1962). plus 5% (w/v) sucrose and 0.05% SilWet L-77 to a final concentration of 10^8 cells per mL (A620 =0.6) before used for Arabidopsis transformation. The Arabidopsis transformation was performed using a modified method developed by (Clough & Bent, 1998). T1 seeds harvested from infiltrated plants were screened on screening plates containing Murashige and Skoog salts and 50 μM/L Bsta (Amresco, USA). Resistant plantlets with two cotyledons were transplanted into pots containing a mixture of vermiculite and soil (1:1) in the growth chamber. T2 seeds were used for genetic analysis and the selection of the homozygous lines for the following experiments. Genomic DNAs of homozygous lines were isolated from fresh leaves using the cetyl-trimethyl-ammonium bromide protocol as described by Weising *et al*., (1995). DNA concentrations were determined using Qubit® fluorometer instrument (Invitrogen, California, USA). The polymerase chain reaction (Q-PCR) was used to confirm transformants by the *IbRCA*-specific primers svF/svR and lvF/lvR. The PCR amplification was carried out in a total volume of 25μl containing 100 ng of genomic DNA, 1 U of KOD FX DNA polymerase (TOYOBO, Shanghai, China) and 500 nM of each primer. The PCR conditions were as follows: denaturation at 98°C for 10 s, annealing at 60°C for 30 s, and an extension step at 68°C for 1 min and a final extension of 5 min at 68°C. The amplified products were electrophoresed on 1.5 % agarose gel.

The primers used in PCR are: for *IbRCAs*-expression lines, svF (5' – A T G G C T A C C T C A G T G T C C A C A A T-3') and svR (5' –C C A G T T T G T T G T C A A G A C T A T A T G T - 3'); for *IbRCAl*- expression lines, lvF (5' – C A G C T G A G C C C G A G A T T G A - 3') and lvR (5' – T T A A A C C T G G T A C G T G C A G G T T C - 3').

Preparation of antibodies: *E.coli* BL21(DE3) harboring the pET32-IbRCAc plasmids (expressing the common sequence of two IbRCA isoforms, a 73 amino acid peptide) was cultivated at 37°C in 20 mL LB medium, supplemented with 100 μg ampicillin/mL until an OD600 of 0.5, respectively. The culture was then transferred into 1 L fresh LB medium and grown until an OD600 of 0.5. IPTG was added to a final concentration of 0.5 μM. After further cultivation for 16 h at 18°C, the culture was centrifuged and the cells were washed twice with phosphate-buffered saline (PBS), resuspended in 30 mL PBS and cracked by sonication on ice. The lysate was centrifuged for 30 min at 15,000 g at 4°C. Recombination protein of IbRCAc was then purified from the supernatant using HisTrap HP affinity columns, according to manufacturer's instructions (GE Healthcare). The Purified

recombination protein of IbRCAc was quantified by the Bradford assay23). The recombination protein of IbRCAc was injected into male New Zea-land rabbits to produce Anti-IbRCAc antibodies.

Protein gel blots analysis: Protein extracts were subjected to SDS-PAGE analysis using 12% acrylamide resolving gel (Mini Protean II System; Bio-Rad). The separated proteins were then transferred to polyvinylidene difluoride membranes, and nonspecific binding of antibodies was blocked with 5% nonfat milk in phosphate-buffered saline solution (pH 7.4) for 2 h at room temperature. Membranes were then incubated overnight at 4°C with polyclonal anti-IbRCAc antibodies (recognize two IbRCA isoforms). The Anti-rabbit IgG antibodies were used as the secondary antibodies (Huaan biotechnology, Hangzhou, China). The enhanced chemiluminescence (ECL) Western-blotting detection kit (Tiangen, Beijing, China) was used for color development.

Gas exchange: A portable photosynthesis system (LICOR-6400, LICOR, Lincoln, NE) with a red/blue LED light source (LI6400-02B) mounted onto a Whole Plant Arabidopsis chamber (LI-6400-17) was used to determine the gas exchange. The chamber is designed to measure whole plant CO_2 exchange on small rosette-type plants such as A. thaliana. Gas exchange measurements were carried out from 8:00 to 18:00 (light cycle). The conditions of gas exchange measurements were consistent with the corresponding growth conditions of these plants.

Statistical analysis: All results were presented as means ± SE. The statistical significance of differences in the mean values between the transgenic and wild-type plants was determined using the Student's t test. And set $p<0.05$ as a threshold.

Results and discussion

Clone of *IbRCA*: In plant species, Rubisco activase (RCA) is present either as a long isoform (RCAl) and a short isoform (RCAs) or only RCAs (Portis 2003). Unlike the two *RCAs* in Arabidopsis and spinach, which are produced by alternative splicing at the 3' end of one transcript (Salvucci *et al*., 2003), sweet potato leaf contains *IbRCAs* and *IbRCAl*, which are derived from two separate genes (Fig. 1B). This conclusion is supported by the following evidence: (1) *IbRCAs* contains four introns, while *IbRCAl* contains six introns. Although the distribution and the position of these introns are conserved in *IbRCAs* and *IbRCAl*, the length and sequence of these introns are quite different; (2) the difference in amino acid composition between *IbRCAs* and *IbRCAl* could not be resolved by the current *RCA* transcript splicing mechanism observed in Arabidopsis, spinach, and rice; (3) the amino acid sequences of IbRCAs and IbRCAl showed only approximately 75% identity. In addition, there were three highly homologous genes for both *IbRCAs* and *IbRCAl* (Fig. 1). Although nucleotide sequences of three similar isoforms had different numbers of point mutations, most of them occurred in the first or third base of codons, so amino acid sequences changed rarely (Table 1). Similarly, it was found that several homologous genes for sucrose transporter genes and 14-3-3 protein genes were present in sweet potato, which was likely from two or three subgenomes of hexaploid sweet potato (Chang *et al*., 2012; Li *et al*., 2010). The high homologies of these genes, which should be from three similar subgenomes, imply that the sweet potato is probably an autohexaploid origin. However, this inference cannot be confirmed at this time, because the wild ancestors of sweet potato have not been determined. Sequences data from this article have been deposited at GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers JQ923423 - JQ9234230.

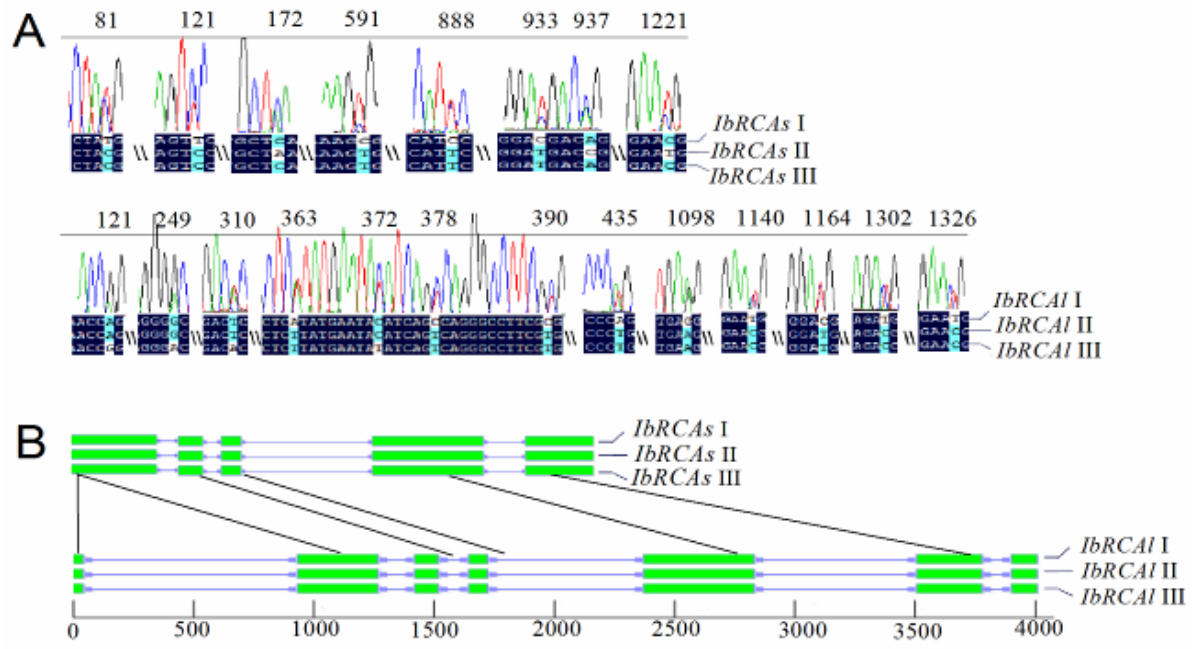

Fig. 1. Structural features of the two *IbRCA* isoforms. A. Nucleotide differences of the three *IbRCAs* homologous genes (named *IbRCAs* I, II, and III) and the three *IbRCAl* homologous genes (named *IbRCAl* I, II, and III). B. Genomic structures of *IbRCAs* and *IbRCAl* genes. Exons (shown in gray boxes) and introns (shown in white lines) are drawn to scale.

Table 1. Difference among three similar isoforms of *IbRCAs* and *IbRCAl*.

Mutational site	**81**	**121**	**172**	**591**	**600**	**735**	**870**	**888**	**933**	**936**	**965**	**1221**
Ib-RCAs I	T	T	C	C	G	T	A	C	C	A	G	C
Ib-RCAs II	C	C	A	T	C	C	T	T	T	C	G	T
Ib-RCAs III	C	C	C	T	G	T	T	T	T	A	A	C
Codon[a]	3	3	1	3	3	3	3	3	3	3	2	3
Amino acid[b]	N	P to S	Q to K	N	N	N	N	N	N	N	R to K	N
Mutational site	**121**	**142**	**249**	**310**	**336**	**363**	**372**	**378**	**390**	**411**	**435**	**624**	**627**
Ib-RCAl I	A	T	G	T	T	A	C	C	C	A	A	T	T
Ib-RCAl II	A	C	G	T	C	T	T	T	T	G	T	C	C
Ib-RCAl III	G	C	A	A	C	T	T	T	T	G	T	C	C
Codon[a]	3	3	3	1	3	3	3	3	3	3	3	3	3
Amino acid[b]	N	P to S	N	S to T	N	N	N	N	N	N	N	N	N
Mutational site	**714**	**720**	**734**	**747**	**792**	**939**	**951**	**1044**	**1098**	**1140**	**1164**	**1302**	**1326**
Ib-RCAl I	T	T	G	G	A	C	A	A	G	T	C	T	T
Ib-RCAl II	T	C	A	A	T	T	T	C	A	C	T	C	C
Ib-RCAl III	T	C	G	A	T	T	T	C	A	C	T	C	C
Codon[a]	3	3	1	3	3	3	3	3	3	3	3	3	3
Amino acid[b]	N	N	G to E	N	N	N	N	N	N	N	N	N	N

[a] = Position in a codon.

[b] = N, No change in amino acid. P to S, proline to serine change

Transformation: The line of RCA-deletion mutant (ΔRCA), salk_118831 of Arabidopsis was used to investigate the role of two *IbRCA* isoforms in photosynthesis and/or in plant growth. Two *RCAs* in Arabidopsis are produced by alternative splicing at the 3' end of one transcript (Yin *et al*., 2010), so the ΔRCA lines express neither of two RCA isoform proteins. As we know, the RCA mutant of Arabidopsis is isolated as a mutant which required high CO_2 concentration for plant growth, because of incapability of maintaining sufficient activation of Rubisco to grow under the atmospheric CO_2 concentration (Eckardt *et al*., 1997; Salvucci *et al*., 2006). So, we grew these plants in a climate box (RZH-500C-CO_2, HuiEr, HangZhou) and controlled the CO_2 concentration as 600 ppm. The RCA mutant of salk_118831 with a T-DNA insertion in the first intron, 333bp downstream of the start codon was verified by PCR (Fig. 2A). Wild-type Arabidopsis expresses approximately equal amounts of the 42 kDa non-redox-regulated short isoform and 46 kDa redox-regulated long isoform of activase (Fig. 2C) (Henderson *et al*., 2011). The absence of the short and long isoforms in plants homozygous for the deletion by PCR and Western blot analysis confirmed that the ΔRCA abrogated the expression of both endogenous RCAs and RCAl (Figs. 2B, C).

The RCA catalyzes the activation of Rubisco by removing inhibitory sugar phosphate (Andrews *et al*., 1995). This enzyme is required to maintain Rubisco activity in higher plants grown at ambient CO_2 concentration. It was shown that RCA may exert considerable control over photosynthesis (Hendrickson *et al*., 2008; Kim & Portis, 2005; Portis, 2003; Salvucci & Crafts-Brandner, 2004; Weston *et al*., 2007) and plant growth (Haigler *et al*., 2007; Kurek *et al*., 2007) especially under heat stress. In order to investigate the role of two *IbRCA* isoform genes, p35S-*IbRCAs* and p35S-*IbRCAl* were inserted into the genome of salk_118831, respectively, by floral dip of Agrobacterium cells. A total of 14 independent resistant lines in *IbRCAs* transformation and 8 independent resistant lines in *IbRCAl* transformation were obtained. And then the genomic DNAs were isolated from these 22 plants and the PCR reactions were performed using the svF/ svR and lvF/ lvR primers. The results displayed that only three independent resistant lines (S1, S8, and S13) could detect an expected 389 bp DNA fragment and three independent resistant lines (L1, L5 and L7) could detect an expected 1262 bp DNA fragment (Fig. 2B). These six independent lines were then used for Western blot assay, and all the six transgenic plants expressed only one type of IbRCA, that is, IbRCAs or IbRCAl (Fig. 2C).

Expression of *IbRCAs* gene in Arabidopsis Δ*RCA* mutant recovered the ability of photosynthesis: Under normal growth conditions (22°C, atmospheric CO_2 concentration), *IbRCAs*-expressing lines could grow normally and had the similar phenotype with wild-type plants (Fig. 3A). In order to confirm the functions of *IbRCAs* in photosynthesis, three-week-old *IbRCAs*-expressing and wild-type plants were carried out to measure the photosynthetic rate. The results showed that there were almost no differences in photosynthetic rate between wild-type and *IbRCAs*-expressing plants (Fig. 5A). In addition, the fresh weight of wild-type and *IbRCAs*-expressing plants were measured, and the result displayed that the wild-type and *IbRCAs*-expressing plants had the similar fresh weight (Table 2). These data suggested that the expression of *IbRCAs* in Arabidopsis is sufficient for functional complementation to recover the ability of photosynthesis by significantly decreasing the threshold value of CO_2 concentration for catalyzing the carbamoyl reaction.

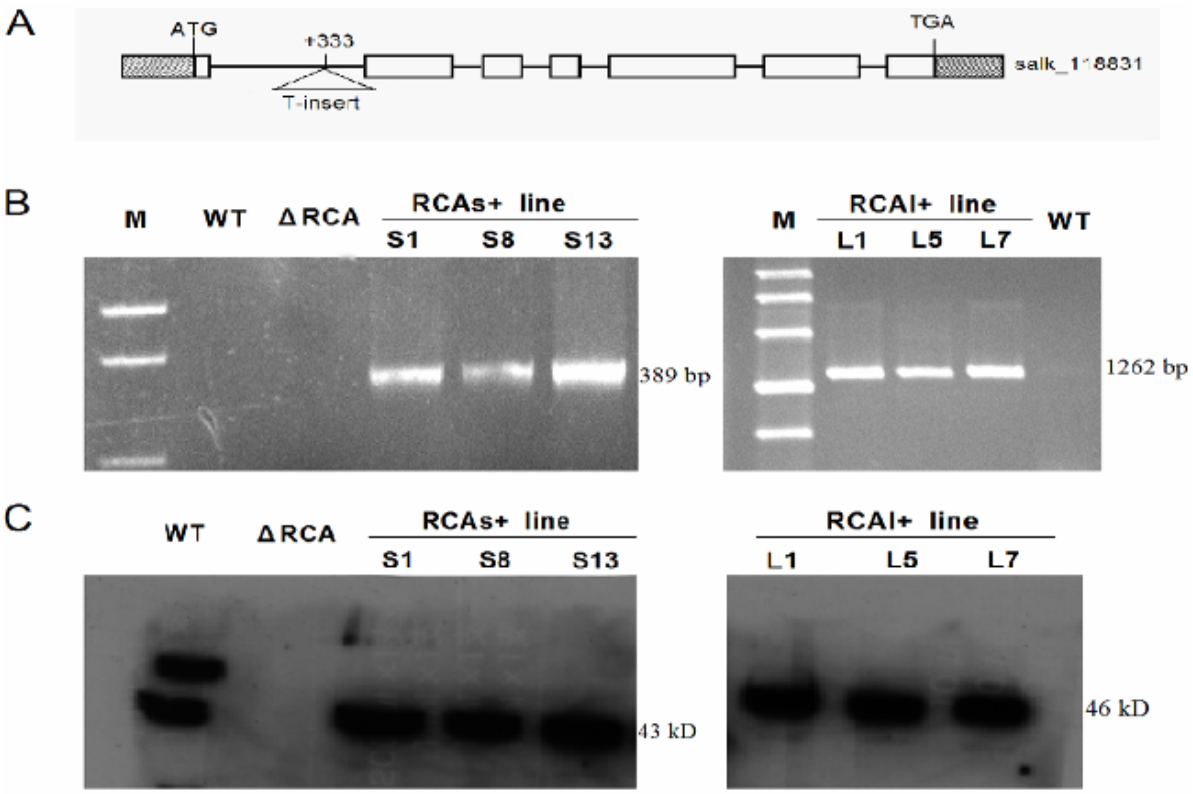

Fig. 2. Molecular analyses of *IbRCA*-transgenic Arabidopsis. A. Schematic diagram of the T-DNA insertions into the Arabidopsis RCA gene (At2g39730.1). Numbers indicate the positions of T-DNA insertion relative to the start codon ATG. The open triangle shows the T-DNA insertion. B. PCR analysis of transgenic Arabidopsis plants. M, DNA molecular weight markers; WT, wild-type plant; RCAs+ lines, *IbRCAs*-expressing plants; RCAl+ lines, *IbRCAl*-expressing plants. *IbRCAs*-specific and *IbRCAl*-specific primers were used in the PCR analysis. C. Western blots analysis of IbRCA-transgenic plants.

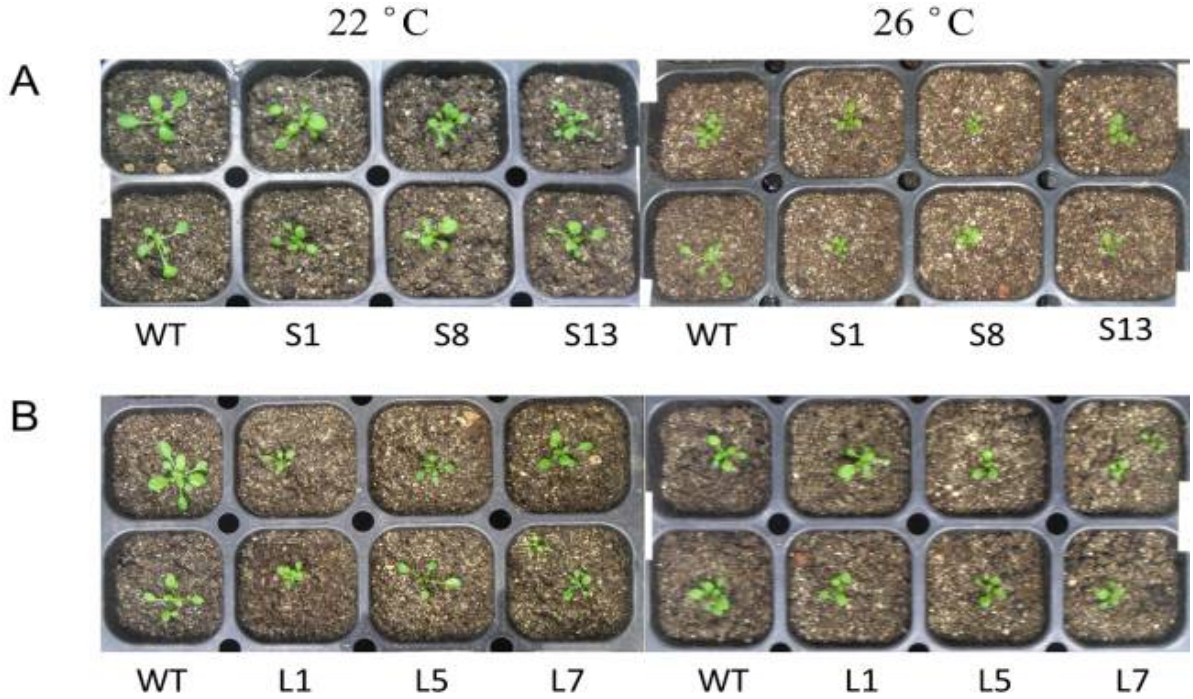

Fig. 3. Growth status of three-week-old Arabidopsis. A. Wild-type and *IbRCAs*-expressing plants were grown under 22°C or 26°C and ambient CO_2 concentration conditions. B. Wild-type and *IbRCAl*-expressing plants were grown under 22°C or 26°C and high CO_2 concentration (600 ppm) conditions.

Table 2. Fresh weight of the whole Arabidopsis (three-week-old). *n* = (6) ± SE. *indicated significant differences between grown at 22°C and 26°C.

Plant line	22°C (mg fresh weight /plant)	26°C (mg fresh weight /plant)	Mean of differences (mg fresh weight /plant)	P value (Significant)
WT	4.93 ± 0.143	3.20 ± 0.058	1.733	<0.0001**
S1	4.57 ± 0.109	2.32 ± 0.037	2.267	<0.0001**
S8	4.50 ± 0.086	2.27 ± 0.042	2.233	<0.0001**
S13	4.38 ± 0.108	2.60 ± 0.037	1.783	<0.0001**
L1	2.92 ± 0.101	2.78 ± 0.101	0.133	0.3461
L5	2.93 ± 0.149	2.83 ± 0.076	0.106	0.2063
L7	3.13 ± 0.067	2.65 ± 0.065	0.582	0.0489*

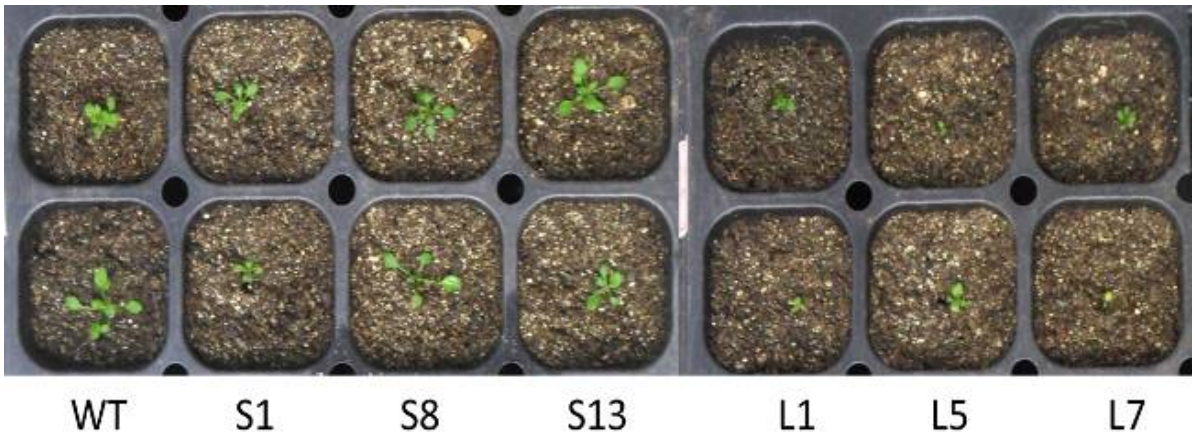

Fig. 4. Arabidopsis growth status under ambient CO_2 concentration and 22°C conditions.

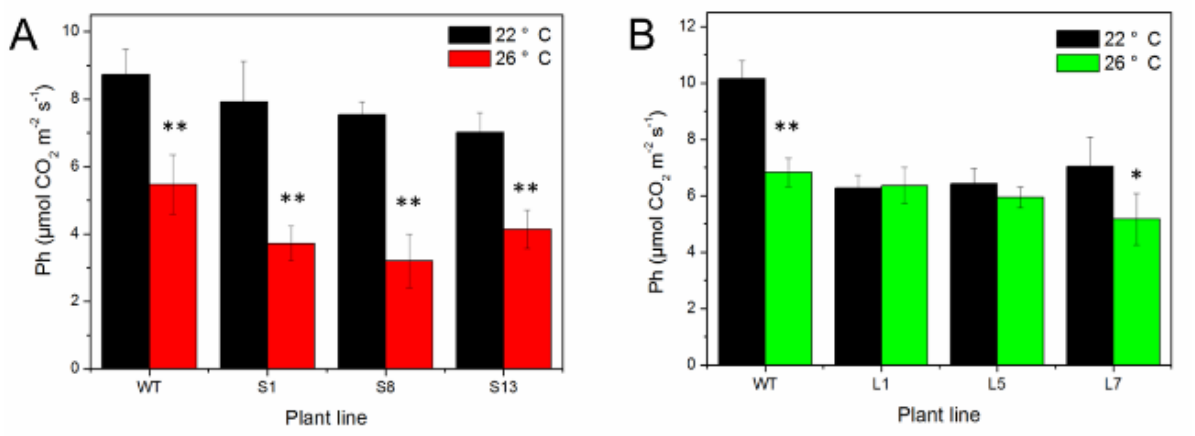

Fig. 5. Photosynthetic rate of Arabidopsis. A. Wild-type and IbRCAs-expressing plants were grown under 22°C or 26°C and ambient CO_2 concentration conditions. B. Wild-type and IbRCAl-expressing plants were grown under 22°C or 26°C and high CO_2 concentration (600 ppm) conditions. Bars marked with a * indicated significant differences between grown at 22°C and 26°C. Bars indicate mean and error bars are SE. Six plants were used in these experiments.

The photosynthetic performance of wild-type and *IbRCAs*-expressing lines under heat stress was also measured, and the data were collected after these plants were grown at 26°C for three weeks. Both *IbRCAs*-expressing lines and wild-type plants began to turn yellow and exhibited slow growth rates. Although *IbRCAs*-expressing plants could recover the ability of photosynthesis under normal conditions, the photosynthetic rate was decreased sharply compared to that of wild-type plants when the temperature was at 26°C (Fig. 5A). The average photosynthetic rate of *IbRCAs*-expressing plants was 7.49 ± 0.72 μmol CO_2 m^{-2} s^{-1}, and was about 86% of wild-type plants (8.73 ± 0.75 μmol CO_2 m^{-2} s^{-1}) at 22°C However, its photosynthetic rate was declined drastically and dropped by 50.7%, when the temperature increased to 26°C. In contrast, the photosynthetic rate of wild-type plants was only decreased by 37.3% at 26°C compared to that at 22°C (Fig. 5A). Besides, the fresh weight of the whole plant among different lines displayed the same results (Table 2). Crafts-Brandner & Salvucci, (2000) also demonstrated that the Rubisco of higher

plant was a thermostable enzyme with enhanced catalytic turnover rates as temperature increase. Thus, we infer that the decrease of photosynthesis might be caused by the thermolability of IbRCAs under heat stress.

Expression of *IbRCAl* enhanced heat tolerance: Unlike the *IbRCAs*-expressing plants, under normal growth conditions, *IbRCAl*-expressing plants could not grow normally (Crafts-Brandner & Salvucci, 2000) (Fig. 4). So we grew *IbRCAl*-expressing plants in a climate box under 22°C with a high CO_2 concentration. The average photosynthetic rate of three-week-old *IbRCAl*-expressing plants was 6.59 ± 0.67μmol CO_2 m^{-2} s^{-1} and only 65% of wild-type plants (Fig. 5B). Compared to at 22°C, the wild-type plants displayed lower growth rate under 26°C. However, *IbRCAl*-expressing plants were hardly changed in appearance between grown at 22°C and 26°C (Fig. 3B). In order to further study whether the *IbRCAl*-expressing lines could improve heat tolerance, three-week-old plants grown at a high CO_2 concentration (600 ppm) and 26°C were measured their photosynthetic rate. The results revealed that the photosynthetic rate of *IbRCAl*-expressing plants exposed heat stress were 5.82 ± 0.64 μmol CO_2 m^{-2} s^{-1}, and was 88.3% of the unexposed heat stress plants, that is, their photosynthetic rate decreased by 11.7%. However, the photosynthetic rate of wild-type plants exposed heat stress was 6.83 ± 0.51μmol CO_2 m^{-2} s^{-1}, and declined by 32.7% compared with unexposed heat stress plants (Fig. 5B). These data showed that the expression of *IbRCAl* cannot improve the photosynthetic rate directly under ambient CO_2 concentration, but it could markedly enhance the thermostability of the plants and may constitute a mechanism of photosynthetic acclimation to heat stress. Numerous previous studies supported this notion. These studies revealed that RCAl was more thermostable than RCAs and its optimum temperature for ATP hydrolysis was much higher (Crafts-Brandner *et al*., 1997; Salvucci, 2008; Salvucci & Crafts-Brandner, 2004). Furthermore, we found that both two RCA isoforms were present in sweet potato at any conditions, and the RCAl was also markedly unregulated by heat. Based on the relationship between activase isoform and photosynthesis described above, it would be expected that the plants should exhibit greater photosynthesis with amounts of IbRCAs increasing under normal conditions, but less inhibition by moderate heat stress on amounts of IbRCAl increasing.

Conclusion

We cloned and characterized two *IbRCA* cDNAs encoding the short and long isoforms in sweet potato and found that these two *IbRCA* isoform mRNAs were transcripts from two different genes. Interestingly, there are three highly homologous isoforms (homology > 98%) in both short and long *IbRCA*, which were likely from three similar subgenomes of the hexaploid sweet potato, implying that sweet potato is probably an autohexaploid origin. Based on the relationship between activase isoform and photosynthesis described above, it would be expected that plants should exhibit greater photosynthesis with amounts of IbRCAs increasing under normal conditions, but less inhibition by moderate heat stress on amounts of IbRCAl increasing.

Acknowledgements

The work was supported by the "Eleven-Five" Key Project of Sichuan Province (Grant No. 07SG111-003-1), National Science &Technology Pillar Program of China (Grant No. 2007BAD78B03) and the Key Program for forestry of Chongqing University of Art and Science (Grant No. 201302).

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(Received for publication 15 March 2013)