Overexpression of the rubisco activase gene improves growth and low temperature and weak light tolerance in Cucumis sativus

Huangai Bi, Peipei Liu, Zhensheng Jiang and Xizhen Ai

State Key Laboratory of Crop Biology/key laboratory of crop biology and genetic improvement of horticultural crops in huanghuai region/College of Horticulture Science and Engineering, Shandong Agricultural University, Tai’an, 271018, China
Research and Development Department, Golden Ma Ma Agricultural Science and Technology Company, Qingdao, 266600, China
Academy of Agricultural Sciences, Kingenta Ecological Engineering Group Co., Linyi, 276700, China

Correspondence
*Corresponding author,
e-mail: axz@sdau.edu.cn
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Rubisco activase (RCA) is an important enzyme that can catalyze the carboxylation and oxygenation activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is involved in the photosynthetic carbon reduction cycle. Here, we studied the effects of changes in RCA activity on photosynthesis, growth and development, as well as the low temperature and weak light tolerance of RCA overexpressing transgenic cucumber (Cucumis sativus) plants. CsRCA overexpression increased the plant height, leaf area and dry matter, and decreased the root/top ratio in transgenic cucumber plants compared with the wild-type (WT) plants. Low temperature and low light stress led to decreases in the CsRCA expression and protein levels, the photosynthetic rate (Pn) and the stomatal conductance (Gs), but an increase in the intercellular CO2 (Ci) concentration in cucumber leaves. The actual photochemical efficiency and maximal photochemical efficiency of photosystem II in cucumber seedlings also declined, but the initial fluorescence increased during low temperature and weak light stress. Transgenic plants showed a lower decrease in the CsRCA expression level and actual and maximal photochemical efficiencies, as well as increases in the Ci and initial fluorescence relative to the WT plants. Low temperature and low light stress resulted in a significant increase in the malondialdehyde (MDA) content; however, this increase was reduced in transgenic plants compared with that in WT plants. Thus, the overexpression of CsRCA may promote the growth and low temperature and low light tolerance of cucumber plants in solar greenhouses.

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, 4.1.1.39) is an important photosynthetic enzyme that catalyzes the carboxylation and oxygenation of ribulose-1,5-bisphosphate (RuBP), the first committed step in the competitive metabolic pathways of photorespiration and photosynthetic CO2 fixation in higher plants (Spreitzer 1999, Hong et al. 2005). Rubisco activity is dependent on Rubisco activase (RCA), a nuclear-encoded chloroplast regulatory protein in plant leaves, because the carboxylation and oxygenation activities of internal Rubisco can only be activated by RCA. RCA may control the release of RuBP and other inhibitors from Rubisco and thus regulate its activation state (Brooks and Portis 1988). Therefore, the RCA activity is an important factor for regulating Rubisco activity (Gao et al. 2008).

Abbreviations – MDA, malondialdehyde; PSII, photosystem II; RCA, Rubisco activase; RT-PCR, reverse transcription-polymerase chain reaction; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WT, wild-type.
Since RCA was discovered in 1985 (Salvucci et al. 1985), the enzymatic characteristics, activation in vivo, and self-regulating mechanism have been extensively investigated (Zhang et al. 2005, Portis et al. 2008, Li et al. 2010). However, the cooperative mechanisms of RCA and Rubisco in higher plants remain unclear in the ever-changing environment. With the development of biotechnology, the RCA genes of many plants have been cloned (Werneke et al. 1989, Rundle and Zielinski 1991, To et al. 1999, Salvucci et al. 2003). To reveal the cooperation between RCA and Rubisco, and the role of RCA in regulating photosynthesis, a series of studies have been performed to determine whether the Rubisco activity would be improved by changing the RCA's catalytic ability using transgenic plants. As we know, RCA exists in $\alpha$- and $\beta$-subunit form in higher plants and the $\beta$-subunit was about 30 amino acid residues smaller than the $\alpha$-subunit, which was caused by different alternative splicing of the RCA gene. The existence of RCA can be very different from one plant to another. Portis (2003) found that there was only $\beta$-subunit in cucumber plants.

Zhang et al. (2002) found that in Arabidopsis plants expressing only the shorter RCA isoform, the Rubisco activity was as high as in the wild-type (WT) under saturating light, but the activity was not downregulated at light intensities that were limiting for photosynthesis. In contrast, in plants expressing only the longer isoform, the Rubisco activity was downregulated at limiting light intensities, but the activity was slightly lower and increased much more slowly at saturating light intensities when compared with WT Arabidopsis plants. Wu (2004) obtained transgenic rice plants containing the larger sense RCA isoform in which the Rubisco activity, net photosynthetic rate (Pn), apparent quantum yield, electron transfer rate and grain quality were all improved in the transgenic rice plants.

In northern China, low temperatures and low light intensities are the most common abiotic stresses for cucumber plants cultivated in solar greenhouses. These conditions often limit the growth and yield of cucumber. One strategy to overcome these stresses is the engineering of cucumber plants with suitable genes that allow the plants to adapt to the specific solar greenhouse environment (Bi et al. 2013). Although the RCA gene of cucumber was cloned in 1992 (Preisig-Muller and Kindl 1992), it was not known if biotechnology could be used to improve the photosynthetic capacity and alleviate the adverse effects of low temperature because cucumber is difficult to transform.

Recently, we obtained cucumber plants overexpressing the RCA gene (CsRCA) through Agrobacterium tumefaciens-mediated transformation. CsRCA's overexpression increased the Pn and carbohydrate contents significantly in cucumber leaves (Liu et al. 2012). We investigated the role of overexpressed CsRCA in growth, photosynthetic carbon metabolism and the chilling tolerance of cucumber plants. This work will help provide information for future research on improving the stress resistance and yields of cucumber and other horticultural crops.

Materials and methods

Plant materials and growth

Transgenic plants with overexpression of RCA gene (CsRCA) were grown from seeds collected from selfed T0 progeny of cucumber (Cucumis sativus ‘08-1’) transformed using the A. tumefaciens system (Liu et al. 2012). The seeds of three T0 progeny lines named T1-2, T1-3 and T1-7 with 2.0-fold, 1.8-fold and 1.6-fold of WT RCA were selected and used for this study. Untransformed ‘08-1’ cucumber plants were used as the control. A total of 50 seeds of each transgenic line and WT were germinated on moist filter paper in the dark at 28°C for 24 h, and then, the seeds were sown in 10-cm diameter plastic pots filled with sterilized soil (one plant per pot) and grown in solar-greenhouse with sunlight during the day (maximum of 800–1000 μmol m$^{-2}$ s$^{-1}$ PFD) and 24–30°C/12–20°C day/night temperature under a 13 h photoperiod. On September 13, the seedlings with three leaves were planted at the experimental station of Shandong Agricultural University, Tai’an, China, with a density of 55 000 plants per hectare (row and plant spacings were 60 and 30 cm, respectively). All plants received the same management under typical solar-greenhouse conditions; 70 days after sowing (October 25), the plant growth of transgenic and WT cucumber was investigated.

Low temperature and weak light treatment

We sowed 50 seeds of each transgenic line and WT cucumber and the seedlings were grown at the same condition as above. When the third leaf was fully expanded, a group of the seedlings were transferred to a growth chamber simulated low temperature (5°C) and weak light (90–100 μmol m$^{-2}$ s$^{-1}$). The remaining seedlings were kept at normal conditions in solar-greenhouse as a control. The photoperiod was 13 h. Young fully expanded leaves were sampled for analysis at 0, 6, 12 and 24 h after transferring from control to stress condition.

Growth analysis

The effects of CsRCA overexpression on growth of cucumber plants were measured over the 70 days of
Real-time quantitative RT-PCR assay

Total RNA from cucumber leaves was extracted using an RNA extraction kit (Trizol; TaKaRa, Dalian, China) and reverse transcribed using the PrimeScript® RT Master Mix Perfect Real Time (TaKaRa). The relative mRNA expression of the gene of RCA in cucumber plants was analyzed by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) using a SYBR® Premix Ex Taq™II (TaKaRa) according to the manufacturer's instructions. The expression level for each gene was calculated as 2-ΔΔCt, where Ct represents the cycle number when the fluorescence signal in each reaction reaches the threshold. All samples were repeated three times.

SDS-PAGE and immunological analysis

A section of the CsRCA coding region in the pMD18-T vector, approximately 562 bp, was subcloned into the pET-30 a(+) vector between the BglII and EcoRI sites. A recombinant of the prokaryotic expression vector pET-CsRCA was constructed and transformed into Escherichia coli BL21, and then expression was induced with 0.5 mM isopropylthio-β-D-galactoside (IPTG) for 4 h at 37°C. Insoluble aggregates were solubilized in the presence of 2× SDS loading buffer and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% separating gels and 5% concentrating gels containing 10% SDS. The strongly induced fusion protein bands were collected in a phosphate buffer solution (PBS) and were used to immunize BALB/c female mice (6 weeks old) with four subcutaneous injections at 1-week intervals to obtain an antiserum. The secondary antibody was peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Bio-technology, Inc.).

Rubisco and RCA activity assays

Assays for the activities of Rubisco and RCA were performed using the NaH14CO3 method, as described by Jiang et al. (2010).

Measurement of gas exchange and fluorescence parameters

Net photosynthetic rates (Pn), stomatal conductance (Gs) and intercellular CO2 concentration (Ci) were measured for the second apical leaves using a portable photosynthetic system (Ciras-2, PP Systems International, Hitchin, Hertfordshire, UK). Constant PFD (600 μmol m−2 s−1), CO2 concentration (350–360 mg l−1) and leaf temperature (25 ± 1°C) were maintained throughout all measurements. Each measurement was repeated at least three times. Fluorescence was measured at 25°C with five replications using a portable fluorometer (FMS-2; Hansatech, Norfolk, UK). Leaves were dark adapted for 30 min before measurement of maximal fluorescence (Fm), variable fluorescence (Fv) and initial fluorescence (F0) in darkness. Afterwards, the steady state fluorescence (Fs), initial fluorescence (F0′) and maximum fluorescence (Fm′) under 600 μmol m−2 s−1 effective light were detected. Fluorescence nomenclature was used and calculated according to Demmig-Adams and William (1996) and service manual of FMS-2 chlorophyll fluorometer as follows: maximal photochemical efficiency of photosystem II (PSII) in darkness: ΦPSII = (Fm − F0)/Fm.

Measurement of MDA content

The MDA content was determined by thiobarbituric acid (TBA) method, as described by Zhao et al. (2002).

Statistical analysis

The data are presented as the means ± 1 standard deviation (SD) of three replicates. Statistical analysis, such as ANOVA, was performed using DPS software. The Duncan’s multiple range test (DMRT) was applied to compare significant differences between treatments.
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Results
Molecular characterization of the transgenic plants
Transgenic plants infected with A. tumefaciens carrying CsRCA were determined by PCR after an initial screening with 50 mg l⁻¹ hygromycin. Seven individual hygromycin-resistant lines were obtained from tissue culture (Liu et al. 2012). These initial hygromycin-resistant plants were named T0, and the progeny obtained from T0 were named T1. Three lines, T1-2, T1-3 and T1-7, were selected for real-time PCR and western blot analyses. Real-time PCR showed that the level of CsRCA mRNA increased 0.92-fold, 0.78-fold and 0.52-fold in the T1-2, T1-3 and T1-7 plants, respectively, compared with the WT plants (Fig. 1A). A western blot analysis with an antiseraum against CsRCA revealed the presence of strong protein signals in transgenic plant leaves, whereas a weak signal was found in WT cucumber leaves (Fig. 1C).

Activities of RCA and Rubisco in transgenic cucumber leaves
The activities of RCA in WT and T1-2, T1-3 and T1-7 transgenic cucumber leaves were measured. Fig. 1B shows that the activity levels of RCA in the three selected transgenic cucumber lines (T1-2, T1-3 and T1-7) were 41, 34 and 15% higher than the activity in WT plants (P < 0.05). Thus, the overexpression of CsRCA increases the activity of RCA in transgenic plants relative to the activity in WT plants.

To further evaluate the cooperation of RCA and Rubisco, the activity of Rubisco in the WT and transgenic plants was also determined. The Rubisco total and initial activities (Fig. 1D, E) in the T1-2, T1-3 and T1-7 plants were significantly higher than those in the WT plants (P < 0.05).

The linear relationship between photosynthetic rate and RCA activity
The Pn in transgenic plants with increased RCA activities was promoted in RCA sense plants compared with equivalent WT tomato plants (data not shown) (Liu et al. 2012). To further explore the relationship between RCA activity and photosynthesis, we performed a preliminary analysis by plotting photosynthesis against RCA activity (Fig. 2A), Rubisco initial activity (Fig. 2C) and Rubisco total activity (Fig. 2D). The RCA activity
was linearly correlated ($R^2 = 0.96$) with photosynthesis in transgenic cucumber plants. The initial and total activities of Rubisco, as the key enzyme in the Calvin cycle, were linearly correlated with photosynthesis (Fig. 2C, D). Thus, we speculated that $CsRCA$ regulated the photosynthesis of cucumber plants through Rubisco as indicated by the data in Fig. 2B.

**Growth in transgenic cucumber plants**

The growth in transgenic and WT cucumber plants grown in solar greenhouses for 70 days are shown in Fig. 3. The plant heights were significantly higher in T1-2 and T1-3 compared within WT ($P < 0.05$). The leaf areas also increased in T1-2 and T1-3 ($P < 0.05$), but no remarkable differences were observed between T1-7 and WT in either plant height or leaf area. There were no obvious differences in stem diameter between the transgenic and WT plants. Compared with WT plants, the T1-2 and T1-3 plants increased 31% and 25% in root dry matter, respectively, and increased 42% and 35% in top dry matter, respectively. No significant differences were found between T1-7 and WT in root or top dry matter. T1-2 and T1-3 presented significantly lower root to top ratios than WT plants, but T1-7 showed no remarkable difference compared with WT plants.

We also analyzed the linear correlation ($R^2 = 0.95$ for $Pn$; $R^2 = 0.98$ for RCA activity) between $Pn$ or RCA activity and total dry matter accumulation, implying the importance of RCA in carbon assimilation. Fig. 4 shows that at a higher $Pn$, the total dry matter increased, in agreement with the higher RCA activity. This suggested that $CsRCA$ overexpression improves the growth attributed to the higher $Pn$, which increased the accumulation of shoot photosynthetic products in transgenic cucumber plants with increased the RCA activity. The higher the activity level, the greater the positive effects.

**Response of $CsRCA$ to low temperature and weak light stress in WT and transgenic seedlings**

Changes in $CsRCA$ expression under chilling stress are shown in Fig. 5B. The protein signals decreased as the time under stress increased both in WT and transgenic plants; however, the signal was always stronger in transgenic plants than in WT plants. After 24 h of chilling stress, protein signals still appeared in transgenic plants, while none were observed in WT plants. This demonstrated that RCA is sensitive to low temperature and weak light, and that short-term chilling stress can decrease its gene expression level.

**Effect of low temperature and low light on gas exchange parameters in WT and transgenic seedlings**

Low temperature and low light led to significant decreases in $Pn$, but the extent of the decrease...
Fig. 3. Plant height (A), stem diameter (B), leaf area (C), tip dry weight (D), root dry weight (E) and root top ratio (F) of wild-type and transgenic cucumber plants (measured on October 25). All values shown are mean ± SD (n = 3). a and b indicate that mean values are significantly different among samples (P < 0.05).

Fig. 4. The linear analysis between total dry matter and photosynthetic rate and RCA activity in WT and transgenic cucumber leaves. (A) Total dry matter as a function of photosynthetic rate. (B) Total dry matter as a function of RCA activity.

varied. After a treatment at 5°C for 6 h, the Pn decreased 26, 32 and 38% in T1-2, T1-3 and T1-7, respectively, while it decreased 44% in the WT. Afterwards, the Pn continually decreased both in transgenic lines and WT with the increase in time under stress, but the decrease in Pn was more obvious in WT than in transgenic plants. The Gs of transgenic and WT leaves decreased in synchrony with Pn under low temperature and light intensity stress, whereas the Ci increased as time under stress increased. This indicated that the decrease in Pn under low temperature and light intensity stress was mainly attributed to non-stomatal limitations. Compared with
Stress time (h)

WT

T1-2

T1-3

T1-7

Fig. 5. CsRCA mRNA abundance and protein levels in WT and transgenic cucumber seedlings (T1-2, T1-3 and T1-7) under low temperature (5°C) and low light intensity (90–100 μmol m⁻² s⁻¹) for 0, 6, 12 and 24 h. (A) CsRCA mRNA abundance, total RNA was separately isolated from fully expanded leaves in Cucumis sativus, and subjected to real-time PCR analysis. (B) CsRCA protein levels, 2 μg purified protein (positive control) and 25 μg protein samples from different treatment leaves were separated by SDS-PAGE and polyclonal antibodies were used to detect RCA protein. The dilution of the primary and secondary antibodies was set at 1:500. All values shown are mean ± SD (n = 3). a–d indicate that mean values are significantly different among samples (P < 0.05).

Changes in the maximal photochemical efficiency (Fv/Fm) were not noticeable, and no significant differences occurred between WT and transgenic seedlings during the first 6 h of low temperature and light intensity. When the treatment time was more than 12 h, the Fv/Fm in all of the seedlings decreased. At the end of the experiment, the decrease in Fv/Fm in transgenic seedlings was less than in WT seedlings.

There were no significant changes in initial fluorescence (F₀) both in WT and transgenic seedlings during the first 6 h of low temperature and light intensity. Afterwards, however, the F₀ increased as the time under stress increased. The increase in F₀ in transgenic seedlings was less than in WT seedlings. This result indicated that more than 6 h of low temperature and light intensity stress caused photoinhibition in cucumber leaves. The overexpression of CsRCA alleviated the injuries caused by low temperature and light intensity stress in the reactive center of PSII.

Effect of low temperature and low light on MDA content in WT and transgenic seedlings

Malondialdehyde (MDA) is the final product of lipid peroxidation, and its content can reflect the stress tolerance of plants. Fig. 7 shows that the MDA content was significantly higher after 24 h exposure to low temperature and light intensity stress (P < 0.05), indicating that lipid peroxidation in cucumber seedlings was caused by the chilling stress. The increase in the MDA content in transgenic seedlings was lower than in WT seedlings (P < 0.05). This suggested that the overexpression of CsRCA increased the tolerance of cucumber plants to low temperature and light intensity stress.

Discussion

Overexpression of CsRCA improves growth by increasing photosynthesis

Rubisco is the key factor that limits the carbon assimilation in plants through the C3 cycle; nevertheless, changes in Rubisco activity are unable to alter photosynthesis as expected (Mann 1999, Spreitzer and Salvucci 2002, Parry et al. 2003). Only Rubisco that is activated by RCA can affect the normal operation of the Calvin cycle, resulting in higher yields (Singh et al. 2014, Yin et al. 2014). In this study, the overexpression of CsRCA not only increased the RCA activity, but also enhanced the initial and total activities of Rubisco in cucumber leaves (Fig. 1). The data using transgenic plants clearly demonstrated a correlation between RCA activity and the Pn (Fig. 2A, R² = 0.96), as well as a correlation between
Rubisco initial or total activity and the Pn (Fig. 2C, \( R^2 = 0.98 \); Fig. 2D, \( R^2 = 0.94 \)). These results indicate that increasing the \( CsRCA \) expression promotes the carbon assimilation by enhancing the Pn (Liu et al. 2012) of cucumber plants, in accordance with a previous study in which the photosynthetic capacity was increased as a result of the increase in RCA mRNA abundance (Yin et al. 2010).

To further understand the relationship between RCA activity and growth, we measured the relevant growth indices of transgenic and WT plants. The morphological characteristics of the transgenic plants were similar to those of the WT plants, except for a higher plant height, larger leaf area and greater dry matter. The total dry matter gradually increased as the Pn and RCA activity increased in sense-oriented \( CsRCA \) transgenic plants (Fig. 4, \( R^2 = 0.95 \) and \( R^2 = 0.98 \), respectively). The transgenic plants showed a lower root-shoot ratio than the WT plants. These results may be attributed to the higher Pn that resulted from the accumulation of photosynthetic products in sense-oriented transgenic plants. This corroborated prior analysis on the accumulation of starch and sugar in \( CsRCA \) sense-oriented cucumber plants in our laboratory (Liu et al. 2012).

Overexpression of \( CsRCA \) improves low temperature and low light tolerance by increasing photosynthetic capacity and decreasing the degree of membrane lipid damage

The reduction in Pn caused by abiotic stress is attributed to an inhibition of Rubisco activity (Brüggemann
et al. 1992, Weng et al. 2002). As a molecular chaperone, RCA not only promotes and maintains the catalytic activity of Rubisco, but also is involved in the mechanisms of plant photosynthesis in response to temperature and light (Yin et al. 2010, Chen et al. 2015). Thus, the effect of abiotic stress on RCA is becoming a hot research topic. Yuan et al. (2016) confirmed that the mRNA expression and activity of RCA decreased dramatically under chilling stress. In agreement with the increased levels of RCA expression and activity, the high temperature tolerance of cucumber plants was enhanced, with a higher Rubisco activity (Bi et al. 2016). Here, the expression of CsRCA in mRNA and its protein levels, as well as the Pn in the transgenic lines, were significantly greater than in the WT plants after low temperature and low light stress for 24 h (Figs 5 and 6). In parallel, a reduced Gs and increased Ci in both transgenic and WT plants were observed under chilling intensity, implying that the reduced Pn was caused by non-stomatal limitations. The cucumber plants with increased CsRCA levels showed a greater Gs but a lower Ci. Thus, it can be hypothesized that under chilling stress, the Pn decrease in cucumber plants was associated with the lower photosynthetic enzyme activity as reported by Jurczyk et al. (2016), which was compensated for by overexpressing CsRCA in sense-oriented transgenic cucumber plants.

The chlorophyll fluorescence parameters reflect light energy absorption and utilization of plant leaves (Demmig-Adams and William 1996). We found a gradual decline in Fv/Fm and ΦPSII and an increase in F0 in WT cucumber plants exposed to chilling stress in our study. As the chilling period increased, the decline or increase was relieved in CsRCA sense plants, suggesting that overexpressing CsRCA could maintain relatively greater light energy absorption and utilization levels, which could restore part of the reduction in photosynthesis caused by the chilling stress.

Moreover, in response to a 24-h period of low temperature and weak light stress, cucumber plants showed a surge in the MDA content, indicating severe disruptions of the cell membranes (Fig. 7). However, the MDA contents of transgenic plants with increased RCA activities were consistently lower than those of WT plants under low temperature and low light. Thus, higher RCA mRNA expression may help cucumber plants mitigate low temperature stress by increasing the reaction center activity of PSII and decreasing the degree of membrane lipid damage.

In summary, the overexpression of CsRCA increased growth by promoting photosynthesis and carbohydrate accumulation. In addition, an increase in CsRCA expression improved the low temperature and weak light tolerance, which may be ascribed to greater Pn, Gs, Fv/Fm and ΦPSII values, as well as lower Ci and F0 values, and MDA contents. These findings are of practical importance for relieving the low temperature and low light stress on cucumber plants and improving their adaptation to the solar greenhouse environment.

Authors’ contributions
H. G. B. performed most part of the experiment, analyzed the data and completed the first draft. X. Z. A. designed the experimental plan and edited the manuscript. P. P. L. and Z. S. J. worked together with H. G. B. to accomplish the experiment.

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