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REGULAR PAPER

Arabidopsis thaliana expressing a thermostable chimeric Rubisco activase exhibits enhanced growth and higher rates of photosynthesis at moderately high temperatures

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Abstract Temperature is one of the most important factors controlling growth, development, and reproduction in plants. The rate of photosynthesis declines at moderately high temperatures in plants and particularly in temperate species like *Arabidopsis thaliana*. This can be attributed to a reduced ability of Rubisco activase to achieve optimum activation of Rubisco, leading to reduced Rubisco activity. In order to overcome this problem, we transformed the Arabidopsis *rca* mutant with a more thermostable, chimeric activase where a Rubisco recognition domain in the more thermostable tobacco activase was replaced with that from Arabidopsis. Transgenic lines expressing this activase showed higher rates of photosynthesis than the wild type after a short exposure to higher temperatures and they also recovered better, when they were returned to the normal temperature. Moreover, under extended exposure to moderately elevated temperature, the transgenic lines had higher biomass and seed yield when compared with the wild type plants.

Keywords Genetic modification · Functional characterization · Photosynthesis · Rubisco activase · Activase · Thermostable activase · Transgenic

Abbreviations

ATP	Adenosine 5′-triphosphate
ATPase	ATP hydrolysis
ECM	Activated Rubisco, carbamylated with magnesium bound
ER	Inactive Rubisco bound with inhibitor RuBP
rca	Regulation carboxylation activity or the Rubisco activase gene
Rubisco	Ribulose 1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose 1,5-bisphosphate

Introduction

The population of the world is projected to increase from approximately 6.5 billion this year to over 9.0 billion in 2050 (UN 2007) and the global mean temperature is also expected to rise by several degrees celsius by the end of this century (IPCC 2007). Keeping these projections in mind, there is an urgent need not only to increase global crop productivity and yield, but also to make crop plants more tolerant to the projected increases in temperature by implementing various strategies on an urgent basis (Evans 1998; FAO 2007).

Photosynthesis is one of the most sensitive biological processes that are negatively affected by higher temperatures (Berry and Björkman 1980; Quinn and Williams 1985). The optimum temperature range for photosynthesis for most C3 plants falls between 20°C and 35°C and in many cases, the peak rate of CO_2 assimilation and net photosynthesis (Pn) occurs well below 30°C. As photosynthesis is arguably

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one of the most important but heat sensitive metabolic process of plants, it is one of the factors targeted for possible improvement. A recent advance in understanding the factors contributing to the temperature response of photosynthesis is the finding that the inhibition of photosynthesis can be due to the deactivation of Rubisco under moderately high temperatures (<40°C). This is attributed to the failure of Rubisco activase to maintain a high Rubisco activation state because of its thermolability (Crafts-Brandner and Salvucci 2000; Salvucci and Crafts-Brandner 2004a, b).

Rubisco activase, which is a member of the AAA^+ family (Neuwald et al. 1999), regulates the activation state of Rubisco (Portis 2003) and is quite temperature sensitive in most plant species that have been examined thus far (Robinson and Portis 1989; Salvucci et al. 2001; Salvucci and Crafts-Brandner 2004a). Activase generally is present as a multimeric complex consisting of two isoforms of different molecular mass, resulting from alternative splicing of pre-mRNAs. However, Salvucci et al. (2003) reported that the two isoforms are products of separate genes in cotton and some species, like tobacco, apparently express only one isoform (Portis 2003). In Arabidopsis and presumably most species, the large isoform is more sensitive than the smaller isoform to ADP and is also regulated by the redox state of the chloroplast Zhang and Portis 1999; Zhang et al. 2002). Activase facilitates activation and maintains Rubisco activity by using ATP hydrolysis to release tightly bound sugar phosphates from the catalytic sites of Rubisco (Portis 2001).

Despite its thermolability, activases from species endemic to contrasting environments can exhibit a large difference in thermostability (Salvucci and Crafts-Brandner 2004b). For example, the activases from cotton and tobacco are considerably more stable at elevated temperatures than that from spinach when assayed in vitro (Crafts-Brandner et al. 1997; Eckardt and Portis 1997; Salvucci et al. 2001). The existence of more thermostable activases raises the possibility of using these activases to improve the photosynthesis and growth of species less tolerant of high temperatures. A possible problem with this approach is that the activation of Rubisco by activase can exhibit species specificity (Wang et al. 1992). For example, tobacco activase, a potential candidate does not activate spinach Rubisco very well. However, a domain in activase that contains specific residues contributing to the specificity of the interaction between these proteins was recently identified in part by using chimeric tobacco/spinach activase proteins (Li et al. 2005).

We took advantage of this research to explore the possibility of creating Arabidopsis plants that are more tolerant to elevated temperatures. We created a chimeric activase comprised of the tobacco activase, but containing an Arabidopsis Rubisco recognition domain. In vitro studies revealed that the chimeric activase retained the thermostability of tobacco activase and activated Arabidopsis Rubisco effectively. We then transformed *rca* mutant plants to express this chimeric activase, as the wild type activases are absent. The higher rates of photosynthesis, improved growth, and productivity of the transgenic plants at moderately high temperatures that we observed with these plants clearly demonstrate that this is a possible strategy for improving the thermotolerance and yield of other plants. For example, this technique might readily be applied to the important temperate crop, canola which is a close relative of Arabidopsis.

Materials and methods

Rubisco and other materials

Arabidopsis wild type Rubisco was isolated following protocols reported by Wang and Portis (1992). Primers for site-directed mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA, USA) and a site-directed mutagenesis kit (QuikChange) was obtained from Stratagene (La Jolla, CA, USA). Other high purity chemicals were from Sigma (St. Louis, MO, USA) or other leading manufacturers of laboratory chemicals.

Construction of chimeric activase, its expression and purification

A PstI site and a XhoI site were introduced at the 5′ end and the 3′ end, respectively, of the Rubisco recognition domain (amino acids 267–344 using spinach numbering) in the mature protein of Arabidopsis. Similarly, at the 3′ end of the corresponding position in tobacco activase, a XhoI site and at 5′ end a PstI site was introduced. Then the modified activase cDNA of Arabidopsis was digested for 2 h with XhoI/PstI. This was followed by gel purification of Rubisco recognition domain fragment. Simultaneously, the modified tobacco activase cDNA was also treated with XhoI/PstI and then the gel purified Rubisco recognition domain fragment of Arabidopsis was ligated with it to produce the chimeric Tob-Arab activase mature cDNA. This construct was then sequenced to confirm its fidelity as a chimeric activase. The protocols for expressing and purifying recombinant chimeric activase from bacteria were followed as described previously (Wang et al. 1992; Zhang and Portis 1999).

Rubisco and activase assays at elevated temperature

The activase ATPase activities at all temperatures were assayed following protocols as reported by Esau et al.

(1996) but the concentrations of NADH and PEP were increased to 0.9 mM and 6 mM, respectively, to maintain adequate levels for the longer duration (20 min) of assay. To accommodate the high NADH concentration, the decrease in absorption at 370 nm was recorded. The protocol described by Esau et al. (1996) was followed for spectrophotometric assay of Rubisco activation. After the pre-incubation of the assay media at the desired temperature, activase was added 2 min prior to initiation of the assay by addition of inactive Rubisco (ER). Rubisco (2.5 μg ml^{-1}), RuBP (8 mM) and 10 U carbonic anhydrase (CA) were used for the assay. The maintenance of activity by previously activated Rubisco (ECM) at elevated temperature was assayed as described by Crafts-Brandner and Salvucci (2000).

Construction of binary vector with the chimeric cDNA and transformation of agrobacterium

An Arabidopsis activase 5′UTR and signal peptide gene fragment was ligated with the chimeric activase (Tob-Arab) cDNA at the 5′ end utilizing an NcoI site. To this cDNA, a tobacco activase fragment containing the 3′ end of the sensor 2 domain (with XhoI site) and 3′ UTR was ligated using the XhoI site. Site-directed mutagenesis was used to eliminate the NcoI site in order to leave a sequence for signal peptide cleavage ($LA^{\downarrow}VK$). This cDNA construct (Tob-Arab) was then cloned into the PBluescript II SK (−) using the XbaI and SalI sites.

The binary vector pBCAB was constructed for transforming *Agrobacterium tumefaciens* using a HindIII/EcoRI fragment of binary vector pDS9 containing the CAB3 promoter and NOS terminator and ligating it to another binary vector pBIN121 from Clonetech (Palo Alto, CA, USA). Then the Tob-Arab cDNA was digested by XbaI/KpnI and ligated into pBCAB creating the final construct for transformation, pBCAB-Chimeric.

The construct, pBCAB-Chimeric was cloned into DH5α competent cells, and then cultured. This was then used to isolate and purify the plasmid and the fidelity of the construct was confirmed by DNA sequencing. Then in a tube containing 100 μl *A. tumefaciens* GV1101 competent cells, 1 μl of the plasmid was added and incubated in ice for 30 min. This was followed by electroporation using an Eppendorf Electroporator 2510 using the protocol provided by the manufacturer. This mixture containing electroporated cells was then mixed with 900 μl S·O.C Media. The cells were then plated on selective YM plates containing two antibiotics, kanamycin and rifampicin both at the concentration of 50 μg ml^{-1} and incubated at 30°C for 48 h. A single clone was inoculated in a tube containing 5 ml of LB media again containing two antibiotics, kanamycin and rifampicin (50 μg ml^{-1}) and incubated for 48 h at 30°C. Then a sample of this culture was verified by PCR using primers designed to include the 5′ and 3′ ends of the Tob-Arab cDNA.

Growth and transformation of Arabidopsis *rca* mutant plants and selection of transformants and homozygous lines

The seeds of Arabidopsis *rca* mutant were treated with cold temperature of 4°C and then spread in 3.5 inch pots filled with Sunshine mix #1 purchased from Sun Gro Horticulture (Bellevue, WA, USA), covered with transparent plastic and cold treated again for 2 days at 4°C. The pots were then transferred to growth chambers maintained at high CO_2 (1,000 ppm) and 150 μE m^{-2} s^{-1} of light irradiance. A day/night cycle of 16/8 h was maintained for promoting flowering. In order to facilitate many secondary bolts the primary bolts of the plants were cut.

A standard protocol of floral dipping was utilized for transformation of Arabidopsis (Clough and Bent 1998). After 1 week, a second round of dipping was carried out. The plants were then allowed to grow until the siliques matured, and then the seeds were collected, cleaned and dried for selection of transformants.

Then seeds were cold treated at 4°C for several days and used for selection. First, the seeds were surface sterilized with 75% ethanol for 5 min and then another 5 min in a solution containing 5% bleach and 0.1% Tween 20 and then rinsed in sterilized deionized H_2O five times. The sterilized seeds were then suspended in sterilized 0.1% agarose, and plated on agar selection plates containing 0.8% agar, 1/2 MS salts, 1% sucrose, and kanamycin (30 μg ml^{-1}). The plates were then transferred to cold room maintained at 4°C for 2 days in the dark before transfer to a growth chamber. Seedlings with dark green colored and healthy secondary leaves and roots were recognized as transformants, transferred to pots containing autoclaved Sunshine mix #1, and grown under long day conditions (16 h light) to induce early flowering. The seeds from the transformants then were harvested and germinated on selection plates. From these seedlings, total protein was isolated and immuno-blotting was performed for verification of transgenic lines expressing Tob-Arab cDNA.

For selection of homozygous lines, a classical genetic approach was followed. Seeds from the T1 plants were plated on selection plates containing 30 μg ml^{-1} of kanamycin. Four sets of plates with 100 seeds per set were analyzed. The number of healthy seedlings with dark green leaves and the chlorotic ones were counted and the ratios were recorded. Selected transgenic seedlings were transferred to pots and from these plants seeds were harvested and subjected to selection again. Homozygous plants were obtained after the selection process was repeated for several rounds.

Growth, productivity and measurements of rate of photosynthesis

The homozygous transgenic lines and the wild type Arabidopsis plants were grown in growth chambers at 22°C temperature, and 150 μE m^{-2} s^{-1} of light intensity with a 10 h/14 h light/dark cycle. For exposure to long-term moderate heat stress, the plants were subjected to 27°C for 8 h during the day, keeping all other conditions the same. Humidity for all growing conditions was kept at 72% except during the measurements of photosynthesis when it was increased to a minimum of 75%. All the plants were kept in the short-day conditions for 6 weeks until measurements of rates of photosynthesis and biomass were made. The plants used for characterization of reproductive development were grown in a chamber with 16 h of light. Light intensity, night-time temperature regime and RH were kept same as before but the duration of higher temperature exposure was increased to 16 h daily.

The analyses of net photosynthesis rate (Pn) at the normal temperature of 22°C, as well as at higher temperatures were carried out using the LI-COR Li6400 portable infrared gas analyzer (LI-COR Biosciences, Lincoln, NE, USA) at 350 μE m^{-2} s^{-1} of illumination and 300 ppm of CO_2. For all Pn measurements, the standard fluorescence leaf chamber (2 cm^2) was used. Plants were exposed to temperatures of 30°C and 38°C for 2 h before measuring the Pn at those temperatures, and the 38°C treated plants were returned to 22°C for 2 h to measure the recovery of Pn. At least four independent plants were used for Pn measurements at all temperatures. The experiment was repeated twice with similar results.

For measurements of root length, photographs were taken 1 week after germination at 27°C on agar plates using a digital camera and then root length were measured using Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA). Each set of pictures consisted of at least four seedlings of each line and several sets of plants were analyzed for root length. For leaf area measurements, plant photographs were taken 3 weeks after germination and then analyzed using ImageJ software, an image processing program developed at National Institutes of Health (Abramoff et al. 2004). Pictures of at least four seedlings of each line were analyzed and the process was repeated at least two times.

Fresh weight of root and shoot of four or more independent plants of wild type and the two transgenic lines were taken at 6 weeks after germination. The measurements were repeated two or more times with similar results. Numbers of siliques were counted when all the plants were mature and included at least four independent plants of each line and the countings were repeated two times. Seeds were harvested from these respective plants, dried and then weights were taken.

For calculation of percentage germination, seeds of the wild type and the transgenic plants previously grown at 22°C or 27°C were cold treated, sterilized, and then plated on agar plates with 1/2 MS salts, 1% sucrose, and 0.8% agar but no kanamycin, and incubated at 22°C. The numbers were counted 2 days after germination. Each plate had 100 seeds and four were used for each line.

Rubisco activation assay

Assays were performed after plants were exposed to 30°C for 8 h during the day following protocols as previously described by Kim and Portis (2005).

Results

Enhanced thermostability of the chimeric activase (Tob-Arab) and activation of Rubisco at elevated temperatures

Our initial studies showed that tobacco activase was less sensitive to exposure to elevated temperatures than the corresponding small isoform of Arabidopsis activase (Arabidopsis 43). A chimeric activase, Tob-Arab, in which a domain consisting of residues 267–334 in tobacco activase (spinach numbering) were replaced by the Arabidopsis domain, was created, successfully expressed in *E. coli* and purified using procedures described previously (Li et al. 2005).

The temperature stability of the ATPase activity of recombinant tobacco, Arabidopsis 43, and Tob-Arab were then compared at various temperatures over 20 min time period (Fig. 1). At 25°C, the activities of all three enzymes were comparable and declined only minimally. When assayed at 37°C, the activities were higher and initially comparable but the activity of Arabidopsis 43 declined slightly faster over the first 7 min and after 20 min, only 33% of the initial activity remained as compared to 67% for tobacco and surprisingly 83% for Tob-Arab. When assayed at 40°C, the activity of Arabidopsis 43 declined very rapidly and minimal activity remained by 10 min. In marked contrast, both tobacco activase and Tob-Arab retained substantial activity after 20 min, about 40% for tobacco and 50% for Tob-Arab. Based on ATPase activity, the structure of the chimeric activase was not drastically disrupted and its thermostability was comparable to the tobacco enzyme.

Retention of ATPase activity is a necessary but not sufficient condition for its ability to activate Rubisco. For example, with an analogous replacement of the Rubisco

recognition domain in spinach activase with the tobacco domain, while about 25% of the spinach ATPase activity was retained, there was no Rubisco activation activity (Li et al. 2005). Thus, the activation of the Arabidopsis Rubisco–RuBP complex (ER) by both the Arabidopsis and Tob-Arab activases were compared. At 25°C, the activity of Arabidopsis Rubisco was increased equally by Arabidopsis and Tob-Arab activase over the 5 min assay period (Fig. 2), which demonstrates that the specificity of the wild type enzyme was completely transferred to the chimera. At 40°C, the activity of Rubisco (ER) increases quite rapidly in the absence of activase (Kim and Portis 2006), making comparisons of initial rates difficult. Nevertheless, there was at best only a little increase in the rate and more importantly, the final extent of Rubisco activation by the wild type Arabidopsis 43 activase at 40°C. In contrast, Rubisco activation was greatly enhanced (by about 1.9 fold) by Tob-Arab activase after about 5 min at this higher temperature.

When fully carbamylated (activated) Rubisco (ECM) is incubated with RuBP in vitro, its activity declines gradually. This inhibition is due in part to the formation of inhibitors (Edmondson et al. 1990), which increases with temperature (Salvucci and Crafts-Brandner 2004a; Kim and Portis 2006). By removing various inhibitors, activase is able to maintain the activation state of Rubisco at normal temperatures (Robinson and Portis 1988; Wang and Portis

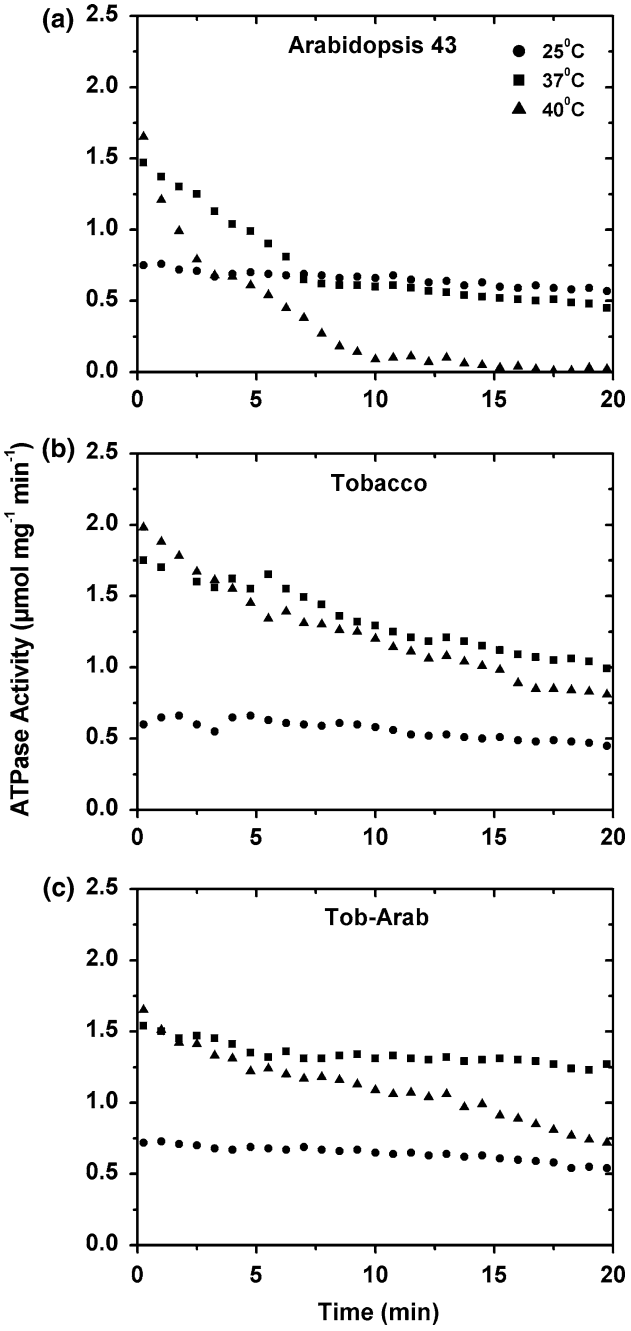

Fig. 1 Temperature stability of the ATP hydrolysis activity of Arabidopsis 43, Tobacco, and the chimeric Tob-Arab activases. Activity was measured continuously at the indicated temperatures in a spectrophotometer. Data shown are averages of two independent experiments

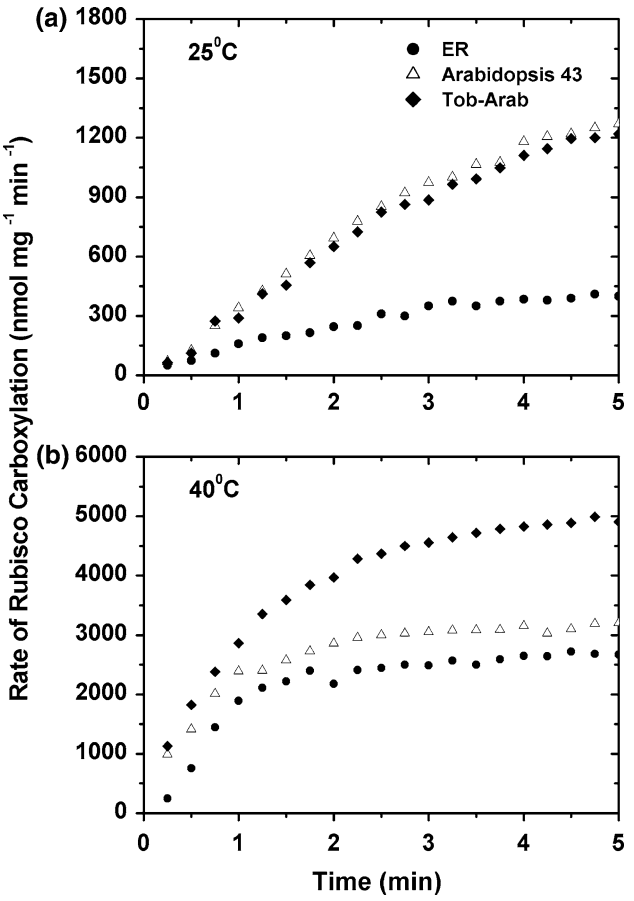

Fig. 2 Increase in Rubisco activity in the absence (ER) or presence of Arabidopsis 43 or the chimeric Tob-Arab activase at 25°C and 40°C. Activases were added 2 min before the assay was initiated by adding the inactive Rubisco-RuBP complex (ER). Data shown are averages of two independent experiments

1992). An important determination of whether Tob-Arab activase could avert this decline in the Rubisco activity in comparison to Arabidopsis activase was performed at 40°C (Fig. 3). From the beginning of the assay, the activity of Rubisco declined by about 28% to reach a plateau within 5 to 10 min. Whereas Arabidopsis 43 could not arrest this decline, the Tob-Arab activase not only reversed the small decrease that occurred before it was added, but subsequently increased the Rubisco activity by 20% in less than 9 min and maintained it at a level, about 50% greater than otherwise, during the duration of the assay.

Thus, based on all of these in vitro assays, the Tob-Arab chimeric activase has better thermostability than the native Arabidopsis activase isoform, making it a suitable candidate for possibly improving the thermotolerance of Arabidopsis via transformation.

Engineering and initial characterization of transgenic Arabidopsis expressing the chimeric activase

The chimeric activase gene was introduced into a binary transformation vector, behind a chlorophyll a binding protein promoter (CAB3) to provide light-regulated gene expression to mimic native activase gene expression. The *rca* mutant of Arabidopsis, which does not express the native activase isoforms, was transformed by floral dipping (Clough and Bent 1998) and transformed lines obtained by selection on kanamycin. Immunoblot analyses from the leaves of several transgenic lines were performed and lines H2, H3-1, H3-2, H4-1 and H5, with similar levels of expression of this chimeric activase to that of wild type were retained (Fig. 4). Following further selection to obtain homozygous plants, two independent lines (H3-2 and H4-1) were chosen for comparison with the wild type plants.

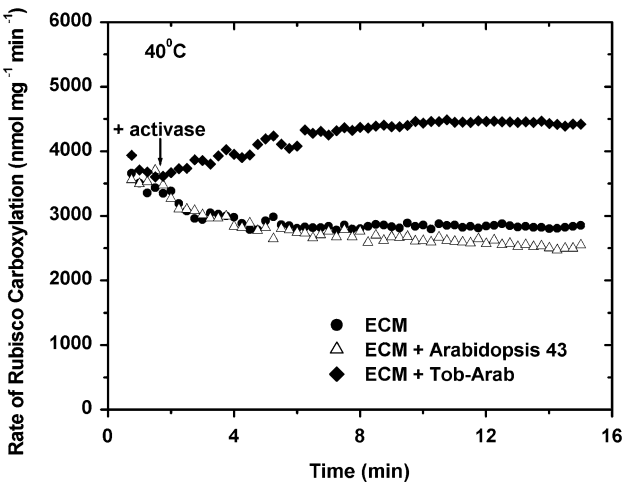

Fig. 3 Ability of the Arabidopsis 43 and chimeric Tob-Arab activase to maintain the activity of activated Rubisco (ECM) at 40°C. Rubisco was incubated at room temperature for 20 min with 10 mM $MgCl_2$ and 10 mM $NaHCO_3$ to activate it to the ECM form. The activases were added 2 min after starting the assay by addition of ECM. Data shown are averages of two independent experiments

Growth and development of transgenic plants expressing the thermostable chimeric activase under moderately high temperature

When grown at a moderately high temperature of 27°C, seeds of wild type Arabidopsis and the two transgenic lines expressing the more thermostable Tob-Arab activase exhibited a difference in root growth (Fig. 5). The average root length of the plants 1 week after germination was about 22% greater for line H3-2 and 20% for line H4-1 than the wild type plants.

Enhanced growth at 27°C for the transgenic lines as compared to the wild type was also observed on a long-term basis. Three weeks after germination, the transgenic plants had similar leaf color and overall phenotype as compared to the wild type plants, but the leaf areas of plants of the lines H3-2 and H4-1 were significantly larger ($P < 0.05$) by 15 and 18%, respectively, as compared to the wild type (Fig. 6). At 6 weeks, higher root, shoot, and total plant biomass were observed (Fig. 7). The root mass of plants of line H3-2 and H4-1 were significantly greater ($P < 0.05$) than the wild type plants by about 17 and 14%, respectively, and increases (about 22% and 21%) in shoot mass were also observed. Thus, the transgenic lines H3-2 and H4-1 obtained greater total biomass in long-term growth at the moderately higher temperature of 27°C.

The better growth exhibited by the transgenic lines was most likely due to the maintenance of higher rates of photosynthesis at moderately high temperatures by the transgenic plants (Fig. 8). Since small differences in photosynthesis at 27°C were very difficult to establish (data not shown), we exposed the plants to higher temperatures for measurements. At 22°C, the rates of net photosynthesis for all the plants were similar. However, after a 2 h exposure to 30°C the rate of photosynthesis (Pn) in the transgenic plants at this temperature was 18% (H3-2) and 20% (H4-1) greater than the wild type. The difference was even more

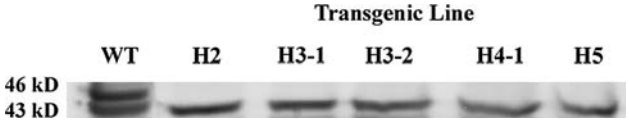

Fig. 4 Western blot showing expression levels of activase in wild type (WT) and various transgenic Arabidopsis plants with the chimeric Rubisco activase. Equal amounts of protein extracted from the leaves were loaded on an SDS-PAGE gel. Antibody raised against Rubisco activase was used and standard blotting methods were followed

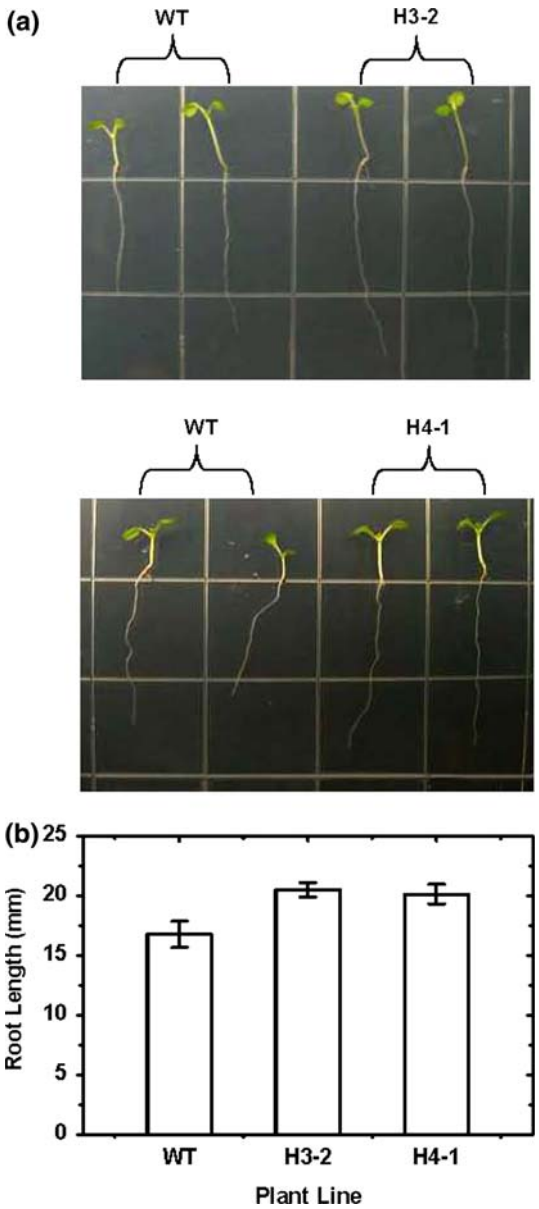

Fig. 5 Effect of a moderately high temperature of 27°C on root growth of wild type (WT) and two transgenic lines. **a** Sample photographs taken 1 week after germination. **b** Averages of root length (means ± SE) of the seedlings (four of each line were used). The seedlings were exposed to 27°C for 8 h during the middle of the 10 h day

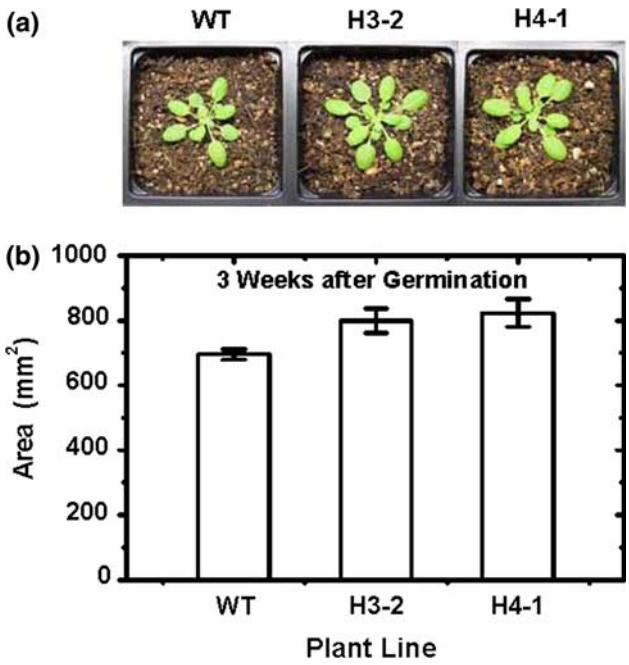

Fig. 6 Effect of moderately high temperature of 27°C on shoot growth of wild type (WT) and two transgenic lines. **a** Sample photographs taken 3 weeks after germination. **b** Averages of leaf area (means ± SE) measured from photographs of four seedlings of each line. The plants were exposed to 27°C for 8 h during the middle of the 10 h day

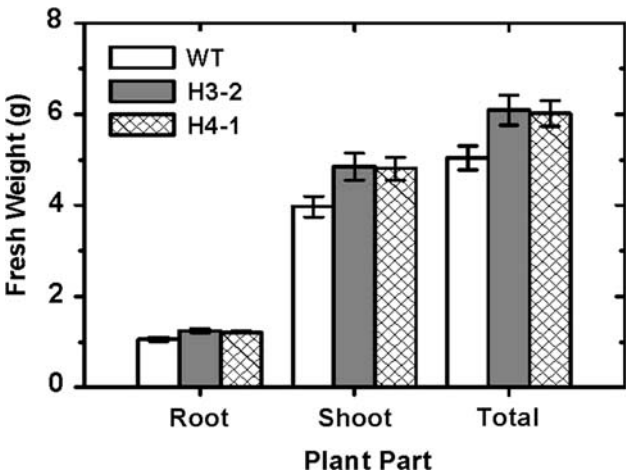

Fig. 7 Effect of a moderately high temperature of 27°C on the biomass of wild type (WT) and two transgenic lines. After germination, the plants were exposed to 27°C for 8 h during the middle of the 10 h day for 6 weeks. Values are means ± SE of four seedlings of each line

pronounced following a brief exposure and assay at 38°C with the Pn of plants belonging to transgenic lines H3-2 and H4-1 greater by 46 and 38%, respectively, than that of the wild type plants. The recovery in Pn was also 15% better in the transgenic lines H3-1 and H4-1, when the plants were returned to 22°C after an exposure to high temperature. The % Rubisco activation was also slightly higher in the transgenic plants as compared to the wild type plants when they were exposed to an elevated temperature of 30°C for 8 h. The % activation values recorded for WT were 68.6 ± 1.29, and that of H3-2 and H4-1 were 72.9 ± 1.52, and 73.7 ± 1.67, respectively, (Table 1). The % activation were similar at 25°C.

Based on previous studies of the effect of moderately high temperatures on photosynthesis and Rubisco activation in Arabidopsis (Kim and Portis 2005; Salvucci et al.

2006) and the in vitro studies, and % activation measurements presented above, the differences in growth and development are most likely due to the higher activation state of Rubisco in the transgenic lines leading to slightly higher rates of photosynthesis.

Reproductive performance of the transgenic plants expressing the thermostable chimeric activase under moderate high temperature

When the plants belonging to wild type and the transgenic lines were evaluated for their reproductive performance in terms of the number of siliques per plant and overall seed yield after being grown at the moderately high temperature of 27°C for 16 h during each light period, the differences were quite large (Fig. 9). The average number of siliques in the transgenic lines H3-2 and H4-1 were over three times more than the wild type (Fig. 9a). The siliques of the transgenic lines were larger and thus contained more seeds (data not shown). Most importantly, the total seed weight per plant recorded for lines H3-2 and H4-1 were about four times greater than the wild type (Fig. 9b).

Seed viability is also a very important measure of reproductive performance. The germination rates of seeds collected from plants that were grown at 22°C was similar at about 95% (Fig. 9c). However, the difference in the germination rates was large for seeds collected from plants grown at 27°C. While the seeds of lines H3-2 and H4-1 maintained a relatively healthy germination rate of over 70%, the seeds from the wild type germinated poorly at a rate of 23%.

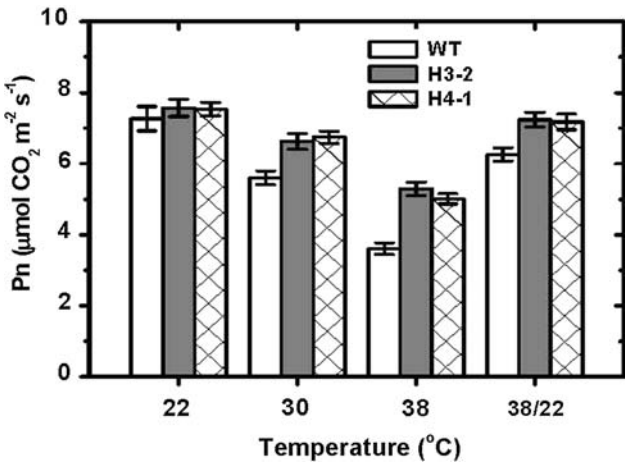

Fig. 8 Effect of temperature on net photosynthesis rate (Pn) of 6 week old wild type (WT) and two transgenic lines. Pn was measured using 350 μmol m^{-2} s^{-1} of light and 300 ppm of CO_2. The plants were exposed to the indicated temperatures for 2 h before measuring the Pn at that temperature. Recovery (38/22) after exposure to 38°C for 2 h was determined following the return of the plants to 22°C for 2 h. Values are means ± SE from four plants

Table 1 Effect of a moderately high temperature of 30°C on the % activation of Rubisco in wild type (WT) and two transgenic lines

	Plant line		
	WT	H3-2	H4-1
% Activation at 25°C	71.8 ± 1.26	72.3 ± 1.58	72.5 ± 1.80
% Activation at 30°C	68.6 ± 1.29	72.9 ± 1.52	73.7 ± 1.67

The plants were exposed to 30°C for 8 h during the middle of the 10 h day before the tissue were collected for % activation assay. Values are means ± SE of four biological replicates (seedlings) of each line

Our results demonstrating improved reproductive performance of the plants expressing a more thermostable activase are similar to those reported by Kurek et al. (2007) who used gene shuffling technology to generate more stable activases. Whereas, the wild type plants exhibit marked declines in silique/seed production and seed germination, the transgenic plants are able to maintain reproductive performance at this moderately higher temperature.

Discussion

We took advantage of research that identified a Rubisco recognition domain in activase (Li et al. 2005) to improve the thermostability of the native Arabidopsis activase. The more thermostable tobacco activase was modified to recognize Arabidopsis Rubisco by replacing its Rubisco recognition domain with that of Arabidopsis. In vitro studies (Figs. 1, 2 and 3) confirmed that the chimeric activase retained the thermostability of tobacco and activated Arabidopsis Rubisco as efficiently as the Arabidopsis isoform. We could have chosen to replace only the two amino acids that were shown to be directly responsible for determining Rubisco preference, but decided to replace the larger domain initially used in the previous study because there are a large number of differences (20 of 67 residues) between tobacco and Arabidopsis in this region. Pending further studies and/or further optimization of the chimera we created, these differences could contribute in subtle ways ensuring the efficient recognition and activation of Arabidopsis Rubisco that we observed with the chimera. We also could have chosen to use the equally (as compared to tobacco) thermostable cotton activase (Salvucci and Crafts-Brandner 2004b), and not utilize domain replacement. This might have worked but there are 15 differences between cotton and Arabidopsis in the replaced region and the relative efficiency of cotton activase in the activation of Arabidopsis Rubisco has not been determined. In the absence of a structure for activase, domain replacement was a conservative choice and it is potentially widely

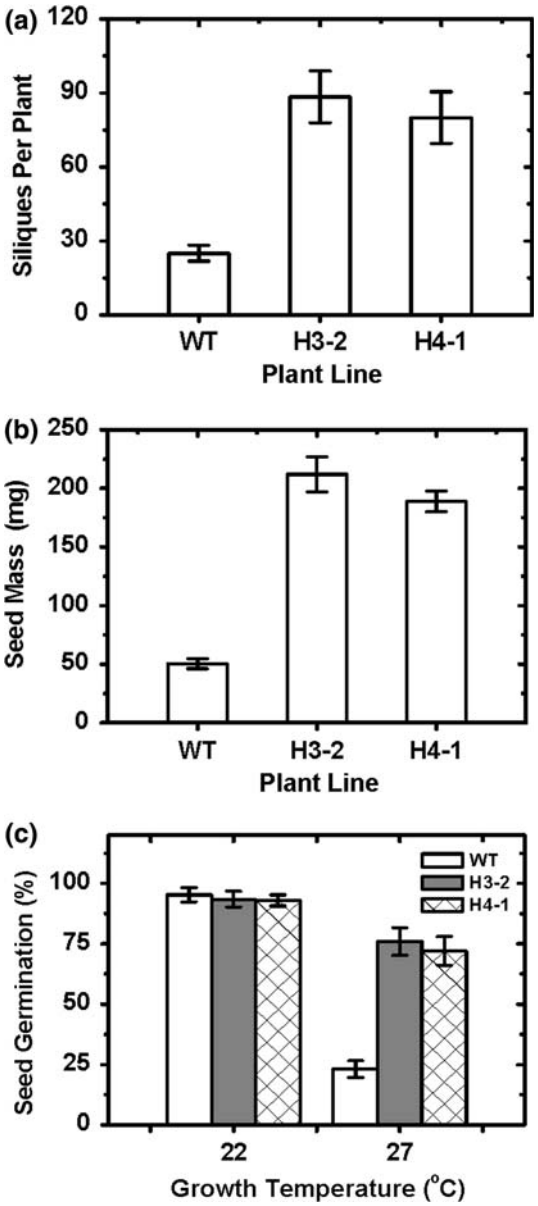

Fig. 9 Effect of moderately high temperature of 27°C on the reproductive development of wild type (WT) and two transgenic lines. After germination, the plants were exposed to 27°C for 16 h during each light period until maturity and **a** numbers of siliques, **b** total seed mass were measured. In **c** the germination rates of seeds collected from plants grown at 22°C and 27°C were determined at 22°C. Values are means ± SE from four plants

applicable for improving the thermotolerance of activase and thus plants.

The transgenic plants expressing the more thermostable chimeric activase were clearly more tolerant of higher growth temperatures than the wild type. Root (Figs. 5 and 7) and vegetative growth (Figs. 6, 7) was improved at all stages examined and most importantly, more seeds with better germination rates were produced (Fig. 9). We attribute the improved growth to the greater tolerance of photosynthesis to moderately high temperatures that we observed with the transgenic plants (Fig. 8). However, the effects on seed production and viability were quite dramatic as compared to the effects on photosynthesis. The importance of Rubisco in the metabolism of oil seed plants (like Arabidopsis) has recently become apparent (Schwender et al. 2004). The role and importance of activase in seed metabolism is not clear. It is possible that coping with the exceptional thermolability of activase imposes a critical burden on some aspect of reproductive development. Clearly, the dramatic effects on seed development with the observed small effects on Rubisco activity at the temperatures we used, merits further studies.

The transgenic plants we studied do not express the larger activase isoform that confers redox regulation to both the ATPase and Rubisco activation activities of the native complex, which consists of equal amounts of both isoforms (Zhang and Portis 1999; Zhang et al. 2002). As the thermostability and productivity of plants with just the shorter isoform were shown to be similar to wild type (Kurek et al. 2007; Salvucci et al. 2006), we did not include the larger isoform in present study.

Recent research has resulted in the idea that the heat lability of activase leading to a reduced activation state of Rubisco is a major factor responsible for the inhibition of photosynthesis at moderately high temperatures (Crafts-Brandner and Salvucci 2000; Salvucci and Crafts-Brandner 2004a, b). This hypothesis is controversial because other studies (Cen and Sage 2005; Kubien and Sage 2008; Schrader et al. 2004) present evidence that in some species the regeneration of RuBP becomes limiting because of the temperature sensitivity of other processes. Species differences may be possible because activases in plants adapted to contrasting thermal environments (for example, cotton that grows well in hot climates as compared to Antarctic hairgrass that grows in a cold environment) exhibit different sensitivities to moderate heat stress (Salvucci and Crafts-Brandner 2004b). Previous research with plants expressing different forms of activase suggests that in Arabidopsis, lower activase activities leading to inhibited Rubisco activity are the basis for the reduced photosynthesis rates at moderately high temperatures (Kim and Portis 2005; Salvucci et al. 2006). This hypothesis is strongly supported by our studies because the introduction of a more thermostable activase to Arabidopsis did have a significant impact on improving growth and yield under moderately stressful temperatures.

Recently, Kurek et al. (2007) also generated more thermostable Arabidopsis activases and found that transgenic plants expressing these activases had improved vegetative and reproductive growth under growth conditions very similar to ours. They used an elegant but laborious gene shuffling method, which resulted in only one to

three amino acid differences in the final variants chosen for analysis. Interestingly, one of the two residues that affected thermostability in the shuffled Rubisco activase occurs in the sensor-2 region that we used to create the more thermostable activase for Arabidopsis. It is not clear to what extent the mutations that they identified would be applicable to other temperate species.

Previously, other researchers have also reported increased activase stability under heat stress for tobacco plants accumulating glycinebetaine in the chloroplasts (Yang et al. 2005) and rice plants over-expressing the chloroplast sedoheptulose 1,7-bisphosphatase enzyme (Feng et al. 2007). Other important factors in determining the growth and photosynthesis of a plant like the heat stability of the thylakoid membrane, site of conversion of light energy in the chloroplast, have also been reported (Murakami et al. 2000; Sharkey 2000). When Murakami et al. (2000) silenced the *FAD7* gene in Arabidopsis, which encodes a chloroplast-localized ω-3 fatty acid desaturase and thus reduced the level of membrane lipid unsaturation, it allowed the transgenic plants to grow much better at higher temperatures. These studies along with ours clearly indicate that plants tolerant to higher temperatures can be engineered using a variety of approaches.

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