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CHARACTERIZATION OF RUBISCO ACTIVASE FROM THERMALLY CONTRASTING GENOTYPES OF *ACER RUBRUM* (ACERACEAE)[1]

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The lability of Rubisco activase function is thought to have a major role in the decline of leaf photosynthesis under moderate heat (<35°C). To investigate this further, we characterized Rubisco activase and explored its role in the previously demonstrated thermal acclimation and inhibition of two genotypes of *Acer rubrum* originally collected from Florida (FL) and Minnesota (MN). When plants were grown at 33/25°C (day/night) for 21 d, the FL genotype compared to the MN genotype maintained about a two-fold increase in leaf photosynthetic rates at 33–42°C and had a 22% increase in the maximal rate of Rubisco carboxylation (V_{cmax}) at 33°C under nonphotorespiratory conditions. Both genotypes had two leaf *Rca* transcripts, likely from equivalent alternative splicing events. The RCA1 and RCA2 proteins increased modestly in FL plants under warmer temperature, while only RCA2 protein increased in MN plants. Rubisco large subunit (RbsL) protein abundance was relatively unaffected in either genotype by temperature. These results support the idea that Rubisco activase, particularly the ratio of Rubisco activase to Rubisco, may play a role in the photosynthetic heat acclimation in *A. rubrum* and may have adaptive significance. This mechanism alone is not likely to entirely explain the thermotolerance in the FL genotype, and future research on adaptive mechanisms to high temperatures should consider activase function in a multipathway framework.

Key words: heat stress; photosynthesis; red maple; Rubisco activase; Rubisco large subunit; warming.

Trees comprise the majority of the carbon sequestering biomass in terrestrial ecosystems and their response to environment and climate change is a key determinate of global net primary production and carbon sequestration (e.g., Barford et al., 2001; Breshears et al., 2005). Because of the importance of temperature to global forest productivity, understanding photosynthetic responses to rising temperature is critical for predicting forest growth. To correctly characterize the physiological adjustments to climate gradients in predictive models, we need to decipher the interactive effects of environmental components while accounting for potential physiological acclimation and genetic differences. Unfortunately, because of the environmental and physiological dynamics in forest canopies, there is no consensus about the mechanisms responsible for leaf photosynthetic acclimation (Frak et al., 2002; Kosugi and Matsuo, 2006). Consequently, in the present study we set out to characterize and understand the molecular aspects of the genetic difference in photosynthetic temperature response in two thermally divergent genotypes of a common temperate forest species.

At the leaf level in forest ecosystems, the spatial and temporal heterogeneity of temperature and the variability in a species' physiological response to temperature have complicated process-based model predictions. Consequently, models that use the Farquhar et al. (1980) 25°C photosynthesis parameters may inaccurately predict physiological parameters at temperatures below and above 25°C (Bernacchi et al., 2001, 2003). Therefore, the need to understand the underlying molecular mechanism of heat-limited photosynthesis, particularly in response to moderate heat (<35°C), has been emphasized by predictions of accelerated climate change. However, determining the effects of temperature on photosynthesis, particularly the most heat-limiting traits, has been difficult to discern due to the highly regulated and coordinated processes of photosynthesis. For example, there is evidence that thylakoid membranes become permeable at moderate heat, resulting in proton leakage and reduced electron flow (Pastenes and Horton, 1996; Bukov et al., 1999), followed by a reduction in ribulose 1,5-bisphosphate (RuBP) regeneration (Wise et al., 2004). Alternatively, there is evidence that the enzyme responsible for Rubisco's catalytic proficiency, Rubisco activase, is heat labile (Robinson and Portis, 1989; Eckardt and Portis, 1997; Salvucci et al., 2001), and the enzyme has even been suggested as the primary cause for reduced photosynthetic performance in response to moderate heat (Salvucci and Crafts-Brandner, 2004a). Supporting results for this view were recently reported in a study comparing *Arabidopsis* with modified thylakoid membrane fluidity and Rubisco activase (Kim and Portis, 2005). Results from this study indicate that even at moderate heat, photosynthesis is compromised because of reduced carboxylation with a concomitant decrease in electron transport.

Rubisco activase is a stromal protein usually present as two isoforms of 41–43 kDa and 45–46 kDa that arise from one alternatively spliced transcript (Portis, 2002; Spreitzer and Salvucci, 2002). However, deviations from this generalization exist. For example, cotton and maize encode both small and large isoforms of activase on two separate genes (Salvucci et al., 2003; Ayala-Ochoa et al., 2004). Barley has a separate

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RCAB gene that encodes a 41-kDa isoform in addition to having the alternatively spliced *RCAA* gene (Rundle and Zielinski, 1991). There also are a number of species that produce only the smaller 41–43-kDa protein isoform (e.g., tobacco, common bean, cucumber, maize, and mung bean [Portis, 2002]). Still, the specific physiological role of a given isoform with respect to heat stress is generally not understood. In wheat plants exposed to elevated temperatures, the 42-kDa isoform accumulates, but a new 41-kDa Rubisco activase also is produced (Law and Crafts-Brandner, 1999). This is in contrast to spinach in which the large isoform of Rubisco activase is inherently more thermal stable than the small isoform (Crafts-Brandner et al., 1997). In addition, a novel 46-kDa isoform accumulated under prolonged exposure to moderate heat (Law et al., 2001).

In a previous study, we addressed the observation that a Florida genotype of *Acer rubrum* L. (red maple) had a higher growth rate and greater net photosynthesis compared to a Minnesota genotype under elevated acclimation and measurement temperatures (Weston and Bauerle, 2007). Analysis with coupled gas exchange and chlorophyll-*a* fluorescence suggested that the higher rates of net photosynthesis were due to a greater maximal rate of Rubisco carboxylation (V_{cmax}) and reduced photorespiration as determined by the quantum requirement of PSII per CO_2 (lower Φ_{PSII}/Φ_{CO2}). Because Rubisco activase function can affect V_{cmax}, it is possible that the genotypic differences in photosynthetic performance may be the result of activases with different physiochemical properties. In fact, the heat susceptibility of Rubisco activase has previously been correlated to habitat origin (Salvucci and Crafts-Brandner, 2004b), and it is possible that our prior results reflect intraspecific genetic variation in the Rubisco to Rubisco activase relationship.

To date, there is very limited information concerning Rubisco activase in woody plants. A search of NCBI's GenBank database yielded only one full-length cDNA clone, *Malus domestica* (Z21794), and partial sequences or expression sequence tag (EST) clones for *Pinus halepensis* (AJ271896), *P. pinaster* (AL749921), *Populus deltoidies* hybrid (BU102304), and *Prunus dulcis* (BU645562). As a step toward elucidating the potential function of Rubisco activase in the thermal tolerance of woody plants, we investigated specific physiological and molecular characteristics of Rubisco activase from the Florida and Minnesota genotypes of red maple. Herein, we report the cloning and sequencing of full-length cDNAs of the large and small transcripts of a Rubisco activase gene from red maple (designated *Rca1* and *Rca2*, respectfully), and we provide data regarding the gene structure, nuclear organization, transcript splicing, and protein accumulation in relation to growth temperature.

MATERIALS AND METHODS

Plant material and growth conditions—Red maple 'Northwood' and 'Florida Flame' genotypic lines, known in the horticultural trade as sensitive and insensitive to heat (Sibley et al., 1995a, b; Weston and Bauerle, 2007), were used in this study. The genotypes were originally collected from Minnesota (46°55′44″ N, 92°55′10″ W) and Florida populations (30°33′34″ N, 84°19′33″ W), respectively. Collection sites were 11 390 km apart and differed considerably in their local climatic conditions according to NRCS (Natural Resources Conservation Service) climate information retrieval reports (http://www.wcc.nrcs.usda.gov/climate/climate-map.html). For example, the county where the Minnesota genotype was collected has a yearly average temperature and rainfall of 3°C and 763 mm. In contrast, the county where the Florida genotype was collected reports 22.6°C and 1369 mm for yearly average temperature and rainfall. Average growing season length (days with minimum temperature above 0°C) is 310 d in the Florida county compared to 102 d in the Minnesota county.

Plants were propagated as dormant rooted cuttings in 2002 and grown in 3-L plastic pots containing a mixture of sand, peat, and silt loam (1 : 2 : 1, v/v). After bud break in the spring of 2004, plants were transferred to a greenhouse set to 27/25°C day/night conditions and ambient humidity. Plants were kept well-watered and fertilized twice a week (Peters 20-10-20) with micronutrients, Masterblend, Chicago, Illinois, USA). After 3 wk of greenhouse growth, plants were randomly assigned to Mylar (Laird Plastics, Charlotte, North Carolina, USA) chambers within the greenhouse (1.5 m H × 1 m W × 4 m L). Mylar is the recommended material for chamber construction (Hari et al., 1999), and we found light transmittance and quality results similar to Corelli-Grappadelli and Magnanini (1993), in which light was more than 90% of greenhouse incident light with midday levels exceeding 1800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the spectral composition was unchanged over the 400–900-nm range (Corelli-Grappadelli and Magnanini, 1993). The chambers were maintained under either ambient or warm temperature conditions (33/25°C, day/night). Each chamber had independent environmental control, where chamber temperatures were sampled at 30-s intervals with type T, fine wire thermocouples (CR21X, Campbell Sci., Logan, Utah, USA), and a control switch triggered the air conditioners to either heat, cool, or run at ambient temperature (fan only) to maintain the temperature within 1°C of the set point (model YSO9, Friedrich, Inc., San Antonio, Texas, USA). In addition, air within each chamber was humidified, and an additional data logger monitored relative humidity on a 15-min time step in each chamber (model Hobo Pro, Onset Computer Corp., Pocasset, Massachusetts, USA) to verify that vapor pressure deficit effects that co-vary with temperature were kept below values that influence red maple stomatal responses (Bauerle et al., 2004). The study was conducted from May to August of 2004 with photoperiod and light quantity similar to natural local conditions (34°41′0″ N, 82°50′15″ W).

Experimental design—A randomized block design was used to test temperature effects on net photosynthesis. Specifically, 10 plants of each genotype were randomly selected and placed in Mylar chambers set at 27/25°C (day/night) or 33/25°C. Two chambers were set at 27/25°C, and an additional two chambers were set at 33/25°C. Each week throughout the study, plants were transferred within chambers to minimize chamber effects. The actual number of plants sampled varied according to parameter tested and is indicated in the figure legend for that specific experiment.

Gas exchange analysis—After plants had acclimated to 33/25°C (day/night) conditions for 21 d, they were brought from the temperature controlled chambers to the laboratory, and gas exchange characteristics of the first fully expanded leaves were measured using a CIRAS-1 portable gas exchange system (PP systems, Amesbury, Massachusetts, USA). Leaf measurements were made at light-saturating conditions of photosynthetic photon flux density (PPFD) of 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, a value determined in our preliminary experiments that also agreed with Bauerle et al. (2003). The cuvette atmosphere was maintained at 360 $\mu mol \cdot mol^{-1}$ CO_2 and 21% O_2. Temperature response curves were constructed by changing leaf temperature with the CIRAS-I cuvette Peltier blocks in a manner similar to that of Sharkey et al. (2001) with an initial measurement at 25°C and then stepwise increases in temperature to 42°C. Specifically, leaf temperature within the cuvette was increased to 27, 30, 33, 36, 39, and finally to 42°C. Incubation at each temperature ranged from 10–15 min, until CO_2 fixation and stomatal conductance were stabilized. The recovery phase decreased from 42 to 25°C following the reverse order of the induction phase. An additional measurement at 25°C was made after 30 min to determine long-term recovery effects.

The maximal rate of Rubisco carboxylation (V_{cmax}) was estimated in vivo under nonphotorespiratory (2.0% O_2; Edwards and Walker, 1983) conditions so that Rubisco properties, as inferred from this assay, could be correlated to Rubisco activase function without the confounding effects of oxygenation reactions. Thus, leaves opposite to those used for temperature response curves were acclimated to a C_a (cuvette atmospheric CO_2 concentration) of 360 $\mu mol \cdot mol^{-1}$ CO_2, 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD, and 2.0% O_2. The CO_2-limiting linear phase of the response curve was constructed by changing the C_a from 200, 150, 100, 75, and 50 $\mu mol \cdot mol^{-1}$. At the end of the measurements, C_a was again stabilized at 360 $\mu mol \cdot mol^{-1}$, and A_{net} was recorded. This procedure

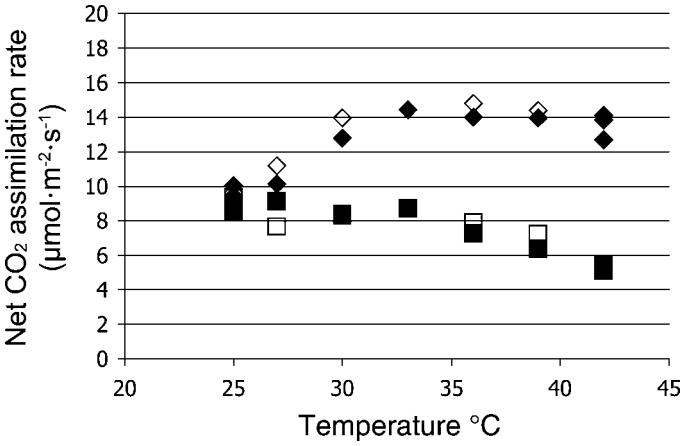

Fig. 1. Leaf photosynthetic response to temperature of Florida (diamond) and Minnesota (square) genotypes of *Acer rubrum* after 21 d of acclimation to 33/25°C (day/night) conditions. Carbon dioxide was 360 μmol·mol^{-1}, oxygen 2%, light was 1200 μmol·m^{-2}·s^{-1} PAR, and $N = 6$. Temperature was increased up to 42°C (solid symbols) and then decreased back down to 25°C (open symbols).

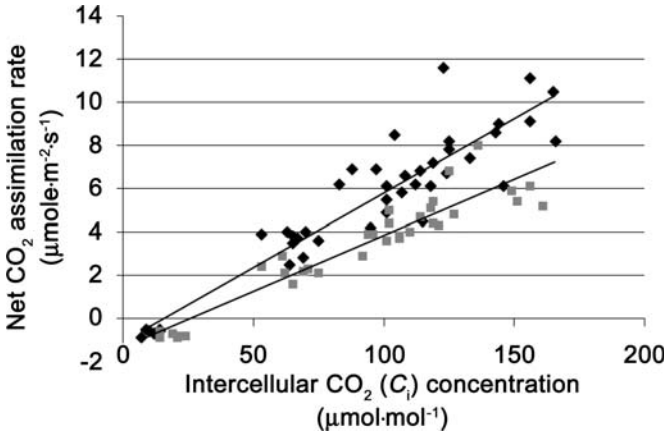

Fig. 2. The response of CO_2 assimilation rate to intercellular CO_2 concentration (C_i) for leaves of Florida (♦; $y = 0.069x - 1.1$; $R^2 = 0.88$) and Minnesota (■; $y = 0.052x - 1.3$; $R^2 = 0.90$) genotypes of *Acer rubrum* acclimated to 33/25°C (day/night) and measured at 33°C. Measurements were taken at 360 μmol·mol^{-1} CO_2, 2% oxygen, light was 1200 μmol·m^{-2}·s^{-1} PAR, and $N = 6$.

allowed us to compare post- and pre-A_{net} values at 360 μmol·mol^{-1} to ensure that stomata were open and to verify the stability of photosynthesis.

Cloning and sequencing—Total nucleic acid was isolated from leaf tissue using a CTAB method optimized for recalcitrant woody plant material (Lefort and Douglas, 1999) and modified with the use of an alternative extraction buffer (50 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 1.1 M NaCl, 0.4 M LiCl, 1% CTAB, 2% PVP 25 000, 0.5% Tween 20; F. Lefort, personal communication). Isolated nucleic acid from both ecotypes was treated with RQ1-DNase (Promega, Madison, Wisconsin, USA) and reverse transcribed using SuperScript (Invitrogen, Carlsbad, California, USA) following the manufacturer's instructions. The resulting cDNA was PCR-amplified using forward primer 5′-CCYGCYTTYATGGGACAAGC-3′ and reverse primer 5′-GCCCAGTAGAACTTCTCC-3′. The primers were designed in regions of high sequence conservation based on an alignment of Rubisco activase sequences available in GenBank (http://www.ncbi.nlm.nih.gov), resulting in the amplification of a PCR product spanning the 390- to 890-bp region of Rca. The 5′ and 3′ untranslated regions were cloned using 5′- and 3′-RACE protocols from Sambrook et al. (1989). This resulted in two full-length cDNA sequences termed *Rca*1 and *Rca*2. Maple Rubisco large subunit (RbcL) was similarly PCR-amplified using forward 5′-ATGTCACCACAAACAGAGAC-TAAAG-3′ and reverse primer 5′-TGCATTACGATCGGAACGCCCA-3′ primers.

All amplification products were cloned into pGEM-T Easy (Promega), and inserts were sequenced at the Clemson University Core Sequencing Facility using a LiCor 4200 Sequencer (LI-COR, Lincoln, Nebraska, USA). Primer pairs specific to red maple and designed for a 3′ region of the cDNA (5′-GGAGAAGTTCTACTGGGC-3′) and the 3′-untranslated region (UTR) (5′-ATCTGACATACTTAGAAGTG-3′) were used to amplify genomic DNA spanning the suspected alternative splice site. Derived nucleotide and deduced amino acid sequences were compared with sequences from the GenBank database using BLASTn.

Western analysis of Rubisco and Rubisco activase protein contents—Leaf tissue samples (5 cm^2) were extracted in 2× SDS-PAGE loading buffer, and protein was measured using the RC-DC assay (Bio-Rad, Hercules, California, USA). Clarified supernatants were heat treated at 95°C for 5 min prior to electrophoresis in 10% SDS-PAGE gels. The samples were transferred to an Immobilon membrane (Millipore, Billerica, Massachusetts, USA) and probed with a polyclonal cotton anti-Rubisco activase (from M.E. Salvucci, USDA, Western Cotton Research Lab, Phoenix, Arizona, USA), followed by a horseradish peroxidase-conjugated secondary antibody and chemiluminescence (West Pico Chemiluminescence, Pierce Biochemicals, Rockford, Illinois, USA). Blots were stripped and reprobed with anti-Rubisco (from R. Sage, University of Toronto, Canada). When plants were grown under warm temperature conditions, native protein extracts were recalcitrant for enzyme activity assays.

Southern gel blot analysis—Red maple total nucleic acid was isolated from 2 g of young leaf tissue (<1.5 cm) using a modified CTAB method (Lefort and Douglas, 1999) as previously described. RNase-A was used to remove RNA in the samples. Ten micrograms of purified DNA was digested to completion using *Eco*RI, *Hind*III, or *Bam*HI restriction enzymes and size-fractionated in a 1% agarose gel. The DNA was transferred from the gel onto Hybond N+ membrane (Amersham, Piscataway, New Jersey, USA) in 5× SSPE (0.9 M NaCl, 50 mM sodium phosphate pH 7.7, and 0.5 mM EDTA) containing 0.4 M NaOH, and re-natured in neutralization buffer (1 M Tris pH 7.4 and 1.5 M NaCl). The DNA was then cross-linked to the membrane with UV light irradiation (Cross Linker 1800, Stratagene, La Jolla, California, USA)

The entire *Rca*2 cDNA clone was radiolabeled by PCR using sequence-specific primers in a 50-μL reaction containing 20 ng of plasmid DNA, 100 nM of each primer, 40 μC of ^{32}Pα-dCTP, 2 μM of each dNTP, and 5 U of *Taq* polymerase (Promega). The PCR reaction was initially denatured at 95°C for 5 min and then continued for 30 cycles of 30 s denaturation at 95°C, 30 s annealing at 57°C, and 120 s extension at 72°C. The PCR reaction was incubated at 72°C for 5 min to complete extension. The labeled probe was purified through a Micro Bio-Spin 30 chromatography column (BIO-RAD, Hercules, California, USA). The entire 50-μL reaction was used for hybridization, which followed standard methods (Sambrook et al., 1989). The membrane was washed one time for 15 min in 2× SSC and 0.1% SDS at 65°C followed by another washing at 1× SSC and 0.1% SDS at 65°C, then a final 15 min wash at 0.5× SSC and 0.1% SDS at 65°C.

RESULTS

Thermotolerance of red maple—Under photorespiratory conditions (ambient O_2), net photosynthetic CO_2 assimilation rate (A_{net}) of Minnesota plants was relatively unaffected by temperature increases from 25 to 33°C. At temperatures above 33°C, however, A_{net} decreased gradually with higher temperature throughout the response curve (Fig. 1). In contrast, plants representing the Florida genotype maintained optimal photosynthetic rates up to ~40°C with only a slight decrease at 42°C (Fig. 1). Following temperature reduction, photosynthetic recovery for the Minnesota and for the Florida genotypes was nearly immediate, and the recovery curve closely paralleled the induction curve (Fig. 1). The maximal rate of

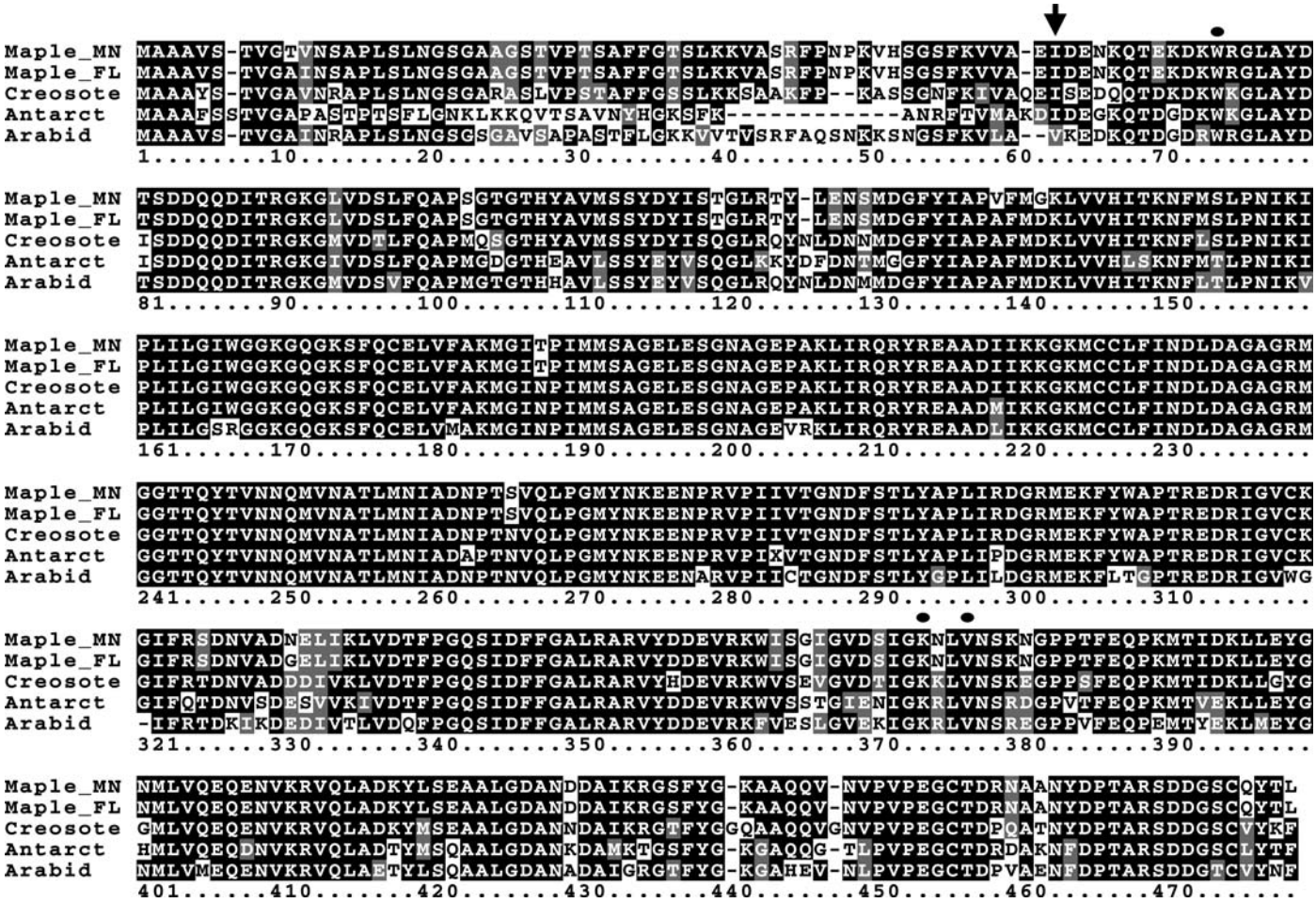

Fig. 3. Amino acid sequence alignment of the large Rubisco activase from *Acer rubrum* genotypes from Minnesota (Maple-Mn; GenBank DQ915976) and Florida (Maple-Fl; GenBank DQ915974); from *Arabidopsis thaliana* (GenBank X14212, Werneke and Ogren, 1989); and from species of thermally contrasting habitats including creosote bush (GenBank AY312575) and Antarctic hairgrass (GenBank AY312573). Identical amino acids appear in black boxes, similar amino acids appear in gray boxes, and differences appear in white boxes. The arrow denotes the start of the mature *Arabidopsis* peptide, and ovals represent conserved regions discussed in the text.

Rubisco carboxylation (V_{cmax}), as estimated under nonphotorespiratory conditions (2.0% O_2) in vivo by constructing photosynthetic assimilation (A) to intercellular CO_2 (C_i) curves at 33°C, was significantly higher (22%) for the Florida than for the Minnesota genotype (Fig. 2).

Cloning and sequence analysis of Rca and RbcL cDNAs— Leaf cDNA from the Florida genotype was created and screened by PCR using degenerate oligonucleotide primers designed from conserved sequences of the Box II and Box VII′ motifs of *Rca* sequences available in the literature (Portis, 2002). This amplification resulted in an ~800-bp fragment for cloning. Preliminary sequence analysis using the BLASTn algorithm from NCBI indicated that the clone had strong homology to Rubisco activase. The full-length cDNA clone was obtained by 5′- and 3′-RACE, and this clone was designated *Rca*1-Fl. Additional RT-PCR reactions using leaf RNA as the template and maple-specific primers isolated a second *Rca* cDNA, designated *Rca*2-Fl (GenBank DQ915973). The Florida sequences were subsequently used to PCR amplify two full-length Rubisco activase clones from Minnesota red maple cDNA (*Rca*1-Mn and *Rca*2-Mn; GenBank for *Rca*2-Mn: DQ915975).

The full-length cDNA of *Rca*1-Fl and of *Rca*1-Mn are nearly identical at the nucleotide level (98% similarities) with a length of 1662 bp and an open reading frame of 1416 bp. The predicted precursor protein is composed of 472 amino acids, where the first 56 residues presumably encode a transit peptide targeting the protein to the chloroplast stroma. This prediction is based on available gene annotation/prediction programs (http://psort.nibb.ac.jp/; http://www.cbs.dtu.dk/services/ChloroP/; http://hc.ims.u-tokyo.ac.jp/iPSORT/) and previous characterizations of *Rca* sequences from other species. The mature RCA1 protein is predicted to be 47 kDa (Fig. 3).

Similar to what was found for *Rca*1, the *Rca*2 cDNAs from both genotypes were identical in length (i.e., 1710 bp) with an open reading frame of 1305 bp that encodes a predicted precursor protein of 435 amino acids and a mature protein with a molecular mass of 42 kDa. In addition, and identical to what was determined for *Rca*1, *Rca*2 is predicted to have a transit peptide of 56 amino acids for targeting to the chloroplast. Overall, *Rca*2 from the Florida and the Minnesota genotypes

Fig. 4. Amino acid sequence alignment of the Rubisco large subunit from Minnesota (Maple-Mn; GenBank DQ459381) and Florida (Maple-Fl; GenBank DQ915972) genotypes of *Acer rubrum*, tobacco (GenBank AAD15025.1), and spinach (GenBank CAA23473.1). Ovals denote key residues implicated in recognition and interaction with Rubisco activase as discussed in Li et al. (2005) and in the text.

had 99% sequence identities at the nucleotide level and 98% predicted amino acid sequence identities.

To check for possible polymorphisms at known binding residues between activase and Rubisco, a partial cDNA for Rubisco large subunit (*RbcL*) was similarly cloned and sequenced from both the Minnesota and the Florida genotypes (Fig. 4). Alignment of the predicted amino acid sequences indicted strong homology between maple genotypes (98%) and to spinach and tobacco *RbcL* as well (93% amino acid identities in both cases).

The organization of activase in the red maple genome was investigated using genomic blot (Southern) analysis (Fig. 5). Genomic DNA from Florida and Minnesota genotypes was digested with three separate endonucleases, then size-fractionated and probed with radiolabeled *Rca*1-Fl cDNA. The near identical nucleotide sequence homology between Florida and Minnesota Rubisco activase genes alleviated the need to reprobe the blots with the three other *Rca* cDNAs. Maple *Rca* cDNA contains two *Nco*I sites, four *Hind*III sites, and two *Eco*RI sites. As illustrated in Fig. 5, the *Nco*I digest for both genotypes revealed the presence of only two bands instead of the expected three. However, the maple *Rca* cDNA has an *Nco*I site 51 bp from the 3′-UTR, and it is unlikely that such a small fragment remained on the gel. The *Hind*III digest revealed the presence of four bands in the Minnesota genome and three in the Florida genome, suggesting a possible polymorphism (at ~3.2 kb) as well as increased signal for the ~2.6 kb band, indicating a possible doublet (Fig. 6).

To investigate a role for alternative splicing in red maple activase gene expression, genomic DNA from the 3′ portion of Florida and Minnesota *Rca* was analyzed. With a forward PCR primer complementary to a 3′/C-terminal coding domain and a reverse primer specific to the 3′-UTR of the *Rca*1 cDNA, a single 450-bp fragment was amplified and sequenced from the genomic DNA template. When the nucleotide sequence of this genomic band was compared with *Rca*1 and *Rca*2 cDNA sequences, it appears that the last intron can be selectively removed using a common 3′ acceptor splice site and either of two different 5′ donor splice sites (Fig. 7). The alternative splicing event would produce two mRNAs differing by a 39-bp insertion–deletion. Interestingly, the longer transcript, *Rca*2, produces the smaller protein (43 kDa) because of the presence of a stop codon in the 39-bp insert. Alternatively, the *Rca*1 intron can be completely removed, allowing full-length translation of a 46-kDa polypeptide.

Rubisco and Rubisco activase abundance in relation to temperature—The role of Rubisco activase in photosynthetic acclimation to warm temperature was explored by growing Florida and Minnesota genotypes at ambient (27/25°C; day/night) or warm (33/25°C) temperature conditions for 21 d (Fig. 8). Protein was isolated from the first fully expanded leaf, similar to those used for gas exchange analyses. Exposure to warm temperature had a negligible effect on Rubisco large subunit protein quantity (Fig. 8). However, the Florida genotype had a modest increase in the two RCA isoforms, while the Minnesota genotype had an increase in only the small RCA isoform.

DISCUSSION

The highly regulated and coordinated processes of photosynthesis have complicated efforts to understand the molecular control points of photosynthetic responses to elevated temperature. However, the Rubisco to Rubisco activase relationship is proving to be interesting in this regard because studies indicate that activase can be exceptionally sensitive to heat (Robinson and Portis, 1989; Eckardt and Portis, 1997; Salvucci et al., 2001). These findings suggest that activase may contribute to adaptive traits that are advantageous in high temperature habitats. In a previous study, growth and photosynthetic characteristics of red maple genotypes known for their sensitivity or insensitivity to heat were characterized (Weston and Bauerle, 2007). This controlled experiment under different levels of temperature stress indicated that differences in photorespiration and Rubisco activity were apparent between genotypes and may contribute to an important mechanism in

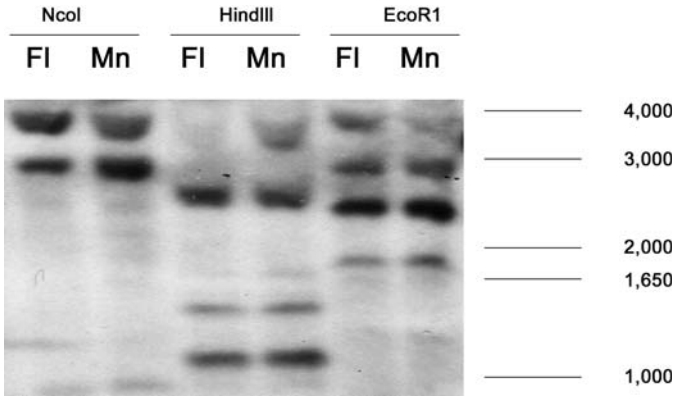

Fig. 5. Southern blot analysis of the *Rca* gene from *Acer rubrum*. Genomic DNA was digested with *Nco*I, *Hind*III, or *Eco*RI and hybridized with radiolabeled full-length *Rca2* cDNA. The positions of molecular size markers in kilobase pairs are indicated at the right. The Florida genotype is represented by "Fl" and the Minnesota by "Mn."

the photosynthetic heat acclimation process. Consequently, the present study set out to characterize and understand the molecular aspects of the genetic difference in temperature response. On the basis of our findings and the impending effects of global warming on terrestrial ecosystems, the ratio of Rubisco activase to Rubisco and photorespiration are important constraints to consider and could influence genotype fitness in an environment with elevated atmospheric temperature.

Photosynthetic response to elevated temperature—We found that red maple genotypes originally collected from Florida or Minnesota differed markedly in their photosynthetic response to heat. These results confirm those previously reported for 1-yr-old plants, with the Florida genotype having a broader temperature optimum and higher V_{cmax} than the Minnesota genotype (Weston and Bauerle, 2007). Similar results were observed previously among species from diverse habitats (e.g., reviewed in Berry and Björkman, 1980; Salvucci and Crafts-Brandner, 2004b) and in ecotypes along temperature gradients (e.g., Slatyer, 1977a, b; Ferrar et al., 1989), which were speculated to be consequences from past adaptation events. In the former case, Salvucci and Crafts-Brandner (2004b) found that the thermal properties of activase from warm habitat plants (e.g., desert creosote bush) were more robust to heat than activase derived from cold habitat plants (e.g., Antarctic hairgrass), thereby identifying a candidate gene and mechanism for increased V_{cmax} and thus for photosynthetic performance under elevated temperatures.

Molecular characterization of Rubisco activase and Rubisco—To investigate the association of Rubisco activase with the photosynthetic responses observed in this study, the genes were cloned and characterized. The RCA1- and RCA2-predicted proteins are highly similar between maple genotypes, as well as to Rubisco activase sequences from other species including *Arabidopsis thaliana* (Werneke and Ogren, 1989), creosote bush (*Larrea tridentata*), and Antarctic hairgrass (*Deschampsia antarctica*) (Salvucci and Crafts-Brandner, 2004b) with 79.2%, 85.8%, and 76.7% amino acid identity, respectively. The near identity of *Rca*1 and *Rca*2 between maple genotypes suggests that the encoded activase proteins have similar thermal properties. To elucidate potential differences within possible amino acid binding residues, a partial clone of the Rubisco large subunit was sequenced. Although a protein crystal structure of Rca is not available, previous studies found that activase K374 and V377 (K311 and V314 of the mature peptide) and W74 are involved in the recognition of Rubisco (van de Loo and Salvucci, 1996; Li et al., 2005). Additionally, the authors found evidence for the direct interaction of RCA K374 and V377 with RbcL E94 and P89, respectively. As noted in Figs. 3 and 4, these key residues are conserved in RCA and RbcL for both maple genotypes. Although only two directly interacting residues have been identified to date, these results suggest that the two proteins have equal potential for protein recognition and interaction.

Next, we compared red maple activase to those from *Arabidopsis* (Werneke and Ogren, 1989), spinach (Werneke et al., 1989), rice (To et al., 1999), and to *RcaA* of barley (Rundle and Zielinski, 1991). These species all have an activase encoded by a single nuclear gene that is alternatively spliced to produce two immunologically different proteins (i.e., isoforms). Red maple appears to be similar, because the *Nco*I digest and gene copy number reconstructions are consistent with the presence of a single *Rca* gene in the *A. rubrum* genome. However, a final determination of gene copy number would require the construction and screening of a red maple genomic library, an area for future research. Although there is evidence that the isoforms of activase that arise from alternative splicing have different thermal properties, for example, RCA1 from spinach and pea is much more tolerant to heat than is RCA2 (Crafts-Brandner et al., 1997; Rokka et al., 2001; Salvucci et al., 2001), this is not always the case. In fact, isoforms of activase from *Arabidopsis* seem to have little difference in sensitivity to heat (Kallis et al., 2000). In addition, in a recent study with transgenic *Arabidopsis* plants expressing reduced amounts of RCA1 or RCA2, photosynthetic inhibition in response to moderate heat was similar regardless of activase form (Salvucci et al., 2006). Thus, we are unable to fully explain the sensitivity of red maple to heat through thermal properties or activase isoforms.

Accumulation of Rubisco and Rubisco activase upon long-term exposure to moderate heat—The immunological results suggest that there is a modest differential temperature effect between genotypes in the ratio of Rubisco to RCA protein abundance. This is in contrast to ozone- and drought-treated Aleppo pine (*Pinus halepensis*), which had coordinated changes in Rubisco and activase protein quantities (Pelloux et al., 2001). In our study, the Minnesota genotype only increased the small RCA1 isoform, while the Florida genotype equally increased both RCA1 and RCA2 isoforms in response to warm temperature growth conditions. The importance of this potential acclimation mechanism is difficult to discern because the thermal properties of activase isoform are often species specific (see previous text) or possibly related to other mechanisms. For example, RCA1 is redox sensitive, and stromal oxidation has been previously reported under moderate heat stress as a possible result of thylakoid membrane leakiness (Schrader et al., 2004). However, Kim and Portis (2005) used transgenic *Arabidopsis* modified in fatty acid composition to show that plants with reduced thylakoid membrane leakiness did not have increased thermotolerance. Thus, a distinct role for activase isoform in regard to photosynthetic heat acclimation is currently unknown. Interestingly though, previous

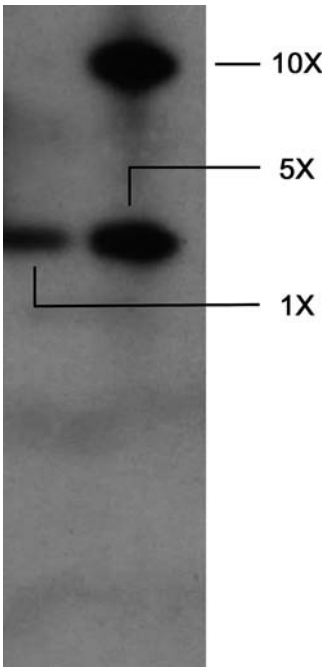

Fig. 6. Gene copy-number reconstructions. The genome size of *Acer rubrum* was estimated from *Acer pseudoplatanus* DNA C-values reported in the Royal Botanical Gardens at Kew database (http://www.rbgkew.org.uk/cval/homepage.html). Reconstructions were run with one, five, and 10 copy equivalents of the *Rca*1-Fl probe.

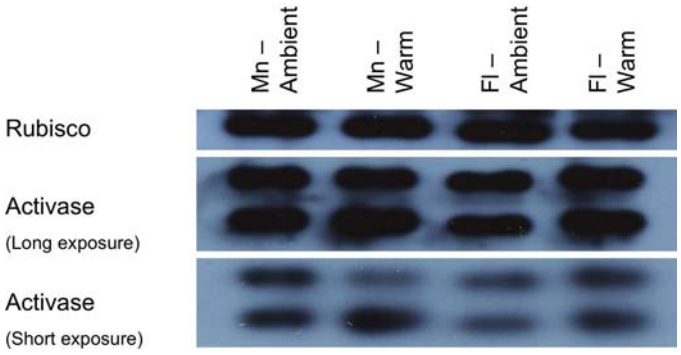

Fig. 8. Rubisco activase and Rubisco large subunit polypeptide accumulation after 21 d of acclimation to warm 33/25°C (day/night) or ambient (27/25°C) conditions. Total protein equal to 0.025 mg (Rubisco) or 0.1 mg (Rubisco activase) was loaded onto gels.

research has shown that the larger the Rubisco activase oligermeric complex, the greater is its ability to hydrolyze ATP at elevated temperatures (Portis, 2002) and thus contribute to the maintenance of Rubisco activity under conditions of high temperature stress. Perhaps even the modest increase that we observed in the amount of activase to Rubisco protein in both genotypes has significant temperature implications with respect to the observed photosynthetic adjustments. For instance, this shift could allow each Rubisco enzyme to be protected by a larger Rubisco activase oligermeric complex.

Conclusions—Understanding the molecular response and physiological constraints of photosynthesis to elevated temperatures is imperative in the face of both documented climate changes and predicted changes that could alter ecosystem structure and function. Our results show that the decline in V_{cmax} under warm acclimated temperature in the Minnesota genotype could be partly due to the temperature-dependent function of Rubisco activase and its affects on Rubisco activation. However, the marked difference in photosynthetic rates between genotypes in response to increased temperatures is not due to obvious differences in activase sequences or splicing potential, Rubisco sequences, or the known binding residues between Rubisco and activase. Also, it is doubtful that the modest increase in activase protein at warmer growth temperatures can explain the photosynthetic differences between the two genotypes. While red maple Rubisco activase likely contributes to the overall heat acclimation of the photosynthetic apparatus, we conclude that activase is not solely responsible for the increased thermotolerance of the Florida genotype over that of the Minnesota genotype.

Future research should consider the consequences of activase function in a multipathway framework when scaling genotype or species-specific molecular responses to temperature from the plant to the population level. For example, Rubisco heat deactivation via Rubisco activase may be an adaptive response protecting against photorespiratory metabolites (Sharkey et al., 2001; Sharkey, 2005). Furthermore, an additional role for activase has been hypothesized in protecting the thylakoid protein synthesis machinery under heat stress (Rokka et al., 2001). This role, along with the competing processes of photorespiration and carboxylation, raises inter-

Fig. 7. Putative alternative splicing pattern of maple Rubisco activase. The top strand represents the genomic DNA sequence of the last intron with flanking exon DNA sequence. The middle strand represents *Rca*2 mRNA that contains an early stop codon (underlined) because of a 39-bp insertion. The bottom strand represents *Rca*1 mRNA, which has a different 5′-splice site resulting in complete removal of the intron and its early stop codon.

esting questions concerning the trade-offs in activase function in response to temperature and how trade-offs relate to plant performance and fitness characteristics in terrestrial ecosystems.

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