The molecular and ecophysiological roles of Rubisco activase and Rubisco activase likes in photosynthetic thermal regulation of Acer rubrum and Arabidopsis thaliana

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THE MOLECULAR AND ECOPHYSIOLOGICAL ROLES OF
RUBISCO ACTIVASE AND RUBISCO ACTIVASE LIKES
IN PHOTOSYNTHETIC THERMAL REGULATION
OF *Acer rubrum* L. AND *Arabidopsis thaliana*

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Plant and Environmental Science

by
David Joseph Weston
December 2006

Accepted by:
Dr. William L. Bauerle, Committee Chair
Dr. Wm. Vance Baird
Dr. Brandon d. Moore
Dr. Douglas Bielenberg
ABSTRACT

Determining the consequences of human impact on our current and future climate is proving to be one of the greater challenges in modern biology. Current global models employing coupled atmosphere and terrestrial carbon feedbacks range in their predictions from the inconsequential to that of vast food shortages and species extinctions. However, there is hope that the emergence of new molecular approaches will revolutionize our understanding of plant and animal responses to a changing environment, allowing for higher predictive power when scaling up from the level of the individual to ecosystem-scale processes. This dissertation is an initial attempt for the melding of molecular biology with ecophysiology and acts as an entry point for the emerging field of ‘ecogenomics.’ The first chapter gains insight into the physiological control points of heat-limited photosynthesis between ecotypes of the common forest tree species’, red maple (Acer rubrum L.). The second chapter investigates the molecular contribution of a single enzyme, Rubisco activase, in the inhibition of photosynthesis between heat- sensitive and -insensitive ecotypes of red maple. The final chapter continues to delve further with molecular biology techniques and employs the use of a well-known model species’ (Arabidopsis thaliana) to discover novel molecular and physiological linkages for heat-limited photosynthesis.
DEDICATION

This dissertation is dedicated to my wife Jen for her unyielding encouragement, understanding, and love. This degree would not have been completed without the supportive smiles and love from Jaiden and Joey.
ACKNOWLEDGMENTS

I owe a great debt of gratitude to Dr. William L. Bauerle, whose unselfish encouragement and financial support allowed for the pursuit of this truly novel and collaborative research effort. Your kindness will be remembered.

Many thanks go out to Dr. Wm. Vance Baird, for welcoming me so warmly to your lab and introducing me to the nuances of molecular biology. Your kindness and guidance is much appreciated.

I also wish to extend a sincere thank you to Dr. Brandon d. Moore. I deeply appreciate your unyielding guidance and encouragement that has fostered into a continuing mentorship.

This dissertation would not have been the same without the critical scientific discussions and ideas from Dr. Douglas Bielenberg. I also wish to thank Dr. Shu-Hua Cheng, Dr. Kathy Sparace, Abhijit Karve, Rucha Karve, and Ginger Swire-Clark for friendship and scientific expertise. My graduate studies would not have been the same without the inspiration from John Seketa, John & Wonda Maddox, and the Bodines.
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PREFACE

This dissertation is written in journal style and is formatted into three main chapters, each intended for publication. Each chapter is comprised of: Abstract, Introduction, Materials and Methods, Results and Discussion, and Literature Cited.
Inhibition and acclimation of C₃ photosynthesis to moderate heat: a perspective from thermally contrasting genotypes of *Acer rubrum* (red maple)

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Abstract

The effects of moderate heat on growth and photosynthesis were investigated in clonal genotypes of *Acer rubrum* L. originally collected from the thermally contrasting habitats of Florida and Minnesota, USA, and known in the horticulture trade for sensitivity and insensitivity to heat, respectively. Under common garden and warm greenhouse conditions (33 °C/25 °C; day/night), the Florida genotype exhibited more growth. To determine the physiological parameters associated with this response, plants were acclimated to ambient (27 °C/25 °C, day/night) or warm (33 °C/25 °C) temperatures for 21 days before measurement of net photosynthesis. In vivo measurements of gas exchange and chlorophyll-α fluorescence on ambient acclimated plants revealed that the Florida genotype maintained a higher photosynthetic rate that was accompanied by higher stomatal conductance, more open PSII reaction centers, a greater PSII quantum yield, and a lower quantum requirement for photosystem II (Φ_{PSII}) per CO₂ (Φ_{CO₂}) throughout the measurement range (25 °C - 48 °C). When both genotypes were acclimated to and measured at 33 °C/25 °C, analysis of the response of net photosynthesis to calculated intercellular CO₂ concentration indicated that the maximal rate of Rubisco carboxylation (V_{cmax}) decreased more for the Minnesota genotype in response to elevated temperature. Additionally, Φ_{PSII}/Φ_{CO₂} at 33 °C was markedly higher for Minnesota plants under photorespiratory conditions, but similar to Florida plants under non-photorespiratory conditions. Taken together, the higher net photosynthetic rates at 33 °C/25 °C for the Florida genotype is due to a number of possible mechanisms including the maintenance of a higher V_{cmax}, and a more efficient quantum requirement of PSII per CO₂ that is likely the result of lower photorespiration.
Introduction

The increase in *Acer rubrum* L. (red maple) dominance among softwood and hardwood stands marks one of the most dramatic changes in post-colonized eastern North American forests (Abrams 1998). Currently, the range of red maple is bound to the north by Newfoundland, the south by Florida and the west by Minnesota and eastern Texas (Burns and Honkala 1990). Included in this range are habitats differing dramatically in light, nutrition, moisture status and temperature. As a result, red maple has segregated into specific ecotypes or races adapted to a wide range of local endemic environments (Abrams and Kubiske 1990, Bauerle et al. 2003). However, the leaf physiological characteristics (e.g. gas exchange, osmotic adjustment and N levels) of red maple are relatively modest and fail to explain its newfound prominence among such diverse habitats. Therefore, the species’ range expansion is not explained by its leaf physiology and has been appropriately termed “the red maple paradox” (Abrams 1998).

Understanding the physiological constraints of a species’ ability to adapt to changing environments, particularly those reflective of elevated temperatures, is currently exemplified by predictions of climate warming. Temperature trends in the United States show an overall warming in the mean temperature (Houghton et al. 2001). Forest ecosystems are thus subjected to distributional and phenological changes, and species’ range shifts (Easterling et al. 2000). A recent study investigating the responses of diverse terrestrial ecosystems to climate change, found that estimates of species ‘committed to extinction,’ will range from 18 to 32 percent by 2100 depending on the climate-warming scenario (Thomas et al. 2004). Such studies, while insightful, have failed to consider the physiological traits that allow plants to acclimate to warmer habitats.
One possibility whereby plants could moderate otherwise negative effects of local environment is the response of photosynthesis to temperature. It has been known for quite some time that even moderately elevated temperatures (< 35 °C) inhibit photosynthesis, the relationship of which is considered a major ecological driving force in plant distributions (Berry and Björkman 1980). Despite decades of investigation into heat-limiting photosynthetic processes, the traits selected for in high temperature habitats remain elusive. Traditionally, photosystem II (PS II) has been considered the most heat labile component of photosynthesis (Berry and Björkman 1980), but PS II damage is usually restricted to temperatures above 40 °C (Berry and Björkman 1980). However, more recent studies call attention to the effects of moderate heat on thylakoid membranes, where leaky membranes have an indirect effect on electron transport that reduces ATP and NADPH availability (Bukov et al. 1999). Support for this hypothesis was obtained by Wise et al. (2004), where the functional photosynthetic limitation to moderate leaf temperatures was explained by ribulose-1, 5-bisphosphate (RuBP) regeneration via electron transport limitation. In a follow-up study, Schrader et al. (2004) reported that moderate heat stress affected thylakoid membrane permeability, resulting in stimulated cyclic photosystem I activity at the expense of stromal reductants. Subsequently, Rubisco was deactivated as a possible protective mechanism to reduce photorespiratory metabolite accumulation.

One enzyme responsible for Rubisco’s catalytic activity, Rubisco activase, is heat labile (Robinson and Portis 1989, Eckardt and Portis 1997, Salvucci et al. 2001) and it has even been suggested as a primary cause for reduced photosynthetic performance under moderate heat (Salvucci and Crafts-Brandner 2004a). Supporting evidence for this
hypothesis was recently reported in a study comparing transgenic Arabidopsis with improved membrane integrity (lower membrane lipid saturation) and various isoforms of Rubisco activase (Kim and Portis 2005). In that study, plants subjected to heat (including moderate heat 38 °C) were measured for detailed photosynthetic characteristics that included metabolites, gas exchange and fluorescence parameters. The results led the authors to conclude that inhibition of photosynthesis was due to Rubisco deactivation, which concomitantly lowers electron transport. Taken together with investigation of thermally contrasting species (Salvucci and Crafts-Brander 2004c), these results suggest that physiochemical properties of Rubisco activase may affect the geographic distribution of higher plants.

To investigate the underlying photosynthetic traits that may infer adaptation to elevated temperatures in a long-lived forest species, in vivo gas exchange and chlorophyll-a fluorescence measurements were used to determine mechanisms of heat-induced reductions of photosynthesis in two red maple genotypes known in the horticultural trade for their sensitivity and insensitivity to heat (Sibley et al. 1995a, 1995b, J. Ruter personal communication). We tested the hypothesis that the heat insensitive Florida genotype would have higher rates of net photosynthesis in comparison to the heat sensitive Minnesota genotype at moderately elevated temperatures.
**Materials and Methods**

*Plant material and treatments*

Dormant rooted cuttings of red maple were transplanted into 3L pots containing a mixture of sand, peat and silt loam (1:2:1, v/v) substrate and fertilized twice weekly with soluble fertilizer (Peters 20-10-20 N,P,K with micronutrients, Masterblend, Chicago, IL) at the Clemson University Biosystems Research Complex (Clemson, SC). The study was conducted from June to early November of 2002 and replicated during the same time frame in 2003. We used two genotypic lines of red maple known in the horticultural trade as ‘Northwood’ and ‘Florida Flame’, which were originally collected from indigenous populations from Minnesota (46° 55’ 44’’ N, 92° 55’’ 10’’ W) and Florida (30° 33’’ 34’’ N, 84° 19’’ 33’’ W), respectively.

To control temperature, four Mylar® chambers (4 m x 1 m x 1.5 m) were constructed within a Biosystems Research Complex glasshouse. A Campbell 21X data logger (Campbell Scientific, Logan, UT) monitored the temperature of each chamber with type T thermocouples and controlled chamber temperature via a heat exchange pump (model YSO9, Friedrich Inc., San Antonio, TX, USA). Photoperiod and light quantity were similar to natural local conditions (34°41’ 0’’ N, 82°50’15’’ W) for the duration of the measurement period (June-September). To minimize potential chamber effects, plants were alternated among chambers on a weekly basis. After three weeks of 27 °C/25 °C growth conditions, plants were randomly assigned to treatment (33 °C/25 °C) or control (27 °C/25 °C, day/night) temperatures, with ambient humidity and light.
Gas exchange

Net assimilation ($A_{net}$) versus calculated intercellular CO$_2$ ($C_i$) response curves were constructed between June 14 and September 24. Calculations of gas exchange parameters were performed according to (Farquhar et al. 1980) and derivation of photosynthetic parameters was described previously (Bauerle et al. 2003). To ensure steady-state activation of Rubisco prior to measurement with a Ciras-1 gas analyzer (PP systems, Haverhill, Mass., USA), the leaf within the cuvette was acclimated to a CO$_2$ concentration of 360 µmol mol$^{-1}$ and a saturating Photosynthetic Photon Flux Density (PPFD; 1200 µmol m$^{-2}$ s$^{-1}$) for 10-15 minutes. To determine $V_{cmax}$ and the CO$_2$ compensation point ($\Gamma^*$), the CO$_2$ limiting linear phase of the response curve was constructed by changing the cuvette atmospheric CO$_2$ concentration ($C_a$) from 200, 150, 100, 75, and 50 µmol mol$^{-1}$. At the end of the measurements, $C_a$ was again stabilized at 360 µmol mol$^{-1}$ and $A_{net}$ recorded. This procedure allowed us to compare post- and pre-$A_{net}$ values at 360 µmol mol$^{-1}$ to ensure open stomata and verify the stability of the photosynthetic apparatus. Lastly, the light source was turned off and the cuvette was shrouded by a black cloth for leaf dark respiration ($R_d$) measurement. Response curves were obtained under 21% and 2% O$_2$.

With a parallel Ciras-1 gas analyzer, light response curves were generated on the opposite leaf of that measured for the $A$ versus $C_i$ response. Leaf cuvette CO$_2$ ($C_a$) was held at 360 µmol mol$^{-1}$ while vapor pressure deficit (VPD) and O$_2$ concentrations were set identical to the $A_{net}$ versus $C_i$ experiment. Light response curves were used to estimate quantum yield of CO$_2$ ($\Phi_{CO2}$), which can be easy to underestimate (Singsaas et al. 2001). Therefore, a number of precautions were taken that included the use of both 21% and 2%
O₂ concentrations (interested readers are referred to Singsaas et al. 2001). When using 21% O₂, Cₐ was adjusted to maintain a Cᵢ of 230 to 250 µbar to compensate for errors incurred by photorespiration and at least three data points were collected below 100 µmol m⁻² s⁻¹ PPFD to avoid the incorporation of non-linear data points (Singsaas et al. 2001).

Fluorescence

Chlorophyll-a fluorescence parameters were measured with an OS-500 pulse amplitude modulated fluorometer (Opti-Science, Westand, Mass., USA). The fiber optic cable from the fluorometer was coupled to the Ciras-1 gas exchange system by a specially constructed light source, PLC 5(B). Actinic light was provided by the integrated Ciras-1 leaf chamber. In order to estimate Fo’ (minimal fluorescence) under fully oxidized conditions (Qₐ), the actinic light was terminated and a far-red pulse was generated from the fluorometer for 5 s⁻¹ to fully oxidize Qₐ. Steady-state fluorescence (Fₛ) was measured after excitation by a weak modulated red light and maximum fluorescence (Fₘ') was determined after a saturating pulse (8000 µmol m⁻² s⁻¹ for 1 s⁻¹) of white light. To ensure a fully reduced Qₐ, maximal photosynthetic efficiency was determined at predawn and directly before the response curve by using dark adaptation clips. Results indicated that 20 min of dark adaptation produced similar readings as compared to predawn measures.
Light absorbtance

Leaf light absorbtance ($\alpha$) was estimated as described in Bauerle et al. (2004), where readings from a Minolta SPAD 502 Meter (Spectrum Technologies Inc., Plainfield, IL) were non-linearly correlated to red maple light absorbtance, reflectance, and transmittance.

Statistics

A randomized complete block design with sampling 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 °C/25 °C (day/night) or 33 °C/25 °C. Two chambers were set at 27 °C/25 °C and an additional two chambers were set at 33 °C/25 °C. Each week throughout the study, plants were transferred within chambers to minimize chamber effects. Actual number of plants sampled varied according to parameter tested and is indicated in the figure legend for that specific experiment. For each photosynthetic parameter reported in Table 2, a 2-way ANOVA (JMP 6.0, SAS Institute, Cary, NC) was performed with genotype and temperature as independent variables. Error terms were pooled and reported as standard error of the difference of the means.
Results

Growth characteristics

Whole plant growth characteristics were measured on Florida and Minnesota genotypes of red maple under greenhouse conditions set at 33 °C/25 °C (day/night) temperature and ambient relative humidity and photoperiod. After 42 days of greenhouse growth conditions, the Minnesota genotype failed to show any measurable sign of increased stem height, leaf number, or branch number (Table 1.1). This is in contrast to the Florida genotype, which showed increases in all measured parameters. Under common garden conditions (at Clemson University, South Carolina, USA), both genotypes showed increased stem height from May until September; however, the magnitude of the increase was 46% higher for the Florida genotype compared to that of the Minnesota genotype (Table 1.1).

Table 1.1  Growth characteristics of *Acer rubrum* genotype Florida and Minnesota. Plants were grown under 33 °C/25 °C temperatures for 42 days in growth chambers or under a common garden at Clemson University, Clemson, South Carolina, USA from May until September. Plants under both environmental conditions were well-watered and provided with supplemental nutrition. ND means non detectable. Means ± standard errors (in parentheses) represent growth increase per genotype for the common garden and growth chamber study (n = 8 per genotype per environment).

<table>
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<tr>
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<tr>
<td></td>
<td>Fl</td>
<td>Mn</td>
</tr>
<tr>
<td>Stem height increase (mm)</td>
<td>20 (5.0)</td>
<td>ND</td>
</tr>
<tr>
<td>Branch # increase</td>
<td>0.5 (0.2)</td>
<td>ND</td>
</tr>
<tr>
<td>Leaf # increase</td>
<td>1.5 (0.3)</td>
<td>ND</td>
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Temperature response under 21% O<sub>2</sub> and 27°C/25°C (day/night)

Simultaneous measurements of Gas exchange and chlorophyll-a fluorescence were used to evaluate and assess the intrinsic photosynthetic response of Minnesota and Florida genotypes of red maple to elevated temperature. Leaf net photosynthetic characteristics differed between genotypes and in response to temperature. Under 27 °C/25 °C (day/night) growth conditions, Florida plants exhibited a higher net CO<sub>2</sub> assimilation rate in comparison to Minnesota (Figure 1.1a). Stomatal conductance was higher for Florida plants and even displayed a small increase until 30 °C, before declining with increasing temperature. Minnesota plants did not show an initial rise in stomatal conductance like Florida plants, but rather maintained a declining trend with increasing temperature throughout the response (Figure 1.1b). Mean average intercellular CO<sub>2</sub> partial pressure was higher for Florida plants in comparison to Minnesota until 42 °C. Above this temperature, intercellular CO<sub>2</sub> partial pressure rose sharply for both genotypes as a subsequent response to reduced net photosynthesis, with the magnitude of the response being greater for Minnesota (Figure 1.1c).
Figure 1.1 The temperature responses of the rates of net CO₂ assimilation (a), stomatal conductance (b), and intercellular CO₂ partial pressure (c) in Florida ♦ and Minnesota ■ genotypes of Acer rubrum L. Photosynthesis was measured at 500 µmol m⁻² s⁻¹ PPFD, 360 µbar CO₂ and 21% O₂. Each point represents the mean (± SE) where n = 5.
Quantum yield of PSII ($\Phi_{\text{PSII}}$) provided an estimate of the proportion of electrons passing though PSII per quanta absorbed and was higher for the Florida genotype throughout all measurement temperatures (Figure 1.2a). The temperature optimum for $\Phi_{\text{PSII}}$ was broader than that for net CO$_2$ assimilation, with declining rates not apparent until $\sim 37$ °C. The proportion of open and undamaged PSII reaction centers, as measured by photochemical quenching (qP), was also higher for Florida plants at temperatures above 25 °C (Figure 1.2b). Interestingly, non-photochemical quenching (qN), which is an indication of the amount of absorbed energy dissipated as heat, was similar between genotypes regardless of temperature (Figure 1.2c).
Figure 1.2 The temperature responses of the quantum yield of PSII (a; $\phi_{\text{PSII}}$), photochemical quenching (b; $q_P$), and non-photochemical quenching (c; $q_N$) in Florida ♦ and Minnesota ■ genotypes of *Acer rubrum* L. Measurements were made at a PPFD of 500 $\mu$mol m$^{-2}$ s$^{-1}$, 360 $\mu$bar CO$_2$, and 21% O$_2$. Each point represents the mean (± SE) where $n = 5$. 

![Diagram showing the temperature responses of the quantum yield of PSII (a; $\phi_{\text{PSII}}$), photochemical quenching (b; $q_P$), and non-photochemical quenching (c; $q_N$) in Florida ♦ and Minnesota ■ genotypes of *Acer rubrum* L. Measurements were made at a PPFD of 500 $\mu$mol m$^{-2}$ s$^{-1}$, 360 $\mu$bar CO$_2$, and 21% O$_2$. Each point represents the mean (± SE) where $n = 5$.](image)
The ratio of $\Phi_{\text{PSII}}$ to $\Phi_{\text{CO}_2}$ provides an estimate of the moles of electrons passing through PSII needed to reduce one mole of CO$_2$. When measured under photorespiratory conditions (21% O$_2$) this estimate also includes electrons contributing to the photorespiratory pathway. We used this parameter as a proxy for carboxylation efficiency in response to heat (Figure 1.3). Under photorespiratory conditions, the quantum requirement of PSII per CO$_2$ ($\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$) was higher for the Minnesota genotype under all measurement temperatures (Figure 1.3). In fact, the rate of increase for the Minnesota genotype, as measured by difference in slope, was 51% higher than that of the Florida genotype (Figure 1.3).
Temperature response of warm-acclimated plants under 21% and 2% O2

The photosynthetic response of red maple to prolonged periods of elevated temperature was investigated by acclimating plants to warm (33 °C/25 °C, day/night) and ambient (27 °C/25 °C) temperature conditions for 21 days. Under photorespiratory conditions, maximum net photosynthesis ($A_{\text{max21%}}$) decreased for both genotypes when acclimated and measured at 33 °C/25 °C (Table 1.2). Specifically, the mean difference between $A_{\text{max21%}}$ from ambient to warm temperatures significantly decreased 11% and 25% for Florida and Minnesota, respectively. However, when warm-acclimated plants were analyzed under non-photorespiratory conditions ($A_{\text{max2%}}$), net photosynthesis again decreased, but the magnitude of the decline was less severe at 9% and 18% for Florida and Minnesota, respectively.

The maximal rate of Rubisco carboxylation, $V_{c_{\text{max}}}$, under photorespiratory conditions decreased for both genotypes at warm acclimation and measurement temperature by 26% and 34% for Florida and Minnesota, respectively (Table 1.2, Figure 1.4). The percent decrease was significant for both genotype and temperature (Table 1.2). To investigate the intrinsic response of heat on $V_{c_{\text{max}}}$, plants acclimated to both ambient and warm conditions were measured at 33 °C and compared to ambient acclimated and measured plants. As depicted in Figure 1.4, the decline in $V_{c_{\text{max}}}$ was similar for both ambient and warm acclimated plants when measured at 33 °C.
Table 1.2  Means ± standard errors (in parentheses) for gas exchange and fluorescence parameters of leaves of *Acer rubrum* genotype Florida and Minnesota (n = 6 plants per genotype). Symbols are defined in materials and methods. Plants were acclimated to 27 °C/25 °C (day/night) or 33 °C/25 °C temperatures for 21 days and measured at the acclimation day temperature.

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>A. rubrum</em> ‘Fl’</th>
<th><em>A. rubrum</em> ‘Mn’</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>27/25°C</td>
<td>33/25°C</td>
<td>27/25°C</td>
</tr>
<tr>
<td>$A_{\text{max21%}}$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</td>
<td>10.6 (0.4)</td>
<td>9.5 (0.4)</td>
<td>8.7 (0.4)</td>
</tr>
<tr>
<td>$V_{\text{cmax}}$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</td>
<td>79.8 (3.2)</td>
<td>58.7 (3.2)</td>
<td>72.5 (3.2)</td>
</tr>
<tr>
<td>$A_{\text{max2%}}$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</td>
<td>16.3 (0.6)</td>
<td>14.9 (0.6)</td>
<td>14.7 (0.6)</td>
</tr>
<tr>
<td>$R_d$ ($\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$)</td>
<td>0.3 (0.1)</td>
<td>0.5 (0.1)</td>
<td>0.4 (0.1)</td>
</tr>
<tr>
<td>$\Phi_{\text{CO2 at 2%}}$</td>
<td>0.10 (0.01)</td>
<td>0.07 (0.01)</td>
<td>0.08 (0.01)</td>
</tr>
<tr>
<td>$\Phi_{\text{PSII/CO2 at 21%}}$</td>
<td>6.5 (0.25)</td>
<td>7.7 (0.25)</td>
<td>6.6 (0.25)</td>
</tr>
<tr>
<td>$\Phi_{\text{PSII/CO2 at 2%}}$</td>
<td>4.8 (0.15)</td>
<td>5.1 (0.15)</td>
<td>4.5 (0.15)</td>
</tr>
<tr>
<td>$\Gamma^*$ ($\mu$mol mol$^{-1}$)</td>
<td>60.5 (1.8)</td>
<td>79.1 (1.8)</td>
<td>64.5 (1.8)</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>84.0 (0.47)</td>
<td>82.0 (0.47)</td>
<td>84.4 (0.47)</td>
</tr>
</tbody>
</table>
Figure 1.4 The response of CO$_2$ assimilation rate to intercellular $C_i$ concentration for leaves of Florida (a) and Minnesota (b) genotypes of *Acer rubrum* L. acclimated and measured at 27 °C/25 °C (day/night; ♦), or 33 °C/25 °C (▲), or acclimated to 27 °C/25 °C and measured at 33 °C/25 °C (■). Measurements were made at a PPFD of 1,100 µmol m$^{-2}$ s$^{-1}$, 360 µbar CO$_2$ and 21% O$_2$. Each point represents the mean (± SE) where n = 7-10.
The quantum yield of CO$_2$ ($\Phi_{\text{CO}_2}$) measured under 2% O$_2$ conditions did not significantly differ in response to genotype and are similar to those previously reported for unstressed C$_3$ plants (Long et al. 1993, Table 1.1). Photosynthetic efficiency was further investigated by comparing the quantum yields of PSII to that of CO$_2$ ($\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$) in ambient and warm acclimated plants under both photorespiratory and non-photorespiratory conditions (Table 1.2). Under 2% O$_2$, only a slight increase in $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ was noted and was significant for temperature only. This suggests minor partitioning of electrons to alternative electron sinks such as the Mehler and nitrogen pathway. By measuring and comparing photosynthetic efficiency ($\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$) under both photorespiratory and non-photorespiratory conditions, the proportion of electrons contributing to alternative sinks and that of photorespiration can be estimated (Long and Bernacchi 2003). Using this approach, $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ measured under 21% O$_2$ revealed significant increases of 25% and 16% for Minnesota and Florida plants, respectively (Table 1.2). Taken together, these results suggest considerable partitioning of electrons to the photorespiratory pathway, particularly for the Minnesota genotype.

*Effects of a transient heat stress on net photosynthesis and fluorescence parameters of warm-acclimated plants*

To further investigate the mechanistic properties of the photosynthetic apparatus, Florida and Minnesota genotypes acclimated to warm temperatures were subjected to a 10 min heat stress of 42 ℃ and allowed to recover. Net photosynthesis and fluorescence parameters were measured prior to, during, and after the transient heat treatment (Figure 1.5). To compensate for genotypic differences in intercellular CO$_2$ partial pressure ($C_i$), at high temperature (refer to Figure 1.1c), this experiment was conducted under high CO$_2$
(1000 ppm) and 2% oxygen. Net photosynthesis declined for both genotypes immediately following heat stress induction (Figure 1.5d). However, the magnitude of inhibition was greater for Minnesota plants in comparison to Florida plants. Photosynthetic recovery was near that of pre-heat treatment conditions for both genotypes. The quanta passing through PSII ($\Phi_{PSII}$) was also reduced by the heat treatment and was subsequently followed by a recovery to near pre-treatment conditions similar to that of net photosynthesis (Figure 1.5c). In contrast to the photosynthetic response, however, $\Phi_{PSII}$ decline was not severe in the Minnesota genotype and acted quite similar to that of Florida plants. Non-photochemical quenching (qN) is an estimate of the amount of absorbed quanta dissipated as heat rather than that entering the light reactions. This parameter rose for both genotypes upon heat treatment induction (Figure 1.5a), however Minnesota plants exhibited a marked drift up throughout the heat treatment, which was seen in all three replicate curves ($n = 3$). Minimal fluorescence measured in the light, $F_{o'}$, rose for both genotypes upon heat induction, however the rise was again greater in Minnesota plants when compared to Florida plants (Figure 1.5b).
Figure 1.5 Fluorescence and photosynthetic parameters prior to, during, and after a 10 min 42 °C heat stress. Plants were acclimated to 33 °C/25 °C (day/night) conditions. Measurements were made at a PPFD of 500 µmol m⁻² s⁻¹ at 1000 ppm CO₂ and 2% O₂. Results from three replicate experiments (n = 3) are plotted.
Discussion

The inability for leaf level physiology to explain the range expansion of red maple to diverse habitats has puzzled biologists and has even led to the term, ‘red maple paradox’ (Abrams 1998). The understanding of this mystery has relevance beyond that of forest management, and may even reveal key heritable traits for warm temperature habitats. In the present study, we found several within-species characteristics at the leaf level that differed between genotypes known for their sensitivity and insensitivity to heat. Notably, the higher growth rate observed for the heat insensitive Florida genotype was associated with greater net photosynthesis, a higher $V_{c_{\text{max}}}$, and a more efficient quantum requirement of PSII per CO$_2$ (lower $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$).

Although shifts in temperature optimum in relation to growth condition were not measured in this study, the failure of either genotype to maintain maximal rates of net photosynthesis under warm acclimation and measurement conditions indicates that complete photosynthetic acclimation was not observed in this study. In addition, the near identical decline in $V_{c_{\text{max}}}$ for both ambient- and warm-acclimated plants measured at moderate heat also suggests that thermal acclimation of photosynthesis was not complete, and may even be of minor importance for the performance of these two genotypes under heat. As reviewed by Berry and Björkman (1980), the potential for physiological acclimation of photosynthesis to temperature is highly variable ranging from marked shifts in temperature optimum to no change, or even negative adjustments. Results from our study are more similar to those reported for populations of *Eucalyptus pauciflora*, where peak assimilation rates of photosynthesis correlated with endemic habitat and is possibly reflective of past ecotypic adaptation (Slatyer 1977a, 1977b, Ferrar et al. 1989).
This is in contrast to findings in Acer saccharum (sugar maple), *Eucalyptus globules*, *Eucalyptus camaldulensis*, and *Eucalyptus nitens*, where populations exhibited more evidence of physiological adjustments than of ecotypic adaptation (Ferrar et al. 1989, Battaglia et al. 1996, Gunderson et al. 2000).

In this study, we found inherent differences between the two genotypes grown under ambient temperature conditions. Specifically, \( A_{\text{max}} \) was substantially higher for the Florida genotype throughout the temperature response curves and was accompanied by a higher stomatal conductance, more open PSII reaction centers, a greater PSII quantum yield, and a lower quantum requirement of PSII per CO\(_2\). When plants were measured and acclimated to warm temperatures, \( A_{\text{max}} \) declined for both genotypes; however, the declined was more severe for the Minnesota genotype (Table 1.2). Taken together, these results indicate that the Florida genotype has a greater intrinsic ability for higher CO\(_2\) assimilation as well as higher growth temperature tolerance.

To further investigate \( A_{\text{max}} \) decline under heat, we partitioned electron flow between alternative sinks such as Mehler, nitrogen, and the ascorbate-malate shuttle to that of photorespiration. Under non-photorespiratory conditions, a theoretical minimum of 4 electrons and eight photons are needed to fix one molecule of CO\(_2\). Therefore, at 2% oxygen (non-photorespiratory conditions), electrons in addition of 4 are assumed to contribute to alternative electron sinks. Results showed that the \( \Phi_{\text{PSII}} / \Phi_{\text{CO}_2} \) for both genotypes under non-photorespiratory conditions minutely increased under heat and is in accordance with other published studies that report < 10% of electrons participate in alternative sinks (Ruuska et al. 2000), even under heat (Laisk et al. 1998). By performing the same measurements under photorespiratory conditions, contributions to
photorespiration and alternative sinks can be determined. Thus, photorespiration can be estimated by comparing the difference between $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ at photorespiratory and non-photorespiratory conditions (Long and Bernacchi 2003). Using this technique, we found that the Minnesota genotype partitions 2 extra photons per CO$_2$ fixed compared to Florida plants at elevated acclimation and measurement temperature and is most likely the result of increased photorespiration and not respiration (Table 1.2). In fact, a similar response has been reported previously for C$_3$ plants and was also attributed to photorespiration (Oberhuber & Edwards 1993). Since liquid phase diffusive resistance was not measured in this study, it is not clear if the rise in photorespiration is in response to decreased internal CO$_2$ concentrations ($C_i$) alone or a combination of both.

The Minnesota genotype had markedly lower stomatal conductance compared to the Florida genotype. This was reflected by a small but noticeable difference in intercellular CO$_2$ concentrations ($C_i$), which would presumably contribute to higher photorespiration as discussed above. However, the heat induced $A_{\text{max}}$, decline observed in this study, is not completely alleviated when measured at 2% oxygen. Thus, there appears to be additional limitations to photosynthesis at moderately high temperatures.

Another possible contributing factor for the reduced net photosynthesis reported in this study is the decline in the maximal rate of $V_{\text{cmax}}$. This parameter decreased for both genotypes under heat acclimation and measurement with greater repression observed for the Minnesota genotype. In addition, Fo’, increased under transient heat stress more for the Minnesota genotype compared to the Florida genotype. This parameter has been used previously to separate the effects of heat between electron transport and stromal-based processes for Rubisco activase deficient mutants (Sharkey et al. 2001).
Interestingly, the marked rise in Fo’ for the Minnesota genotype is strikingly similar to that reported for Rubisco activase tobacco mutants (Sharkey et al. 2001). In fact, a decrease in Rubisco activation under moderate heat has been widely reported before (Feller et al. 1998, Laisk et al. 1998, Crafts-Brandner and Law 2000, Crafts-Brandner and Salvucci 2000, Salvucci et al. 2001, Salvucci and Crafts-Brandner 2004b, Salvucci and Crafts-Brandner 2004c), and has been attributed to the heat sensitivity of the protein responsible for Rubisco activation, Rubisco activase (refer to Portis 2002, Salvucci and Crafts-Brandner 2004b for reviews).

The difference in genotype growth under warm acclimation conditions (Table 1.1) was dramatic and could therefore complicate interpretation of the data since photosynthesis is highly regulated and can be influenced by internal factors (e.g. sink demand; Thomas & Strain 1991, Maier & Teskey1992) as well as temperature. The general consensus is that an increase in sink demand is positively correlated with an increase in photosynthesis (e.g., Myers et al. 1999, Lavigne et al. 2001, Vaast et al. 2005). Thus, it is possible that some of the photosynthetic processes mentioned above are not in direct response to heat, but an indirect consequence of heat on internal factors. However, data from the transient heat stress experiment (Fig. 1.5) suggests that the Florida genotype is more thermotolerant than the Minnesota genotype, and is most likely in direct response to heat and not internal factors. For example, the marked rise in Fo’ is an indication of PSII acceptor side limitation (see above) and is known to decrease upon nonphotochemical quenching. However, Minnesota plants were not able to quench Fo’ to Florida levels, even with higher relative nonphotochemical quenching. This response was subsequently followed by lower net photosynthesis and PSII quantum yield.
compared to the Florida genotype. These results demonstrate a greater thermotolerance for the Florida photosynthetic apparatus compared to Minnesota and suggests that growth differences were not a critical factor in that particular experiment.

A number of mechanisms have been proposed to explain the expansion in the red maple range and dominance in North America over the last 100 years and include adjustments to variable soil moisture conditions (Bauerle et al. 2003), dark respiration rates (Turnbull et al. 2001), shoot damage recovery (Sipe and Bazzaz 2001), seedling recruitment (Lambers and Clark 2005), herbivores tolerance (e.g. gypsy moths, Jedlicka et al. 2004), and low resource requirement for leaf biomass (Nagel et al. 2002).

However, gas exchange measurements from northeastern forest canopies revealed that red maple has a relatively low maximum photosynthetic rate (Jurik et al. 1988, Kloeppele et al. 1993, Kubiske and Pregitzer 1996, Turnbull et al. 2002). The data presented herein, and specific to the heat sensitive Minnesota genotype, support these previous observations of low maximum photosynthesis. However, in contrast to these studies, the heat insensitive Florida genotype had a relatively high photosynthetic rate. This may be a reflection of the high genetic diversity of red maple and the subsequent taxonomic varieties or subspecies including rubrum, trilobum, and drummondii (Flora of North America Project, http://hua.huh.harvard.edu/FNA/index.html). In fact, the Florida genotype used in this study has morphological characteristics of drummondii, which is endemic to the southeastern USA and may explain its increased growth and photosynthetic performance under heat. Although the use of genotypes in this study precludes generalizations to subspecies, future research should consider comparisons between and among taxonomic varieties and populations of widespread species, such as
red maple, in response to temperature. Such studies are necessary in revealing intraspecific variation in response to a changing climate, the consequences of which could affect predictions of climate-carbon feedbacks (King et al. 2006), species range models (Helman et al. 2006 in review) and even the microevolutionary consequences of climate change (Rice and Emery 2003).

In conclusion, our results indicate that there is intraspecific variation in red maple photosynthetic response to heat at the leaf level which may, in part, explain its success to thermally contrasting habitats. Most notably, the heat insensitive genotype has a greater intrinsic ability to maintain higher net photosynthetic rates in response to elevated temperature. Data from this study suggest that the maintenance of higher net photosynthesis was due to multiple factors including a higher $V_{\text{cmax}}$ and a more efficient quantum requirement of PSII per CO$_2$ that is likely a subsequent response to lower photorespiration.
Literature Cited


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Molecular characterization of Rubisco activase from thermally contrasting genotypes of

*Acerc rubrum* L. (Aceraceae)

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Abstract

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 have characterized Rubisco activase and explored its role in the previously demonstrated thermal acclimation and inhibition of two ecologically distinct genotypes of *Acer rubrum* L. (red maple) collected from Florida and from Minnesota, USA. In this study, when plants were grown at 33 °C/25 °C (day/night) for 21 days, the Florida genotype compared to the Minnesota genotype maintained about a 2-fold increase in leaf photosynthetic rates at temperatures from 33 to 42 °C and had a 22% increase in $V_{\text{cmax}}$ (maximal rate of Rubisco carboxylation) at 33 °C under non-photorespiratory conditions. Using homologous probes from full-length cDNA clones, Southern blot analysis indicated the presence of one Rubisco activase (*Rca*) gene in the *A. rubrum* genome. We further found that both genotypes have two leaf *Rca* transcripts, likely due to equivalent alternative splicing events in each. The *RCA* cDNAs from the Florida and Minnesota genotypes had correspondingly 97% nucleotide identities, with predicted 98% amino acid identities. Western analysis confirmed the presence of two Rca polypeptides of size 47 kDa and 42 kDa, designated RCA1 and RCA2, respectively. After growth at 33 °C/25 °C relative to growth at 27 °C/25 °C (day/night), both RCA1 and RCA2 proteins increased modestly in Florida plants, while only RCA2 protein increased, though again modestly, in Minnesota plants. Rubisco large subunit (RbsL) protein abundance, was relatively
unaffected in either genotype by the warmer growth temperature. Taken together with previous studies, these data indicate that while an increased ratio of Rubisco activase to Rubisco is part of the photosynthetic heat acclimation process in red maple, genotype differences in photorespiration might be even more important.
**Introduction**

Photosynthesis is sensitive to heat, especially in comparison to other cellular and whole plant processes (Berry and Björkman, 1980). 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 and the associated elevated atmospheric temperatures. In fact, a recent study predicted maize and soybean yield losses, two crops known for their heat tolerance, of up to 17% for each degree centigrade above current annual season temperature (Lobell and Asner, 2003). Furthermore, in regard to forest ecosystems, the spatial and temporal heterogeneity of temperature at the leaf level along with 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 not accurately predict physiological parameters at temperatures below and above 25 °C (Bernacchi et al., 2001; Bernacchi et al., 2003).

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 concomitant 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 Jr., 1989; Eckardt and Portis Jr., 1997, Salvucci et al., 2001), and it 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 due to reduced carboxylation with a concomitant decrease in electron transport.

Rubisco activase is a member of the chaperone-like AAA+ protein family [ATPase associated with diverse cellular activities; (Neuwald et al., 1999)], characterized by assisting target protein function usually through conformational change when powered by ATP hydrolysis. Rubisco activase acts similarly, and after ATP hydrolysis binds to Rubisco and releases inhibitory ligands (Portis Jr., 2002). This role under heat stress is particularly important when the balance of the Rubisco population shifts from the activated to the de-activated state (Crafts-Brandner and Salvucci, 2000), which is likely the result of misprotonation of RuBP (Portis Jr. et al., 1995, Salvucci and Crafts-Brandner, 2004b). This process of ‘catalytic misfire’, or ‘fall-over’, inactivates Rubisco and if unassisted, is very slow to recover (Duff et al., 2000, Salvucci and Crafts-Brandner, 2004b for reviews). Thus, Rubisco activase appears to maintain Rubisco’s maximal catalytic efficiency under non-optimal physiological conditions, such as elevated temperature (Salvucci and Crafts-Brandner, 2004a).

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 Jr., 2002, Spreitzer and Salvucci, 2002). However, exceptions do exist. For example, cotton and maize have both small and large isoforms, but these are the products of two separate
genes (Salvucci et al., 2003; Ayala-Ochoa et al., 2004). Barley has a separate 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 Jr., 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, where the large isoform of Rubisco activase is inherently more thermal stable than is the small isoform (Crafts-Brandner et al., 1997). Adding to this diversity is cotton, which produces a novel 46 kDa isoform under prolonged exposure to moderate heat (Law et al., 2001).

In a previous study, we addressed the observation that a Florida Acer rubrum L. (red maple) genotype had a higher growth rate and greater net photosynthesis compared to a Minnesota genotype under elevated acclimation and measurement temperature (Weston and Bauerle, 2007). Analysis with coupled gas exchange and chlorophyll-α fluorescence, suggested that the higher rates of net photosynthesis were due to a greater maximal rate of Rubisco carboxylation ($V_{\text{cmax}}$) and reduced photorespiration as determined by quantum requirement of PSII per CO$_2$ (lower $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$). Because there is evidence that the heat susceptibility of Rubisco activase is correlated to the species’ endemic habitat (Salvucci and Crafts-Brandner, 2004c), it is possible that our previous 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 EST clones for *Pinus halepensis* (AJ271896), *P. pinaster* (AL749921), *Populus deltoides* 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 certain/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’, which are clones from native populations indigenous to Minnesota and Florida, respectively, were propagated as dormant rooted cuttings and grown in 3 L plastic pots containing a mixture of sand, peat and silt loam (1:2:1, v/v). After bud break, the plants were transferred to a greenhouse set to 27 °C/25 °C day/night conditions and ambient humidity. Plants were maintained under well-watered conditions and fertilized twice a week (Peters 20-10-20 N,P,K with micronutrients, Masterblend, Chicago, Illinois, USA). After three weeks of greenhouse growth, plants were randomly assigned to Mylar® chambers within the greenhouse (1.5 m H X 1 m W 4 m L). These were maintained under either ambient or warm temperature conditions (33 °C/25 °C, day/night). Each chamber had independent environmental control via a Campbell 21X data logger (Campbell Scientific, Logan, Utah, USA). The data logger monitored the temperature of type T thermocouples within each chamber and controlled heating and cooling air conditioners (model YSO9, Friedrich Inc., San Antonio, Texas, USA). 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).
**Gas exchange analysis**

Plants acclimated to 33°C/25°C (day/night) conditions for 21 days 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 a photosynthetic photon flux density (PPFD) of 1,200 µmol m$^{-2}$ s$^{-1}$ and atmospheric CO$_2$ was maintained at 360 µmol mol$^{-1}$ CO$_2$ and 21% O$_2$. Temperature response curves were constructed by changing leaf temperature with the CIRAS-I cuvette Peltier blocks. The initial measurement of the induction phase was made at 25 °C and then temperature was increased in steps to 42 °C. The recovery phase decreased in steps from 42 °C to 25 °C. 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 non-photorespiratory 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 µmol mol$^{-1}$ CO$_2$, 1,200 µmol m$^{-2}$ 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 µmol mol$^{-1}$. At the end of the measurements, $C_a$ was again stabilized at 360 µmol mol$^{-1}$ and $A_{net}$ was recorded. This procedure 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, pers. comm.). 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’–CCYGCYTYYATGGGACAAGC–3’ and reverse primer 5’–GCCAGTAGAACTTCTCC–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 Rca1 and Rca2. Maple Rubisco large subunit (RbcL) was similarly PCR-amplified using forward 5’–ATGTCACCACAAAACAGAGACTAAAG–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 Inc., Lincoln, Nebraska, USA). Primer pairs specific to red maple and designed to a 3’ region of the cDNA (5’ GGAGAAGTTCTACTGGGC–3’) and the 3’ 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*

Five cm² leaf tissue samples were extracted in 2x 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 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 re-probed with anti-Rubisco (from R. Sage, University of Toronto, Canada).

*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 µg 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 5X 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 Rca2 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 32P-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 2X SSC and 0.1% SDS at 65°C followed by another washing at 1X SSC and 0.1% 
SDS at 65 °C, then a final 15 min wash at 0.5X SSC and 0.1% SDS at 65 °C.
Experimental design

A randomized block design with sampling 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 °C/25 °C (day/night) or 33 °C/25 °C. Two chambers were set at 27 °C/25 °C and an additional two chambers were set at 33 °C/25 °C. Each week throughout the study, plants were transferred within chambers to minimize chamber effects. Actual number of plants sampled varied according to parameter tested and is indicated in the figure legend for that specific experiment.
Results and Discussion

Thermotolerance of red maple

In a previous study, we found 1-year old saplings of red maple genotypes indigenous to Florida or Minnesota to differ markedly in their growth and photosynthetic thermotolerance (Weston and Bauerle, 2007). To further investigate the temperature sensitivity of Florida and Minnesota red maple genotypes, we compared gas exchange characteristics of 2-year-old Florida and Minnesota plants grown under warm conditions (33 °C/25 °C, day/night) for 21 days before measurement. Results confirm those previously found for 1-year-old plants, with the Florida genotype exhibiting a broader temperature optimum and higher $V_{cmax}$ in comparison to the Minnesota genotype. Specifically, photosynthesis in the Minnesota genotype began to decline immediately after leaf temperature increased to $\sim\!35$ °C, while the Florida genotype maintained optimal photosynthetic rates up to $\sim\!40$ °C (Figure 2.1). Photosynthetic recovery for both Minnesota and Florida plants was nearly immediate and closely followed the induction curve (Figure 1). Previous research has shown that photosynthesis fails to recovery to pre-heat stress levels with activase deficient mutants (Sharkey et al., 2001). Results from our study indicate that the heat stress did not irreversibly damage the photosynthetic apparatus and suggests that activase was functioning for both genotypes.
Figure 2.1 Leaf photosynthetic response to temperature of Florida (diamond) and Minnesota (square) genotypes of *Acer rubrum* L. after 21 Days of acclimation to 33°C/25°C (day/night) conditions. Carbon dioxide was 360 µmol mol⁻¹, oxygen 2% and light was 1200 µmol m⁻² s⁻¹ PAR and n = 6. Temperature was increased up to 42°C (solid symbols) then decreased (open symbols).

The maximal rate of Rubisco carboxylation (*V*<sub>cmax</sub>) was estimated *in vivo* by constructing photosynthetic assimilation (*A*) to intercellular CO₂ (*C<sub>i</sub>*) curves at 33 °C (Figure 2.2). To account for genotypic differences in photorespiration, *A* to *C<sub>i</sub>* curves were constructed under 2.0% O₂. Under these conditions, Florida plants exhibited a 22% higher *V*<sub>cmax</sub> than Minnesota plants. One possibility is that this increased Rubisco carboxylation potential in Florida plants could be due to enhanced Rubisco activase function.
Figure 2.2 The response of CO₂ assimilation rate to intercellular Cᵢ concentration for leaves of Florida (♦; y = 0.069x - 1.1; R² = 0.88) and Minnesota (◼; y = 0.052x – 1.3; R² = 0.90) genotypes of Acer rubrum. L. acclimated to 33°C/25°C (day/night) and measured at 33°C. Measurements were taken 360 μmol mol⁻¹ CO₂, 2% oxygen, and light was 1200 μmol m⁻² s⁻¹ PAR.
Cloning and sequence analysis of red maple Rca and RbcL cDNA

To characterize Rca at the molecular level, 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 plant Rcas (Portis Jr., 2002). This amplification resulted in a clone with an ~ 800 bp insert. Sequence analysis using the BLASTn algorithm from NCBI indicated that the clone displayed strong homology to Rubisco activase. The full-length cDNA clone was obtained by 5’ and 3’ RACE and this clone was designated Rca1-Fl. Additional RT-PCR reactions of leaf RNA using maple specific primers isolated a second Rca cDNA, designated Rca2-Fl (GenBank acc. no. DQ915973). The Florida sequences were subsequently used to PCR amplify two full-length Rubisco activase clones (Rca1-Mn and Rca2-Mn; GenBank acc. no. for Rca2-Mn – DQ915975) from Minnesota red maple cDNA.

The full-length cDNA of Rca1-Fl and of Rca1-Mn are nearly identical at the nucleotide level with a length of 1662 bp and an open reading frame of 1416 bp. The predicted precursor protein is composed of 472 amino acids, with the first 56 residues presumably encoding a transit peptide targeting the chloroplast according to prediction programs (http://psort.nibb.ac.jp/; http://www.cbs.dtu.dk/services/ChloroP/; http://hc.ims.u-tokyo.ac.jp/iPSORT/) and prior characterization of Rca from other species. The mature RCA1 protein is predicted to be 47kDa (Figure 2.3). Rca2 from both genotypes is 1710 bp in length 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 42kDa. Similar to Rca1, Rca2 is predicted to have a transit peptide of 56 amino acids targeting the chloroplast. The Rca1 and Rca2 predicted proteins are highly similar
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, 2004c) with 79.2%, 85.8%, and 76.7% amino acid identity, respectively.

A partial Rubisco large subunit (*RbcL*) was similarly cloned and sequenced from both the Minnesota and the Florida genotypes (Figure 2.4) with results indicating strong amino acid sequence homology between maple genotypes (98%). The clones were highly identical to spinach and tobacco *RbcL*, with amino acid identities in both cases of 93%. Although a protein crystal structure of RCA is not available, previous studies found that activase G373 and V377 (G311 and V314 of the mature peptide), and W75 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 G373 and V377 with *RbcL* D94 and P89, respectively. As noted in Figures 2.3 and 2.4, all of these key residues are conserved in Rca and *RbcL* for both maple genotypes. This suggests the maple proteins have equal potential for protein recognition and interactions.
Figure 2.3  Amino acid sequence alignment of large Rubisco activases. Species include *Acer rubrum*. genotypes Minnesota (Maple-Mn; GenBank acc. no. DQ915976) Florida (Maple-Fl; GenBank acc. no. DQ915974), *Arabidopsis thaliana* (GenBank acc. no. X14212, Wernke and Ogren, 1989), and those from species of thermally contrasting habitats including Creosote bush, (GenBank acc. no. AY312575) and Antarctic hairgrass, (GenBank acc. no. 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 diamonds represent conserved regions discussed in the text.
Figure 2.3  Amino acid sequence alignment of large Rubisco activases (Continued)

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1. Insertion
2. Deletion
3. Match
Figure 2.4  Amino acid sequence alignment of the Rubisco large subunit from Minnesota (Maple-Mn; GenBank acc. no. DQ459381) and Florida (Maple-Fl; GenBank acc. no. DQ915972) genotypes of *Acer rubrum*, Tobacco (GenBank acc. no. AAD15025.1), and Spinach (GenBank acc. no. CAA23473.1). Diamonds denote key residues implicated in recognition and interaction with Rubisco activase as discussed in Li et al. (2005) and the text.

| Maple_Fl | MSPQTETKASVGFKAGVKYKLYTYYTPVYTKTDIILAAFRVTPQPGVPP |
| Maple_Mn | MSPQTETKASVGFKAGVKYKLYTYYTPVYTKTDIILAAFRVTPQPGVPP |
| Tobacco  | MSPQTETKASVGFKAGVKYKLYTYYTPVYTKTDIILAAFRVTPQPGVPP |
| Spinach  | MSPQTETKASVGFKAGVKYKLYTYYTPVYTKTDIILAAFRVTPQPGVPP |

1 .................................. 10  .................................. 20  .................................. 30  .................................. 40

| Maple_Fl | EBAGAAAVABSSTGTWTAVWTDGLTSLDRYKRCYNIEPVAGENQICY |
| Maple_Mn | EBAGAAAVABSSTGTWTAVWTDGLTSLDRYKRCYNIEPVAGENQICY |
| Tobacco  | EBAGAAAVABSSTGTWTAVWTDGLTSLDRYKRCYNIEPVAGENQICY |
| Spinach  | EBAGAAAVABSSTGTWTAVWTDGLTSLDRYKRCYNIEPVAGENQICY |

51  .................................. 60  .................................. 70  .................................. 80  .................................. 90

| Maple_Fl | VAYPLDLFEBSVTNMFTSIVGNVFCAFMRKRLRLSLDLPPAMFSKTFQC |
| Maple_Mn | VAYPLDLFEBSVTNMFTSIVGNVFCAFMRKRLRLSLDLPPAMFSKTFQC |
| Tobacco  | VAYPLDLFEBSVTNMFTSIVGNVFCAFMRKRLRLSLDLPPAMFSKTFQC |
| Spinach  | VAYPLDLFEBSVTNMFTSIVGNVFCAFMRKRLRLSLDLPPAMFSKTFQC |

101 .................................. 110 .................................. 120 .................................. 130 .................................. 140

| Maple_Fl | PPHGIQVERDLNQKRPGPLGCTIKPKLGLSAKNYGRAVECLRGLDFT |
| Maple_Mn | PPHGIQVERDLNQKRPGPLGCTIKPKLGLSAKNYGRAVECLRGLDFT |
| Tobacco  | PPHGIQVERDLNQKRPGPLGCTIKPKLGLSAKNYGRAVECLRGLDFT |
| Spinach  | PPHGIQVERDLNQKRPGPLGCTIKPKLGLSAKNYGRAVECLRGLDFT |

151 .................................. 160 .................................. 170 .................................. 180 .................................. 190

| Maple_Fl | KDDENVSNQPFRWDRFLFCAABIYFAETGBIKGHLNATAGTCSEM |
| Maple_Mn | KDDENVSNQPFRWDRFLFCAABIYFAETGBIKGHLNATAGTCSEM |
| Tobacco  | KDDENVSNQPFRWDRFLFCAABIYFAETGBIKGHLNATAGTCSEM |
| Spinach  | KDDENVSNQPFRWDRFLFCAABIYFAETGBIKGHLNATAGTCSEM |

201 .................................. 210 .................................. 220 .................................. 230 .................................. 240

| Maple_Fl | LKRAVFARELGVPIVM |
| Maple_Mn | LKRAVFARELGVPIVM |
| Tobacco  | LKRAVFARELGVPIVM |
| Spinach  | LKRAVFARELGVPIVM |

251 .................................. 260
The organization of activase in the red maple genome was investigated with genomic blot (Southern) analysis (Figure 2.5). Genomic DNA from Florida and Minnesota genotypes was digested with endonucleases and probed with radiolabeled Rca1-Fl cDNA. The near identical (97%) nucleotide sequence homology between Florida and Minnesota Rubisco activase genes alleviated the need to re-probe the blots with radiolabeled Rca1-Mn cDNA. Maple Rca cDNA contains two NcoI sites, four HindIII sites, and two EcoRI sites. As is observed in Figure 2.5, the NcoI digest for both genotypes revealed the presence of two bands instead of the expected three bands. However, the maple Rca cDNA has an NcoI site 51 bp from the 3’-UTR and it is unlikely that such a small fragment remained on the gel. The HindIII digest revealed the presence of four bands in the Minnesota genome and three in the Florida genome indicating a possible polymorphism at ~3.2 Kb as well as increased signal at ~2.6 Kb, indicating a possible doublet. This interpretation, along with results from the NcoI digest and the gene copy number reconstructions (Figure 2.6) are consistent with the presence of a single Rca gene in the A. rubrum genome. However, the four bands in the EcoRI lane suggest the presence of an additional Rca gene, or more likely, an additional EcoRI site in an intronic portion of the gene. Final determination of gene copy number would require the construction and screening of a red maple genomic library.
Figure 2.5  Southern blot analysis of the \textit{Rca} gene from \textit{Acer rubrum}. Genomic DNA was digested with \textit{NcoI}, \textit{HindIII}, or \textit{EcoRI} and hybridized with radiolabeled full length \textit{Rca2} cDNA. The positions of molecular size markers in kbp are indicated at the right. The Florida genotype is represented by Fl and the Minnesota by Mn.
Figure 2.6 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.rbgkey.org/uk/cval/homepage.html). Reconstructions were run with 1, 5, and 10 copy equivalents of the Rca1-Fl probe.

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. Using a forward PCR primer complementary to a coding domain sequence and a reverse primer specific to the 3’–untranslated region of the Rca1 cDNA, a single distinct 450 bp fragment was amplified and sequenced from genomic DNA template. By comparing the nucleotide sequence of this genomic band with Rca1 and Rca2 cDNA sequences, it appears that the last intron can be entirely or partially removed using a common 3’ acceptor splice site and either of two different 5’ donor splice sites (Figure 2.7). This interpretation is consistent with alternative pre-mRNA splicing of Rubisco activase reported for other species such as Arabidopsis (Werneke and Ogren,
1989), spinach (Werneke et al., 1989), rice (To et al., 1999), and RcaA of Barley (Rundle and Zielinski, 1991). The alternative splicing event would produce two mRNAs differing by a 39 bp intron insertion deletion. Interestingly, the longer transcript, Rca2, produces the smaller protein (43 kDa) due to the presence of a stop codon in the 39 bp insert. Alternatively, the Rca1 intron can be completely removed, allowing full-length translation of a 46 kDa polypeptide.

Figure 2.7 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 Rca2 mRNA that contains an early stop codon (underlined) because of a 39 bp insertion. The bottom strand represents Rca1 mRNA, which has a different 5’ splice site resulting in complete removal of the intron and its early stop codon.
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 °C/25 °C; day/night) or warm temperature conditions (33 °C/25 °C) for 21 days (Figure 2.8). Protein was isolated from the first fully expanded leaf, similar to that used for gas exchange analysis. Exposure to warm temperature had a negligible effect on Rubisco large subunit protein quantity in relation to ambient conditions (Figure 2.8). However, the Florida genotype exhibited a modest increase in both Rca isoforms, while the Minnesota genotype showed increased abundance in only the small isoform. These 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, which showed coordinated changes between Rubisco and activase protein quantities (Pelloux et al., 2001). Interestingly though, previous research has shown that the larger the Rubisco activase oligomeric complex the greater is its ability to hydrolyze ATP at elevated temperatures (Portis Jr., 2002). Perhaps even the modest increase that we have observed in the amount of activase to Rubisco protein in both genotypes has some importance to observed photosynthetic adjustments to warm temperatures. This shift could allow each Rubisco enzyme to be protected by a larger Rubisco activase oligomeric complex.
Figure 2.8 Rubisco activase and Rubisco large subunit polypeptide accumulation after 21 days of acclimation to warm 33°C/25°C (day/night) or ambient (27°C/25°C) conditions. Total protein equal to 0.025 µg (Rubisco) or 0.1 µg (Rubisco activase) were loaded onto gels.
The role of Rubisco activase in red maple photosynthetic response to elevated temperature

The uncertainty of rapid climate change, along with predicted global warming emphasizes the importance of understanding the molecular response and physiological constraints of photosynthesis to elevated temperatures. Our results show that the decline in \( V_{\text{cmax}} \) under warm acclimation and measurement temperature in the Minnesota genotype could be partly due to high temperature affects on Rubisco activation and therefore the temperature-dependent function of Rubisco activase. However, the marked difference in photosynthetic rates between genotypes in response to warm 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 observed 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 multi-pathway framework when scaling molecular mechanism to the plant and population level. For example, it has been proposed that Rubisco heat deactivation via Rubisco activase is an adaptive response protecting against photorespiratory metabolites (Sharkey et al., 2001; Sharkey et al., 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 newfound role along with the competing processes of
photorespiration and carboxylation raise interesting questions concerning the tradeoffs in activase function under heat and how they relate to plant performance and fitness characteristic under warm temperature habitats.
Literature Cited


Characterization and Expression of *Arabidopsis thaliana* Rubisco Activase Genes

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Abstract

Rubisco activase (RCA) is a AAA+ protein that functions to maintain the activation state of the principle photosynthetic enzyme, Rubisco, and which can limit photosynthesis at moderate heat. The Arabidopsis genome also has a putative Rubisco activase (PRCA, At1g73110) that shows a 49% amino acid sequence identity to RCA, but whose function is not known. In this study, we have compared some phylogenetic and expression characteristics of RCA with PRCA. PRCA has an N-terminal plastid localization signal and shares many domains characteristic to RCA and other AAA+ proteins including Walker A and Walker B domains. One striking exception to this generalization is a divergent N-terminal region prior to Box II, which in RCA contains a highly conserved Trp⁴¹ necessary for Rubisco activation, but which in PRCA is specifically absent. Also, PRCA lacks the C-terminal alternative splice site of RCA. A phylogenetic analysis of the structural features of these genes revealed orthologous genes in rice and ice plant, which form a sub-clade under true Rubisco activases.

To examine expression characteristics, we cloned both RCA and PRCA as C-terminal GFP fusion genes, and showed by protoplast transient expression assays that both proteins are expressed in the chloroplast. Transcript abundance by real time PCR showed that PRCA is expressed in different tissues and that RCA and PRCA mRNA generally occur at similar levels in various tissues. Additionally, transcript abundance for both genes was induced as early as 4 h after 37°C whole plant heat treatments. Both promoters were cloned into plant expression vectors as C-terminal luciferase fusion genes and transient expression assays indicated that both promoters are subject to glucose repression. Thus, both proteins have similar structures and sub-cellular expression, and
both genes have similar tissue expression characteristics. However, amino acid sequence comparisons do suggest that plant PRCAs might have a unique function to RCA, perhaps as a chaperone-like function similar to a number of other AAA+ proteins.
Introduction

The AAA+ protein superfamily (ATPases with various cellular activities) is conserved among organisms and is central for a number of cellular processes including membrane fusion, cell cycle regulation, gene expression, organelle biogenesis, as well as general protein repair and degradation processes (Neuwald et al., 1999). In plants, the best characterized AAA+ protein is Rubisco activase (RCA), which is charged with regulating the activation state of Rubisco. Typically, activase is encoded by a single nuclear gene that is alternatively spliced to produce two protein isoforms of 41-43 kDa and 45-46 kDa in spinach (Werneke et al., 1989), Arabidopsis (Werneke and Ogren, 1989), barley (Rundle and Zielinski, 1991), and rice (To et al., 1999). However, exceptions do exist. For example, it appears that mung bean, cucumber, Phaseolus vulgaris, and tobacco only encode the shorter B isoforms (Portis, 2003). Adding to this diversity is an additional small isoform of activase in barley (Rundle and Zielinski, 1991), a second RCA gene in maize which codes for an identical B isoform but has a different 3’UTR (Ayala-Ochoa et al., 2004), and the presence of both large and small isoforms on different genes in cotton (Salvucci et al., 2003).

A key feature of Rubisco activase is its physiological regulation. Activase function is very sensitive to the ADP/ATP ratios in the chloroplast stroma such that it is minimally active under dark conditions (Robinson and Portis, 1989; Zhang and Portis, 1999; Kallis et al., 2000). This light-dependent regulation is thought to provide a coarse adjustment of Rubisco activation state in response to available sinks for reduced carbon. Additionally, the large isoform of activase is relatively more sensitive to regulation by the ADP/ATP ratio, yet its sensitivity is dampened by reduction via thioredoxin-f (Zhang and
Portis, 1999). This mechanism allows the protein to be light/dark regulated (Zhang et al., 2001; Zhang et al., 2002) in a manner consistent with redox regulation of other key photosynthetic enzymes (Schurmann and Jacquot, 2000). Thus, a general view is that the coarse adjustment of activase function obtained by varying ADP/ATP ratios is further fine-tuned via redox regulation of the large activase isoform, thereby synchronizing Rubisco activation state particularly to low light environments (reviewed in Portis, 2003).

At the molecule level, Rubisco catalysis proceeds with carbamylated Rubisco binding its substrates, RuBP and CO$_2$, then ‘closing’ the active site through peptide repositioning. Upon the last phase of catalysis, the peptide shield (loop 6 of an adjacent large subunit) retracts and products are released, enabling Rubisco to be ready for another reaction (Duff et al., 2000). However, RuBP can bind uncarbamylated Rubisco sites, which are very slow to open unassisted, resulting in dead-end complexes (Duff et al. 2000, reviewed in an activase context by Salvucci and Crafts-Brandner, 2004). Such complexes are slow to reverse themselves in the absence of activase. Thus, one suggestion is that activase restores catalytic function by encircling Rubisco with a ring of 16 (or possibly 8) subunits which move in a concerted fashion when powered by ATP hydrolysis to move key Rubisco structural domains, thereby promoting the movement of loop 6 and the release of inhibitory sugar phosphates (Portis, 2002; Portis, 2003).

The efficiency with which the described “activation” process proceeds is compromised at elevated temperatures due to the heat sensitivity of Rubisco activase. Recent research has shown that in several agronomic species Rubisco activation is inhibited below 35°C and that activase forms large molecular aggregates during the first 10 min of a heat treatment (Feller et al., 1998; Rokka et al., 2001). These results were
corroborated in an elegant study by Salvucci et al. (2001), in which protein denaturation was determined for heat treated tobacco and pea protoplasts by rhodanese trapping experiments. These authors found that activase is denatured at temperatures far below those required to denature other photosynthetic enzymes (e.g. Rubisco and phosphoribulokinase). Taken together with the relatively low temperature optimum of activase ATPase activity, Rubisco deactivation likely increases under elevated temperatures because activase is unable to maintain the activated state (Crafts-Brandner and Salvucci, 2000).

Interestingly, the response of Rubisco activase to heat treatment does vary among different species. For example, prolonged heat exposure induces the synthesis of specific heat stress forms of activase in maize (Sanchez de Jimenez et al., 1995), cotton (Law et al., 2001), and wheat (Law and Crafts-Brandner, 2001), likely by post-transcriptional processing of a mature activase. This occurs in contrast to spinach, which constitutively produces a large activase isoform that has a 13°C higher temperature optimum than does its smaller isoform counterpart (Crafts-Brandner et al., 1997). Analyzing across an ecological gradient, recombinant creosote (Larrea tridentate) activase was shown to have a 10°C higher temperature optimum compared to Arctic haregrass (Deschampsia Antarctica) activase (Salvucci and Crafts-Brandner, 2004). These results suggest that the response of activase to temperature might play a role in species’ geographic range distributions. However, this relationship might be evident even more so in the temperature sensitivity of the interaction between Rubisco and activase, but this remains poorly characterized (Portis 2003).
There is evidence that Rubisco activase can also interact with alternate macromolecules. For example, upon heat shock of spinach leaves, activase associates with thylakoid bound ribosomes, perhaps as a protective response (Rokka et al. 2001). Thus, we were intrigued by the presence of a putative Rubisco activase (PRCA, At1g73110) in the sequenced *Arabidopsis* genome and in *Arabidopsis* EST libraries. In the present work, we have initially characterized expression of PRCA tissue transcripts and protein, and have compared some of their responses with RCA expression after short-term heat treatment of *Arabidopsis*. 
Material and methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heyn. ecotype Columbia (Col-0) was grown in Pro-Mix potting medium (Quebec, Canada) in a growth chamber at \(\sim 110 \text{ \mu mol m}^{-2} \text{s}^{-1}\) PAR under a 12 hour photoperiod and at 23°C/20°C (day/night). Plants were fertilized weekly with a quarter-strength nutrient solution. For whole plant heat treatment expression assays, 3 week-old plants were subjected to 37°C temperatures for 6 h and allowed to recover for 72 h. Measurements were taken throughout that period. For plants as a source of protoplasts, leaves were harvested for enzyme digests from 3-4 week-old plants.

RT-PCR and real time RT-PCR detection of activase and putative activase transcripts

Four week-old plants were harvested, divided into mature leaves (> 3 cm), young leaves (between 1 cm and 2 cm), stems, siliques, flowers, and roots, then flash frozen in liquid nitrogen. One hundred mg of tissue was ground in liquid N\(_2\), using a mortar and pestle. RNA was extracted according to the manufacturer’s instructions (Qiagen, Valencia, CA). Five hundred ng of RNA was subsequently used for cDNA synthesis, also according to the manufacturer’s instruction (Superscript reverse transcriptase, Invitrogen). To differentiate between the alternatively spliced variants of Rubisco activase (At2g39730), the same forward primer (5’-TGACTCTGCCTAAATCAAG-3’) was paired with an isoform specific reverse primer of 5’ – GCTCCTTTTCCCTGTTTAC– 3’ or 5’-GCTCCTTTTCCGTAGAAAGTT-3’ for the small and large protein coding transcripts, respectively (see Werneke and Ogren, 1989 for
a complete annotation of *Arabidopsis* activase). Forward and reverse primers for putative activase (At1g73110) were: 5’-GGGAGGTAAAGGACAAGGCAA-3’, 5’-GGATTCAGCTGAGTGTTCCTC-3’; for GAPDH (At3g26650): 5’-TCTCTTCCCTTCGGCAAGAAAC-3’, 5’-GACCATGCCAACATCTCAGGAA-3’; and for β-tubulin (At1g75780): 5’-TACCTCACAGCTTCAGCCATGT-3’, 5’-TTTCCGACAAACGTGGAAGCCA-3’, respectively.

For RT-PCR, cDNA was diluted two-fold and 1 µl was subsequently used in a 25 µl PCR reaction with 0.4 units of taq polymerase (Promega, Madison, WI), 50mM KCl, 10mM Tris-HCl, pH 9.0, 0.1% Triton® X-100, 1.5 mM MgCl₂, and 25 pmol of the primer pairs described above. The reaction mixture was amplified in an MJ thermal cycler at 94°C for 5 min and then cycled 27 times at 94°C 30 sec, 53°C 30 sec, 72°C 1 min, with a final extension at 72°C for 7 min. An equal amount of products were run on a 1.5% agarose gel and visualized by UV light. Real-time RT-PCR used cDNA as prepared above but diluted 10-fold; 1 µl was used in a 25 µl reaction with SYBR Green Supermix kit (BioRad, Hercules, CA) with the following amplification program: 94°C 5 min, 40 cycles at 94°C for 15 sec followed by 53°C for 50 sec. All samples were amplified in triplicate from the same RNA sample and mean critical threshold (Cₜ) values were used to determine PCR efficiency and relative fold change for each gene (Vandesompele et al., 2002). All samples were run on a 1.5% agarose gel to ensure the presence of one visual band.
Protoplast isolation and preparation

*Arabidopsis* leaf protoplasts were isolated as described previously and transfected using polyethylene glycol (Hwang and Sheen, 2001). Briefly, round leaves (~1 g) from 3 to 4 week old plants were collected and cut into 0.5-1 mm strips with a double-edged razor blade. Leaf strips were transferred to a petri dish containing 5-10 ml of enzyme solution (0.5 M mannitol, 10 mM MES pH 5.7, 10 mM ascorbate, 20 mM KCl, 10 mM CaCl₂, 0.1% BSA, and 1.0% R10 cellulase (Yakult Pharmaceutical Co., Tokyo), and vacuum infiltrated for 5 min. After infiltration, the petri dish was covered with aluminum foil and incubated at room temperature for 2.5 – 3 h min. Protoplasts were released with hand shaking, washed by pelleting at 110g, 2 min, and resuspended in 5 ml of W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 5 mM glucose, and 1.5 mM Mes, pH 5.7). Washing was repeated once and the resuspended protoplasts were allowed to settle at room temperature for 10-20 min. After settling, the W5 solution was decanted and protoplasts were resuspended in MMg solution (0.4 M mannitol, 15 mM MgCl₂, and 4 mM MES, pH 5.7).

Typically, 2 X 10⁵ protoplasts in MMg solution were mixed with 10 µg of reporter plasmid DNA plus 5 µg of effector plasmid DNA and 5 µg of ubiquitin10::GUS (Kovtun et al. 1998), the latter to allow for normalization of expression. Procedurally, protoplasts were transfected, transferred to 12 well culture dishes, and then incubated 30 min in the dark at room temperature. Protoplasts were then moved to the light (~40 µmol m⁻² s⁻¹) and 2 mM glucose was added to treatment cultures 90 min after transfection. Protoplasts were harvested at 6-8 h after transfection and assayed for luciferase and Gus activities (Jefferson, 1987; Sheen 1996).
Subcellular localization of activase and putative activase

Carboxy-terminal GFP fusion proteins were created by cloning the corresponding cDNAs between BamH1 and PstI restriction sites of a plant expression vector (Kovtun et al., 1998). The full-length open reading frame with transit peptide of Rubisco activase large isoform (RCA1), Rubisco activase small isoform (RCAs), and putative Rubisco activase (PRCA) were amplified by RT-PCR using RNA from Arabidopsis. Both RCA1 and RCAs used the same forward primer, 5’- CGGGATCCCATGGCGCGCCAGTTTC-3’, which contained a BamHI adaptor. The forward primer was paired with a PstI containing reverse primer of 5’-nnnnn-3’ for RCA1 and 5’- CCTAAAGTTGTAGAAACA GGTTCAT-3’ for RCAs, representing unique areas in the 3’ UTR of mRNA. PRCA cDNA was also cloned into the plant expression vector with 5’- CGGGATCCCATGGCGCTTGCAAACAT-3’ (forward) and 5’-CCTATCGTCCATGTTCTTCATGTA-3’ (reverse) primers containing BamHI and PstI restriction sites, respectively. The constructs were cloned under the control of a 35S CaMV promoter with a PPDK enhancer (Kovtun et al., 1998).

For subcellular localization, 10 µg of plasmid DNA were transfected into protoplasts using PEG, and expression was monitored 6-8 h later. Control proteins targeted to the nucleus (Wrky29-GFP), cytosol (CDPK30-GFP), and mitochondria (HXK1-GFP) were used to facilitate interpretation of localization results. Samples were monitored using an Axiovert 200M fluorescence microscope with Apotome (Carl Zeiss Microimaging Inc., Thornwood, NY) and appropriate filters (Chroma Technology Corporation, Rockingham, VT).
Transient expression of activase and putative activase

Reporter constructs for pea Rubsico small subunit (Rbcs-Luc) and HXK1-HA were as described in Sheen (1996) and Moore et al. (2003). Putative promoters of activase and putative activase were made by cloning 2.76 to 3.1 kB, respectively, of the upstream DNA into luciferase vectors as described above. Forward and reverse promoter amplification primers for activase and putative activase were,

5’- AACTGCAGAATTCGATCCGAATCAC-3’ and
5’- CATGCCATGGCTCATCAAGACTGCGAC-3’, and,
5’- AACTGCAGAATTCAGATTCTGGTCCC-3’ and
5’- CATGCCATGGTAACCACAAATCAC-3’, respectively. Effector constructs used for promoter analysis, RCAs and PRCA, were made by sub-cloning the corresponding cDNA into the plant expression vector that had a C-terminal double HA tag substituted for GFP (Kovtun et al., 1998).
Results

Sequence Analysis of PRCA cDNA

A genome database search of Arabidopsis thaliana (http://www.tigr.org, http://arabidopsis.org) revealed an open reading frame for a putative Rubisco activase (At1g73110, PRCA). The ORF is predicted to encode a 432 amino acid precursor protein with a predicted molecular weight of 48.33 kDa. Subcellular localization programs all predicted that PRCA contains a transit peptide sequence targeting to the chloroplast (http://psort.nibb.ac.jp/; http://www.cbs.dtu.dk/services/ChloroP/; http://hc.ims.u-tokyo.ac.jp/iPSORT/), with possible cleavage sites for the transit peptide ranging from 24 to 39 aa (data not shown). By comparison, Arabidopsis RCA2 encodes a 446 amino acid precursor with a 39 amino acid transit peptide (Werneke et al., 1989).

A broader genome database search of available activase sequences (NCBI, http://www.ncbi.nlm.nih.gov/) revealed additional putative activase sequences from rice and Mesembryanthemum. We then constructed a phylogenetic tree using a number of the available sequences from characterized RCAs and the three PRCAs (Fig. 3.1). The PRCAs interestingly form a separate subclade, which is more closely related to prokaryotic activase than to eukaryotic activase (Fig. 3.1). The PRCAs ranged from 42-49% amino acid identity and 56-69% similarity in relation to AtRCA2.
Figure 3.1 Phylogenetic tree of small isoforms of Rubisco activases. Species (GenBank accession numbers) include Spinacia oleracea (AAD13840.1), Nicotiana tabacum (12643758), Larrea tridentata (AAP83929.1), Oryza sativa (BAA97583.1), Arabidopsis thaliana (AAA20203.1), Deschampsia antarctica (AAP83927.1), Cucumis sativus (CAA47906.1), Chlorococcum littorale (CAA71667.1), Chlamydomonas reinhardtii (AAR23425.1) Zantedeschia aethiopica (AAK25801.1), Phaseolus vulgaris (AAC12868.1), and putative activases from Arabidopsis thaliana (NP_177454.1), Oryza sativa (19387266), and Mesembryanthemum crystallinum (71834884). Alignment and tree building performed on full-length amino acids according to (Leclercq et al., 2005).
A direct amino acid alignment of all predicted PRCAs and representative RCAs was constructed to further define gross similarities between the peptides (Fig. 3.2). AtPRCA is 24 amino acids smaller than its RCA2 counterpart. However, this observation cannot be extended as an overall generalization to all PRCAs since the rice PRCA seems to be a partial sequence and *Mesembryanthemum* PRCA is 130 amino acids longer than AtRCA (Fig. 3.2). Nevertheless, some trends are apparent. Most notable is the divergence in the N and C termini of PRCA to RCA, as well as the absence of the two C-terminal cys residues conserved in larger form of RCA (Fig. 3.2). There was also considerable homology of PRCA to previously identified conserved regions of RCA and AAA+ proteins (Neuwald et al., 1999; Portis, 2002). For example, the highly conserved P-loop region in the Walker A motif is necessary for activating Rubisco and for ATPase functions, and is also conserved in AtPRCA (Fig. 3.3, Table 3.1). As further indicated in Figure 3.3, residues necessary for AtRCA2 interaction with the bound nucleotide, including D244 in the Walker B motif, N299 in the sensor 1 motif, and R363 in the Sensor 2 motif are retained in PRCA (Portis, 2002). AtPRCA also has the E/D287 residue in Box VI and R313 in Box VII, both of which have been implicated in interacting with an adjacent bound nucleotide subunit. This suggests that AtPRCA, like RCA2, has the ability to form oligomers or supercomplexes. In fact, all known highly conserved residues necessary for RCA function, as denoted by diamonds in figure 3.2, are represented in PRCA except for W83. This residue in particular, along with the first 50 amino acids of the mature peptide is specifically involved in Rubisco activation, with no known effect on ATPase activity.
Figure 3.2 Amino acid sequences of putative activases from *Arabidopsis thaliana* (NP_177454.1), *Oryza sativa* (19387266), and *Mesembryanthemum crystallinum* (71834884) aligned with the small isoform of RCA from *Spinacia oleracea* (AAD13840.1).
Figure 3.3 Amino acid sequence alignment of Arabidopsis thaliana PRCA with RCA. Conserved domains (brackets) and basic amino acid residues (diamonds) for RCA are noted as described in Portis (2002, see also text). Arrow denotes the transit peptide cleavage site for RCA.
Table 3.1 Percent identity and similarity of putative activase to Rubisco activase. Amino acid sequences for *Arabidopsis thaliana* putative activase and Rubisco activase were aligned and percent identities and similarities were recorded for conserved regions as reported by Portis (2002).

<table>
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<tr>
<th>Protein Region</th>
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<th>Percent Similarity</th>
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<tbody>
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<tr>
<td>Walker A</td>
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<td>88</td>
</tr>
<tr>
<td>Box IV</td>
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<tr>
<td>Box IV’</td>
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<td>Sensor 2 (Box VIII)</td>
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**Cloning and subcellular localization of PRCA protein**

We next cloned PRCA from a Col leaf expression library. This was cloned as C-terminal YFP- and CFP-fusion proteins. The subcellular localization of PRCA was investigated by transiently expressing constructs of PRCA-YFP and PRCA-CFP in Arabidopsis leaf protoplasts (Fig. 3.4 and 3.5). With a long-pass emission filter, excitation at either 458 or 490 nm allowed visualization of CFP and YFP fluorescence, as well as chlorophyll autofluorescence at the longer red wavelengths. This system allowed us to visualize chloroplasts apart from PRCA-YFP (Fig. 3.4.A) and RCAL (Fig. 3.4.B) within the protoplast. Thus, this assay indicated that both PRCA and RCAL fusion proteins were targeted to chloroplasts. To substantiate this interpretation, control transfections with CDPK30-GFP, HXK1-GFP, and WRKY29-GFP showed expected targeting to the cytosol, mitochondria, and nucleus, respectively (data not shown). In some cases, PRCA was observed to form a punctate mass on the chloroplast membrane (Fig 3.5).
Figure 3.4 Subcellular localization of (A) PRCA-YFP and (B) RCAL-YFP after transient expression in Arabidopsis leaf protoplasts.
Figure 3.5 PRCA-CFP expression in Arabidopsis leaf protoplasts. (A) Chlorophyll autofluorescence, (B) PRCA-CFP (note the accumulation of protein on outer chloroplast surfaces, and (C) overlay of A onto B.
Transcript expression patterns of PRCA and RCA

Tissue specificity of PRCA was investigated by comparing transcript abundance among different organs of 3-week old plants using tubulin as an internal control. Results from real time RT-PCR revealed that PRCA mRNA was in leaves and siliques (Table 3.2). In order to compare transcript abundance, unnormalized values of the critical threshold (fluorescence within the linear range of amplification and above background) for target genes and the tubulin control are reported (Table 3.2). Quantitatively, the relative expression in young leaves was highest, closely followed by siliques and then mature leaves (Table 3.2). Flowers, stems and roots were nearly void in PRCA or RCA expression with at least a 20-fold decrease in expression compared to young leaves. Interestingly, PRCA transcript abundance resembled that of RCA in all tissue types except for mature leaves. In this case, PRCA had an 18% lower C$_t$ value, which translates to nearly a 12 fold increase in expression.
Table 3.2. Tissue specificity as determined by Real-Time RT-PCR. Critical threshold for each gene was determined in triplicate with the mean reported and standard deviation in parentheses.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>RCA</th>
<th>PRCA</th>
<th>Control</th>
</tr>
</thead>
<tbody>
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<td>Siliques</td>
<td>25.4 (.3)</td>
<td>22.1 (.4)</td>
<td>32.5 (.3)</td>
</tr>
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<td>Young Leaf</td>
<td>22.0 (.4)</td>
<td>21.7 (.4)</td>
<td>32.1 (.9)</td>
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<td>Flowers</td>
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<td>32.7 (.6)</td>
<td>32.5 (.5)</td>
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<td>32.5 (.2)</td>
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<td>32.4 (1.0)</td>
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</tbody>
</table>
The transcript abundance of RCA is known to rapidly accumulate in response to heat (Law and Crafts-Brandner, 2001). To investigate the response of PRCA expression to heat, plants were grown under 23°C/20°C (day/night) for 3 weeks before plants were subjected to 37°C heat for the 12 h night period. Above ground tissue was collected prior to heat treatment (−12 h on Fig. 3.6), 4 h into the heat treatment (−8 h on Fig. 3.6), at the end of heat treatment, (corresponding to the start of the photoperiod; 0 h on Fig. 3.6), and during recovery at 24 h, 48 h, 144 h. Transcripts encoding heat shock proteins (HSP) 18.2 and HSP 101 were very responsive to the treatment, displaying a near 18- and 14-fold increase in abundance after 4 h of heat, respectively. The control transcript, tubulin, was relatively constant throughout the experiment and never varied by more than 1 fold change. The Rubisco small subunit (RbcS) expression remained relatively constant as well, and only showed a slight increase at the start of the photoperiod (Fig. 3.6).

Increased PRCA transcript abundance was apparent within 12 h of heat treatment (Fig. 3.6), and mirrored that of the RCAs except for a slight lag in accumulation at the start of the photoperiod. Increased transcript abundance was still observed 48 h after heat stress for both RCAs and PRCA and didn’t return to pre-stress conditions until 144 h. To substantiate these results, a separate set of plants were analyzed under the same treatment conditions (note the ‘B’ samples in Fig. 3.6). Overall trends were similar to that of the first set of samples except that recovery to pre-stress conditions was noted earlier at 48 h. In addition, PRCA expression did not show the initial lag in increased transcript abundance, but rather mirrored that of RCA-S quite closely (Fig. 3.6).
Figure 3.6 Transcript abundance in response to heat. Real time RT-PCR of RCAS, RCAL, PRCA, heat shock proteins (HSP101 and HSP18.2), Rubisco small subunit (RbcS), and Tubulin prior to 37°C heat treatment (-12 h), 4 h into heat treatment, end of heat treatment (Also corresponding to start of photoperiod; 0 h) and recovery points at 24 h, 48 h, and 144 h. Transcripts with ‘B’ denote biological replicates.
Transient expression of PRCA promoter

RCA is one of many photosynthetic genes thought to be down-regulated by glucose signaling as part of a feed-back regulatory network in response to whole plant carbohydrate demand (Rolland et al., 2002; Price et al., 2004), and hexokinase is a key glucose sensor/transducer in this network (Moore et al., 2003). To determine the responsiveness of PRCA to sugar suppression, ~2.8 kb of DNA upstream from the PRCA start codon sequence was cloned into Luc expression vectors. The putative promoter was then transiently expressed in Arabidopsis protoplasts and promoter activity was determined under varying conditions by quantifying luciferase. All transfections contained a ubiquitin-GUS construct allowing the normalization of transfection efficiencies. The Rubisco small subunit promoter (RbcS-LUC), was previously cloned and is known to be glucose responsive (Moore et al., 2003). Under control conditions, PRCA displayed 82% relative promoter expression compared to RbcS suggesting that most, if not all, promoter elements were included in their respective constructs. When PRCA-LUC was coexpressed with hexokinase cDNA, expression was repressed similar to that of RbcS-LUC (Fig. 3.7, and Moore et al., 2003). Promoter repression was further enhanced when glucose was added to the protoplast incubation medium. This response was previously reported for RbcS (Moore et al., 2003) and reflects the role of hexokinase as a flux sensor of increasing glucose. Interestingly, PRCA displayed similar trends as RbcS, with hexokinase repression amplified with the addition of glucose (Fig. 3.7). These data suggest that PRCA is a target gene regulated by the plant glucose sensing and signaling network.
Figure 3.7 Relative promoter activities of RCA, PRCA and RbcS transiently expressed as luciferase constructs into Arabidopsis leaf protoplasts. Activities were normalized according to the expressed GUS activity from co-transfected UBQ10-GUS. HXK1-HA was co-transfected in some experiments as effector cDNA. After transfection, protoplasts were incubated in the light ± 2 mM glucose, and harvested after 8 h.
Discussion

AAA+ proteins are conserved among organisms and are characterized by their energy dependent chaperone-like activity for diverse cellular activities. RCA is by far the most studied AAA+ protein in plants and is responsible for maintaining the catalytic efficiency of Rubisco. The importance of this relationship is most evident under suboptimal environmental conditions such as moderate heat stress, for which activase has been implicated as the primary photosynthetic limitation (Salvucci et al., 2001; Salvucci and Crafts-Brandner, 2004). The consequences of this relationship extend beyond molecular curiosity and effect topics relevant to crop improvement as well as ecological aspects such as species dynamics in response to predicted global warming.

When comparing amino acid sequences of PRCA to RCA, the most dramatic mismatch between activase and putative activase is at the N-terminus of the mature protein before the Box II region. Although not well-characterized, earlier work with RCA indicted that this region is necessary for Rubisco activation (van de Loo and Salvucci, 1996) and is considered the generic targeting sequence region for AAA+ proteins (Frickey and Lupas, 2004). In fact, the conserved Trp83 (Figure 3.1, and van de Loo and Salvucci, 1996) for RCA is the only single conserved amino acid among the activases that is not present in PRCA. Trp 83 has been directly implicated in the activation of Rubisco. van de Loo and Salvucci (1996) found that recombinant RCA with an Ala, Cys, Phe, or Tyr substituted for Trp had reduced initial rates of Rubisco activation by 10, 11, 53, and 68%. These results suggest that PRCA may not be efficient at activating Rubisco. However, the same study (von de Loo and Salvucci, 1996) also
found that mixed populations of recombinant RCA and wildtype RCA had little effect on initial Rubisco activation.

While N-terminal divergence is apparent between RCA and PRCA, a comparison of *Arabidopsis* PRCA to rice and ice plant putative activases (the only sequenced putative activase’s to our knowledge) revealed marked similarity in the N-terminal region (Fig. 3.3). When this conserved region (amino acids 90-145; Fig 3) was aligned with all NCBI sequences using the blastp program, only the rice and ice plant sequences were retrieved, suggesting that PRCA is specific to plants. Further support for this interpretation was found when the same region was aligned to the newly sequenced *Populus* genome (version 1.1) and one sequence was retrieved (gw.1.L.G852.1; http://shake.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). Interestingly, this sequence was not activase. Although a comparison of only four plant species, the conservation within this region among diverse species raises questions concerning the evolutionary history and thus the molecular function of this gene.

One possibility is that PRCA is the result of a gene duplication event. According to this idea, the functional redundancy of a gene duplicate is likely to be lost to pseudogenization (Zhang, 2003). In cases where duplicate genes retain function within the genome it is predicted that an entirely new role was gained (neofunctionalization), or that part of the parental gene function was adopted (subfunctionalization). In the case of pseudogenization, the daughter gene incurs deleterious mutations until it is rendered expressionless as a gene or non-functional as a protein. The 49% amino acid identity of putative activase to activase indicates considerable overall divergence. However, structural domains characteristic of AAA+ proteins seem to be conserved relative to that
observed among Rubisco activases from different genera. This evidence suggests that PRCA is not evolving towards pseudogenization as the mis-sense sequences are not random but specific to regions.

As evidenced within this study, PRCA shares many general molecular features with RCA including amino acid identity, sub-cellular localization, transcript profiles in relation to organ specificity, as well as shared regulation of promoter activity in response to heat and glucose treatments. One possibility is that RCA is unable to produce enough protein to accomplish its function and needs additional protein coded for by a second gene. However, anti-activase plants do not exhibit a phenotype until protein levels are less than 10% of wild type concentrations (Hammond et al., 1998), suggesting that RCA protein is in abundance or that PRCA has the ability to assist RCA.

The subfunctionalization model predicts that duplicate genes will share overlapping redundant functions and then proceed towards functional divergence (Moore and Purugganan, 2005). Depending on putative activase’s evolutionary progression, it could overlap with RCA in its association with Rubisco or it may have already diverged into a former or currently unrealized role of RCA. Interestingly, a study by Rokka et al. (2001) found that RCA has dual temperature-dependent functions. Under optimal temperatures, the authors suggest that RCA protein carries on with its well-defined role in releasing inhibitory sugar phosphates from Rubisco. However, under heat shock conditions, they found that RCA binds to thylakoid-bound polysomes, possibly acting as a metabolic chaperone protecting the associated translation machinery. This formerly unknown and relatively uncharacterized role of activase leaves open the possibility for PRCA associations with other macromolecules.
In addition to the possibility of PRCA participating in a newly found activase function, there are a number of subtle differences between the two genes, which may provide insight into different functional roles. One such difference is PRCA’s high transcript abundance in mature leaves compared to RCA. This could reflect a number of roles including Rubisco breakdown for nitrogen remobilization or reveal the need for extra chaperone activity in older leaves. A second difference between the two proteins is in their amino acid identity in the N-terminal region. This region has previously been associated with specificity factors between RCA and Rubisco and infers that PRCA may be associated with protein other than Rubisco.

In summary, PRCA shares many molecular features with RCA, yet differs in a context consistent with the subfunctionalization model of gene duplication. This is supported by minimal functional divergence as inferred from (1) non-random amino acid substitutions specific to the sensor regions on the mature protein, and (2) organ specific divergence in gene expression. Additionally, PRCA shares many of RCA’s, which are also characteristic of the subfunctionalization model. These include the retention of amino acid domains necessary for ATP hydrolysis, sub-cellular localization to the chloroplast, heat responsive expression, and promoter elements necessary for the recognition of sugar signaling events. Further experimentation, particularly a complete phylogentic analysis of the two genes complimented by biochemical approaches, including protein-protein interactions, are necessary to define the functional role of PRCA.
Literature Cited


