

The differential response of photosynthesis to high temperature for a boreal and temperate *Populus* species relates to differences in Rubisco activation and Rubisco activase properties

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Summary Significant inhibition of photosynthesis occurs at temperatures only a few degrees (≤ 10 °C) above the optimum, resulting in a considerable loss of potential productivity. Most studies of heat stress have focused on crop or weedy annual plants, whereas similar studies with trees have been limited in number. As temperature is a major factor limiting the geographic ranges of most plants, the aim of this study was to use two *Populus* species adapted to contrasting thermal environments for determining the factors that constrain photosynthetic assimilation (*A*) under moderate heat stress in tree species. Consistent with its native range in temperate regions, *Populus deltoides* Bartr. ex Marsh. exhibited a significantly higher temperature optimum for *A* than did *Populus balsamifera* L., a boreal species. The higher *A* exhibited by *P. deltoides* at 33–40 °C compared to that for *P. balsamifera* was associated with a higher activation state of Rubisco and correlated with a higher ATPase activity of Rubisco activase. The temperature response of minimal chlorophyll *a* fluorescence for darkened leaves was similar for both species and was not consistent with a thylakoid lipid phase change contributing to the decline in *A* in the range of 30–40 °C. Taken together, these data support the idea that the differences in the temperature response of *A* for the two *Populus* species could be attributed to the differences in the response of Rubisco activation and ultimately to the thermal properties of Rubisco activase. That the primary sequence of Rubisco activase differed between the species, especially in regions associated with ATPase activity and Rubisco recognition, indicates that the genotypic differences in Rubisco activase might underlie the differences in the heat sensitivity of Rubisco activase and photosynthesis at moderately high temperatures.

Keywords: heat stress.

Introduction

Poplars (*Populus* sp.) are rapidly growing tree species that make an important contribution to meeting the global need for paper, timber and other wood-based products (Harlow et al. 1991, Taylor 2002). The genus *Populus* has an extensive latitudinal and altitudinal range throughout much of the temperate and boreal regions of the northern hemisphere, with species that tolerate either high or cool summer temperatures (Harlow et al. 1991, Roden and Pearcy 1993). *Populus* species provide useful and commercially important subjects in global climate change research due to their dynamic response to different environmental conditions and ability to sequester large amounts of carbon (Gielen and Ceulemans 2001). An understanding of the physiological responses of poplars to climate change is crucial for designing future management practices to optimize growth and carbon sequestration by forests and plantations in the future.

The vast boreal forest of Canada is an important area for carbon sequestration (Sage et al. 2008), and poplar species are major components of this forest, especially in the mixed-wood stands of western Canada. One of these species, *Populus balsamifera* L., can be found across northern North America in the boreal forest and even north of the Arctic Circle in northwestern Canada (Harlow et al. 1991). Given the prediction for the extent of warming in the boreal forest in this century (Intergovernmental Panel on Climate Change 2007), one might expect that the contribution to carbon sequestration by *P. balsamifera* would decrease, depending on how sensitive its photosynthesis is to moderately high temperatures.

High temperature adversely affects various cell functions, but photosynthesis is well known to be particularly sensitive to heat stress (Berry and Björkman 1980, Salvucci and Crafts-Brandner 2004a, 2004b). The temperature optimum for plant CO₂ assimilation (*A*) is usually rather broad and

generally matches the average daytime temperature encountered in the natural environment (Berry and Björkman 1980). However, significant inhibition of photosynthesis occurs at temperatures above the optimum, resulting in a considerable loss of potential productivity (Lobell and Asner 2003). For example, Law and Crafts-Brandner (1999) found that gradually increasing the leaf temperature from 35 to 42.4 °C for cotton and from 30 to 39.2 °C for wheat caused a 50% reduction in the net CO₂ assimilation (*A*). On the other hand, respiration, at least for cotton, sweet potato, rice and black spruce, a boreal species, has been shown to have a much higher temperature optimum than that for photosynthesis (Salvucci and Crafts-Brandner 2004a, Cen and Sage 2005, Makino and Sage 2007, Sage et al. 2008), leading to the potential for a considerable reduction in diurnal carbon gain for the plant when the temperatures rise above the optimum for photosynthesis.

The inhibition of *A* by high temperature occurs under both photorespiratory and non-photorespiratory conditions (Kobza and Edwards 1987, Crafts-Brandner and Salvucci 2000). Therefore, the reduction in *A* cannot be simply explained by the greater rate of photorespiration at high temperatures, which results from changes related to the differential solubility of CO₂ and O₂ and the kinetic properties of the CO₂ assimilating enzyme of the Calvin–Benson cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Jordan and Ogren 1984, Sage and Sharkey 1987).

A common response to moderate heat stress among various species, including perennials and annuals (both C₃ and C₄), is the enhanced inactivation of Rubisco (Weis 1981a, b, Kobza and Edwards 1987, Law and Crafts-Brandner 1999, Crafts-Brandner and Salvucci 2000, 2002, Bukhov and Dzhilbladze 2002, Haldimann and Feller 2004, Salvucci and Crafts-Brandner 2004a, Cen and Sage 2005, Kim and Portis 2006, Salvucci et al. 2006, Makino and Sage 2007, Hendrickson et al. 2008, Kubien and Sage 2008). This inactivation could be a consequence of a reduction in ATP synthesis resulting from an increased fluidity of the thylakoid membranes and their increased permeability to H⁺ with increasing temperatures (Schrader et al. 2004, Wise et al. 2004, Cen and Sage 2005, Sage et al. 2008, Zhang and Sharkey 2009, Zhang et al. 2009). The lower chloroplastic ATP:ADP value would indirectly lead to Rubisco inactivation via reduced Rubisco activase activity (an ATPase enzyme required in the process of Rubisco activation, Portis 1995, 2003). Alternatively, Rubisco deactivation at a high temperature could result from the heat sensitivity of Rubisco activase, even in the absence of changes in ATP:ADP, and is exacerbated by the increasing need for higher activase activity to counteract increased rates of Rubisco inactivation at high temperature (Salvucci and Crafts-Brandner 2004a, 2004b).

Considering the commercial and environmental importance of *Populus* species, such as *P. balsamifera*, as an important component of the boreal forests of North America,

knowledge of their response to increasing temperature would be valuable for predicting their CO₂ assimilation capacity and productivity, as well as to improve their productivity in the future. Information on the response of photosynthesis to a high temperature for tree species is limited, with little information on *Populus* species, in particular. Studies of northern and southern *Acer rubrum* L. (red maple) plants indicated that these two populations have distinctly different responses to temperatures above the optimum (Weston and Bauerle 2007, Weston et al. 2007). The Florida genotype (heat resistant) maintained a higher *A*, a higher stomatal conductance and more open photosystem (PS) II reaction centers compared to the Minnesota genotype within the temperature range of 25–48 °C. Naturally growing oak (*Quercus*) seedlings were found to be quite sensitive to high temperature, exhibiting a 90% reduction in *A* as temperature increased either rapidly or gradually from 25 to 45 °C under conditions that do not result in a stomatal limitation to *A* (Haldimann and Feller 2004). This reduction in *A* was associated with a decline in the activation state of Rubisco from about 90% at 25 °C to < 30% at 45 °C, but this was not accompanied by a phase change in the thylakoid membrane lipids, as assessed by minimal chlorophyll fluorescence analysis of darkened leaves. Also for oak, heat stress did not substantially increase PSII inactivation. Interestingly, the decline in Rubisco activation was not associated with the aggregation of Rubisco activase at high temperature. However, Haldimann and Feller (2004) did not present data on the temperature response of activase activity, per se.

There is one report that two *Populus* species growing in contrasting thermal environments differed with respect to *A* at supra-optimal temperatures. For a western North American cottonwood, *Populus fremontii* Wats., growing in lowlands at high summer temperatures, *A* was less sensitive to high temperatures than that for the aspen, *Populus tremuloides* Michx., growing in the same region but at high elevation with cool summers (Roden and Pearcy 1993). However, no one has investigated the temperature response of *Populus* species from different environments grown under the same environment to determine whether adaptive (as opposed to acclimatory) differences exist.

The objective of this study was to determine whether differences exist in the heat response of photosynthetic parameters for *Populus deltoides* Bartr. ex Marsh. and *P. balsamifera* and to characterize any differences in the photosynthetic response to moderately high temperatures. Studying the effect of moderate heat stress on *P. balsamifera* and a *Populus* species adapted to contrasting thermal environments could shed some light on the mechanism of *Populus* adaptation to high temperature and provide a tool for understanding the biochemical constraints to *A* at moderately high temperatures for improving the response of poplar photosynthesis to heat stress in the future.

Materials and methods

Plant materials

Greenhouse-grown plant material was used for all gas-exchange analyses. *P. deltoides* cuttings and seeds were collected from Marshall County, IN (latitude 41.4, longitude 86.1 and elevation ~ 220 m). The seeds of *P. balsamifera* were obtained from the Western Boreal Aspen Corporation of Edmonton, Alberta, Canada (latitude 53.3, longitude 113.3 and elevation ~ 705 m). The cuttings were rooted during the late winter in a commercial potting soil (Ball Growing-on Mix), and the seeds were germinated in the same type of soil that was kept continually moist. The plants were grown for at least 2 months in 46-l pots before they were used in experiments. All plants were watered daily and fertilized weekly with Hoagland's solution. The greenhouse temperature was maintained at ~ 25/17 °C (day/night). To keep the plants producing vegetative growth in December of each year, the photoperiod was prolonged by 5 h with 400 W metal halide lamps.

Gas-exchange analyses

Photosynthetic gas-exchange measurements were done in the greenhouse using a Li-Cor model Li-6400 (Li-Cor, Lincoln, NE) photosynthesis system at a photon flux density of 1700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and at a relative humidity of 40–45% in the leaf cuvette. The values of A at 27, 33, 36 and ~ 40 °C were determined on the leaves that had just reached full expansion. Preliminary temperature response curves (20–36 °C) for A indicated that 27 °C was at or slightly below the optimum for these *Populus* species (data not shown). The leaf was left at each temperature for 30–45 min before the gas-exchange measurements were commenced. The CO_2 partial pressure was 37 Pa and the O_2 partial pressure was 21 kPa.

The response of A to calculated internal leaf CO_2 concentration (C_i) at 27 and 36 °C was performed on four to five plants of both species under the conditions stated above. The values of A were determined at 14 $p\text{CO}_2$ values from 0 to 220 Pa. Values of the carboxylation efficiency (CE) were estimated using the Photosynthesis Assistant software (Version 1.1, Dundee Scientific, Dundee, UK) (Singh et al. 2005).

Chlorophyll *a* fluorescence analysis

The minimal chlorophyll *a* fluorescence (F_0) from dark-acclimated leaf disks of 1.5 cm in diameter was monitored in the dark as temperature was slowly raised from 25 to 48 °C in the chamber of a Hansatech Oxygen Electrode system (Hansatech, Ltd., Pentney, King's Lynn, UK). Fluorescence measurements were made using a PAM 101/103 fluorometer (Waltz GmbH, Effeltrich, Germany) through a port in the oxygen electrode chamber (Korniyev et al. 2004).

Molecular analyses

The total RNA from *P. deltoides* and *P. balsamifera* was extracted from young leaves using a TRIzol Reagent kit protocol (Ambion cat. # 9738). Total RNA quality was evaluated using a 0.9% formamide agarose gel. First-strand cDNA was synthesized using a M-MLV reverse transcriptase and oligo dT (Promega, Madison, WI) and was used to amplify the predicted *P. deltoides* and *P. balsamifera* Rubisco activase cDNAs. The forward primer (5'-atggcagcaaccatctc-3') and a reverse primer (5'-gagaggaacttctatggttag-3') were designed using publicly available sequencing databases (<http://www.populus.db.umu.se/contig>, POP-LAR.1563.C2). The amplified PCR products were then ligated into the pGEM-T Easy vector system (Promega) and confirmed by sequencing.

To express the *Populus* Rubisco activase proteins in *Escherichia coli*, the cDNAs from both species were amplified using a high-fidelity DNA polymerase *Pfu* hotstart (Stratagene) and cloned into the pET 100 vector to produce an in-frame fusion with a 6xHis tag following the procedures of the Champion pET Directional TOPO Expression Kits (Nitrogen, Life Technologies Corporation, Carlsbad, CA). As the recombinant Rubisco activase did not contain the chloroplast transit peptide (cTP), the primers, 5'-caccatggctgaggagtacgatgag-3' for *P. balsamifera* and 5'-caccatggcagatcagatgagaag-3' for *P. deltoides*, as well as the same reverse primers above were used to generate the PCR products. A culture of *E. coli* (BL 21) harboring plasmids pET100-*Pbactivase* and pET100-*Pdactivase* was grown in LB medium containing 50 $\mu\text{g/mL}$ Ampicillin at 37 °C to an optical density of 0.6 at 600 nm. Gene expression was induced by adding isopropyl β -D-thiogalactopyranoside to a final concentration of 1 mM, and growth was allowed to continue for 18 h in LB medium at 25 °C. The cells were harvested by centrifugation at 4 °C, resuspended in a solution containing 20 mM potassium phosphate buffer (pH 7.0), 10 mM imidazole and 500 mM NaCl. They were passed three times through a French press at 122.76 MPa and centrifuged at 55,000g for 1 h at 4 °C. The supernatant was applied to a Ni^{2+} affinity column (HiTrap Chelating HP, GE Healthcare Bio-Sciences Corp., formerly Amersham Biosciences, Piscataway, NJ) incorporated into a BioCAD perfusion chromatography system (PerSeptive Biosystems, GMI, Ramsey, MN). The column was washed with 200 ml of 20 mM phosphate buffer (pH 7.0) containing 500 mM NaCl (buffer A) supplemented with 25 mM imidazole. The His-tagged activase was then eluted with a gradient from 25 to 250 mM imidazole in 40 ml of buffer A. All solutions used for the Ni^{2+} affinity chromatography were degassed and purged with helium gas. The imidazole was removed by buffer exchange against 20 mM phosphate buffer using an Amicon YM-10 membrane. The protein concentration was determined by the Bradford method (Bradford 1976) using BSA as a standard.

The partial purification of Rubisco activase from the leaves of the two poplar species was based on the protocol described by Robinson et al. (1988). All procedures were performed at 0–4 °C. The protein was extracted from freshly sampled leaves in 100 mM borate buffer, pH 7.6, 10 mM MgCl₂, protease inhibitor cocktail (Sigma, St. Louis, MO), 1 mM ATP, insoluble poly(vinylpyrrolidone) (2% w/v) and 5 mM dithiothreitol (DTT). The solution was centrifuged at 16,000g for 10 min before (NH₄)₂SO₄ was added to a concentration of 35% (w/v). This solution was stirred on ice for 30 min, centrifuged, and the pellet was dissolved in 50 mM HEPES-KOH, pH 7.2, 5 mM MgCl₂, 5 mM DTT and protease inhibitor cocktail. The redissolved solution containing the suspended cell membrane fragments was centrifuged in an ultracentrifuge for 60 min at 66,000g. The protein in the supernatant was concentrated using an Amicon YM-10 membrane.

For western blot analyses, proteins were separated by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels. The recombinant Rubisco activase was detected using the His-tagged antibodies according to the protocol of the alkaline phosphatase immunoblot assay kit (Novagen, EMD Chemicals, Inc., Gibbstown, NJ) or using a primary polyclonal antibody to tobacco Rubisco activase followed by a secondary antibody conjugated to horseradish peroxidase. Native Rubisco activase from the crude leaf extracts was identified using the polyclonal antibody to the tobacco enzyme. The blots were analyzed by adding the detection solution consisting of 12 ml of 50 mM Tris-HCl, pH 7.5, 250 µl of 3,3'-diaminobenzidine and 10 µl of H₂O₂ (9.79 M).

Enzyme activity analyses

To measure the activity of Rubisco in crude leaf extracts and its *in vivo* activation state, leaf samples were collected using a freeze-clamp device at liquid N₂ temperature after the leaf had been exposed to the desired temperature (27, 36 and 40 °C) for at least 35 min and steady-state photosynthesis had been reached in the chamber of the Li-6400 photosynthesis system. The samples were kept at –80 °C not > 15 days before extraction and assay for Rubisco activity. The frozen leaf disks were ground at liquid N₂ temperature followed by homogenization at 4 °C in a glass tissue grinder containing the extraction solution as described by Law and Crafts-Brandner (1999), except that the buffer was Bicine. The initial activity of Rubisco (not activated *in vitro*) was determined < 1 min after the beginning of the extraction process. The Rubisco activity was determined as the amount of ¹⁴CO₂ incorporated into acid-stable products at 30 °C in 0.5 ml containing 100 mM Bicine, pH 8.0, 10 mM MgCl₂, 10 mM NaH¹⁴CO₃ (0.1 µCi/µmol) and 0.4 mM RuBP. The reaction was stopped after 45 s using 4 N formic acid/1 N HCl (1:1). To determine the total activity (activated *in vitro*), 50 µl of the extract were incubated for 5 min in the assay solution without RuBP at

21 °C to fully activate the enzyme followed by the addition of RuBP to initiate the reaction.

The ATPase activity of Rubisco activase was determined at different assay temperatures ranging from 10 to 50 °C in 1 ml of a solution containing 100 mM Tricine-NaOH (pH 8.0), 10 mM MgCl₂, 2 mM DDT, 20 mM KCl, 2 mM ATP, 2 mM phosphoenolpyruvate, 3 mM NADH, 1.7 units of pyruvate kinase and 2.5 units of lactate dehydrogenase. The reaction was initiated by the addition of Rubisco activase, and changes in the concentration of NADH were measured using a spectrophotometer at 340 nm (Salvucci 1992). The concentration of protein in extracts was determined by the Bradford method (Bradford 1976).

Statistical analyses

A two-way analysis of variance followed by mean separation using the Bonferroni test was performed using GraphPad Prism 5.0 software. For some data sets, the Student's *t* test was used to analyze for significant differences of pairs of means.

Results

Response of gas-exchange parameters to moderately high temperature for the Populus species

Values of *A* measured at a CO₂ partial pressure of 37 Pa and at an O₂ partial pressure of 21 kPa were significantly higher for *P. deltoides* than for *P. balsamifera* across the tested temperatures ($P < 0.01$) (Figure 1A). However, rates of dark respiration measured during the middle of the photoperiod were not significantly different for both species between 27 and 40 °C (data not shown). Also, *A* for *P. balsamifera* was more sensitive to temperatures between 33 and 40 °C than that for *P. deltoides*, as indicated by the significantly greater percentage reduction in *A* for *P. balsamifera* ($P < 0.01$ at 33 and 36 °C and $P < 0.05$ at 40 °C) (Figure 1B). Compared to *A* at 27 °C, exposure to 36 °C reduced *A* for *P. balsamifera* by 25% but only by 11% for *P. deltoides*, and raising the temperature to 40 °C caused *A* to decline by 45% for *P. balsamifera* and by 24% for *P. deltoides*. We noted a similar response for plants grown outside of the greenhouse (data not shown). Values of *C*_i and stomatal conductance to water (*g*_s) varied about 10% over this temperature range.

Sage et al. (2008) suggested that the initial slope of the *A*/*C*_i curve (the CE) could be a valuable tool to determine whether a particular species is limited by Rubisco (via Rubisco activase) or by electron transport at a high temperature. From *A* versus *C*_i curves, such as the representative ones shown in Figure 2, the derived CE declined significantly with an increase from 27 to 36 °C for both *Populus* species [from 1.99 ± 0.38 to 1.58 ± 0.23 µmol m⁻² s⁻¹ Pa⁻¹ for *P. balsamifera* ($P < 0.01$) and from 2.4 ± 0.45 to 1.63 ± 0.46 ($P < 0.01$) for *P. deltoides*].

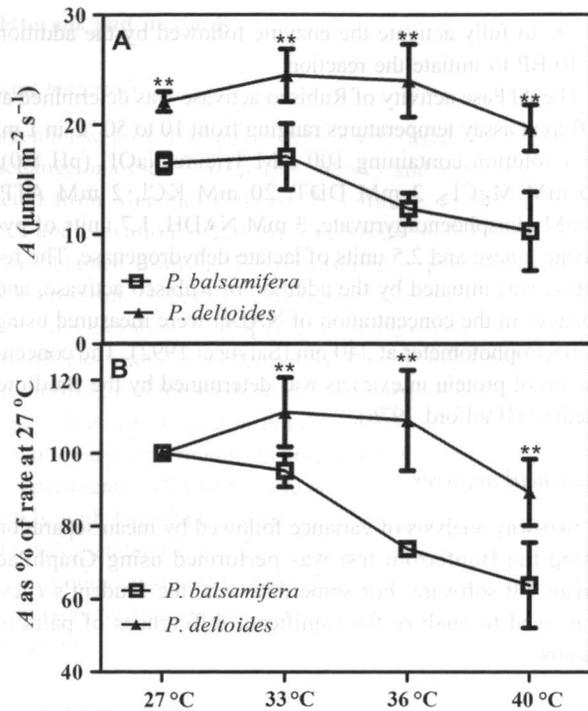


Figure 1. The response of CO_2 assimilation (A) to temperature for *P. deltoides* and *P. balsamifera* at 37 Pa CO_2 and 21 kPa O_2 (A) and A relative to that at 27 °C for both *Populus* species (B). The values are means \pm standard deviation, $n = 4-8$. Asterisks indicate significant differences in species mean values at each temperature as determined by a paired Student's t test ($*P < 0.05$ and $**P < 0.01$). ns, not significant.

The activation state of Rubisco

Changes in CE are valuable estimates of changes in carboxylation, but a more direct method of determining the effect of temperature on carboxylation is to determine the activation state of Rubisco in leaf extracts. A significant ($P < 0.0001$) reduction in the activation state of Rubisco did occur for both species as leaf temperature increased from 27 to 40 °C in the leaf chamber of the Li-6400 photosynthesis system (Figure 3). However, the greater reduction occurred for *P. balsamifera* Rubisco, such that its activation state was $> 10\%$ points less than that for *P. deltoides* Rubisco at 36 and 40 °C.

Phase change of thylakoid membrane lipid with increasing temperature

We used the minimal fluorescence emitted by chlorophyll a molecules (F_0) in the dark from leaf disks of both *Populus* species to estimate the threshold temperature at which a major change in the phase of thylakoid membrane lipids occurred. For both species, F_0 remained constant from 27 to 43.5 °C (Figure 4). When the temperature reached 48.5 °C, F_0 rose to the same extent for both species, indicating that they may not be substantially different with respect to the temperature at which a major lipid phase change occurred in the thylakoid membranes. Note that this temperature was considerably higher than that at which A declined for both species (Figure 1).

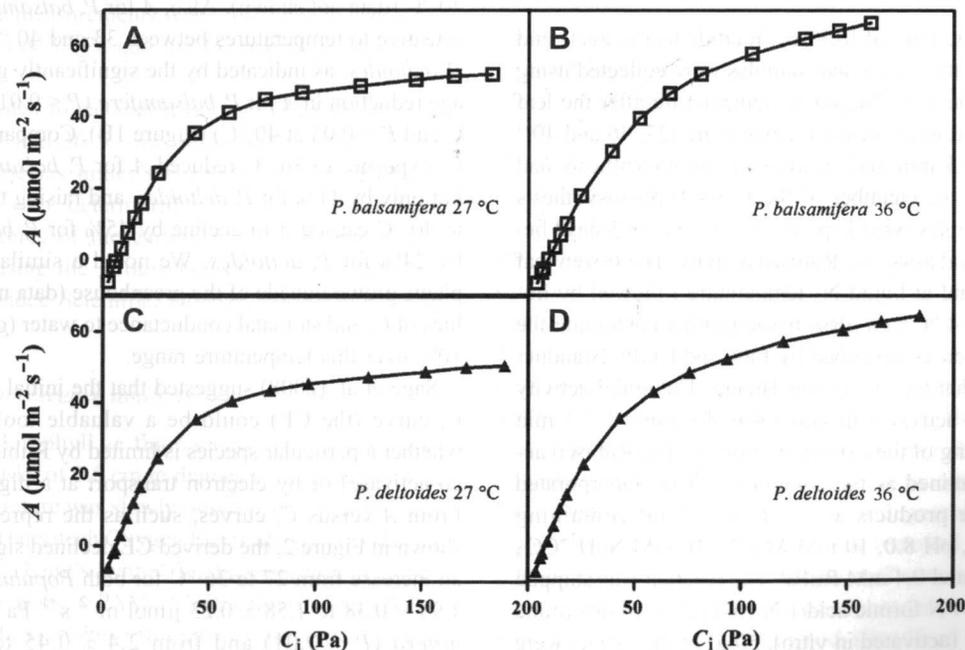


Figure 2. Representative responses of CO_2 assimilation (A) to leaf internal CO_2 concentration (C_i) for *P. balsamifera* (A and B) and *P. deltoides* (C and D) at 27 and 36 °C.

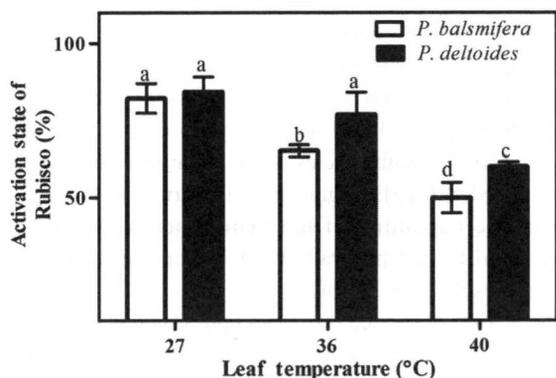


Figure 3. The in vivo activation state of Rubisco based on the initial (not activated in vitro) and total (activated in vitro) activity in leaf extracts for *P. deltoides* and *P. balsamifera* at three leaf temperatures. The values are means \pm standard deviation, $n = 5$. Letters indicate significant differences in mean values ($P < 0.05$) according to the Bonferroni multiple comparison tests. Rates of Rubisco initial activity at 27, 36 and 40 °C were 483 ± 110 , 431 ± 96 and 348 ± 74 , respectively, for *P. deltoides* and 500 ± 177 , 338 ± 74 and 233 ± 111 , respectively, for *P. balsamifera*. Rates of Rubisco total activity at 27, 36 and 40 °C were 574 ± 129 , 570 ± 170 and 582 ± 135 , respectively, for *P. deltoides* and 614 ± 225 , 520 ± 121 and 464 ± 198 , respectively, for *P. balsamifera*.

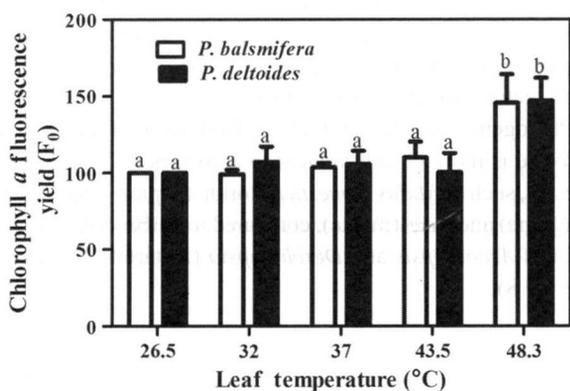


Figure 4. The minimal chlorophyll *a* fluorescence yield (F_0) from dark-acclimated leaf disks of *P. deltoides* and *P. balsamifera* as temperature was slowly raised in the chamber of a Hansatech Oxygen Electrode system. Letters indicate significant differences in mean values ($P < 0.05$) according to the Bonferroni multiple comparison tests.

Forms of Rubisco activase in Populus

To determine whether one or two forms of the Rubisco activase were present in *Populus* leaves, proteins from the crude leaf extracts of *P. balsamifera* and *P. deltoides* were subjected to SDS-PAGE western blot analysis. Probing with a polyclonal antibody for tobacco activase revealed two forms of activase subunits in leaf extracts from the two *Populus* species with molecular masses of about 46 and 43 kD (Figure 5A).

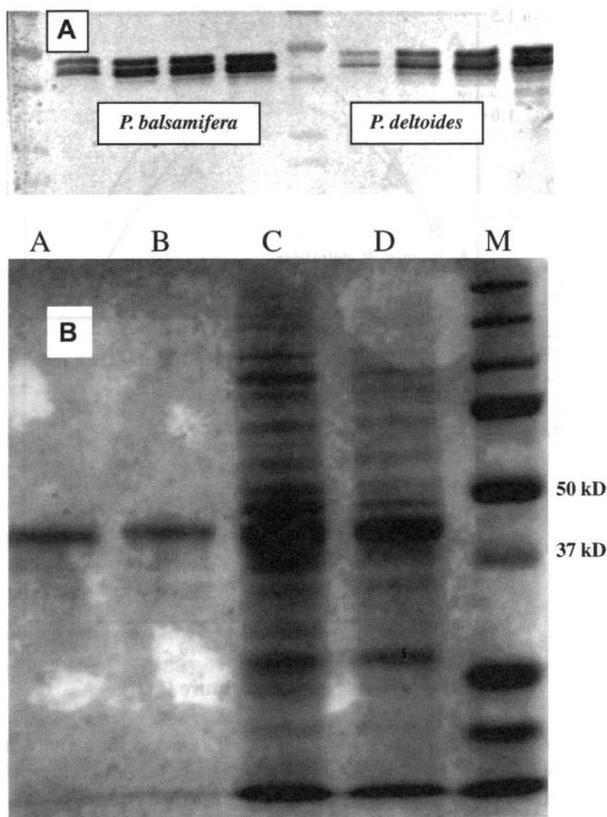


Figure 5. Panel A: SDS-PAGE of a crude leaf extract for *P. balsamifera* and *P. deltoides* probed with a polyclonal antibody to tobacco Rubisco activase. The lanes were loaded with increasing amounts of protein from left to right for each species. The molecular masses are about 46 and 43 kD for the upper and lower bands of activase, respectively. Panel B: SDS-PAGE of recombinant activase from *P. deltoides* (A, purified and C, crude) and *P. balsamifera* (B, purified and D, crude) expressed in *E. coli*. Purification was accomplished with a perfusion chromatography system (FPLC) using a Ni^{++} affinity column.

Temperature responses of partially purified and recombinant Rubisco activase activity for P. balsamifera and P. deltoides

Recombinant Rubisco activase protein without the cTP was derived from the cDNA cloned from each species. As described below, the recombinant protein from both species was the short or β form of Rubisco activase, the more thermo-sensitive form in some species (Crafts-Brandner et al. 1997). After purification, the proteins were judged to be 90% pure based on the SDS-PAGE gels stained with coomassie blue (Figure 5B).

The ATPase activity for the recombinant activases from both species increased as temperature increased from 10 to 27 °C without any significant ($P > 0.05$) species difference (Figure 6A). However, from 30 to 50 °C, the ATP hydrolysis activity of *P. balsamifera* activase was significantly ($P < 0.001$) lower than that for *P. deltoides* activase at each assay temperature. The ATPase-specific activity declined at

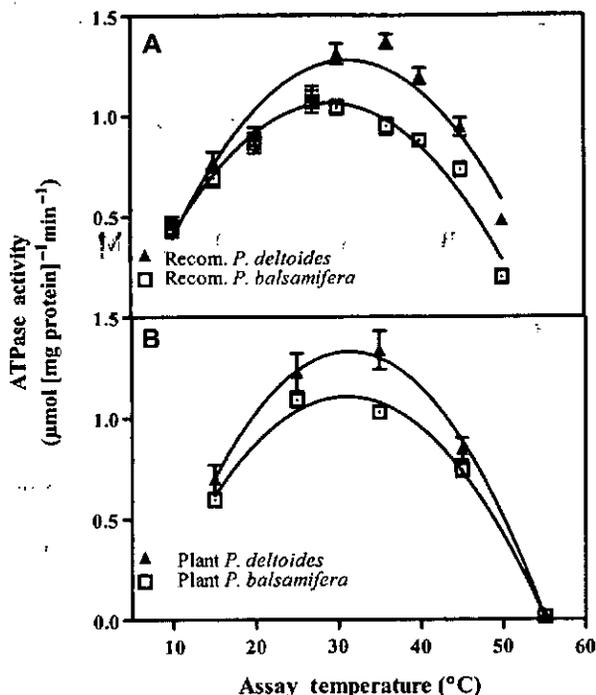


Figure 6. The temperature response of the ATPase-specific activity of recombinant Rubisco activase for *P. deltoides* and *P. balsamifera* (A) and partially purified Rubisco activase from *P. deltoides* and *P. balsamifera* leaves (B). The values are means \pm standard deviation, $n = 4$.

temperatures above 30 °C for *P. balsamifera* activase and above 35 °C for *P. deltoides* activase.

The activity of partially purified Rubisco activase from *P. balsamifera* and *P. deltoides* leaf extracts containing both α and β forms exhibited a response to temperature similar to that for the recombinant β counterpart (Figure 6B). These results indicated that the temperature response of the recombinant β form of activase was representative of that for both forms.

Molecular analyses of the β form of Rubisco activase from *P. balsamifera* and *P. deltoides*

The cDNAs for the mature Rubisco activase gene without the cTP from *P. balsamifera* and *P. deltoides* migrated similarly, and only one band was detected for each species. Sequence alignment and the length of the PCR fragment showed that the cDNA for both species was that for the short form (β isoform) of Rubisco activase (Crafts-Brandner et al. 1997). Full sequences of the cDNA for this β isoform showed that the open reading frame of the gene is 1323 bp (1149 bp without the cTP) in *P. balsamifera* and 1320 bp (1146 bp without the cTP) in *P. deltoides*, data not shown.

The expected immature proteins consist of 440 amino acids in *P. balsamifera* and of 439 in *P. deltoides* with 94% homology at the amino acid level (Figure 7). When the cTP sequences are removed, the mature protein for *P. balsamifera*

is composed of 382 amino acids and that for *P. deltoides* is composed of 381 with 94.2% sequence homology (Figure 7). The predicted protein size according to the bioinformatic software program, <http://us.expasy.org/cgi-bin/protparam>, was 42.5 kD for both species, slightly smaller than that estimated by SDS-PAGE (Figure 4B). The arrow in Figure 7 indicates the beginning of the mature activase protein as determined by the program available online, <http://www.cbs.dtu.dk/services/ChloroP/>.

The β form of the activase from both *Populus* species consists of 11 domains (Figure 7). Starting from the N-terminus of the protein these domains are Box II; Walker A (ATP binding site); Box IV; Box IV'; Walker B (involved in metal ligand binding and ATP catalysis, Wang and Portis 2006); Box VI (ATP binding site); Sensor I (ATP binding site); Box VII (ATP hydrolysis); Box VII'; Box VII'' and Sensor II (Rubisco binding region). The critical lysine and valine residues (K374 and V377) that interact with the Rubisco enzyme (Li et al. 2005) are conserved in the activases from both *Populus* species, as is the aspartate residue (D174 in *Arabidopsis*) in the Walker B motif that is required for activase aggregation (van de Loo and Salvucci 1998). The *Populus* proteins also contain the highly conserved tryptophan residue (W15 in *P. balsamifera* and W14 in *P. deltoides*, van de Loo and Salvucci 1996) as indicated by the symbol, n, in Figure 7. The asterisk (*) in Figure 7 refers to a highly conserved lysine residue in Box VII' (Salvucci and Klein, 1994) found in the activase from both *Populus* species. The greatest differences in the sequences of the two *Populus* activases occur in the Box VII'' sector and near the C-terminus.

Phylogenetic analysis showed that the β form of the *Populus* activase is more closely related to activases in other woody species, such as *Malus domestica* Borkh. (apple), *Vitis vinifera* L. (grape) and *Acer* (maple), compared to herbaceous species, such as *Arabidopsis* and *Deschampsia* (Antarctic hair grass) (Figure 8).

Discussion

Species differences in CO₂ assimilation at high temperature

It is widely accepted that photosynthetic CO₂ assimilation (*A*) is sensitive to heat stress (Berry and Björkman 1980, Haldimann and Feller 2004, Salvucci and Crafts-Brandner 2004a). Species adapted to contrasting thermal environments have different thermal optima for photosynthetic processes (Salvucci and Crafts-Brandner 2004b). Species adapted to a high temperature environment often have higher temperature optima for *A* compared to optima of species, such as boreal species, growing in cool environments. For example, the optimum temperature for *A* was found to be 10 °C higher for creosote bush (*Larrea tridentata* (DC.) Coville) when compared to that for Antarctic hair grass (*Deschampsia antarctica* E. Desv.) (Salvucci and Crafts-Brandner 2004b). A similar pattern was ob-

TRANSIT PEPTIDE

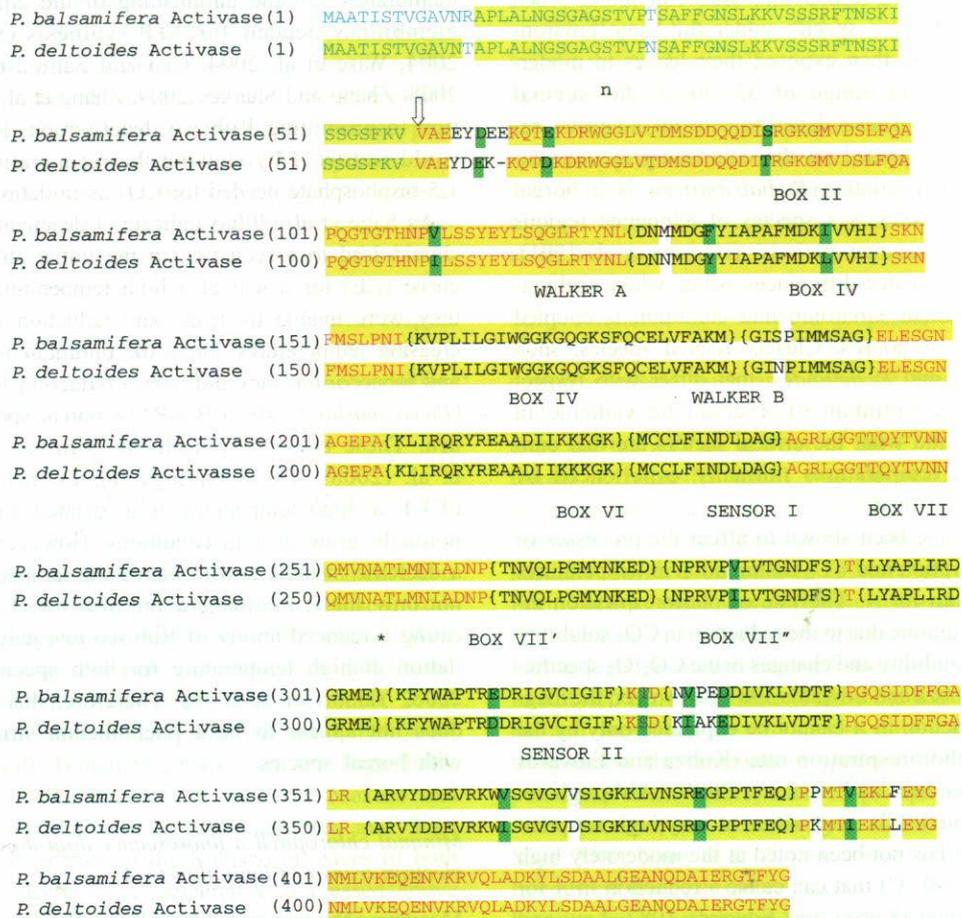


Figure 7. Alignment of the derived amino acid sequences of Rubisco activase homologs from *P. balsamifera* and *P. deltoides*. The sequences include the cTP and are 94% identical (yellow regions). The green regions indicate a change in a neutral amino acid to another neutral amino acid, whereas white indicates a change to a different group. The arrow indicates the start of the mature protein. Marked with an 'n' is an important tryptophan residue for Rubisco recognition mentioned in the text.

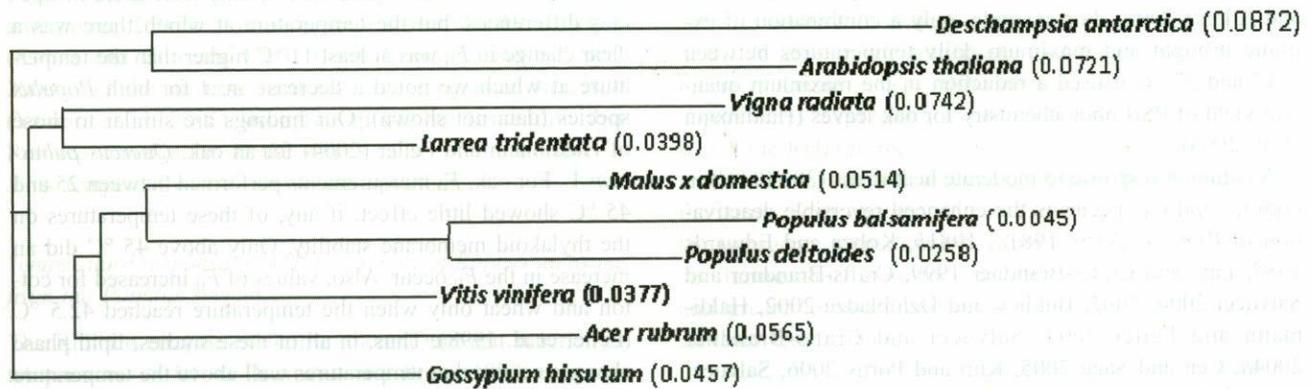


Figure 8. A phylogenetic tree for Rubisco activase from several plant species based on the amino acid sequence alignments after removing the transit peptide.

served for populations of a single species, *A. rubrum*, growing in Minnesota and Florida (Weston and Bauerle 2007, Weston et al. 2007). Gas-exchange measurements

showed that the Florida genotype (heat resistant) maintained a higher *A* and a higher stomatal conductance compared to the Minnesota genotype within the temperature

range of 25–48 °C. In this study, we found that A for *P. balsamifera* declined more than that for *P. deltoides* when we grew plants of these species under the same environmental conditions and then exposed their leaves to moderate heat stress in the range of 33–40 °C for several minutes (Figure 1). These data indicate an adaptive response to heat stress based on the contrasting thermal environments of their ranges (*P. balsamifera* is a boreal species and *P. deltoides* is a species of temperate regions with warm to hot summer periods) (Harlow et al. 1991). Presumably, even greater differences occur when acclimation to the indigenous environmental condition is coupled to these adaptive responses. Closely related species, such as *P. balsamifera* and *P. deltoides*, that differ with respect to the temperature optimum of A could be valuable in studies to understand what the critical factors are that constrain A at high temperatures normally experienced by crop species in the field.

Many factors have been shown to affect the processes of light energy absorption and CO₂ assimilation at temperatures above the optimum for A . The rate of photorespiration increases with temperature due to the reduction in CO₂ solubility compared to O₂ solubility and changes in the CO₂/O₂ specificity of Rubisco (Kobza and Edwards 1987). However, the magnitude of the reduction in A cannot be explained only by the increase in the photorespiration rate (Kobza and Edwards 1987, Crafts-Brandner and Salvucci 2000). Although researchers have noted PSII inactivation at very high temperatures (> 42 °C), it has not been noted at the moderately high temperatures (30–40 °C) that can cause a reduction in A for most species studied (Kobza and Edwards 1987, Law and Crafts-Brandner 1999). In fact, tree species, such as the *Populus* species we studied, are considered to be more protected against photoinhibition compared to short-lived, annual species due mainly to their perennial nature that exposes them to widely fluctuating conditions of light and temperature in their natural environment (Demmig-Adams and Adams 2006). For example, only a combination of extreme drought and maximum daily temperatures between ~ 32 and 37 °C caused a reduction in the maximum quantum yield of PSII photochemistry for oak leaves (Haldimann et al. 2008).

A common response to moderate heat stress (30–40 °C) in both C₃ and C₄ species is the enhanced reversible deactivation of Rubisco (Weis 1981a, 1981b, Kobza and Edwards 1987, Law and Crafts-Brandner 1999, Crafts-Brandner and Salvucci 2000, 2002, Bukhov and Dzhiladze 2002, Haldimann and Feller 2004, Salvucci and Crafts-Brandner 2004a, Cen and Sage 2005, Kim and Portis 2006, Salvucci et al. 2006, Makino and Sage 2007, Hendrickson et al. 2008, Kubien and Sage 2008). Whether this deactivation is a secondary consequence of a low chloroplastic ATP level in the chloroplast that actually constrains A at high temperature or is the direct constraint on A has been debated (Salvucci and Crafts-Brandner 2004a, Sage et al. 2008). It is thought that a low ATP level could develop at a high

temperature from an increased fluidity of the thylakoid membranes and the diminishing of the ΔpH across these membranes needed for ATP synthesis (Schrader et al. 2004, Wise et al. 2004, Cen and Sage 2005, Sage et al. 2008, Zhang and Sharkey 2009, Zhang et al. 2009). In addition to promoting Rubisco deactivation, low ATP levels could restrict A by slowing the regeneration of ribulose-1,5-bisphosphate needed for CO₂ assimilation.

As Sage et al. (2008) indicate, a deactivation of Rubisco should lead to a reduction in the initial slope of the A/C_i curve (CE) for a leaf at a high temperature. Interestingly, they were unable to detect any reduction in CE with increasing temperatures above the optimum for sweet potato and tobacco, but they did note a reduction for black spruce (*Picea mariana* (Mill.) B.S.P.), a boreal species, and spinach. These data on spinach are corroborated by Yamori et al. (2008). These findings suggest that the reduction of CE at high temperature is associated with species that normally grow in cold conditions. However, in this study, a decrease in the CE with increasing temperatures occurred not only for *P. balsamifera* but also for *P. deltoides*, indicating a reduced ability of Rubisco to catalyze CO₂ assimilation at high temperature for both species (Sage et al. 2008, Yamori et al. 2008). Therefore, this decline in CE does not appear to be a phenomenon strictly associated with boreal species.

Minimal chlorophyll a fluorescence data do not support an electron transport limitation to A

Measuring the minimal chlorophyll *a* fluorescence (F_0) in the dark as the temperature of a leaf is increased has been used as a method to determine at what temperature a major lipid phase change occurs in the thylakoid membranes (Haldimann and Feller 2004). We found no substantial difference in the temperature (~ 44 °C) at which F_0 increased for *P. balsamifera* and *P. deltoides* (Figure 4). Not only were there no species differences, but the temperature at which there was a clear change in F_0 was at least 11 °C higher than the temperature at which we noted a decrease in A for both *Populus* species (data not shown). Our findings are similar to those of Haldimann and Feller (2004) for an oak, *Quercus pubescens* L. For oak, F_0 measurements performed between 25 and 45 °C showed little effect, if any, of these temperatures on the thylakoid membrane stability. Only above 45 °C did an increase in the F_0 occur. Also, values of F_0 increased for cotton and wheat only when the temperature reached 42.5 °C (Feller et al. 1998). Thus, in all of these studies, lipid phase changes occurred at temperatures well above the temperature at which A declined, indicating that this lipid phase change could not be the factor contributing to the decline in A in the range of 30–40 °C.

Recently, Sharkey's group (Zhang and Sharkey 2009, Zhang et al. 2009) used electrochromic shift analyses to estimate the effect of moderately high temperatures on the ΔpH across the thylakoid membranes in light-acclimated

leaves of *Arabidopsis* and tobacco. Switching from 23 to 40 °C caused a significant reduction in the ΔpH for either species, leaving the possibility open that heat caused a leakage of protons from the thylakoid lumen to the stroma of the chloroplasts. However, the researchers were not able to distinguish between the effects of possible proton leakage, possible increased conductance of protons via ATP synthase or a potential increase in ATP synthase activity at high temperature. Although it cannot be discounted that some proton leakage may have occurred during the exposure of the two *Populus* species to moderate heat stress in this study, given the F_0 data, it is not likely that they differed with respect to the extent of this leakage between 27 and 40 °C, making this process an improbable explanation for the species differences in the response of A to heat.

Activation state of Rubisco and the relationship to A at high temperature

A more direct estimate of carboxylation ability than CE is a measure of the *in vivo* activation state of Rubisco. Consistent with several previous reports on other species (Weis 1981a, 1981b, Kobza and Edwards 1987, Law and Crafts-Brandner 1999, Crafts-Brandner and Salvucci 2000, 2002, Bukhov and Dzhibladsze 2002, Haldimann and Feller 2004, Salvucci and Crafts-Brandner 2004a, Cen and Sage 2005, Kim and Portis 2006, Salvucci et al. 2006, Makino and Sage 2007, Hendrickson et al. 2008, Kubien and Sage 2008), the activation state of the Rubisco in leaves of both *Populus* species declined as temperatures increased above the optimum for A (Figure 3). It is noteworthy that the activation state of Rubisco for *P. balsamifera* was lower than that for *P. deltoides*, in keeping with the species differences in A at high temperature. The findings for several species that the changes in the activation state of Rubisco coincided with the changes in A (Haldimann and Feller 2004, Salvucci and Crafts-Brandner 2004a, Haldimann and Feller 2005), a rise in the ratio of RuBP to PGA (Kobza and Edwards 1987, Crafts-Brandner and Salvucci 2000) and the increase in the C_i as temperature increased (Yamori et al. 2008) strongly support the contention that Rubisco inactivation is the direct cause of the decline in A as temperatures rise above the optimum.

Temperature response of ATPase activity of Rubisco activase from the Populus species

The Rubisco enzyme is thermo-tolerant and can function at temperatures > 50 °C (Eckhardt and Portis 1997, Salvucci and Crafts-Brandner 2004b). However, its activation state is sensitive to high temperatures far below those that denature the enzyme. This reduction in the activation state of Rubisco at high temperature is thought to be due mostly to a failure of the Rubisco activase to keep pace with the increased Rubisco deactivation rate at moderately high temperatures (Feller et al. 1998, Law and Crafts-Brandner 1999, Crafts-

Brandner and Law 2000, Crafts-Brandner and Salvucci 2000, 2002, Salvucci and Crafts-Brandner 2004a). It is now well established that Rubisco activase is sensitive to inactivation and even denaturation at moderately high temperatures (Salvucci et al. 2001, Salvucci and Crafts-Brandner 2004a, 2004b). The thermal sensitivity of activase has led to the proposal that activase, through its regulatory control of Rubisco, may determine the geographic distribution of plant species (Salvucci and Crafts-Brandner 2004b, Sage et al. 2008).

In this study, the temperature response of the ATPase activity correlated well with the temperature responses of the Rubisco activation state and of A , as has been noted for cotton, creosote bush and Antarctic hair grass (Salvucci and Crafts-Brandner 2004a, 2004b). The optimum temperature for the ATPase activity of the *P. balsamifera* recombinant activase (β form) was between 27 and 30 °C, whereas it was between 30 and 37 °C for the *P. deltoides* activase. A similar difference was observed in the case of partially purified enzyme (a mixture of α and β forms) from *Populus* plant leaves (Figure 6A and B). It is notable that these optima correspond closely to the optima for A in Figure 1 and also correlate well with the species differences in Rubisco activation state (Figure 3). It is also notable that the activases from the two species do not differ in their activity below the temperature optimum. At suboptimal temperatures, other factors, such as phosphate availability for ATP synthesis, are more likely a constraint to A (Cen and Sage 2005, Sage et al. 2008). These findings support our hypothesis that Rubisco activation is constrained by the Rubisco activase activity at high temperature and are similar to the results for other plant species from contrasting thermal environments (Salvucci and Crafts-Brandner 2004b). Further support of this hypothesis comes from the creation of transgenic *Arabidopsis* plants that express genes encoding more heat-tolerant Rubisco activase. Using DNA shuffling technology, Kurek et al. (2007) developed several activase genes with critical sequence changes that improved heat tolerance in the transgenic *Arabidopsis* plants. More recently, Kumar et al. (2009) developed transgenic *Arabidopsis* lines expressing a chimeric Rubisco activase, in which the Rubisco recognition domain of the more heat-tolerant tobacco activase was replaced with the domain from the *Arabidopsis* enzyme. In comparison to wild-type plants, the transgenic plants exhibited higher rates of photosynthesis during short exposures to supra-optimal temperatures and greater biomass and seed production when grown at elevated temperature.

Molecular analyses of Rubisco activase from P. balsamifera and P. deltoides

Molecular characterization of Rubisco activase is extremely important to understand its catalytic properties, interaction with Rubisco and to potentially improve its thermal stability to withstand higher temperatures in an era of global

warming. Identifying the functional motif and the key residue(s) in the activase protein that improves its thermostability would be valuable information toward producing transgenic plants that were able to withstand high temperatures. From their DNA shuffling experiments, Kurek et al. (2007) have already identified the key residue(s) in the activase protein that improves the thermostability of the enzyme and have produced transgenic *Arabidopsis* plants that were able to withstand higher temperatures. Studying the primary activase structure of both *P. balsamifera* and *P. deltoides* provides information about natural variations in activase structure that can be used to improve the thermal properties of this crucial enzyme.

Based on the primary sequence analysis, the Rubisco activase cloned from both *Populus* species was the short or β form (Figure 7). From the nucleotide sequences, we determined that the mature activase protein was about 42.5 kD after the removal of the chloroplast-targeting peptide. All plant species that express the β form have a molecular weight of 41–43 kD (Portis 2003). We found no additional amino acids at the C-terminus typical of the long or α form of Rubisco activase (Zhang and Portis 1999, Salvucci et al. 2003). Portis 2003 noted that most plant species studied for activase forms, such as *Arabidopsis*, barley, spinach and rice, produce two isoforms of activase, which are the result of alternative splicing from a single gene. An exception is cotton in which the two forms are encoded by two separate genes. In contrast, the tobacco genome contains three separate genes, all of which produce β forms of activase (Qian and Rodermel 1993, Salvucci et al. 2003). Weston et al. (2007) were able to clone both forms of activase from *A. rubrum* populations in Minnesota and Florida. We searched the *Populus* genome data set based on *Populus trichocarpa* and were able to find sequence information only for the β form of the enzyme. However, our immunoblot analysis of partially purified activase from *P. balsamifera* leaves demonstrated the presence of both α and β forms of Rubisco activase.

The β form of the *Populus* activase protein contains all of the eleven domains found in other activases, namely, Box II; Walker A (ATP binding site); Box IV; Box IV'; Walker B (involved in metal ligand binding and ATP catalysis, Wang and Portis 2006); Box VI (ATP binding site); Sensor I (ATP binding site); Box VII, which contains an arginine finger to interact with the γ phosphate bound to an adjacent subunit (Li et al. 2006); Box VII'; Box VII'' and Sensor II (Rubisco binding region) (Figure 7). Sequence analysis of the mature *P. balsamifera* and *P. deltoides* activase proteins showed a difference of 6% in the amino acid composition (Figure 7), whereas the variation found in activases from the *A. rubrum* ecotypes, Florida and Minnesota, was only 2% (Weston et al. 2007). Despite this small amino acid variation, it may be sufficient, especially for the *Populus* species, to explain the variation in the enzyme's activity in vitro and in vivo at moderately high temperature (Figure 6A and B). Crafts-Brandner et al. (1997) proposed that the thermal sensitivity of the Rubisco activase depends on the primary struc-

ture of the enzyme and/or the polypeptide forms. Much of the variation between the activase from the *Populus* species occurred in Box VII'' and in the Sensor II region of the activase protein (Figure 7) toward the carboxyl end, which are domains involved in ATPase activity and recognition of Rubisco (Li et al. 2005). Directed mutagenesis to convert glycine 111 in *Arabidopsis* to glutamic acid increased in vitro activase activity up to 250% but without improvement in thermal stability of the enzyme (Kallis et al. 2000). Kurek et al. (2007) achieved improved heat tolerance in transgenic *Arabidopsis* lines expressing shuffled activase genes having from three to as little as one amino acid substitution (threonine 274 to arginine in Box VII'). These domains are generally in the section of the protein where we noticed the least sequence homology between the activases of the two *Populus* species (Figure 7).

Conclusions

We have shown that *A* for *P. balsamifera* is more sensitive to moderate high temperature compared to *A* for *P. deltoides* under photorespiratory conditions. The differences in *A* between ~33 and 40 °C coincided with the differences in CE associated with differences in Rubisco activation. As in the case of a study with oak seedlings, we detected no problems with factors that could result in an ATP limitation to *A* for either species in this temperature range. Given that the temperature response for the Rubisco activases from both species correlated well with the response for *A* and the Rubisco activation state, we conclude that the greater sensitivity to heat for the *P. balsamifera* activase than for the enzyme from *P. deltoides* is a major contributing factor to the greater heat sensitivity of *A* for *P. balsamifera*. A sufficient difference (6%), much of it in the regions associated with catalytic activity and Rubisco recognition, in the amino acid sequences of the Rubisco activases from these two species exists to provide a tool for determining amino acids that are critical to heat tolerance.

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