Comparative Studies of C₃ and C₄ Atriplex Hybrids in the Genomics Era: Physiological Assessments

Jason Oakley
Master of Science
Department of Ecology and Evolutionary Biology
University of Toronto
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Abstract
The C₃ species Atriplex prostrata was crossed with the C₄ A. rosea to produce F₁ and F₂ hybrids. All hybrids exhibited C₃-like δ¹³C values, and had reduced rates of net CO₂ assimilation compared to A. prostrata. The activities of three major C₄ cycle enzymes were at most 36% of the C₄ values. Photosynthetic CO₂ compensation points (Γ) were generally midway between the C₃ and C₄ values, and were generally accompanied by low, C₃-like activities in one or more of the major C₄ enzymes. Their anatomy resembled that of C₃-C₄ intermediate species using a glycine shuttle to concentrate CO₂ in the bundle sheath. These results demonstrate the C₄ cycle was disrupted in the hybrids, and that most hybrids use a photorespiratory glycine shuttle. Progeny of these hybrids should further segregate C₃ and C₄ traits and in doing so assist in the discovery of C₄ genes using high-throughput methods of the genomics era.
Acknowledgments

I would like to begin by thanking my supervisor, Rowan Sage. He first captured my imagination with this project, and his guidance and support have made its completion possible. I also thank my committee members Tammy Sage and Stephen Wright for their advice and guidance. In particular, I thank Tammy Sage for her guidance and direction on the anatomical portion of the work.

I thank the many members of the R. Sage and T. Sage labs for providing assistance as well as friendship. In particular, I thank Corey Stinson and Patrick Friesen for their assistance with gas exchange measurements; Stephanie Sultmanis and Roxana Khoshravesh for performing the anatomical work, and Jeff Harsant for assistance with photography.

I also thank Bruce Hall, Andrew Petrie, and Debbie Tam for helping to keep my plants happy and for assistance with the growth chambers.

Professor Olle Björkman was involved in the creation of the original Atriplex hybrids and I thank him for his help in collecting seeds of A. prostrata from the same collection site used in their hybrid trials. I also thank Dr. Chris Root for his help in acquiring seeds of A. rosea.

Finally, I would like to thank family and friends for their support and encouragement.
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Declaration

I did all of the experimental work except for the leaf anatomy. I wrote the first draft of the manuscript that has been published in the Journal of Experimental Botany (see reference below). My supervisor, Rowan Sage revised this draft for publication. This published manuscript represents the main text of the thesis document. The appendix was not included in the publication.

Chapter 1
Main Chapter
1 Main Chapter

1.1 Introduction

C₄ photosynthesis is a carbon concentrating mechanism that evolved from C₃ progenitors at least 65 times (Sage et al., 2012). During C₄ evolution, a coordinated series of anatomical and biochemical adjustments established the compartmentation and enzyme activities required to efficiently concentrate CO₂ around Rubisco (Monson and Rawsthorne, 2000). In the process, dozens to hundreds of genes have been altered (Bräutigam et al., 2011a, b; Gowik et al., 2011). A number of the modifications to key biochemical enzymes such as PEP carboxylase have been identified, although most remain unknown, particularly the genes controlling the anatomical modifications (Kajala et al., 2011; Ludwig, 2013). Identification of these elements are essential to efforts to improve C₄ photosynthesis and potentially engineer the C₄ pathway into C₃ crops, as is now being attempted with rice (von Caemmerer et al., 2012; www.C4rice.org).

Gene discovery is most efficient when researchers can apply forward and reverse genetic approaches using genetic model organisms (Meinke et al., 1998). Unfortunately, in the case of the C₄ pathway, ideal model organisms have not been developed, although *Setaria viridis* is a potential candidate (Li and Brutnell, 2011; Covshoff et al., 2014). The lack of tractable genetic models for C₄ photosynthesis requires that alternative means of gene discovery are considered. One option is to generate hybrids between closely related C₃ and C₄ species, and then use a genetic mapping strategy to associate genes with segregating traits. A number of congeneric pairs of C₃ and C₄ species have been hybridized since the discovery of the C₄ pathway. The first C₃ x C₄ hybrids were produced by Malcolm Nobs and Olle Björkman (Figure 1-1) between *Atriplex rosea* (C₄) and *Atriplex prostrata* (formerly termed *A. patula* ssp. *hastata* and *A. triangularis*; Kadereit et al., 2010), and *A. rosea* and *A. glabriuscula* (C₃) (Björkman et al., 1969; Osmond et al., 1980). Subsequent efforts created hybrids between C₃ and C₄-like *Flaveria* species (Apel et al., 1988), and C₃-C₄ intermediate and C₄ *Flaveria* species (Brown et al., 1986; Brown and Bouton, 1993). Hybrids have also been generated between C₃ and C₃-C₄ intermediate *Panicum* species (Bouton et al., 1986). In many of the *Flaveria* crosses, the F₁ hybrids were sterile (Brown and Bouton, 1993). In cases where F₂ hybrids were generated and segregation of traits observed, problems associated with chromosome abnormalities and pairing were evident,
such that mapping populations could not be formed (Osmond et al., 1980; Covshoff et al., 2014). All hybrid studies were abandoned, and the hybrids eventually perished.

With the advent of high-throughput sequencing and bioinformatics, the ability to evaluate genetic differences between hybrid offspring has dramatically improved, such that the requirement for a mapping population can be relaxed. Of particular promise is sequencing of transcriptomes (RNA-Seq), which can quantify gene expression over a large dynamic range and does not require prior knowledge of the genome sequence (Bräutigam and Gowik, 2010). Comparative transcriptomics has already been used to identify genes that are differentially expressed in leaves of closely related C_3 and C_4 plants (Bräutigam et al., 2011a, b; Gowik et al., 2011). By using a comparative transcriptomics approach with segregating F_2 hybrids, the C_4 genes controlling the segregating traits may be identified.

C_3 x C_4 hybrids can also provide novel insights for understanding C_4 structure, function and evolution. With advances in photosynthetic methodology, the development of theoretical models of C_3 and C_4 photosynthesis, and an improved appreciation of how structural adaptations enhance C_4 function, we are now in a much better position to interpret patterns observed in C_3 x C_4 hybrid lines than was the case a generation ago (Dengler and Nelson, 1999; von Caemmerer, 2000; Sage et al., 2013). Predictions from theoretical models of C_4 photosynthesis developed since the hybrid era can also provide valuable insights that will aid the interpretation of C_3 x C_4 hybrid studies (von Caemmerer, 2000; Ubierna et al., 2013). In addition, models describing the function of C_3-C_4 intermediate species (Rawsthorne et al., 1988; von Caemmerer 1989, 1992) appeared near the end of the hybrid studies (Brown and Bouton, 1993). With the modern understanding of C_3-C_4 intermediacy, it is now possible to address the degree to which C_3 x C_4 hybrids express the physiology of C_3, C_4 or C_3-C_4 intermediate species (Sage et al., 2012). In C_3-C_4 intermediates, the major physiological trait is a CO_2 concentrating mechanism (CCM) that shuttles photorespiratory glycine from mesophyll (M) to bundle sheath (BS) tissues where the photorespiratory enzyme glycine decarboxylase is localized (Monson and Rawsthorne, 2000). This CCM is now termed C_2 photosynthesis (Sage et al., 2012)

In reviewing the literature on C_3 x C_4 hybrids, the most attractive system appears to be the cross between A. rosea and A. prostrata (Björkman et al., 1969). An appealing aspect of this system is that the axile inflorescences of A. rosea are entirely composed of female flowers. This facilitates
cross-pollination with *A. rosea* as the maternal parent because the bisexual inflorescences at the branch tips can be easily removed (Osmond *et al*., 1980). The F₁ offspring of the *A. rosea* x *A. prostrata* cross are fertile, although with reduced pollen fertility and seed set. The F₂ offspring exhibit a gradation in many C₄ traits, with independent assortment (Boynton *et al*., 1970). For example, no correlation is apparent between leaf anatomy and expression of C₄ enzymes (Boynton *et al*., 1970). These findings were the first to demonstrate that multiple genes are involved in the expression of C₄ photosynthesis, and show that the loss of any one C₄ trait led to breakdown of the C₄ CCM (Björkman, 1976; Osmond *et al*., 1980). However, chromosomal abnormalities were observed, with only four out of nine chromosomes regularly pairing at meiosis (Nobs, 1976). This precluded traditional genetic analysis, since forming a linkage map was impossible. The use of high-throughput genomics can potentially overcome this constraint (Bräutigam and Gowik, 2010).

To exploit the potential of C₃ x C₄ hybrids in the genomics era, it is necessary to produce new hybrid lines to replace those lost decades ago. We therefore regenerated hybrids between *Atriplex rosea* and *Atriplex prostrata* through to the F₂ generation. Here, we describe the physiology and leaf anatomy of these hybrids using gas exchange and biochemical assays, and interpret the results in light of current theory for the function of C₃-C₄ intermediate and C₄ systems.
Figure 1-1. A) Malcolm Nobs pollinating *Atriplex rosea*, with the pollen donor, *Atriplex prostrata*, to his right. Photo supplied by Olle Björkman. B) Olle Björkman standing behind a clump of *Atriplex prostrata* at the collection site (arrow), December 15, 2010 (Photo by R.F. Sage).
1.2 Methods

1.2.1 Generation of F₁ and F₂ hybrids

With the assistance of Olle Björkman (Figure 1-1B), seeds of *Atriplex prostrata* were collected from a salt marsh along San Francisco Bay in Baylands Park, Palo Alto, California USA (37°27′38.65″N x 122°06′19.63″W). This is the same collection site for this species in the first hybrid trials (Björkman *et al.*, 1969). Seeds of *A. rosea* were collected in a corral along Ball’s Canyon road, 30 km northwest of Reno, Nevada, USA by Dr. Chris Root (39°39′20.68″N x 120°03′19.89″W). All plants used for crosses were grown from these collections in a rooftop greenhouse located at the University of Toronto. Plants were grown in a mixture of sand, Pro-Mix (Premier Tech Ltd., Rivière-du-Loup, Québec, Canada), and sterilized topsoil (2:2:1 by volume) in either 7.6 l or 3.8 l pots. Plants were watered as necessary to avoid drought and fertilized weekly with a mixture containing 1.8 g l⁻¹ of 24-8-16 Miracle-Gro All Purpose fertilizer, 1.2 g l⁻¹ 30-10-10 Miracle-Gro Evergreen Tree and Shrub fertilizer (Scotts Miracle-Gro Co., Marysville, Ohio, USA), 4.0 mM Ca(NO₃)₂, and 1.0 mM MgSO₄. The daytime temperature during growth was 26° to 32°C depending on outdoor temperature and solar insolation, and night temperature was approximately 23°C.

In *A. rosea*, bisexual inflorescences are produced at the branch tips while only female inflorescences are produced in the leaf axils of mature stems (Osmond *et al.*, 1980). *Atriplex prostrata* has only bisexual inflorescences. By removing the bisexual inflorescences from *A. rosea*, we were able to protect the axile flowers from self-pollination and ensure they would receive only pollen produced by *A. prostrata*. Flowers of *A. rosea* were pollinated using an extra-fine paintbrush from August to October, 2011. F₁ hybrid seed was mature when plants senesced in mid-to-late November, 2011. F₁ hybrids were grown in identical environments as the parents, using high pressure sodium lamps to maintain photoperiod at 14 h. These plants flowered beginning in mid-August and were allowed to self-pollinate, with seeds maturing by late-October.

The F₂ hybrids, along with F₁, *A. rosea* and *A. prostrata* plants were grown in a plant growth chamber (Conviron PGC-20, Conviron Ltd., Winnipeg, Manitoba, Canada) at 27°C day/22°C night using the same soil, watering, and fertilizer regime as described above. Photoperiod was 18 hr with a light intensity near 700 μmol m⁻² s⁻¹ during the central 8 hour portion of the
photoperiod, and 200 μmol m\(^{-2}\) s\(^{-1}\) for 4 hours on each side of the high light period. One hour of incandescent light provided 20 μmol m\(^{-2}\) s\(^{-1}\) during the first and last hour of the photoperiod. We selected this photoperiod after preliminary trials showed plants flowered in a 14 h photoperiod. (See Appendix)

1.2.2  Gas exchange, leaf nitrogen and enzyme assays

Gas exchange measurements were conducted on 6 to 10 week old plants, using a recently expanded leaf for all measurements. Leaf disks for enzyme and nitrogen assays were sampled from the leaves used for gas exchange. Carbon isotope ratios of leaf disks from adjacent leaves were determined by the University of Washington Isotope Facility ([http://depts.washington.edu/isolab/](http://depts.washington.edu/isolab/)). Whole leaf gas exchange parameters were measured using a LI-6400 portable photosynthesis system (Li-Cor, Inc., Lincoln, Nebraska, USA) at a leaf temperature of 30 °C (Vogan et al., 2007). For determination of the response of net CO\(_2\) assimilation rate (\(A\)) to intercellular CO\(_2\) content (\(C_i\)), a saturating light intensity of 1500 μmol m\(^{-2}\) s\(^{-1}\) was used for \(A.\) prostrata and 1800 μmol m\(^{-2}\) s\(^{-1}\) for \(A.\) rosea. In the measurement of the \(A/C_i\) response, leaves were first equilibrated to saturating light (1500 μmol m\(^{-2}\) s\(^{-1}\) for \(A.\) prostrata and 1800 μmol m\(^{-2}\) s\(^{-1}\) for \(A.\) rosea) and then measurements were recorded. Subsequently, ambient CO\(_2\) concentration was raised to near 1000 μmol mol\(^{-1}\) to determine the maximum assimilation rate and then reduced in steps to 35 μmol mol\(^{-1}\) for \(A.\) prostrata, 10 μmol mol\(^{-1}\) for \(A.\) rosea and 20 μmol mol\(^{-1}\) for the F\(_1\) and F\(_2\) hybrids. The CO\(_2\) compensation point was calculated using the x-intercept of a linear regression through the lowest 4 to 6 CO\(_2\) concentrations that fell on a linear response of \(A\) versus \(C_i\). This regression was also used to calculate the initial slope of the \(A/C_i\) curve, which is an estimate of carboxylation efficiency (CE). Leaf nitrogen was assayed using a Costech ESC 4010 C:N analyzer by the University of Nebraska Ecosystem Analysis lab, Lincoln, Nebraska (biosci.unl.edu/facilities).

Enzyme assays were conducted at 30 °C for Rubisco and three C\(_4\) cycle enzymes: phosphoenolpyruvate carboxylase (PEPCase), NAD malic enzyme (NAD-ME), and pyruvate phosphate dikinase (PPDK) (Sage et al., 2011). Leaf samples were extracted into 50 mM HEPES buffer (pH 7.8) containing 10 mM MgCl\(_2\), 2 mM MnCl\(_2\), 1 mM EDTA, 2% PVPP (w/v), 1% PVP, 1% BSA, 10 mM DTT, 0.5% (v/v) Triton X-100, 10 mM 6-aminocaproic acid, and 2 mM benzamide. Enzyme activities were assayed with a diode array spectrophotometer by measuring
at 340 nm the reduction of NAD$^+$ (for NAD-ME), or the oxidation of NADH in a coupled enzyme assay (Rubisco, PEPCase, PPDK). NAD-malic enzyme and PEP carboxylase were assayed according to Sage et al., (2011). Rubisco was assayed according to Ashton et al., (1990), with the extract being incubated in the reaction mixture for 10 minutes before the assay to ensure full activation of the enzyme. The PPDK assay was modified from Ashton et al., (1990), with 10 mM KHCO$_3$ replacing NaHCO$_3$ and the concentration of PEPCase being increased to 3 units ml$^{-1}$. All chemicals for enzymes assays with the exception of PEPCase were obtained from Sigma-Aldrich, St. Louis, USA. PEPCase was obtained from Bio-Research Products, North Liberty, Iowa, USA.

1.2.3 Leaf anatomy

For light and transmission microscopy, 2 mm$^2$ samples were cut from the middle region of recently expanded leaves and prepared for microscopy as described by Sage and Williams (1995). Briefly, sections were fixed in 2% glutaraldehyde and 0.5M sodium cacodylate buffer solution (pH 6.9) and post-fixed with a 2% osmium tetroxide solution. Samples were then dehydrated in ethanol increments and embedded in Spurr’s resin. The microscopy samples were obtained from leaves adjacent to those used for gas exchange analyses, and were harvested in the middle of the four-week period when gas exchange data were acquired.

1.3 Results

1.3.1 Generation and growth of the F$_1$ and F$_2$ hybrids

Approximately 80% of $A. \text{rosea}$ flowers that were hand-pollinated with $A. \text{prostrata}$ pollen yielded seed. By contrast, Nobs et al., (1970) reported seed set near 10%. Seedlings of F$_1$ plants were easy to identify as they lacked the red colour present on the bottom $A. \text{rosea}$ leaves. The F$_1$ hybrids produced 50-100 F$_2$ seeds each, similar to the results of Nobs et al., (1970). The germination rate for F$_1$ seeds was over 80%. The growth habit and leaf shape of the F$_1$ hybrids was intermediate between that of the parents and uniform compared to each other, while the F$_2$ hybrids were also intermediate in growth habit but exhibited variable leaf shape (Figure 1-2). Notably, all F$_1$ and F$_2$ hybrids retained female-only inflorescences in the leaf axils, as seen in the maternal parent $A. \text{rosea}$. 
Figure 1-2. Photographs of the *Atriplex* parents, F1 hybrid and F2 hybrids from this study.
1.3.2 Gas exchange results

The F₁ hybrids exhibited a CO₂ compensation point (Γ) near 30 μmol mol⁻¹, in contrast to near 0 μmol mol⁻¹ in *A. rosea* and 50 μmol mol⁻¹ in *A. prostrata*, at 30 °C (Figure 1-3). Representative \( A/C_{i} \) responses for the parents and all hybrids are presented in Figure 1-4. In Figure 1-3A, we show normalized \( A/C_{i} \) responses of the C₃ and C₄ parents, three hybrids, and for comparison, the C₃-C₄ intermediate species *Flaveria floridana*. The normalized curves demonstrate the F₁ and F₂ hybrids had a similar qualitative response as *A. prostrata* and *F. floridana*, with the major exception being that the hybrids had a lower carboxylation efficiency (CE) and CO₂ compensation point (Γ) than *A. prostrata* (Figure 1-3B; Figure 1-5A). The Γ values of the F₂ hybrids ranged from a C₄-like value of 4 μmol mol⁻¹ in F₂-114 to 45 μmol mol⁻¹ in F₂-123; Γ in most F₂ hybrids clustered between 25 to 35 μmol mol⁻¹ (Table 1-1; Figure 1-5). At current air levels of CO₂ (about 400 μmol mol⁻¹ in Toronto), \( A_{400} \) values in the F₂ hybrids ranged between 48% and 67% (average 57%) of the *A. prostrata* value (Table 1-1). At CO₂ saturation, the difference between the mean \( A \) value (\( A_{\text{max}} \)) of the F₂ hybrid lines and *A. prostrata* were less: \( A_{\text{max}} \) in the hybrids ranged between 68% and 89% (mean 77%) of the C₃ values (Table 1-1). The difference in the \( A_{400} \) values between the hybrids and *A. prostrata* was largely due to reduced carboxylation efficiency in the hybrids. The CE values ranged from 32% to 53% (average 44%) of the C₃ value in the F₁ and F₂ hybrids (Table 1-1), and exhibited no relationship with variation in Γ (Figure 1-5A). The δ¹³C of the F₁ and F₂ hybrids ranged from -29.3‰ to -27.6‰, and were consistently more positive than the C₃ mean of -32.2‰ (Figure 1-5B). These values were shifted more negative by approximately 2 ‰ units due to an enriched fossil fuel signature in downtown Toronto, where the growth facilities are located. No relationship was apparent between δ¹³C and either Γ, \( A_{400} \), or \( A_{\text{max}} \), and the CE value (not shown).
Table 1-1. Summary of leaf gas exchange, nitrogen and nitrogen use efficiency parameters for C₃ x C₄ hybrids and their parents grown in plant growth chambers. Means ± SE. N=3 to 6 for gas exchange except for A. rosea (N=2). Abbreviations: ATPR, A. prostrata; ATRO, A. rosea; A₄₀₀, assimilation rate at an ambient CO₂ of 400 μmol mol⁻¹; A_max, assimilation rate at 800 μmol mol⁻¹ CO₂; Cᵢ/Cₐ, ratio intracellular to ambient CO₂ concentration; N, nitrogen content. Carboxylation efficiency is equal to the initial slope of the A versus Cᵢ response. Superscripted x, y or z indicate differences at p <0.05 between the ATPR, ATRO, F₁ hybrid and the pooled mean of all F₂ hybrids. The a, b, c or d letters after each value indicate statistical groups at p <0.05 when all genotypes were compared. Statistical differences at were tested using a one-way ANOVA followed by a Student-Newman-Keuls post-hoc test.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>A₄₀₀</th>
<th>A_max</th>
<th>Cᵢ/Cₐ @ 400</th>
<th>CO₂ compensation point (Γ)</th>
<th>Carboxylation efficiency</th>
<th>Leaf N content</th>
<th>Leaf nitrogen use efficiency (NUE) (=A₄₀₀/leaf N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPR-C₃</td>
<td>31.6±1.2a⁸</td>
<td>37.7±0.7ax</td>
<td>0.80±0.02a⁸</td>
<td>50.5±0.3a²</td>
<td>0.19±0.007a²</td>
<td>175±12a³</td>
<td>182±7ab⁵</td>
</tr>
<tr>
<td>ATRO-C₄</td>
<td>31.2±0.0ax</td>
<td>32.8±0.6abc³⁻</td>
<td>0.57±0.09b⁹</td>
<td>-2.2±0.2d⁶</td>
<td>0.783±0.085c⁸</td>
<td>143±14a³</td>
<td>221±22a³</td>
</tr>
<tr>
<td>F₁</td>
<td>16.0±1.0bc³⁻</td>
<td>25.6±2.0ce⁻</td>
<td>0.81±0.01ax</td>
<td>32.1±4.9c⁸</td>
<td>0.065±0.008c⁸</td>
<td>156 (n=1)</td>
<td>115 (n=1)</td>
</tr>
<tr>
<td>F₂-107</td>
<td>20.2±1.1bc</td>
<td>31.2±1.1bc</td>
<td>0.80±0.02a</td>
<td>25.1±1.5c</td>
<td>0.100±0.007b</td>
<td>130±7a</td>
<td>131±5b</td>
</tr>
<tr>
<td>F₂-108</td>
<td>17.9±1.0bc</td>
<td>30.7±1.0bc</td>
<td>0.76±0.02a</td>
<td>27.8±1.2c</td>
<td>0.088±0.004b</td>
<td>164±14a</td>
<td>110±19b</td>
</tr>
<tr>
<td>F₂-109</td>
<td>20.5±0.8bc</td>
<td>32.1±0.9abc</td>
<td>0.81±0.01a</td>
<td>31.1±2.3c</td>
<td>0.094±0.004b</td>
<td>130±18a</td>
<td>164±26ab</td>
</tr>
<tr>
<td>F₂-112</td>
<td>15.3±2.1c</td>
<td>27.7±2.8bc</td>
<td>0.79±0.07a</td>
<td>31.1±1.5c</td>
<td>0.069±0.007b</td>
<td>147±25a</td>
<td>104±47b</td>
</tr>
<tr>
<td>F₂-114</td>
<td>16.0±0.4bc</td>
<td>26.6±0.3c</td>
<td>0.80±0.01a</td>
<td>3.8±2.4d</td>
<td>0.061±0.004b</td>
<td>125±4a</td>
<td>125±3.6b</td>
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<tr>
<td>F₂-118</td>
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<td>25.4±1.3c</td>
<td>0.76±0.06a</td>
<td>31.2±1.1c</td>
<td>0.084±0.007b</td>
<td>133±6a</td>
<td>124±15b</td>
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<tr>
<td>F₂-119</td>
<td>15.9±3.1bc</td>
<td>28.6±4.2bc</td>
<td>0.71±0.06a</td>
<td>38.3±0.8bc</td>
<td>0.077±0.010b</td>
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<td>110±16b</td>
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<td>F₂-120</td>
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<td>26.5±3.8c</td>
<td>0.083±0.004b</td>
<td>154±6a</td>
<td>115±8b</td>
</tr>
<tr>
<td>F₂-123</td>
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<td>33.5±0.7ab</td>
<td>0.81±0.01a</td>
<td>44.6±1.0ab</td>
<td>0.102±0.004b</td>
<td>149±7a</td>
<td>147±8b</td>
</tr>
<tr>
<td>All F₂</td>
<td>18.0±0.5⁷</td>
<td>29.2±0.6⁷</td>
<td>0.79±0.01⁷</td>
<td>29.5±2.0⁷</td>
<td>0.08±0.003⁷</td>
<td>145±4⁸</td>
<td>126±5⁸</td>
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</tbody>
</table>
Figure 1-3. The response of net CO₂ assimilation rate, $A$, to intercellular CO₂ ($C_i$) at 30°C and 1500 μmol photons m⁻² s⁻¹ for the two *Atriplex* parents, an F₁ hybrid and F₂ hybrids. (A) Normalized net CO₂ assimilation rate for the *Atriplex* parents, the C₃-C₄ intermediate *Flaveria floridana*, an F₁ hybrid, and the F₂ hybrids 114 and 123. (B) The low CO₂ portion of the $A$ versus $C_i$ response illustrating CO₂ compensation points and initial slopes for all hybrids in the study. Results shown are representative responses of 3 to 6 $A$ versus $C_i$ measurements for the hybrids and *A. prostrata*, and two measurements of *A. rosea*. See Figure 1-4 for the non-normalized $A/C_i$ responses of each hybrid.

Figure 1-4. The response of net CO₂ assimilation rate to intercellular CO₂ partial pressure for the *Atriplex* parents and $C_3 \times C_4$ hybrids in this study.
Figure 1-5. The relationship between (A) the carbon isotope ratio as a function of the CO$_2$ compensation point of $A$ ($\Gamma$) and (B) the carboxylation efficiency (CE) and $\Gamma$ in *Atriplex prostrata* (C$_3$, ■), *A. rosea* (C$_4$, ▲), an F$_1$ hybrid (♦), and F$_2$ hybrids (○). Some error bars are obscured by the symbols.
1.3.3 Leaf nitrogen content and nitrogen use efficiency

Although the C₄ parent and all hybrids lines exhibited lower leaf nitrogen content than the C₃ parent, none of their means were significantly different (Table 1-1). Differences in leaf nitrogen use efficiency (NUE) between the C₃ and C₄ species could not be statistically resolved, while each hybrid line except F₂-109 has a significantly lower NUE than the C₄ parent (Table 1-1). On average, the mean NUE of all the F₂-hybrids was 31% less than the C₃ mean and 43% less than the C₄ value.

1.3.4 Enzyme activity

The Rubisco activity of the F₁ and F₂ hybrids was 30% to 50% of the C₃ value (Table 1-2). When the CE of each hybrid was plotted against its corresponding Rubisco activity, the hybrid values cluster around the theoretical relationship between Rubisco and CE in a C₃ species (Figure 1-6). The activities of the three major C₄ cycle enzymes – PEPC, NAD-ME, and PPDK – were generally low in the hybrids and in many cases approached the activity of the C₃ parent (Table 1-2). The F₁ hybrid had significantly higher NAD-ME, PEPC and PPDK activity than the C₃ parent. Five of the nine F₂ hybrids had significantly higher NAD-ME activities than the C₃ parent, while just three had significantly higher PPDK activities than A. prostrata. Differences in PEPC between the F₂ hybrids and the C₃ parent could not be resolved using a one-way ANOVA at p<0.05; however, low statistical power in the test weakened our ability to resolve differences in PEPC activity. Four hybrids exhibited mean PEPC activities that were over twice the C₃ value, and one of these, the F₂-114 with the C₄-like low Γ value, also had elevated activities of NAD-ME and PPDK (Table 1-2).
Table 1-2. The *in vitro* activity of NAD-malic enzyme (NAD-ME), PEP carboxylase (PEPC), pyruvate-phosphate dikinase (PPDK) and Rubisco at 30°C. Mean ± SE, N=4. Abbreviations: ATPR, *A. prostrata*; ATRO, *A. rosea*. Statistical differences between ATPR, ATRO, F₁ and the pooled F₂ means at *p*<0.05 were tested using one-way ANOVA followed by a Student-Newman-Keuls post-hoc test and are shown as superscripts x, y and z. The “*” beside a value indicates means are significantly different from the ATPR activity using a one-way ANOVA followed by a Holm-Sidak post-hoc test where the ATPR mean was treated as the control value.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NAD-ME</th>
<th>PEPC</th>
<th>PPDK</th>
<th>Rubisco</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPR-C₃</td>
<td>2.0±1.1d²</td>
<td>12.5±1.4z²*</td>
<td>2.2±1.2z</td>
<td>156.7±5.1x</td>
</tr>
<tr>
<td>ATRO-C₄</td>
<td>39.9±4.3x*</td>
<td>223.2±19.1xy*</td>
<td>43.0±5.1x²</td>
<td>42.3±3.4z²*</td>
</tr>
<tr>
<td>F₁</td>
<td>7.8±0.8y²*</td>
<td>55.9±7.9z²*</td>
<td>16.0±0.6z²*</td>
<td>74.0±6.3y²*</td>
</tr>
<tr>
<td>F₂-107</td>
<td>11.2±0.2*</td>
<td>32.9±6.3</td>
<td>3.1±1.4</td>
<td>79.9±6.8*</td>
</tr>
<tr>
<td>F₂-108</td>
<td>11.8±1.1*</td>
<td>27.4±5.9</td>
<td>2.8±0.7</td>
<td>69.5±8.9*</td>
</tr>
<tr>
<td>F₂-109</td>
<td>8.2±1.6</td>
<td>15.8±3.8</td>
<td>3.2±0.9</td>
<td>48.4±7.9*</td>
</tr>
<tr>
<td>F₂-112</td>
<td>9.7±0.8*</td>
<td>15.3±4.7</td>
<td>4.0±1.7</td>
<td>65.7±12.5*</td>
</tr>
<tr>
<td>F₂-114</td>
<td>9.6±1.3*</td>
<td>26.8±3.0</td>
<td>15.3±1.8*</td>
<td>68.2±5.4*</td>
</tr>
<tr>
<td>F₂-118</td>
<td>4.5±1.8</td>
<td>20.7±2.1</td>
<td>11.7±3.7*</td>
<td>68.4±6.0*</td>
</tr>
<tr>
<td>F₂-119</td>
<td>9.1±1.1</td>
<td>28.8±6.6</td>
<td>2.8±1.0</td>
<td>64.5±4.8*</td>
</tr>
<tr>
<td>F₂-120</td>
<td>12.0±0.8*</td>
<td>27.7±2.0</td>
<td>3.7±0.6</td>
<td>77.3±2.1*</td>
</tr>
<tr>
<td>F₂-123</td>
<td>6.6±0.7</td>
<td>13.2±3.3*</td>
<td>72.7±0.3*</td>
<td></td>
</tr>
<tr>
<td>All F₂</td>
<td>9.1±0.6y</td>
<td>24.7±1.7²</td>
<td>6.9±1.1z</td>
<td>69.5±2.9y</td>
</tr>
</tbody>
</table>
Figure 1-6. The carboxylation efficiency of photosynthesis as a function of *in vitro* Rubisco activity for the C₃ species *Atriplex prostrata*, an F₁ hybrid and all F₂ hybrids in the study. Carboxylation efficiencies were calculated as the initial slope of the \(A\) versus \(C_i\) response for each genotype. Mean ± 3 to 6. The line is the theoretical carboxylation efficiency predicted for C₃ Rubisco activity using the model of von Caemmerer (2000) and assuming the Rubisco activation state is 80\%, \(\Gamma^*\) equals that of spinach at 30°C, (Brooks and Farquhar, 1985) and the Rubisco kinetics and activation energies for the C₃ *Atriplex glabriuscula* equal those of *A. prostrata* (von Caemmerer and Quick, 2000).
1.3.5  Leaf anatomy

The leaf anatomy (Figure 1-7A, B) and ultrastructure (Fig. 1-8A, B) of *Atriplex prostrata* and *A. rosea* were typical for C3 and C4 members of the genus (Downton *et al.*, 1969; Dengler *et al.*, 1995). *Atriplex rosea* has well-developed BS cells that are discontinuous on the abaxial side of the vein (see also Liu and Dengler, 1994). In cross-section, BS cells are triangular in shape which allows them to be tightly packed against the vein. Enlarged chloroplasts occupy the centripetal half of the BS cells in *A. rosea*, while no chloroplasts occur the outer-most region of the cells (Figs. 1-7B, 1-8B). This is typical for the Atriplicoid-type of Kranz anatomy (Dengler and Nelson, 2000). In *A. prostrata*, BS chloroplasts are smaller than in the C4 plants and the chloroplasts are generally positioned along the outer periphery of the BS cell opposite intercellular air spaces. In cross section, chloroplasts were infrequent along the inner, centripetal wall of the BS cells of *A. prostrata*, and the individual BS cells were generally circular in outline. The BS cells of the F1 and F2 hybrids are variable in size and shape yet typically intermediate in structure between the C3 and C4 condition (Figure 1-7, 1-8; 1-9). Many of the BS cells of both F1 and F2 hybrids are oval in cross section, in contrast to the circular BS cells of *A. prostrata* and the triangular BS cells of *A. rosea*. This pattern resembles that observed in an immature leaf in *A. rosea* (see Fig. 4 in Liu and Dengler, 1994). In all hybrids, BS chloroplasts were numerous and arrayed all around the BS cell periphery (Figure 1-7, 1-8). Chloroplast size and shape in the BS of the F2 hybrids was similar to what was observed in the BS of *A. prostrata* (Figure 1-7, 1-8). In the BS cells of the hybrids, mitochondria occurred between chloroplasts, but did not form distinct ranks between elongated chloroplasts as observed in *A. rosea* (Figure 1-8).
Figure 1-7. Light micrographs of cross-sections through leaves of (A) *Atriplex prostrata*, (B) *Atriplex rosea*, (C) their F₁ hybrid, (D) F₂-108, (E) F₂-114, and (F) F₂-123. See Figure 1-9 for light micrographs of leaf cross sections for the other six hybrids in the study. The “*” delineates bundle sheath cells, the “m” mesophyll cells and the “v” vascular bundles. Bars = 50 µm.
Figure 1-8. Transmission electron micrographs of bundle sheath cells in cross section of (A) *Atriplex prostrata*, (B) *Atriplex rosea*, (C) F2-114, and (F) F2-123. Arrows delineate mitochondria. Abbreviations: c, chloroplasts, m, mesophyll cells, n, nucleus, and v, vascular tissue. Bars = 0.5 µm.
Figure 1-9. Light micrographs of cross-sections through leaves of six *Atriplex prostrata* x *Atriplex rosea* F₂ hybrids from this study. The “*” delineates bundle sheath cells, the “m” mesophyll cells and the “v” vascular bundles. Bars = 50 µm.
1.4 Discussion

Results from this study and prior research with C₃ x C₄ hybrids demonstrate that C₄ photosynthesis is disrupted in the hybrids, as shown by a general increase in the CO₂ compensation point, a reduction in CE and NUE, and the expression of a C₃-like δ¹³C (Björkman *et al*., 1971b, Björkman, 1976; Osmond *et al*., 1980; Brown and Bouton, 1993). In the hybrids generated here, we observed that the F₁ and most F₂ hybrids exhibited Γ values in the mid-range between C₃ and C₄ species. However, one F₂ line (#114) exhibited Γ values that overlap with those of C₄-like species such as *Flaveria brownii* that have a fully functional C₄ cycle (Ku *et al*., 1991). A second F₂ (#123) had Γ approaching the C₃ range. In prior studies, F₁ hybrids exhibit intermediate Γ values; these were interpreted to reflect a mix of C₃ and C₄ biochemistry in the F₁ leaf (Pearcy and Björkman, 1971). The F₁ hybrids are diploid with one set of chromosomes from each parent, and therefore have one C₃ copy and one C₄ copy of each gene, resulting in the mixed physiology (Osmond *et al*., 1980). In F₂ lines, trait segregation is apparent, and hybrids likely lose one or more of the genes essential for C₄ function (Osmond *et al*., 1980; Brown and Bouton, 1993). In F₃ lines, further segregation leads to most hybrids exhibiting C₃-like photosynthetic characteristics (Björkman *et al*., 1971a, b). Occasionally, however, F₃ hybrids exhibit Γ values close to the C₄ value (Björkman, 1976), which is consistent with results from F₂-114. Previous hybrid studies indicate that all parts of the C₄ biochemical cycle and Kranz anatomy must be present for efficient C₄ function (Björkman, 1976; Brown and Bouton, 1993). Since these traits independently segregate (Brown and Bouton, 1993), the probability of an F₂ hybrid acquiring all of the C₄ traits is low, and hence, it is unlikely that full C₄ photosynthesis can occur. However, Γ values in the mid-range between C₃ and C₄ plants demonstrate the existence of a CO₂ concentrating mechanism (CCM) in the F₂ lines. This could result from either a modest C₄ metabolic pump or a C₂-type CCM where photorespiratory glycine is shuttled into the BS cells (Brown and Bouton, 1993). With new hybrids, we are now in a position to evaluate these possibilities and develop working hypotheses to guide future hybrid studies. In our discussion of the F₂ hybrids, we mainly focus on F₂-114, whose C₄-like Γ value indicates greater CCM activity.

In F₂-114, the low, C₄-like Γ is indicative of significant C₄ cycle activity and/or a highly effective C₂ CCM. F₂-114 had activities of PEPC, PPDK and NAD-ME that were 12% to 30% of the C₄ values, indicating the potential for a modest C₄ cycle that could contribute to a reduction in Γ by
supplying some CO₂ to the BS. All other F₂ hybrids in this study had C₃-like activities in at least one of these enzymes, indicating low potential for more than minor C₄ cycle activity. As shown by Type II C₃-C₄ intermediates (those with significant C₂ photosynthesis and C₄ metabolism; Edwards and Ku, 1987), modest C₄ cycle activity combined with a C₂-type of glycine shuttle is sufficient to reduce Γ below 10 µmol mol⁻¹. In the Type II C₃-C₄ intermediate *F. ramosissima*, for example, a Γ of 7 µmol mol⁻¹ was associated with C₄ enzyme activities between 12% and 18% of C₄ values (Ku et al., 1983). A 3.5‰ increase in δ¹³C in F₂-114 relative to *A. prostrata* is also evidence for modest C₄ cycle activity, and is consistent with observed δ¹³C values of Type II intermediates such as *F. ramosissima*, and with modelled increases in δ¹³C assuming a 20% to 30% contribution by PEPC to the BS CO₂ pool and moderate CO₂ leakage (Monson et al., 1988; von Caemmerer, 1992; Sudderth et al., 2007). However, C₄ metabolism could not contribute a large amount of carbon to the final pool of photosynthate in F₂-114, because the δ¹³C values would shift more towards the C₄ values than observed (von Caemmerer, 1992). We therefore hypothesize that the low Γ in F₂-114 reflects a large contribution of a glycine shuttle to the CO₂ pool of its BS cells.

Because C₂ species with no C₄-cycle activity (the type I C₃-C₄ intermediates; Edwards and Ku, 1987) exhibit Γ values above 15 µmol mol⁻¹ (Edwards and Ku, 1987; Ku et al., 1991; Vogan et al., 2007) it seems unlikely that a C₂-type of glycine shuttle could reduce Γ to 4 µmol mol⁻¹ by itself. However, according to the von Caemmerer’s model of C₃-C₄ intermediate photosynthesis (von Caemmerer, 1989), a C₄-like Γ could occur in a pure C₂ species if there is an elevated (20%) fraction of leaf Rubisco in the BS cells, the conductance to CO₂ leakage in the BS is low, and nearly all of the photorespired CO₂ is released into the BS cells. Given the segregation of traits in the F₂ lines (Osmond et al., 1980), it is probable that these criteria could be met in a few hybrids. All of the F₂ hybrids here exhibited Rubisco activities that are a third to a half that of the *A. prostrata* parent, indicating some C₄-type control over Rubisco expression is present in the hybrid lines. C₄ species produce 25%-35% as much Rubisco as C₃ species (Sage et al., 1987), as is demonstrated by lower Rubisco activity in *A. rosea* relative to *A. prostrata*. Although we do not know where the Rubisco is distributed in our hybrids, prior work demonstrates F₁ and F₃ hybrids of *A. rosea* x *A. prostrata* express Rubisco in all chlorenchymatous cells (Hattersley et al., 1977). The high number of plastids in the BS of the F₂ hybrids also indicates significant amounts of the Rubisco are present in their BS chloroplasts. With respect to BS conductance, we
hypothesize that some hybrids, perhaps including F2-114, have inherited traits contributing to low, C₄-like conductance in the BS, such as thick BS cell walls (von Caemmerer and Furman, 2003). It is also likely that there is a high fraction of photorespiratory CO₂ released in the BS of most hybrids. In C₄ plants, photorespiratory glycine decarboxylase (GDC) is localized to BS cells, while in C₃ plants, GDC and the photorespiratory cycle is expressed in both BS and M tissues (Muhaidat et al., 2011; Sage et al., 2011; Schulze et al., 2013). In an F₂ hybrid, there is a good chance that one or more of the GDC subunits exhibit a C₄ pattern and are not expressed in the M cells, whereas their expression in the BS cells would occur if either the C₄ or C₃ pattern were inherited. Hence, it is probable that GDC activity is low in the M tissues of the F₂ hybrids and high in the BS, so that much of the photorespiratory glycine would have to migrate into the BS for decarboxylation. This would explain why most of the F₂ lines have C₂-like Κ values. Certain lines, such as F₂-123 with a more C₃-like Κ may have a leakier BS or relatively less Rubisco in the BS, while other lines with low Κ such as F₂-114 may have proportionally more BS Rubisco or less BS leakiness, plus some contribution from a C₄ cycle. These possibilities point to a need for enzyme localization and leakage assessments in future hybrid studies.

In most hybrids, it is apparent that the BS Rubisco is adequately supplied with CO₂. When carboxylation efficiency is plotted as a function of Rubisco activity, the hybrid values clustered around the theoretical relationship between Rubisco activity and carboxylation efficiency of a C₃ Atriplex-like plant (Figure 1-6). This demonstrates that in most hybrids, Rubisco is on average operating with the same efficiency as in a C₃ leaf. The CE of F₂-109 sits well above the CE versus Rubisco activity plot, which would occur if much of its Rubisco is in a CO₂-enriched environment. The low PEPC and PPDK activity in F₂-109 indicates the reduction of Κ below C₃ values is predominately due to CO₂ influx into the BS via C₂ photosynthesis. Hybrid F₂-114 exhibits the lowest CE relative to the theoretical CE versus Rubisco plot, demonstrating that at least some of its Rubisco is operating with reduced efficiency. Low CO₂ levels in the BS would reduce CE, but this would not result in the low Κ value of F₂-114 because Rubisco oxygenase activity would increase at low CO₂ and raise Κ. Alternatively, Rubisco may be limited by low RuBP regeneration capacity, or a low activation state due to a lack of Rubisco activase. Low RuBP regeneration might result if a C₄ pattern of thylakoid protein expression corresponded to a C₃ pattern of Calvin cycle expression, in which case one of the C₃ compartments could be energy limited. The potential lack of activase expression is an intriguing possibility that could not be
considered in the first era of *Atriplex* hybrid studies, as activase was unknown at the time. In C₄ plants, activase expression is four times higher in the BS than M tissue (Majeran *et al.*, 2005). In the hybrids, a C₄-like pattern of activase expression could leave Rubisco in the M cells in a partially deactivated state. This would explain the low CE in F₂-114, as the M Rubisco could be deactivated and unable to contribute to the CE values.

### 1.4.1 Anatomical patterns

Anatomically, all of the hybrid lines failed to express the well-developed Atriplicoid-type of Kranz anatomy, as has been noted before (Boynton *et al.*, 1970). Atriplicoid Kranz anatomy consists of enlarged BS cells with a surrounding layer of M cells (Liu and Dengler, 1994; Dengler and Nelson, 1999). Chloroplasts in the BS cells of *A. rosea* are elongated and fill the inner two-thirds of the BS, and have many mitochondria distributed along the sides of the chloroplasts (Fig. 6). No chloroplasts or mitochondria occur along the outer BS wall of C₄ *Atriplex* species. This arrangement allows for rapid re-assimilation of CO₂ released by NAD-ME in the mitochondria, with the vacuole of the outer BS providing significant resistance to CO₂ efflux (von Caemmerer and Furbank, 2003). By contrast, the C₃ *Atriplex prostrata* produces small BS chloroplasts that are similar to M cell chloroplasts; these occur along the outer wall of the BS cell against the intercellular air spaces (Boynton *et al.*, 1970). In all the hybrids, the BS chloroplasts are similar in size and shape to those of the C₃ parent, yet their positioning resembles a pattern that is often observed in C₂-type species, where chloroplasts can occur in both a centripetal and centrifugal position (Muhaidat *et al.*, 2011; Sage *et al.*, 2013). Mitochondria still occur between chloroplasts, but to less of a degree than seen in *A. rosea*. Many of the mitochondria in the hybrids also appear between the inner BS wall and the chloroplasts, resembling a pattern apparent in C₃-C₄ intermediate plants using the C₂-type of CCM (Monson and Rawsthorne, 2000; Sage *et al.*, 2011; 2013). These observations further indicate that the BS cells of the F₂ hybrids use the C₂ mode of photosynthesis, although this will depend upon whether enough GDC is present in the BS mitochondria to create a strong sink for glycine produced in the M tissue.
1.4.2 Stomatal control

Previous work with C\textsubscript{3} x C\textsubscript{4} hybrids did not emphasize stomatal regulation, due in part to incomplete understanding at the time of stomatal regulation in C\textsubscript{3} and C\textsubscript{4} species. It is now known that non-stressed C\textsubscript{3} species regulate \(C_i/C_a\) to generally be between 0.7 to 0.8 under humid conditions, while in C\textsubscript{4} plants, \(C_i/C_a\) is maintained between 0.4 to 0.6 (Wong et al., 1979; Taylor et al., 2011; Vogan and Sage, 2011). The lower \(C_i/C_a\) in C\textsubscript{4} species reflects tighter stomatal control and increased carboxylation efficiency of the C\textsubscript{4} pathway relative to C\textsubscript{3} photosynthesis; this explains the greater water use efficiency of C\textsubscript{4} plants (Huxman and Monson, 2003; Vogan and Sage, 2011). Under the relatively low vapor pressure difference between leaf and air in this study, we observed \(C_i/C_a\) to be 0.57 in A. rosea and 0.80 in A. prostrata. In the hybrids, the \(C_i/C_a\) values have largely reverted to the C\textsubscript{3} value (\(C_i/C_a\) of 0.71 to 0.81), indicating that a full complement of C\textsubscript{4} machinery is required for a C\textsubscript{4} pattern of stomatal control.

1.5 Conclusions

With the new C\textsubscript{3} x C\textsubscript{4} hybrids in Atriplex, we have re-established an important system for investigating the genetic control and physiological function of C\textsubscript{4} photosynthesis. In the F\textsubscript{2} lines, we demonstrate a loss of efficient C\textsubscript{4} function, further supporting the hypothesis that all of the components of the C\textsubscript{4} pathway must be in place for C\textsubscript{4} photosynthesis to occur. While impairment of C\textsubscript{4} photosynthesis in the F\textsubscript{2} hybrids is no surprise, an intriguing observation is that improper assembly of the C\textsubscript{3} pathway is also apparent in most F\textsubscript{2} hybrids. This may reflect incomplete expression of the photorespiratory pathway in the M cells of the hybrids, or mismatched compartmentalization of C\textsubscript{3} photosynthetic components. Ironically, with the incomplete assembly of the C\textsubscript{3} and C\textsubscript{4} conditions in the F\textsubscript{2} lines, the default state appears to be C\textsubscript{2} photosynthesis, for what appears to be a rather simple reason. Because both C\textsubscript{3} and C\textsubscript{4} plants express GDC in the BS (Schulze et al., 2013), the probability is high that GDC of the F\textsubscript{2} hybrids is abundant in the BS cells, while GDC levels in the M cells may be low due to inheritance of C\textsubscript{4} expression patterns for at least one of the four GDC subunits. Hence, glycine would have to flow to the BS for decarboxylation, to the benefit of Rubisco in the BS chloroplasts.

We have now successfully generated the F\textsubscript{3} hybrids and will be producing F\textsubscript{4} lines and beyond in order to further segregate traits and possibly create near isogenic lines. With the analytical capabilities provided by modern tools and theory, we are better positioned to evaluate genetic,
biochemical and structural limitations affecting photosynthesis in the hybrids and hence provide critical information that can be utilized to engineer C₄ photosynthesis into C₃ crops as well as understand the evolution of C₄ photosynthesis. These were the initial goals of Olle Björkman, John Boynton, Malcolm Nobs, and Bob Pearcy in the late 1960s when the initial hybrids were created. In the near future, these goals may be realized.
References


Appendix

This Appendix is a more detailed account of photoperiodicity in the *Atriplex* parents and hybrids, as well as the pollination process.

**Photoperiodicity**

Preliminary trials indicated flowering in *A. prostrata* was photoperiodically controlled to begin in late August (photoperiod less than ~13.5 h), while *A. rosea* flowered when plants were approximately 8 weeks old although this is increased when the photoperiod exceeds 15 h and reduced when the photoperiod is less than 13 h. To accommodate these two flowering schedules, we planted *A. prostrata* in early spring and *A. rosea* in mid-summer, thereby allowing for robust flowering by individuals of each species in early September. Another possible way to achieve synchronized flowering would be to grow both parents in a growth chamber under a long photoperiod (16 hours or more) and then reduce this to ~12 hours.

The photoperiod of the F1 hybrid appears to be similar to that of *A. rosea*, although a photoperiod of greater than 15 h appeared to entirely suppress flowering, even reverting flowering stems back to a vegetative state. The photoperiod of the F2 hybrids is highly variable, but many of them are more prone to flowering than either *A. prostrata* or *A. rosea*. A 16 h photoperiod was insufficient for suppressing flowering in all F2 hybrids, but an 18 h photoperiod was sufficient. The 16 h photoperiod did appear to suppress flowering in *A. rosea*.

**Pollination**

To prevent self-pollination by *A. rosea*, bisexual inflorescences must be removed daily, as soon as they became visible at the tips of branches. Daily removal is necessary because bisexual inflorescences continue to form on secondary and tertiary branches of the plant as flowering progressed. In the leaf axils of tertiary branches, single male flowers on a short stalk often formed alongside the female axile inflorescence, and care was taken to remove these as well. The success of this daily removal could be observed by the failure of female flowers to set seed; seed set was indicated by swelling of the ovary within the bracteoles. It was also observed that after more than a week of continued removal of bisexual inflorescences, unfertilized female flowers
grew large, with a spreading of the bracteoles (usually tightly closed) and sometimes with multiple flowers forming within one pair of bracteoles (instead of just one).

A subset of female inflorescences was selected for daily manual pollinations. The same inflorescences were pollinated repeatedly to ensure that all flowers were pollinated at least once; the flowers in a given inflorescence become receptive over a period of at least a few days. The most effective method of transferring pollen by hand from *A. prostrata* was using an extra-fine soft-bristled paintbrush, as judged by observing pollen present on *A. rosea* stigmas after pollen transfer, using a 10x hand lens.

Due to the laborious nature of daily removal of all bisexual inflorescences, it is strongly recommended that a limited number of *A. rosea* plants be used as parents. Given the success rate of the hand pollinations (~80% seed set) one plant in theory should be enough, although having a second plant as insurance would also be fine. Any plants beyond that number would make the daily removal of bisexual inflorescences challenging and increase the risk of *A. rosea* self-pollination.

The F₁ hybrids and relatively small number of F₂ hybrids that I observed retained the flowering habit of *A. rosea*, having female-only axile inflorescences. This property could be utilized in performing backcrosses to the C₃ parent.