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Project Title: "The role of carbonic anhydrase in C_4 photosynthesis*"* – Anthony Studer
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Aims & Objective Update (Year 3)

Aim1: CA mutant analysis in maize
By generating knockouts of maize carbonic anhydrase (CA) genes, we will be able to assay the plant response to limited catalyzation of the first reaction in C_4 photosynthesis. We will use both physiological measurements to assay CA activity and RNA-seq experiments to explore the network of interactions involved in the beginning steps of C_4 photosynthesis.

Objective 1.1 Insertional mutagenesis of CA genes in maize.
Insertions in the tandemly arranged CA genes were generated in funding years 1-2. The results from Objective 1.1 are published in Studer *et al.* 2014 (doi: http://dx.doi.org/10.1104/pp.114.237602). However, while *ca1* single and *ca1ca2* double mutants were identified, a *ca2* single mutant was not recovered. Therefore, to generate a *ca2* single mutant, a screen for recombination events was performed on a population derived from selfing a heterozygous *ca1ca2* double mutant. From this recombination screen, three recombinant plants were identified. Two contained *ca1* single mutants alleles, and one plant contained a *ca2* single mutant allele. The recovered *ca2* single mutant was used for further characterization in Objective 1.2. Three recombination events in 192 plants (384 chromosomes) would indicate that the genetic distance between *Ca1* and *Ca2* in maize is ~0.008cM. An independently derived *ca1ca2* double mutant line was screened for recombination events. Of the 331 plants analyzed (662 chromosomes) seven recombinant chromosomes were identified. Consistent with the previous result, this screen indicated that the genetic distance between *Ca1* and *Ca2* in maize is ~0.01cM.

Objective 1.2 Physiological characterization of *ca* mutants.
The preliminary physiological characterization of the *ca* mutants was completed in funding years 1-2. The results from Objective 1.2 are published in Studer *et al.* 2014 (doi: http://dx.doi.org/10.1104/pp.114.237602). As indicated in the previous annual report, Objective 1.2 was expanded to include a detailed characterization of the *ca* mutants in response to changing environmental conditions. This was a focus in funding year 3.

With funds from this postdoctoral fellowship, Dr. Studer, traveled to Washington State University and trained in Dr. Asaph Cousin's. This not only provided a multidisciplinary training opportunity, but also the means to performed further physiological characterization of the *ca* mutants. Specifically, time course experiments were performed with wildtype, *ca1, ca2,* and *ca1ca2* mutant plants that monitored photosynthesis and stomatal conductance in response to changes in CO_2 and light. Gas exchange experiments were complimented with biochemical assays of enzyme activities and measurements of the stable isotopic composition of leaf material. There were three main results from these experiments. 1) Mutant plants are completely rescued at 800ppm CO_2 (the projected CO_2 concentration at the end of the century), and wildtype plants have similar rates of photosynthesis at 400ppm and 800ppm CO_2 (Figure 1, top panel). Thus, as CO_2 concentrations rise in the atmosphere, maize will not see an increase in rates of

photosynthesis. 2) Unlike *ca* mutants in Arabidopsis, *ca* mutants in maize can rapidly sense changes in CO_2 concentration. However, all mutant plants (but seen most dramatically in the *ca1ca2* double mutant) have a defect in stomatal closing in response to both increased CO_2 and light (CO_2 response shown in Figure 1, bottom panel). This indicates that CA plays an important role in the mechanism of stomatal closure. 3) *ca1* and *ca1ca2* plants have lower rates of photosynthesis and higher stomatal conductance than wildtype plants. Interestingly, rates of photosynthesis in *ca2* mutant plants are similar to wildtype, but *ca2* mutants have an increased stomatal conductance. This indicates that *ca2* does not contribute significantly to photosynthesis, but rather is specific to stomatal movement. With these mutants the demand for CO_2 by the photosynthetic machinery has been decoupled from stomatal control. Further analysis of *Ca2* and its subfunctionalization may provide insight into the link between stomatal movement and photosynthesis in C_4 species.

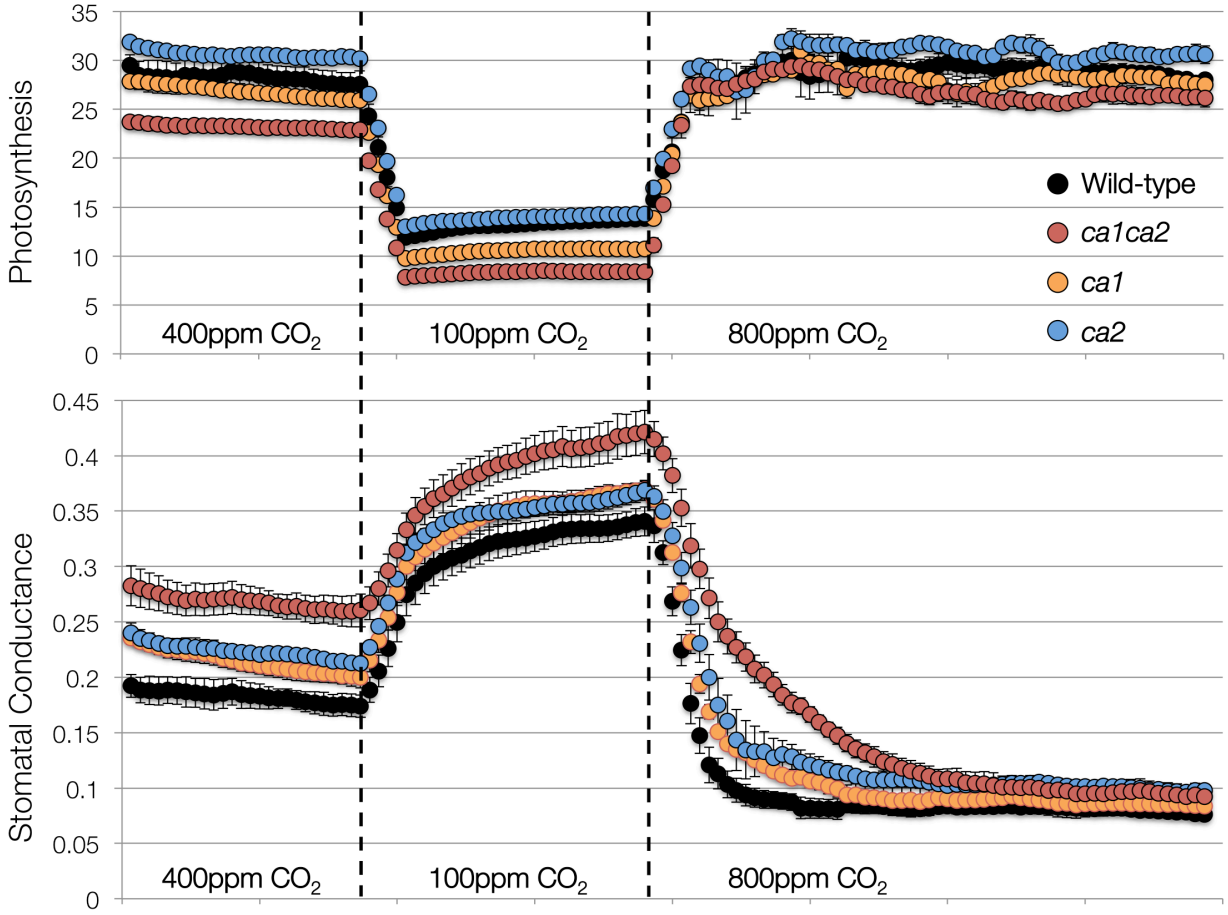

Figure 1: Time course data showing net photosynthesis and stomatal conductance in response to changes in CO_2 concentration. Data was collected for both *ca1* and *ca2* single mutants, as well as the *ca1ca2* double mutant.

Objective 1.3 RNA-seq analysis of *ca* mutants.

To fully leverage the genome of *D. oligosanthes*, which was sequenced with funds from this fellowship, and to fully mine existing RNA-seq data, Objective 1.3 was changed to investigate the expression profile of known C_4 genes across the developing leaf in *D. oligosanthes*, *S. viridis*, and *S. bicolor*. This comparative genomics approach leveraged the close phylogenetic

relationship of the C_3 panicoid grass (*D. oligosanthes*) and two independent evolutions of C_4 photosynthesis *S. viridis* and *S. bicolor*. Several inferences about the evolution of C_4 photosynthesis were made, and will be published with the *D. oligosanthes* genome (manuscript in prep).

Aim 2: Cell biology of CA
Using fluorescently tagged proteins, the localization of different CA paralogs in maize will be assayed. These results will be used in connection with the results from Objective 1 to elucidate the role of CA in C_4 photosynthesis.

Objective 2.1 Subcellular localization of CA
In funding years 1-2, a YFP-tagged *Ca1* cDNA constructs driven with a native promoter was stably transformed into *S. viridis*, and the protein was localized to the cytosol. A YFP-tagged maize *Ca2* cDNA construct was built, however, stable transgenic maize lines were not recovered. Due to the slow development of transgenic lines, other constructs were not pursued because they would fall outside the timeframe of the fellowship.

Objective 2.2 Analysis of CA splice isoforms
It was discovered in funding years 1-2 that the most highly expressed *Ca* transcript undergoes an alternative splicing event that removes the chloroplast transit peptide. Localization of CA to the cytosol of mesophyll cells is a necessary step in the evolution of C_4 photosynthesis. Preliminary data shows that C_3 and C_4 grass species transcribe both splice variants of *Ca1*. YFP-tagged constructs for both isoforms were stably transformed into *S. viridis* in funding year 1-2, but both isoforms were present in the cytosol. It is unclear whether the chloroplast transit peptide is nonfunctional in *S. viridis*, or if the construct backbone is some how interfering with the transcription/translation of the transit peptide. Additional constructs could not be completed within the fellowship timeframe.

However, RNA-seq data was collected on a diverse set of maize inbred lines, and the ratio of the two *Ca1* splice isoforms was calculated (Figure 2). While a large amount of variation was observed, all of the splicing ratios were higher than that observed for C_3 species (<0.2). Because within species variation exists for the splicing ratio, it may be possible to identify the elements that control the difference in the splicing ratio between maize inbreds.

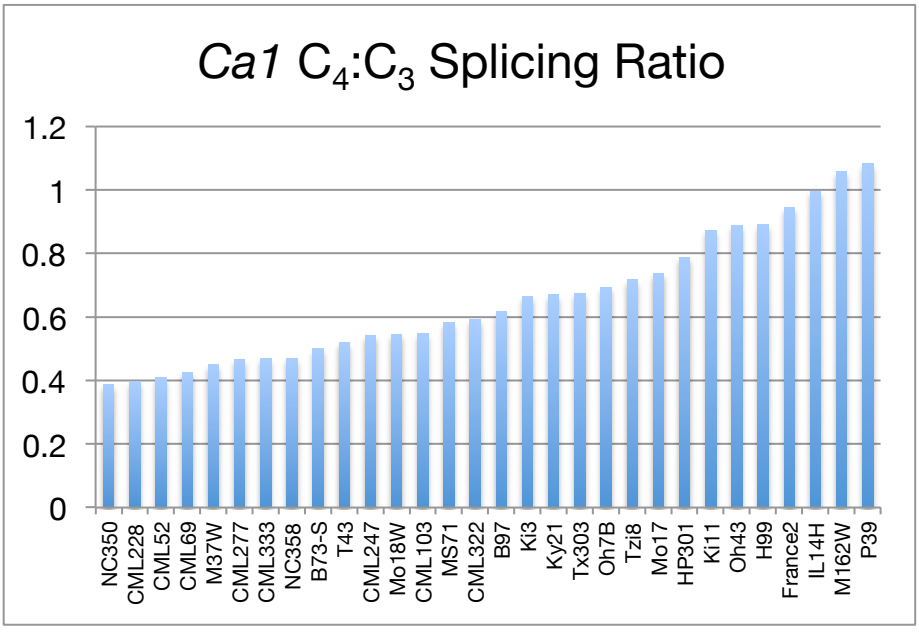

Figure 2: Ratio of the two *Ca1* splice isoforms across a diverse set of maize inbreds. The putative C_4 isoform does not have a chloroplast transit peptide, while the C_3 isoform does.

Aim 3: Utilizing *S. viridis* as a model C_4 grass
Experiments using S. viridis will be performed in parallel with maize in an effort to validate S. viridis as a relevant C_4 grass model. Because experiments in S. viridis can be performed at an accelerated rate compared to maize, a more comprehensive understanding of CA cell biology as well as a functional dissection of CA could be achieved if this system proves applicable.

Objective 3.1 Characterization of CA in *S. viridis*
In funding year 1-2 molecular and *in silico* approaches were used to annotate and characterize the *Ca* gene copies in *S. viridis*. The time needed to generate stable setaria transformants was underestimated, and thus, transgenic experiments aimed at localizing each paralog could not be completed before the end of the fellowship.

Objective 3.2 Co-localization of CA in *S. viridis*
As stated in the last annual report, Objective 3.2 was changed to focus on aquaporin localization. A CFP-tagged aquaporin construct was stably transformed into *S. viridis* to determine if it is localized to the plasma membrane of mesophyll cells. The cDNA sequence of the aquaporin was driven by its native promoter. T_1 plants were recovered and plants positive for the transgene were observed under the confocal microscope. Unfortunately, any potential expression could not be distinguished from background autofluorescence, which has significant overlap with the CFP excitation and emission wavelengths. Native promoters have not produced strong expression of the transgenes and may not be the best way to assay localization.

Objective 3.3 Engineering CA in *S. viridis*
Physiological characterization of the *ca2* single mutant in maize suggests that *Ca2* is expressed in stomata. A construct was designed to express a YFP-tagged maize *Ca2* cDNA sequence driven by its native promoter. This construct was transformed into *S. viridis*, which lacks the *Ca2* gene duplication, to test whether it would be expressed in the same cell type and if it would change the gas exchange profile of the transgenic plants. T_0 plants were examined under the confocal microscope, but no YFP signal was observed. However, analysis of T_0 plants does not always produce reliable results. The T_1 plants need to be evaluated for expression. Unfortunately, T_1 plants could not be propagated before the end of the fellowship.

Budget Summary
Funds were reallocated from *Salary* to *Supplies* because of the early termination of the fellowship. A final balance can be provided after the Danforth Center accounting departments closes the month of May.

Table 1. Budget Expenditures: Years 1-2

Budget Item	Year 1	Year 2	Year 3
Salary	$45,000.00	$50,000.00	$39,586.59
Healthcare/Fringe Benefits	$2,000.00	$2,000.00	$2,000.00
LSRF Annual Meeting	$1,140.44	$443.12	$829.60
DOE			$1,239.42
Supplies	$2,681.00		$11,551.40
Library Construction/Sequencing	$2,530.00	$4,500.00	
Total	**$53,351.44**	**$56,943.12**	**$57,702.33**

Accomplishments: (major activities, significant results, major findings/conclusions, key outcomes, other achievements):

The postdoctoral fellow, Dr. Studer, was offered and accepted an Assistant Professor position at the University of Illinois in Urbana-Champaign. There he will be starting his own research program investigating the genetic control of various aspects of photosynthesis. Initial research, inspired by Objective 1.2, will focus on tradeoff between CO_2 uptake for photosynthesis and H_2O lost through leaf transpiration. Tuning this balance may improve water-use efficiency of important crops, such as maize.

Changes in Approach:

- Objective 1.3 was changed to a comparative analysis of RNA-seq data collected on the developing leaf of C_3 and C_4 species. This provides a broader look at C_4 photosynthesis and is not restricted to CA.

Actual or Anticipated Problems, Delays, or Actions:

As anticipated in the previous annual review, the slow transformation of both *S. viridis* and maize limited the number of transgenic experiments that could be completed within the timeframe of this fellowship.

Project Outputs:

a) Publications:

- Studer, A. J., Schnable, J. C., Weissmann, S., Kolbe, A. R., McKain, M. Ying, S., Cousins, A. B., Kellogg, E. A., and Brutnell, T. P. (In prep). The draft genome of *Dichanthelium oligosanthes*: A C_3 panicoid grass species.
- Studer, A. J., Kolbe, A. R., Brutnell, T. P., and Cousins, A. B. (In prep). Impact of changing CO_2 conditions on *ca* mutants in maize.

b) Websites:

- None

c) Networks/collaborations fostered:

- A collaboration with Dr. Asaph Cousins on the physiological characterization of the CA mutants in maize has been continued.

d) Inventions/patent disclosures:

- An invention disclosure was filed with the Danforth Center (Invention Disclosure DDPSC0063) outlining the identification of transcription factors involved in regulating C_4 photosynthesis using comparative genomics methods in Objective 1.3.

e) Other (data, data production, physical collections, audio or video, software or netware, models, educational aid, or curricula, instruments or equipment)

- None